are the methyls and methylenes of the nonpolar tail. In terms of this picture we may equate $k_{s,eme}$ with the values of K_s calculated from the Setschenow equation (see Hamabata *et al.*, 1973), and calculate free energies of transfer from water to 1 M salt for that fraction of the hexanamide monomers which change their environment on micelle formation, as done previously for the amide solubility data (Hamabata *et al.*, 1973; eq 4). The results of such calculations, *for these data obtained at 50°*, are also shown in Table II.

The last column of Table II, in which we list the average values for the free energy of transfer of a methylene group from water to 1 M $NaClO_4$ or NaCl, as obtained from differential amide solubility data (Table IV; Hamabata *et al.*, 1973), suggests that our picture of the micelle structure may be oversimplified. In the preceding article it was shown that $\Delta G_{tr,CH_2}$ is approximately independent of temperature between 6 and 25°. *If* this independence of temperature of $\Delta G_{tr,CH_2}$ extends to 50°, then these data suggest that a *net* transfer of only *ca.* three methylene group equivalents per hexanamide monomer is taking place on micelle formation. Alternatively, we may speculate that micelle formation *does* involve a fractional burial of the amide group, which results in a *negative* contribution to the total $\Delta G_{tr,eme}$ from water to 1 M salt, and thus partially offsets the larger positive ΔG_{tr} expected for the total nonpolar tail of the hexanamide monomer. A more detailed consideration of the origins of such possible cancellation effects in terms of the location of the "micellar surface" is presented by Ray and Némethy (1971) for the *p-tert*-octylphenoxy(polyethoxy)ethanol–salt systems they have examined. Further experimental work will be required to resolve these ambiguities.

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Minor Components of Cytoplasmic Ribonucleic Acid from Normal and Regenerating Rat Livers†

Suzanne Levin‡ and Nelson Fausto*

ABSTRACT: RNA from rat liver cytoplasm (mitochondria free) was analyzed by sucrose gradient centrifugation followed by electrophoresis in agarose–acrylamide gels. In addition to 28S and 18S ribosomal RNA we detected four minor RNA species of molecular weights ranging from 8×10^5 to 1×10^6. The minor RNA components are included in the "18S peak" of conventional sucrose gradients but can be separated by electrophoresis on 0.5% agarose–3.0% acrylamide gel slabs. These RNA species represent approximately 8% of the total ribosomal (18 + 28S) RNA in rat liver and appear to be a structural component of large ribosomal subunits in which pairs of the minor RNA components may replace intact 28S RNA. Minor RNA bands were detected in polysomes and "native" ribosomal subunits but not in RNA obtained from free monomers. The labeling kinetics of the minor RNA components from livers of partially hepatectomized rats and from fed and starved intact rats argues against these RNA species being precursors to 18S RNA or products of degradation of "old" 28S ribosomal RNA. The results show that the minor RNA species are labeled in parallel with and appear to decay at a slower rate than both 28S and 18S ribosomal RNAs.

Sucrose-gradient centrifugation of rat liver cytoplasmic RNA reveals the presence of three stable species of RNAs with sedimentation coefficients of 28, 18, and 4 S. More detailed analyses of liver cytoplasmic RNA by electrophoresis in polyacrylamide gels show in addition to the major RNA species one or more RNA bands located between 28 and 18S RNA (Dingman *et al.*, 1970; Aaij *et al.*, 1971; Takagi *et al.*, 1971). The biological functions, if any, of such minor RNA species can only be determined after their intracellular distribution and labeling kinetics are known. In this paper we describe a study of these minor RNA species in normal and regenerating rat liver using polyacrylamide–agarose gel electrophoresis.

Experimental Section

Animals. Male albino rats (Holtzman Co., Madison, Wis.) weighing 160–200 g were used in all experiments. The rats were maintained in a temperature-controlled room with alternating 12-hr cycles of light and dark. Partial hepatectomies were performed using the method of Higgins and

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Anderson (1931) and resulted in the removal of approximately 68% of the liver. Intact, sham-operated, or partially hepatectomized rats were starved for 14 hr before killing.

