Pulse-Labeling Patterns of Microsomal Ribonucleic Acid Extracted with Lauryltrimethylammonium Chloride*

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ABSTRACT: A novel method is presented for the extraction of mammalian cytoplasmic ribonucleic acid (RNA) preparatory to sucrose gradient analysis. Microsomes or whole mitochondrial supernatant fractions were treated with the cationic detergent lauryltrimethylammonium chloride (LTAC) in concentrations (1.5–2.0%) sufficient to disrupt the microsomal membranes. RNA, together with an approximately equal quantity of protein, was then precipitated from the mixtures by the addition of 0.04 M magnesium sulfate. The ribonucleoprotein precipitates were dissolved in sodium dodecylsulfate and ethylenediaminetetraacetate (EDTA) and applied directly to sucrose density gradients for fractionation of the RNA. The preparatory procedure was carried out entirely at 0–3°, involved low speed centrifugations only, and required approximately 1 hr. More than 90% of the RNA was recovered, and in pulse-labeled material, the specific activity of the RNA applied to the gradients was identical with that of the RNA in the original cytoplasmic fractions. Evidence is presented that ribonuclease action was minimal during the LTAC–$MgSO_4$ procedure. The *in vivo* labeling patterns of microsomal RNA from a transplantable mouse plasma cell tumor and from the livers of the tumor-bearing mice were studied using the above technique. After a 30-min incorporation of [^{3}H]uridine, radioactivity appeared in both tissues in conjunction with the 18S RNA peak on sucrose gradient analysis, as well as in a heterogeneous distribution throughout the heavier gradient fractions. Definitive labeling of the 28S RNA peak became apparent at 60 min. The high specific activity of the 18S peak relative to the 28S peak was maintained upon exposure of the isolated tumor RNA fractions to lowered ionic strength, additional EDTA, or heating at 60°. Template activity for *in vitro* incorporation of amino acids by *Escherichia coli* ribosomes was associated with sucrose gradient fractions of tumor RNA and appeared to be concentrated in the 18S region. The LTAC–$MgSO_4$ extraction could be conveniently combined with subsequent phenol deproteinization steps for preparative isolation of cytoplasmic RNA.

Secretory protein synthesis is heritably controlled in transplantable murine plasma cell tumors (Potter and Fahey, 1960; Potter *et al.*, 1964). The possibility that this synthesis may be mediated through a specific messenger RNA in each different protein-producing line led us to an investigation of the pulse-labeling patterns of cytoplasmic RNA in these tumors. In an earlier study (Hymer and Kuff, 1964), rapidly labeled RNA had been extracted from plasma cell tumor nuclei without apparent loss of high specific activity components, using an SDS[1] phenol procedure based upon that of Scherrer and Darnell (1962). However, when the same or a related technique (Hiatt, 1962) was later applied to microsome fractions prepared from the tumors 30–60 min after *in vivo* administration of [^{3}H]uridine, we consistently observed that the specific activity of the extracted RNA was 20–30% lower than that of the original microsomal RNA. Since over-all RNA recovery was quite good, it appeared that the extraction procedures produced selective losses of a quantitatively small but metabolically active fraction of RNA.

A fractionating effect of aqueous phenol was first observed in the extraction of RNA from whole cells labeled with ^{32}P (Sibatani *et al.*, 1959; Yamana and Sibatani, 1960), and later shown to occur during the phenol extraction of RNA from both nuclear and cytoplasmic preparations (Sibatani *et al.*, 1960). Rapidly labeled RNA was found to be held preferentially within the phenol phase or at the phenol–aqueous interface. Subsequent modifications in technique (see review by Kirby, 1964), notably the use of SDS during the phenol deproteinization steps, have largely overcome this problem during the extraction of pulse-labeled RNA from isolated nuclei (Hiatt, 1962) or from whole cells in which the labeled RNA is primarily localized within the nuclei (Perry, 1962; Scherrer and Darnell, 1962; Scherrer *et al.*, 1963). The situation is less clear, however, with regard to the phenol extraction of cytoplasmic, and particularly microsomal, RNA. Although rapidly labeled RNA has in fact been pre-

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[1] The following abbreviations are employed: LTAC, lauryltrimethylammonium chloride; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate; DOC, deoxycholate; PCA, perchloric acid.

pared from mammalian microsomes with SDS–phenol techniques (Hoyer *et al.*, 1963; DiGirolamo *et al.*, 1964; Henshaw *et al.*, 1965), data relating to the total recovery of RNA-associated radioactivity during the extraction procedures are generally lacking. Petrovic *et al.* (1965) have recently stated that certain SDS–phenol procedures incompletely extracted a rapidly labeled RNA component of rat liver which they believed to be associated specifically with the microsomal membranes.

The above considerations suggested that there might be value in the development of an additional extraction technique which would permit sucrose density gradient analysis of microsomal RNA without selective loss of rapidly labeled components. Such a procedure, based upon the use of a cationic detergent lauryltrimethylammonium chloride, is described in the present report. It has proved equally applicable to plasma cell tumor and liver microsomes, and may be of particular usefulness in the extraction of RNA from fractions rich in lipoprotein membrane material.

Material and Methods

Tissues. The RPC-20 plasma cell tumor (Kuff *et al.*, 1964) was maintained as a solid tumor by subcutaneous transplantation in female BALB/c mice 20–25 g in weight. Tumors were harvested between 14 and 17 days after transplantation. Livers were obtained from tumor-bearing mice, from normal BALB/c females, or from male Sprague–Dawley rats weighing between 150 and 200 g.

Preparation of Tissue Fractions. All manipulations were carried out between 0 and 4°. In initial experiments with rat liver (Tables I and II), the tissue was homogenized in 4 volumes of 0.25 M sucrose, using 40 strokes in a Potter–Elvejhem homogenizer with a motor-driven Teflon pestle. Nuclei and mitochondria were removed by centrifugation of the homogenates for 10 min at 5000*g* in the No. 296 rotor of the International PR2 centrifuge. The supernatant fluid constituted the mitochondrial supernatant fraction. Microsome pellets were obtained by centrifugation of this fraction for 17 min at 105,000*g* in the No. 40 rotor of the Spinco Model L ultracentrifuge (Kuff and Zeigel, 1960). The pellets were resuspended by vigorous homogenization in 0.25 M sucrose containing 5 mM sodium phosphate, pH 7.4, and 0.5 mM magnesium chloride.

This procedure was modified in the incorporation experiments. The tissues were hand-homogenized (20–25 strokes for tumor; 30–40 strokes for liver) in 0.25 M sucrose containing 4 mM magnesium chloride (Hymer and Kuff, 1964). Under these conditions, nuclear morphology was well preserved as judged by phase microscopy. Between 96 and 98% of the tissue DNA (estimated by diphenylamine reaction) was recovered in the combined nuclear mitochondrial pellets which were sedimented by centrifugation for 10 min at 10,000*g*. The supernatant fluids (mitochondrial supernatant fractions) were centrifuged for 90 min at 105,000*g*. The resultant pellets, which contained free ribosomes as well as the membrane-bounded microsomes (Kuff and Zeigel, 1960), were resuspended by gentle hand homogenization in 0.25 M sucrose containing 10 mM Tris chloride, pH 7.4, and 1 mM magnesium chloride (microsome fractions).

RNA Determination. Recoveries of RNA were based on analysis of the cytoplasmic fractions by a modification of the method of Staehelin *et al.* (1964). The fractions were precipitated at 0° with 0.6 N perchloric acid (PCA) in the presence of 20 mg of Celite (Johns-Manville Hyflo Super-cell), collected by filtration on Millipore membranes (Type HAWP, 25-mm diameter, 0.45 μ pore size), and washed with cold 0.6 N PCA followed by chloroform–ethanol–ether (1:2:2) at room temperature. The precipitates were wet briefly with a small volume of 0.6 N PCA, immediately sucked dry, and transferred on the membranes to conical centrifuge tubes where they were dispersed in a measured volume of 0.6 N PCA and heated with occasional stirring for 20 min at 70°. The membranes were then removed with a stirring rod, and the Celite and protein removed by centrifugation. RNA was estimated from the absorbancy at 260 mμ of the supernatant fluids (Kuff and Zeigel, 1960) after correction for the appropriate Celite-membrane blank. On occasion, the original cold PCA filtrates were collected, constituting the acid-soluble fractions.

Isotope Injection and Radioactivity Assays. [^{3}H]-Uridine and [^{3}H]cytidine (2.5–7 mc/μmole) were purchased as sterile aqueous solutions from the New England Nuclear Corp. The solutions were made to contain 0.15 M NaCl, and 90 μc in a total volume of 0.18 ml was injected into mice either intraperitoneally or intravenously through a tail vein. Radioactivity was assayed in a liquid scintillation system employing Bray's solution (Bray, 1960). Aliquots (0.5 ml) of the PCA extracts described above were pipetted into the vials and neutralized with 50 μl of concentrated ammonium hydroxide before the addition of scintillation fluid (10 ml). Sucrose gradient fractions were counted directly, after preliminary experiments had demonstrated that the small amount of acid-soluble radioactivity contained in the LTAC–$MgSO_4$ precipitates (see Tables III and IV) was confined to the upper 2 or 3 fractions on subsequent gradient analysis. Aliquots (0.5 ml) were treated in the vials with 10 μg of crystallized pancreatic ribonuclease for 2 hr at 37° before addition of scintillation fluid. All samples were corrected to a uniform counting efficiency through the use of an internal standard.

Sucrose Gradient Analysis. Samples (1 or 2 ml) were layered over precooled 5–25% (w/v) linear sucrose gradients (volume 27 ml) prepared with Merck reagent grade sucrose. Centrifugation was carried out at 25,000 rpm and 0° in the SW-25 rotor of the Model L Spinco ultracentrifuge. The ionic compositions of the gradients and the periods of centrifugation are indicated in the figure legends. One-milliliter fractions were collected after puncturing the bottoms of the gradient tubes. Absorbancy was read in a Beckman DU spectrophotometer.

TABLE I: Effect of Lauryltrimethylammonium Chloride (LTAC) on Rat Liver Microsomes.

Microsomes[a] (ml)	LTAC (%)	Fraction of Microsomal Component Sedimentable at Low Speed (15,000g/min)[b] RNA (%)	Protein (%)
0.5	0	(no sediment)	(no sediment)
	.2	103	84
	.5	96	81
	1.0	58	20
	1.5	6.0	4.4
	2.0	5.3	5.1
1.0	0	(no sediment)	(no sediment)
	.2	100	81
	.5	98	80
	1.0	96	58
	1.5	3.3	3.3
	2.0	3.0	2.6

[a] Each milliliter contained the microsomes from 0.33 g of liver resuspended in 5 mM sodium phosphate (pH 7.4)–0.5 mM magnesium chloride–0.25 M sucrose. [b] Mixtures were made to 2.0 ml. After 60 min at 0°, they were centrifuged for 15 min at 1000*g* (Rotor 269, International Centrifuge).

RNA was recovered from the gradient fractions by precipitation with 2 volumes of absolute ethanol at −10° (DiGirolamo *et al.*, 1964). The precipitates were washed once with 95% ethanol at the same temperature, and then either dissolved immediately in buffer (for gradient analysis) or dried *in vacuo* at −10° (for subsequent addition to amino acid incorporating system).

In Vitro Amino Acid Incorporation. RNA fractions recovered from sucrose gradients were tested for their capacity to stimulate amino acid incorporation by a preincubated S-30 fraction from *E. coli* (Nirenberg and Matthaei, 1961). A complete description of the incorporating system and radioactivity assay has been presented (Abell *et al.*, 1965). The reaction mixture (0.25 ml) contained 50 mμmoles of each of the following uniformly labeled L-[^{14}C]amino acids, all at a specific activity of 10 μc/μmole: alanine, arginine, aspartic, glutamic, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine. The incorporation assays were kindly performed by Dr. Creed Abell of the Field Studies Branch, National Cancer Institute.

Materials. LTAC was obtained as a 50% solution (Lot No. 18084L) from K and K Laboratories, Inc., Plainview, N. Y. It consisted of a viscous, clear, slightly yellow solution. Nitrogen analysis was consistent with a 48.5% LTAC content on a weight basis. A 15% stock solution in water was used, without purification, in the present experiments. SDS was obtained from Fisher

TABLE II: Magnesium Sulfate Precipitation of RNA and Protein from LTAC-Treated Liver Microsomes and Mitochondrial Supernatant Fraction.

Fraction[a]	LTAC (%)	$MgSO_4$ (M)	Fraction of Component Sedimentable at 15,000g/min[b] RNA (%)	Protein (%)	RNA RNA + Protein (mg/mg)
Microsomes	0	0	...	...	(0.10)
	1.0	0.001	17	5.8	0.24
		0.005	93	24	0.29
		0.01	102	22	0.33
		0.02	100	20	0.36
	1.5	0.001	9.5	2.2	0.32
		0.005	91	17	0.38
		0.01	103	16	0.41
		0.02	99	14	0.44
		0.03	100	14	0.45
Mitochondrial supernatant fraction	0	0	...	...	(0.04)
	1.5	0.02	101	8.2	0.33
		0.04	101	6.8	0.38
	2.0	0.02	100	7.5	0.35
		0.04	99	6.5	0.39

[a] Fractions representing 0.2 g of rat liver. [b] Magnesium sulfate was added, as a 1 M solution, 5 min after the LTAC. The mixtures (final volume 2.0 ml) were allowed to stand an additional 60 min (microsomes) or 15 min (mitochondrial supernatant) at 0°. They were then centrifuged for 10 min at 1500*g*.