Materials. [6-^{14}C]Orotic acid (sp act. 60.8 mCi/mmol) and [5-^{3}H]orotic acid (sp act. 27 Ci/mmol were purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). L-[*methyl*-^{3}H]Methionine (sp act. 3.27 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, Mass.). Phenol (Fisher Scientific Co., Boston, Mass.) was redistilled and stored frozen until used.

Methods. Sucrose gradients were prepared in a multiple gradient former made by Hoefer Scientific Instruments Co. The sucrose gradients in the appropriate experiments, after centrifugation in a B-60 International Ultracentrifuge (rotor SB-110), were fractionated with an Isco Model D fractionator connected to a recording spectrophotometer equipped with a flow cell. The absorbance profile at 260 nm was thus automatically recorded, and the various fractions of the gradient were collected either manually or with a fraction collector.

Isolation of Polysomes. Livers were excised immediately after decapitation of the rats and homogenized in a Potter-Elvehjem homogenizer using 4 vol of medium A (0.02 M Tris (pH 7.6)–0.25 M sucrose–0.01 M magnesium acetate–0.04 M NaCl–0.1 M KCl–0.006 M mercaptoethanol) (Munro *et al.*, 1964; Jefferson and Korner, 1964). After centrifugation at 10,000*g* for 10 min at 0°, the supernatant was mixed with sodium desoxycholate (1% final concentration) and layered on a discontinuous gradient composed of 2 and 1 M sucrose solutions made up in medium A. Centrifugation at 120,000*g* for 2.5 hr yielded a polyribosomal pellet. When such a pellet is resuspended, layered on a 15–30% sucrose gradient, and centrifuged for 3 hr at 75,000*g*, the absorbance profiles show a clear separation of the large polyribosomal aggregates (Jefferson and Korner, 1964; Fausto, 1972). However, with this method, a variable amount of monosomes is lost, trapped in the interface between 1 and 2 M sucrose.

Isolation of Monomers and Free Polysomes. Livers were excised immediately after decapitation and homogenized (20% w/v) in medium A. After centrifugation at 10,000*g* for 10 min 1 ml of the supernatant was layered on a 15–30% linear sucrose gradient made in 0.02 M Tris (pH 7.6)–0.1 M KCl–0.04 M NaCl–0.005 M magnesium acetate. Sucrose (0.5 ml; 5%) is added to the top of this gradient before layering the cytoplasmic extract. Centrifugation was at 70,000*g* for 4 hr. After absorbancy scanning, the peaks corresponding to monomers and free polysomes were collected separately.

Isolation of Native Subunits. Native subunits are those ribosomal subunits not part of polysomes or monomers. They were isolated using the technique of Wunner *et al.* (1966). Liver was homogenized (20% w/v) in 0.02 M phosphate buffer (pH 7.8)–0.03 M $KHCO_3$–0.025 M KCl–0.35 M sucrose–0.0015 M $MgCl_2$ and centrifuged at 10,000*g* for 10 min. The resulting postmitochondrial supernatant was centrifuged at 105,000*g* for 1 hr. The supernatant from this centrifugation was centrifuged at 105,000*g* for 3 hr. The pellet was composed of native ribosomal subunits.

Isolation of Derived Subunits. The ribosomal subunits obtained by dissociation of polysomes ("derived subunits") were isolated by resuspending the polysomal pellets in 0.01 M Tris buffer (pH 7.6)–0.01 M NaCl–0.01 M EDTA. The subunits were obtained by centrifugation at 75,000*g* for 15 hr at 0°, in a 15–30% linear sucrose gradient prepared with the same buffer.

Isolation of RNA. RNA was isolated from liver postmitochondrial supernatant fractions with phenol at 4° as previously described (DiGirolamo *et al.*, 1964; Fausto and Van Lancker, 1969). Livers were excised immediately after killing the rats and homogenized (20% w/v) in 0.05 M Tris buffer (pH 7.6), 0.25 M sucrose, 0.003 M $CaCl_2$, and 0.04 mg/ml of potassium poly(vinyl sulfate) using a Potter-Elvehjem homogenizer. All procedures were performed in a cold room. After centrifugation at 20,000*g* for 9 min 0.1 vol of a bentonite suspension and sodium dodecyl sulfate (1% final concentration) was added to the supernatant. Phenol containing 0.1% 8-hydroxyquinoline was used for RNA extractions. The aqueous phase resulting from the first extraction was reextracted two more times with phenol and finally precipitated with ethanol overnight. The precipitate was collected by centrifugation, redissolved in 0.001 M Tris-HCl (pH 7.6), and reprecipitated with ethanol. The final precipitate was dissolved in a small volume of 0.02 M Tris-borate buffer (pH 8.3) containing 5×10^{-4} M EDTA. The RNA concentration was measured colorimetrically by the orcinol method (Dische, 1955). The RNA was further fractionated by centrifugation for 14 hr at 75,000*g* on a 5–30% linear sucrose gradient. The samples with absorbance peaks corresponding to 28S and 18S ribosomal RNAs were collected separately and concentrated in a Schleicher and Schuell collodion bag apparatus, under reduced pressure, to a volume of approximately 50 μl. The samples were then analyzed by agarose–acrylamide gel electrophoresis. The same procedure, with minor modifications, was used for RNA extraction from polysomes, monomers, and subunits.

Gel Electrophoresis. Unless otherwise noted 0.5% agarose–3% acrylamide gel slabs were used in a vertical gel apparatus (EC Apparatus Corp.). The slabs were prepared by the method of Peacock and Dingman (1967) as described by Bunting (1971). After a 1-hr prerun the RNA samples were applied and run at 200 V and 0° for approximately 4 hr. Staining of the bands was done with "Stains-All" (Dahlberg *et al.*, 1969) in 50% formamide as described by Bunting (1971). After destaining the gel strips were scanned at 570 nm in a Gilford spectrophotometer.

Determination of Radioactivity. Gels were cut into 1-mm slices. In experiments where the isotope used was ^{14}C, gel slices were placed directly in 4 ml of scintillation fluid in shell vials. In experiments using ^{3}H, gel slices were first solubilized by the addition of 0.1 ml of NCS solubilizer (Amersham/Searle), heated to 55° for 1 hr and cooled to room temperature before addition of 4 ml of scintillation fluid to each vial. The scintillation fluid contained 468 ml of xylene, 468 ml of *p*-dioxane, 280 ml of absolute ethanol, 98.4 g of naphthalene, 6.13 g of 2,5-diphenyloxozole, and 0.16 g of 2-(α-naphthyl)-5-phenyloxazole. Radioactivity was determined in a Nuclear Chicago scintillation counter and corrected for efficiency.

Determination of Specific Radioactivities. For calculation of the specific radioactivity of each RNA fraction, the scan of a gel was traced onto paper and the tracing cut out and weighed to determine the area under each peak. The factor used to convert the weight of the paper to the amount of RNA present was determined as follows: 10, 20, 40, and 50 μl of a solution of 28S RNA, containing 444 μg of RNA/ml, were run in a 0.5% agarose–3.0% acrylamide gel for 1.5 hr at 0°, 200 V. The stained gel was scanned at 570 nm, the absorbance peaks were weighted, and an average conversion was factor calculated.