TABLE III: Recovery of RNA and Radioactivity from RPC-20 Tumor and Mouse Liver Microsomes after *in Vivo* Incorporation of [^{3}H]Uridine.

Fraction	RNA		Radioactivity			
			In RNA			Acid Soluble
	Total (mg)	Recovery (%)	Total (cpm)	Recovery (%)	Specific (cpm/mg of RNA)	Total (cpm)
			Tumor			
30-min incorp[a]						
Whole microsomes	1.72	(100)	32,600	(100)	18,950	11,880
LTAC–$MgSO_4$ precipitate	1.72	100	33,000	102	19,200	550
Same, washed once	1.60	93	31,800	98	19,900	140
180-min incorp[a]						
Whole microsomes	1.11	(100)	66,500	(100)	59,800	4,140
LTAC–$MgSO_4$ precipitate washed once	1.08	97	64,500	97	59,700	22
			Liver			
30-min incorp[a]						
Whole microsomes	1.06	(100)	4,270	(100)	4,030	9,810
LTAC–$MgSO_4$ precipitate	0.97	92	3,920	92	4,040	356
Same, washed once	0.98	92	3,850	90	3,930	54
180-min incorp[a]						
Whole microsomes	0.34	(100)	14,200	(100)	41,800	12,800
LTAC–$MgSO_4$ precipitate washed once	0.31	91	13,000	92	41,900	130

[a] [^{3}H]Uridine (90 μc) was administered intravenously and the animals were sacrificed at the times shown. Livers were obtained from the tumor-bearing mice.

TABLE IV: Recovery of RNA and Radioactivity from RPC-20 Tumor and Mouse Liver Mitochondrial Supernatant Fractions after *in Vivo* Incorporation of [^{3}H]Uridine.[a]

Fraction	RNA		Radioactivity in RNA		
	Total (mg)	Recovery (%)	Total (cpm)	Recovery (%)	Specific (cpm/mg of RNA)
		Tumor			
Mitochondrial supernatant	1.25	(100)	8500	(100)	6820
LTAC–$MgSO_4$ precipitate	1.19	95	8070	95	6780
Same, washed once	1.26	101	8320	98	6600
		Liver			
Mitochondrial supernatant	0.62	(101)	7090	(100)	11,500
LTAC–$MgSO_4$ precipitate	0.60	97	6480	91	10,870
Same, washed once	0.59	95	6510	92	11,080

[a] [^{3}H]Uridine (90 μc) was administered intraperitoneally to tumor-bearing animals 60 min before sacrifice.

Scientific Co. as the USP grade and dissolved just before use.

Results

Solubilization of Microsomes with LTAC. Quaternary ammonium compounds containing aliphatic chains of C_{12} or higher are effective precipitants of nucleic acids from low ionic strength solutions (Jones, 1953; Aubel-Sadron *et al.*, 1961). In our initial studies, LTAC was added in varying concentrations to rat liver microsomes suspended in a dilute phosphate–magnesium buffer (Table I). A biphasic response was observed. At concentrations of 0.5% or less, LTAC caused a rapid aggregation of the microsomes, so that all of the microsomal RNA as well as the bulk of the protein was

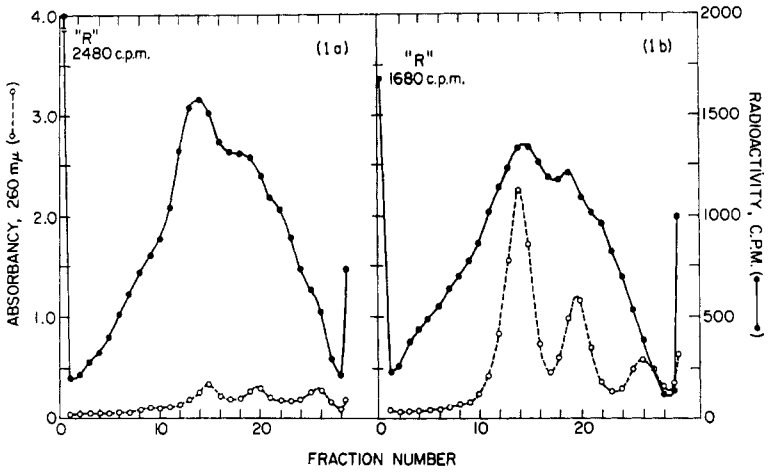

FIGURE 1: The effect of LTAC–$MgSO_4$ extraction upon the sucrose gradient sedimentation pattern of previously isolated pulse-labeled nuclear RNA. (1a) RNA isolated by a phenol procedure from RPC-20 plasma cell tumor nuclei labeled for 30 min *in vivo* with [^{3}H]uridine. (1b) RNA recovered by the LTAC–$MgSO_4$ procedure from a sample of nonradioactive mouse liver microsomes to which pulse-labeled nuclear RNA had been added in an amount equal to that analyzed in (1a). The radioactivity pattern in 1b is thus due entirely to the nuclear RNA and the absorbancy pattern primarily to the microsomal RNA. R represents a residual fraction recovered from the bottoms of the gradient tubes after sampling was completed. See text for further details.

sedimented by low-speed centrifugation of the suspensions. However, with increasing LTAC concentrations, the tendency to aggregation diminished. At levels of 1.5–2.0%, LTAC caused a marked clarification of the microsomal suspensions, similar to that seen with DOC (deoxycholate), and again very little of the microsomal RNA and protein was sedimentable at low speeds. At intermediate LTAC concentrations (*e.g.*, 1%) the effects were apparently sensitive to the relative total amounts of microsomal material and LTAC present in the mixture, and variable results were obtained (compare Tables I and II).

Precipitation of Ribonucleoprotein with Magnesium Sulfate. The addition of magnesium sulfate to LTAC-solubilized rat liver microsomes resulted in a quantitative precipitation of RNA, together with a proportion of the microsomal protein (Table II). Magnesium chloride was found to be much less effective at the same concentrations. The speed with which the nucleoprotein aggregate formed was dependent upon the magnesium sulfate concentration. At 30 mM magnesium sulfate or above, 15 min (at 0°) was sufficient for the formation of a precipitate that could be removed by low-speed centrifugation. The proportion of protein in the magnesium-precipitated pellets appeared to depend somewhat upon both the LTAC and $MgSO_4$ concentrations (Table II); however, this relationship has not been systematically investigated.

Application of the LTAC–$MgSO_4$ procedure to rat liver mitochondrial supernatant fraction, containing soluble fraction as well as microsomes, again resulted in essentially complete precipitation of RNA (Table II). A nearly 10-fold enrichment of RNA with respect to protein was observed.

Washing of Magnesium-Precipitated Nucleoprotein. The low-speed pellets obtained after magnesium sulfate precipitation were insoluble in dilute LTAC–$MgCl_2$ solutions. Precipitates were routinely washed by resuspension in 10 mM Tris chloride, pH 7.4, containing 0.05% LTAC and 1.0 mM magnesium chloride, followed by recentrifugation for 10 min at 1500*g*.