Results

Minor RNA components that migrate between 28S and 18S ribosomal RNAs were found in RNA preparations ob-

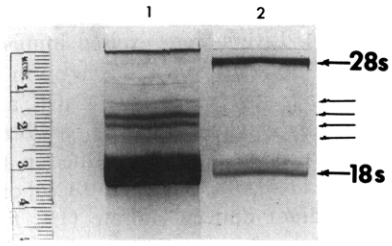

FIGURE 1: Liver cytoplasmic RNA after separation by electrophoresis. RNA was extracted from liver postmitochondrial supernatants of three intact rats. The RNA (1–1.2 mg) was fractionated by sucrose density gradient centrifugation (5–30% sucrose, 14 hr at 70,000*g*) and the absorbance peaks corresponding to 18S and 28S RNAs were collected separately and concentrated. The 18S and 28S RNA peaks (containing 250 and 300 μg of RNA) were concentrated in a collodion bag apparatus to approximately 100 μl. Each concentrated sample (20–40 μl) containing 90 μg (slot 1) and 60 μg (slot 2) of RNA was pipetted into each slot of the gel. The samples were separated by electrophoresis in 0.5% agarose–3% acrylamide gel slabs for 4 hr at 0°. At the end of the electrophoretic run the gels were stained and destained as described under Methods: slot 1, RNA from 18S sucrose gradient peak; slot 2, RNA from 28S sucrose gradient peak. Minor RNA components are indicated by the arrows.

tained from whole cells, postmitochondrial supernatants, or polysomes from rat liver. These minor RNAs are resolvable into four distinct components but the resolution into four bands is dependent on the gel concentration. In 2.0% acrylamide–0.5% agarose gels, the minor components form a single broad peak but increasing the acrylamide concentration to 2.5% allows the resolution of three minor species. In 3% acrylamide–0.5% agarose gels four distinct components are observed and these gels were used in this study. The clearest separation of minor RNA species was obtained from cytoplasmic RNA that had been fractionated by sucrose gradient centrifugation. A picture of an electrophoretic separation on a 3% acrylamide–0.5% agarose gel of 28S and 18S ribosomal RNA peaks obtained after sucrose gradient fractionation of liver postmitochondrial supernatants is presented in Figure 1. The absorbance scanning at 570 nm of a similar gel containing an "18S peak" from a sucrose gradient (Figure 2) shows four

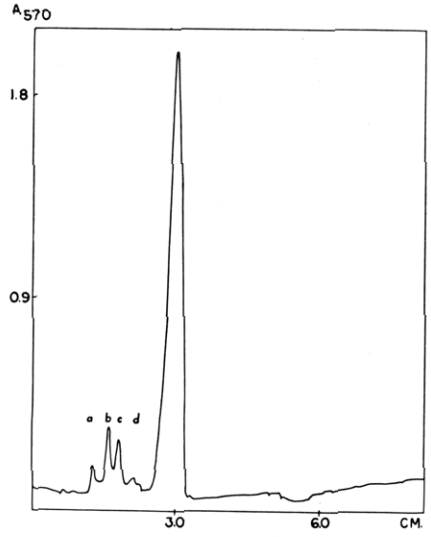

FIGURE 2: Scan at 570 nm of RNA bands of a gel similar to that of slot 1 of Figure 1. (See legend of Figure 1 for the experimental procedure.) Minor RNA components indicated by arrows in Figure 1 are labled a, b, c, and d.

TABLE I: Molecular Weight of Minor RNA Components.[a]

Species of RNA	Obsd Mobility (cm)	Mobility ($cm^2\ V^{-1}\ sec^{-1} \times 10^5$)	Mol Wt
28S	0.25	0.1389	1.6×10^6
Minor band a	0.9	0.5002	$1. \times 10^6$
Minor band b	1.15	0.639	9.2×10^5
Minor band c	1.4	0.778	8.8×10^5
Minor band d	1.6	0.8892	8×10^5
18S	2.5	1.389	6.3×10^5[b]
4S	12.9	7.169	2.45×10^5[b]

[a] Samples of rat liver 4S, 28S, and 18S RNAs containing the minor RNA components obtained by sucrose gradient fractionation were separated by gel electrophoresis (11.76 V/cm for 4 hr at 0°) in a 0.5% agarose–3.0% acrylamide gel slab. The bands were stained as described under Methods. The mobility of each RNA species was measured and the assumed molecular weights were calculated using the equation $\log M = \log M_0 + mu$, where M represents molecular weight of the RNA species, M_0 is the ordinate-intercept molecular weight, m is the slope, and u is the observed mobility (Peacock and Dingman, 1968). [b] Assumed molecular weight.

peaks labeled a, b, c, and d. They represent approximately 8% of the total ribosomal RNA and approximately 5% of the amount of RNA in postmitochondrial extracts of liver. The minor RNA species were found in the same proportion in ribosomes derived from free and bound polysomes.