Recovery of RNA-Associated Radioactivity by LTAC–$MgSO_4$ Procedure. As shown in Table III, the specific activity of RNA precipitated from both tumor and liver microsomes by this method was essentially identical with the specific activity of the RNA in the original microsome preparations. This was true for microsomes isolated after periods of incorporation (30 and 180 min) which gave widely differing distributions of RNA-associated radioactivity on subsequent sucrose gradient analyses (Figures 2 and 3). These facts argue strongly against the loss of any major metabolically distinctive category of RNA during the preparative procedure. Also shown in Table III are the radioactivities appearing in the cold acid-soluble fractions of the microsomes and the LTAC–$MgSO_4$ precipitates. After a single wash, the acid-soluble counts constituted approximately 1% or less of the total radioactivity in the precipitates.

Application of the procedure to mitochondrial supernatant fractions of liver and tumor similarly resulted in excellent recoveries of RNA-associated radioactivity (Table IV). The acid-soluble counts were not determined in these instances.

Solution of Magnesium-Precipitated Nucleoprotein with SDS. SDS is known to dissociate ribosomal RNA from protein (Kurland, 1960). Magnesium-precipitated nucleoprotein pellets were readily soluble at 0° in 10 mM Tris chloride, pH 7.4, containing 0.5% SDS. To

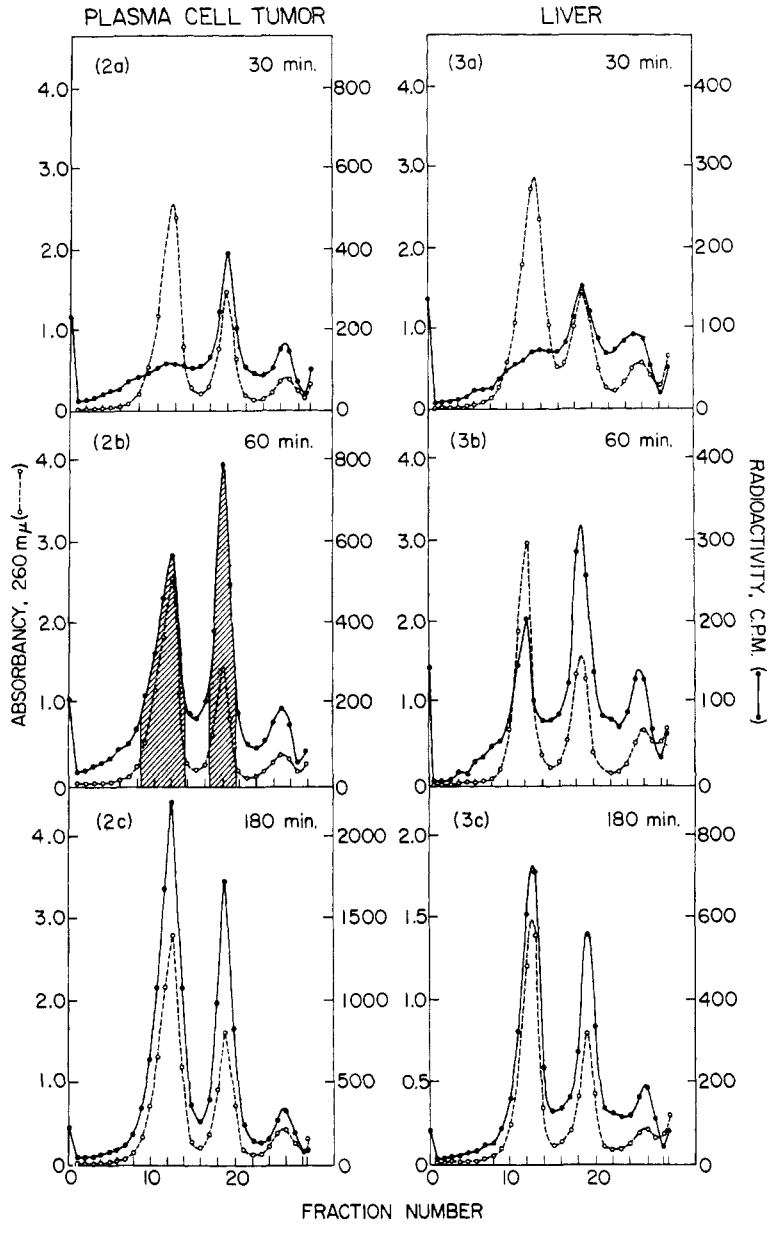

FIGURE 2: Sucrose gradient sedimentation patterns of RNA recovered by the LTAC–$MgSO_4$ procedure from the microsomes of RPC-20 plasma cell tumor. The tissues were taken from tumor-bearing mice at the indicated times after intravenous injection of 90 μc of [^{3}H]uridine. The shaded areas in Figure 2b designate regions that were pooled from replicate gradients for subsequent resedimentation on sucrose gradients (see text and Figure 4). The 5–25% linear sucrose gradients contained 10 mM Tris chloride, pH 7.4, and 50 mM sodium chloride. Centrifugation was for 15 hr at 0° and 25,000 rpm (SW-25 rotor).

FIGURE 3: Sucrose gradient sedimentation patterns of RNA recovered by the LTAC–$MgSO_4$ procedure from the microsomes of mouse liver. See caption to Figure 2 for conditions.

minimize possible magnesium-induced association of diverse RNA components during subsequent sucrose gradient analysis (Staehelin *et al.*, 1964), EDTA (sodium salt), pH 7.5, was added at this step. Experiments on liver microsomal nucleoproteins treated with 0.5% SDS and varying concentrations of EDTA (0–16 mM) indicated that approximately 8 mM EDTA was optimal in terms of the spectral properties of RNA fractions subsequently recovered from sucrose density gradients (see below): absorbancy ratios (A_{260}/A_{230} and A_{260}/A_{280}) varied between 2.05 and 2.15 throughout the major RNA peaks. When lower concentrations of EDTA were used, lower ratios, suggestive of protein contamination, were observed.

Summary of Procedure Preparatory to Sucrose Gradient Analysis. In a conical centrifuge tube, an aliquot of microsome fraction containing between 0.3 and 1.5 mg of RNA was brought to a volume of 1.72 ml with resuspending buffer (see Methods). Two-tenths milliliter of a 15% LTAC solution in water was added, followed in 5 min by 0.08 ml of 1 M $MgSO_4$. The final volume was thus 2.0 ml, the final LTAC and $MgSO_4$ concentrations 1.5% and 0.04 M, respectively. After standing for 15–20 min, the suspension was centrifuged for 10 min at 1500*g*. The supernatant fluid was removed and the pellet agitated vigorously with a stirring rod just prior to the addition of 2.0 ml of 10 mM Tris chloride, pH 7.4–1 mM magnesium chloride–0.05% LTAC. After brief additional stirring, the suspension was recentrifuged and the wash fluid removed. The pellet was again vigorously agitated, and the following additions made in sequence: 1.0 ml of 20 mMTris chloride, pH 7.4; 0.74 ml of water; 0.06 ml of 0.25 M sodium EDTA, pH 7.5; and 0.2 ml of 5% SDS (final volume 2.0 ml; Tris chloride, 10 mM; EDTA, 7.5 mM; SDS, 0.5%). The mixtures were stirred intermittently until solution was complete (3–5 min), and then layered directly over sucrose gradients. Mitochondrial supernatant fractions were treated identically except that the LTAC concentration in the initial step was raised from 1.5 to 2.0%. The entire procedure was carried out between 0 and 3° and required approximately 1 hr. Multiple samples were conveniently treated, and the procedure was easily scaled up as required.

Sucrose Gradient Analysis of LTAC–$MgSO_4$ Treated Material. The SDS-solubilized pellets were analyzed by gradient centrifugation at 0°. Typical absorbancy patterns for liver and tumor microsomal RNA appear in Figures 1–3. In these instances the gradients contained 10 mM Tris chloride, pH 7.4, and 50 mM NaCl. The sedimentation rates of the two main components were determined in the case of the tumor RNA by ultraviolet absorption sedimentation analysis of the peak gradient fractions, using a Spinco Model E analytical ultracentrifuge. Values ($s_{20,w}$) of 29 and 17.5 S were obtained in agreement with the generally accepted values of 28 and 18 S for the major RNA components of mammalian ribosomes (Hall and Doty, 1959; Petermann and Pavlovec, 1963). RNA, protein, and EDTA all contributed to the absorbancy of the small peak near the top of the gradients. This peak was considerably larger in gradient patterns obtained from mitochondrial supernatant fractions. The RNA is assumed to represent transfer RNA, with a sedimentation rate of approximately 4 S.

Patterns very similar to those shown were obtained with gradients containing 10 mM Tris chloride, pH 7.4, and 1 mM magnesium chloride. In gradients containing only 10 mM Tris chloride, the two ribosomal RNA's sedimented at greatly reduced rates, although their relative positions and sizes were maintained.

Test of Ribonuclease Activity during the LTAC–$MgSO_4$ Procedure. The recovery data shown above indicated that gross degradation of RNA did not occur during the procedure. However, they did not eliminate the possibility of an endonuclease attack which might result in considerable size reduction of RNA without loss of acid precipitability (Hymer and Kuff, 1964). This possibility was tested in the following manner. Nuclear RNA was isolated by a phenol–SDS procedure (Hymer and Kuff, 1964) from the RPC-20 tumor after a 30 min *in vivo* incorporation period (90 μc of [^{3}H]uridine administered intravenously). One aliquot was subjected to sucrose gradient analysis without further treatment (Figure 1a). A second aliquot was added to nonradioactive mouse liver microsomes, which were then carried through the LTAC–$MgSO_4$ procedure. Ninety-two per cent of the added radioactivity was recovered. In its major aspects, the radioactivity distribution of the recovered RNA (Figure 1b) was essentially the same as that of the original nuclear RNA.

We regard the above experiment as a fairly rigorous test for ribonuclease activity, since the rapidly labeled RNA of RPC-20 tumor nuclei has been shown to be highly sensitive to intracellular ribonuclease action (Hymer and Kuff, 1964). The results indicate that such action, if it occurs during the LTAC–$MgSO_4$ extraction of microsomes, is not of a magnitude that would result in major size reduction of the RNA.

Early Labeling Patterns of Tumor and Liver Microsomal RNA. Microsome fractions were prepared from RPC-20 tumors and from the livers of the tumor-bearing mice at various times after intravenous injection of [^{3}H]uridine. The microsomes were subjected to the LTAC–$MgSO_4$ procedure as described above, and the resultant material analyzed by sucrose density gradient centrifugation. The absorbancy and radioactivity patterns are shown in Figures 2 and 3.

Labeling of all microsomal RNA components proceded more rapidly in the tumor than in liver after intravenous administration of precursor (see also Table III). There was, however, a general similarity between the labeling patterns of the two tissues. Of particular interest was the appearance, after a 30-min incorporation period, of a radioactivity peak coinciding with the 18 S ribosomal RNA. Definitive labeling of the 28 S ribosomal RNA was not observed at this time in either tissue; rather, radioactivity appeared in a heterogeneous distribution throughout the lower portions of the gradients, with a broad maximum through the 28 S region. A well-defined 28 S radioactivity peak was observed at 60 min in both liver and tumor, apparently superimposed upon the heterogeneous rapidly labeled RNA noted at the earlier time. However, the specific radioactivity in the 28 S peak was still much lower than that in the 18 S RNA region. By 3 hr, this inequality had been greatly reduced by the progressive labeling of both 18 and 28 S components. Longer incorporation periods were not studied.

Radioactivity appeared early in the small peak corresponding to the microsomal transfer RNA. The region of the gradient between this peak and the 18 S RNA encompasses a range of sedimentation constant (4–18 S) frequently ascribed to messenger RNA's of both mammalian and bacterial origin (Gros *et al.*,