It has been shown that the mobility of a species of RNA during electrophoresis in acrylamide gels or mixed agarose–acrylamide gels is inversely proportional to its molecular weight (Peacock and Dingman, 1968; Lewicki and Sinskey, 1970). Peacock and Dingman (1968), using the same apparatus and gels used in this study, found that a plot of mobility against the log of the molecular weight resulted in a straight line. By making a similar plot and assuming molecular weights of 630,000 for 18S ribosomal RNA and 24,500 for 4S RNA it is possible to estimate the molecular weights of each of the four minor band species (Table I). They range from 8×10^5 to 1×10^6, values that are in good agreement with those reported by Dingman *et al.* (1970). The minor RNA components consist of molecules heavier than 18S ribosomal RNA. If our extraction procedure fails to completely remove a tightly bound fraction of protein, it is possible that the minor RNA bands could be composed of 18S (or smaller) RNA bound to a protein moiety and that dissociation of this RNA–protein complex would allow the RNA component to migrate faster. This possibility can be ruled out because treatment of the RNA samples with sodium dodecyl sulfate or electrophoresis in acrylamide–agarose–sodium dodecyl sulfate gels did not change the characteristics of the minor RNA components of cytoplasmic RNA (Table II).

Test for Conformational Isomers and Heat Stability. Although the separation of molecules by polyacrylamide gels occurs primarily on the basis of molecular size, there is some evidence that molecular conformation may also be involved. Dahlberg and Peacock (1971) have shown that treatment of RNA with agents that remove or change secondary and

TABLE II: Effect of Sodium Dodecyl Sulfate Treatment and Heating on Minor RNA Components.[a]

Treatment	Minor Bands (dpm)	18S RNA (dpm)
None	126	786
Sodium dodecyl sulfate	120	895
None	136	918
65°, 10 min	131	761

[a] RNA samples obtained by sucrose gradient fractionation of rat liver cytoplasmic RNA were separated by electrophoresis in 0.5% agarose–3% acrylamide gels. Portions of the RNA samples were analyzed in sodium dodecyl sulfate soaked gels (Dahlberg *et al.*, 1969) or were heated at 65° for 10 min and rapidly cooled before the electrophoretic run. The gels were stained and sliced and the radioactivity was determined as described under Methods. The RNA was extracted from the livers of rats 3 hr after injection of 45 μCi of [^{14}C]-orotic acid.

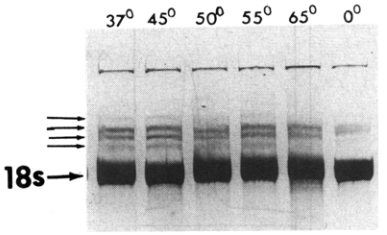

FIGURE 3: Effect of heating in the presence of magnesium on minor RNA components. The 18S RNA fraction obtained after sucrose gradient fractionation of rat liver postmitochondrial RNA was concentrated and divided into six samples. Five samples were dissolved in 0.01 M Tris buffer (pH 8.0)–0.01 M $MgCl_2$ and heated to the indicated temperature. The samples were placed in slots 1–5 (from left to right) of a 0.5% agarose–3.0% acrylamide gel slab. Slot 6 contained RNA dissolved in the standard Tris-borate–EDTA buffer without magnesium. Electrophoresis (200 V) was for 4 hr and 15 min at 0°. The bands were stained with "Stains-All" and destained as described under Methods. Minor RNA components are indicated by the arrows.