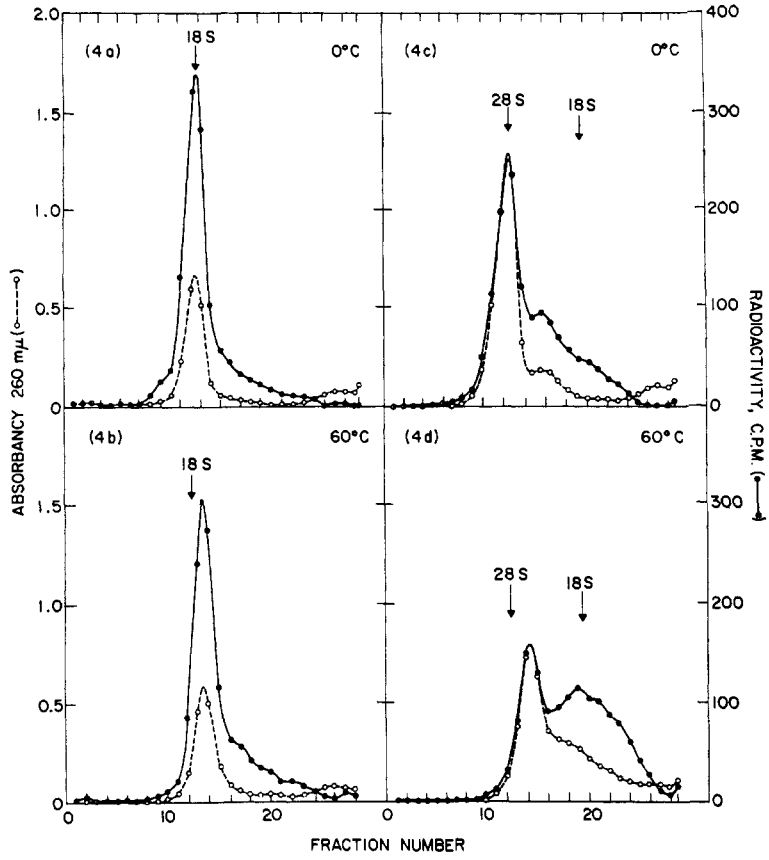

FIGURE 4: Sucrose gradient analysis of isolated 18 S (4a and 4b) and 28 S (4c and 4d) tumor microsomal RNA (labeled 60 min *in vivo* with [^{3}H]uridine). RNA was recovered by alcohol precipitation from an original sucrose gradient fractionation as indicated in Figure 2b. Aliquots of the redissolved RNA's were either held at 0° (4a and 4c) or treated at 60° for 6 min (4b and 4d) before layering over 5–25% linear sucrose gradients containing 5 mM Tris chloride, pH 7.4. The 18 S RNA (4a and 4b) was centrifuged for 33.3 hr at 0° and 25,000 rpm; the 28 S RNA (4c and 4d) for 20.8 hr. Other details of the experiment are given in the text.

1961; Penman *et al.*, 1963; Staehelin *et al.*, 1964; Trakatellis *et al.*, 1964). Inspection of Figures 2 and 3 reveals that the specific radioactivity (cpm/A_{260}) in this region was relatively high in both tissues, particularly at the shorter incorporation period. Generally, this region was not conspicious in terms of the total radioactivity in the gradients. There was a suggestion of a minor peak at approximately the 12 S position in Figure 3b (liver, 60-min incorporation). In these experiments, however, centrifugation was too short to permit optimal resolution of components within the 4–18S range.

Radioactivity distributions similar to those shown in Figures 2a and 3a were observed after 30 min *in vivo* incorporation of [^{32}P]inorganic phosphate and [^{3}H]-cytidine into tumor and liver, respectively. Labeling of the 4 S peak was relatively accentuated in the ^{32}P experiment, as expected from the known turnover of terminal nucleotides in transfer RNA.

Stability of Isolated 28 and 18 S Tumor RNA. It was recognized that the radioactivity distributions may have been influenced by molecular interactions induced by the preparative or gradient techniques. The possibility seemed particularly important with regard to the high specific activity of the 18 S RNA peak, since it has been suggested that pulse-labeled RNA of liver cytoplasm may bind preferentially to 18 S ribosomal RNA in the presence of magnesium ion and elevated ionic strength (Staehelin *et al.*, 1964). Isolated tumor RNA fractions were therefore subjected to a second gradient analysis under conditions that might reverse such association.

The fractions indicated by the shaded areas in Figure 2b (tumor microsomal RNA, 60-min incorporation) were pooled from two replicate gradients. RNA was recovered from the pooled fractions as described under Methods and redissolved at 0° in 5 mM Tris chloride, pH 7.4, containing 0.5% SDS. Sodium EDTA, pH 7.5, was added at a final concentration of 7.5 mM to one aliquot of each RNA preparation which was then layered directly over a precooled sucrose gradient containing 5 mM Tris chloride, pH 7.4, as the sole ionic constituent (Staehelin *et al.*, 1964). A second aliquot (without added EDTA) was heated at 60° for 6 min and then cooled rapidly to 0° before layering over a similar

gradient. The times of centrifugation (see legend, Figure 4) were adjusted to bring the main peaks of the 18 and 28 S RNA preparations to the same position within the gradients.

As shown in Figure 4, the high specific radioactivity of the 18 S RNA peak relative to that of the 28 S persisted throughout all of the above manipulations. Henshaw *et al.* (1965) have also reported that high specific radioactivity associated with pulse-labeled 18 S ribosomal RNA of liver cytoplasm could not be released by heating, changes in salt concentration, or treatment with EDTA. The 28 S ribosomal RNA proved to be relatively labile, as expected from previously reported observations in other systems (Kurland, 1960; Petermann and Pavlovec, 1963; Boedtker *et al.*, 1962). A marked displacement of radioactivity toward lower sedimentation values was apparent in the case of the 28 S fraction, particularly after heating to 60° (Figure 4d). Comparison of the radioactivity and absorbancy curves indicates that this displacement involved the breakdown of high specific activity material in addition to that of the ribosomal RNA itself.

Biological Activity of LTAC–$MgSO_4$ Treated RNA. Fractions from three replicate sucrose gradients of tumor microsomal RNA were pooled as indicated in Figure 5. RNA was recovered from each of the pooled fractions (see Methods), redissolved in 0.1 M Tris chloride, pH 7.6, and tested for its capacity to stimulate amino acid incorporation in a cell-free system derived from *Escherichia coli*. The stimulatory capacities, expressed on a concentration basis with respect to the original gradient fractions, are also shown in Figure 5.

Maximum activity was observed with RNA recovered from the 18 S gradient region, and lesser amounts with the more rapidly sedimenting RNA fractions. Very little RNA was recovered from the upper 2 pooled gradient fractions (see legend, Figure 5), and this material showed no detectable stimulatory capacity for the ribosome system. Similar results were obtained when the incorporation of [^{14}C]threonine alone (rather than a mixture of [^{14}C]amino acids) was studied.

Although the presence of biological activity in certain of the tumor RNA fractions appears to have been unequivocal, additional studies are required in order to quantitate the distribution of this activity throughout the sucrose gradient pattern more precisely. In particular it will be desirable to isolate sufficient RNA from the upper gradient regions so that all fractions can be tested at equivalent RNA levels in the incorporation system. It may be noted, however, that the gradient distribution of stimulatory capacity in the plasma cell microsomal RNA was quite similar to that seen by DiGirolamo *et al.* (1964) in their much more complete study of phenol-extracted liver cytoplasmic RNA.

Effect of Phenol Extraction on Sedimentation Properties of LTAC–$MgSO_4$ Extracted RNA. The nucleoprotein pellets precipitated with $MgSO_4$ and redissolved in SDS–EDTA were directly amenable to phenol extraction. An equal volume of 90% phenol (previously equilibrated with 10 mM Tris chloride, pH 7.4) was added and the suspension stirred vigorously for 10 min. The phases were separated by centrifugation (5 min at 26,000*g*) and the phenol phase reextracted with one-half volume of 10 mM Tris chloride, pH 7.4, containing 0.5% SDS. The combined aqueous phases were then extracted twice more with phenol, stirring for 10 min each time. RNA was recovered from the aqueous phase by alcohol precipitation, redissolved in 10 mM Tris chloride, pH 7.4, containing 50 mM NaCl, passed through a mixed-bed column of Sephadex G-50 and carboxymethyl-cellulose (Hymer and Kuff, 1964) that had been preequilibrated with the same buffer, and finally applied to sucrose density gradients.

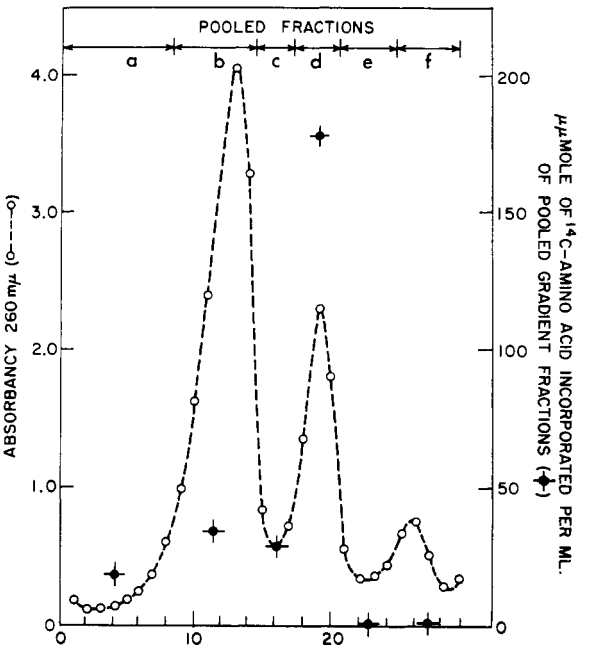

FIGURE 5: Stimulation of *in vitro* [^{14}C]amino acid incorporation in a bacterial ribosome system by sucrose gradient fractions of plasma cell tumor microsomal RNA. The RNA was extracted by the LTAC–$MgSO_4$ procedure and subjected to gradient centrifugation. The fractions from three replicate gradients were pooled as indicated in the figure and the RNA was recovered from each of the pooled fractions (a–f) by ethanol precipitation. Aliquots of the recovered RNA's were tested in duplicate for their capacity to stimulate incorporation of a mixture of 15 [^{14}C]amino acids by a dialyzed *E. coli* S-30 fraction. The quantities of RNA added to the reaction mixtures (total volume 0.25 ml) were: a, 10 μg; b, 150 μg; c, 16 μg; d, 59 μg; e, 2 μg; f, 1 μg. The ratios of counts incorporated in the presence of RNA to the background incorporation (no added RNA) were: a, 1.35; b, 1.46; c, 1.20; d, 2.44; e and f, 1.0. The incorporated radioactivities in excess of background were expressed as μμmoles of amino acid, and in each instance divided by the original volume of the pooled gradient fraction from which the RNA was obtained in order to present the stimulatory activity on the same concentration basis as the A_{260}.

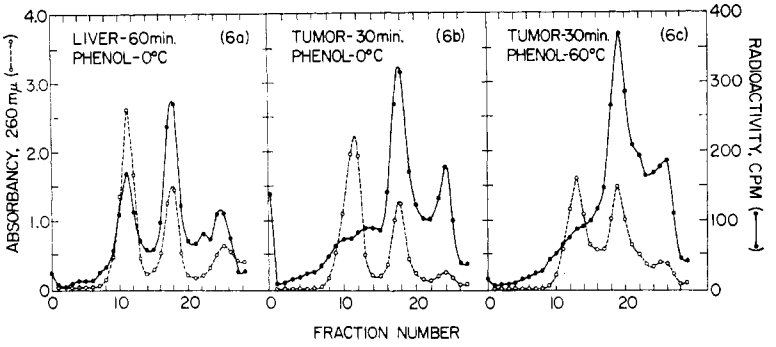

FIGURE 6: Sucrose gradient sedimentation patterns of phenol-treated liver and plasma cell tumor microsomal RNA, labeled *in vivo* with [^{3}H]uridine for 60 and 30 min, respectively. RNA was extracted with the LTAC–magnesium sulfate procedure, dissolved in SDS–EDTA, and then deproteinized with phenol as described in the text. The samples taken for phenol treatment were aliquots of the same preparations used for the direct sucrose gradient analyses shown in Figures 3b (liver) and 2a (tumor), and the conditions of gradient centrifugation were also the same.

The phenol extraction of LTAC–$MgSO_4$ fractions was found to be considerably more convenient than direct phenol extraction of the microsome fractions themselves, since prior elimination of the bulk of the microsomal protein and lipid greatly reduced the quantity of material that collected at the phenol–aqueous interface. Absolute recoveries of RNA were not determined, but the specific activities of the phenol-extracted RNA's ranged between 85 and 95% of those in the LTAC–$MgSO_4$ precipitates, regardless of whether the phenol extractions were carried out at 0 or 60° (see below).

Figure 6 illustrates several sucrose gradients patterns given by phenol-extracted RNA. The LTAC–$MgSO_4$ precipitates from which the RNA was extracted were replicate samples of those used for direct gradient analysis; *i.e.*, Figure 6a may be compared directly with Figure 3b (liver microsomes, 60-min incorporation), and Figures 6b and 6c with Figure 2a (tumor microsomes, 30-min incorporation). When the phenol extractions were carried out entirely at 0° (Figures 6a and 6b), only slight changes in the radioactivity distributions were observed. However, heating to 60° during the phenol extractions markedly altered both the radioactivity and absorbancy distributions of the tumor RNA (Figure 6c). The changes were entirely consistent with those induced in previously isolated 18 and 28 S fractions by heating (Figure 4).

Discussion

Cetyltrimethylammonium bromide (CTAB) has been employed in the isolation and purification of RNA from bacterial, plant, and animal sources (Jones, 1953; Dutta *et al.*, 1953; Aubel-Sadron *et al.*, 1961; Jones, 1963; Ralph and Bellamy, 1964). These preparative methods make use of the strong precipitating action of the quaternary ammonium compound toward nucleic acids in dilute salt solutions and the ease with which the precipitates may be dissociated at elevated ionic strengths. The most recently described procedures as applied to microorganisms (Jones, 1963) and rat liver (Ralph and Bellamy, 1964) involve an initial extraction with phenol followed by precipitation of RNA from aqueous solution with CTAB. The present method is based upon the detergent, rather than the precipitating action of the long-chain alkyltrimethylammonium salts. The membranous elements of the microsomes are apparently disrupted at sufficiently high concentrations of LTAC, releasing associated RNA and/or ribonucleoprotein components that are subsequently precipitated with magnesium sulfate. We have not investigated extensively the use of quaternary ammonium compounds other than LTAC in this context, although CTAB itself has proved unsatisfactory because of its limited solubility in the cold.

The LTAC–$MgSO_4$ procedure is not in itself a complete preparative technique, since the RNA is obtained as a precipitate composed of approximately equal amounts of nucleic acid and protein. However, these precipitates are easily dissociated with SDS, and the resultant solutions are amenable both to direct sucrose gradient analysis of the RNA, and, for general preparative work, to conventional phenol extraction procedures. The rapidity of the LTAC–$MgSO_4$ steps, the low temperature employed, the precipitated state of the RNA during most of the time prior to SDS treatment, probably all serve to minimize ribonuclease action during the extraction procedure.

Although gradient-isolated tumor RNA fractions (particularly the 18 S material) were capable of stimulating amino acid incorporation in a cell-free system, we have no present means of evaluating the preservation of this template activity in terms of that originally contained within the microsome fraction. It may be relevant to note that previously isolated tobacco mosaic viral RNA and yeast transfer RNA fully retained their respective biological activities when subjected to procedures involving precipitation with quaternary ammonium salts (Hirth *et al.*, 1960; Weil *et al.*, 1961; Weil and Ebel, 1962; Ralph and Bellamy, 1964).

The present results clearly confirm (and extend to a new cell type, the neoplastic plasma cell) the preferential labeling of 18 S cytoplasmic RNA which has recently been studied in some detail in both liver (Henshaw *et al.*, 1965a) and Hela cells (Girard *et al.*, 1965). It is not apparent from our own data whether the rapid labeling of the 18 S (as compared to the 28 S) RNA peak results solely from a relative precocity in the nucleo-cytoplasmic transfer of newly synthesized 18 S ribosomal RNA (Girard *et al.*, 1965); whether it is due, at least in part, to a specific association between messenger and 18 S ribosomal RNA (Staehelin *et al.*, 1964; Henshaw *et al.*, 1965a); or finally, whether it reflects some preferred size range of messenger RNA itself. It might be pointed out here that the secretory protein of the RPC-20 plasma cell tumor has a molecular weight of only 24,000 (Kuff *et al.*, 1964), and that the corresponding messenger RNA, if monocistronic, would be expected to have a sedimentation rate considerably less than 18 S.

The 30-min incorporation patterns of both liver and tumor microsomal RNA showed conspicuous distributions of radioactivity through the gradient fractions representative of sedimentation rates above 18 S. It is known that pulse-labeled nuclear RNA isolated from both liver (Hiatt, 1962) and plasma cell tumor (Hymer and Kuff, 1964) appears chiefly in these gradient regions. We have found that our microsome pellets contained only about 10% of the total RNA-associated radioactivity after a 30-min *in vivo* incorporation of [^{3}H]uridine, most of the remainder appearing in the nuclear fraction. The artifactitious transfer of even a small proportion of the highly labeled nuclear RNA to the microsomes fractions could thus strongly influence the radioactivity distributions of the microsomal RNA. Alternatively, the observed patterns might in fact reflect a physiological transfer of rapidly sedimenting nonribosomal RNA from nucleus to cytoplasm.

Finally, it may be suggested that in the case of the plasma cell tumor, both the pulse-labeling patterns and the distribution of the capacity to stimulate *in vitro* amino acid incorporation were consistent with the hypothesis that cytoplasmic messenger RNA sedimented chiefly at rates of 18 S or greater, when analyzed with the LTAC–$MgSO_4$ technique. It is recognized, however, that many factors which may have contributed to the observed patterns, arising from the methods employed or from the special nature of the tissue itself, remain to be evaluated.

Added in Proof

Latham and Darnell (1965) have recently estimated that about 80% of the messenger RNA associated with Hela cell polyribosomes sediments between 12 and 20 S. McConkey and Hopkins (1965) have presented evidence for 15–16 S messenger RNA associated with 45 S ribosomal subunits in Hela cell cytoplasm. The study of Henshaw *et al.* (1965b), previously available in abstract form, has been published *in extenso*. Their results with rat liver indicate that messenger RNA derived either from the 45 S particle or from the total cycloplasm is concentrated in the 18 S region on sucrose gradient analysis.

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Fractionation of Transfer Ribonucleic Acid by Gradient Partition Chromatography on Sephadex Columns*

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ABSTRACT: t-RNA (transfer ribonucleic acid) from *Escherichia coli* may be resolved on partition columns composed of Sephadex G-25 fine beads and a biphasic solvent mixture. The mixture is composed of potassium phosphate pH 6.88 buffer, ethoxyethanol, butoxyethanol, mercaptoethanol, and triethylamine. Linear variation in triethylamine concentration results in an exponential variation of the partition coefficient of t-RNA in the mixture, allowing t-RNA dissolved in aqueous phase immobilized on the Sephadex beads to be extracted by mobile phase containing a gradient of triethylamine. Resolution is sufficient to provide individual amino acid specific t-RNA's of 25–40% purity, as much as 24-fold enriched, in one passage over the column.

Of 13 specific t-RNA's examined, at least 9 are heterogeneous, and 5 separable varieties of t-RNA accept leucine. Results are reproducible for columns of varying diameter, and at least 3.4 g of t-RNA may be fractionated with no decrease in resolution. Except for mechanical losses, recoveries of t-RNA and acceptor activities are complete.

Transfer RNA (t-RNA) is a mixture of many structurally similar polynucleotides each of which participates in the activation and transfer of a specific amino acid in protein synthesis (Berg, 1961; Brown, 1963). Complete resolution of the different t-RNA species and the determination of their primary and secondary structures are needed to establish how each aminoacyl RNA is formed and interacts specifically with an m-RNA–ribosome complex in peptide bond synthesis. Countercurrent distribution (CCD)[1] has proved to be the most successful method to date for resolving different amino acid specific t-RNA's on a preparative scale. First introduced for RNA fractionation by Warner and Vaimberg (1958), it was utilized for the isolation of yeast t-RNA_{tyr}, t-RNA_{val}, and t-RNA_{ala} (Holley *et al.*, 1963) with subsequent total primary sequence determination of the latter (Holley *et al.*, 1965). Others have obtained highly purified yeast t-RNA_{ser} (Tada *et al.*, 1962; Rushizky *et al.*, 1964) and yeast t-RNA_{phe} (Hoskinson and Khorana, 1965). t-RNA from *Escherichia coli* has also been highly resolved (Goldstein *et al.*, 1964) by partitioning through a large number of transfers. However, CCD requires elaborate and expensive equipment, and the isolation of t-RNA from each fraction is time consuming.

Chromatographic methods employing columns of methylated albumin-kieselguhr (Mandell and Hershey, 1960; Sueoka and Yamane, 1962), DEAE-cellulose and DEAE-Sephadex (Cherayil and Bock, 1965; Kawade

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[1] Abbreviations used in this work: t-RNA_{leu} or t-RNA_{ileu}, t-RNA which accepts leucine or isoleucine, respectively. Leu-RNA or ileu-RNA are used to designate the esterified forms of the t-RNA's. Where more than one species of t-RNA_{leu} occurs we have, in the absence of any systematic way of designating the multiple forms of RNA obtained in fractionation procedures, referred to them by the order of elution from the partition column, *e.g.*, t-$RNA_{leu,1}$ is the first peak of t-RNA_{leu}. Other abbreviations: PPO, 2,5-diphenyloxazole; dmPOPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; CCD, countercurrent distribution; AMP, adenosine monophosphate; CTP, cytidine triphosphate; ATP, adenosine triphosphate.