tertiary structure can allow formerly separable molecules to migrate as a single band. They conclude that molecules that can be made to have identical electrophoretic mobilities by removal of secondary or tertiary structure are conformational isomers, not differing in primary sequence. The question considered in this experiment was whether the four minor band species were actually conformational isomers of some number less than four. Heating RNA in the presence of Mg^{2+} has been shown to change secondary and tertiary structure, resulting in a more disordered, random conformation of the RNA. The presence of Mg^{2+} stabilizes new conformations of the RNA molecules as they cooled. Samples of 18S RNA obtained from a sucrose gradient of liver cytoplasmic RNA were maintained for 5 min at various temperatures in Tris-borate buffer containing Mg, allowed to cool, and then analyzed by acrylamide gel electrophoresis (Figure 3). It can be seen that changes in secondary or tertiary structure do not cause minor bands to migrate with a single velocity in electrophoretic gels and that they probably represent four distinct molecular classes. It may be noted that heating to 65° does cause some lack of resolution of minor bands but this appears to be the result of degradation.

Henshaw *et al.* (1965) have suggested that a large fraction of messenger-like RNA in rat liver cytoplasm may be present in the 18S peak. This mRNA fraction is degraded to 6–14S material under conditions that scarcely affect ribosomal RNA. Since the minor RNA fraction described in this paper sedimented close to 18S RNA in sucrose gradients it was possible that these minor components would be more sensitive to heat than the bulk of the 18S fraction and thus be responsible for the heterogeneity found by Henshaw *et al.* (1965). Labeled liver cytoplasmic RNA from partially hepatectomized rats was separated by sucrose gradient centrifugation and the 18S fraction collected and concentrated as described under Methods. A sample of this RNA was heated at 65° for 10 min, cooled in ice, and analyzed by electrophoresis. Table II shows that while, as expected, heating caused some loss of labeled material from the 18S RNA, essentially no radioactive label was displaced from the minor RNA bands. The RNA of these minor bands appears to be as stable as 28S ribosomal RNA.

Takagi *et al.* (1971) demonstrated that treatment of rat liver polysomes with low concentrations of ribonuclease *in vitro* does not increase the proportion or amounts of minor RNA components obtained from these preparations. It could be argued that this procedure lacks specificity because exogenous ribonuclease was added to the polysomes. To determine if degradation of ribosomal RNA by endogenous ribonuclease could produce or increase the amounts of the minor RNA components described in this study, we kept liver polysomes at −20° for a period of 2 weeks. The ribosomes were dissociated into subunits; RNA was extracted from the small and large subunits and analyzed by gel electrophoresis. The products of the partial degradation of ribosomes by endogenous ribonuclease migrated differently than the minor RNA components that are the subject of this study. The minor RNA bands (corresponding to RNA species of 8×10^5 to 1×10^6 mol wt) were not increased in these preparations of partially digested ribosomal RNA.

Subcellular Localization of Minor RNA Species. Minor RNA bands are present in RNA extracted from whole cells, postmitochondrial supernatants, and polysomes from rat liver. We isolated RNA from ribosomal subunits obtained by the dissociation of polysomal pellets (Figure 4e) in an EDTA-containing buffer. Subunits were separated by sucrose gradient centrifugation (Figure 4a) and the peaks representing the large and the small subunits were collected separately. RNA was isolated from each subunit using a modified phenol extraction procedure. The RNA obtained from the small subunit formed a single symmetrical peak when centrifuged in a sucrose density gradient (Figure 4b). Gel electrophoresis of this RNA peak reveaeld only an 18S component (Figure 4d). On the other hand two peaks were obtained after sucrose density gradient centrifugation of RNA isolated from the large subunit (Figure 4c). Electrophoretic analyses of these peaks (Figure 5) revealed that one of them (peak A) contained a large amount of 28S ribosomal RNA but no clearly resolvable minor bands while the other (peak B) contained the minor RNA bands in addition to some 28S and 18S RNAs. These results are in agreement with the data presented for liver by Takagi *et al.* (1971) and the results of Nair and Knight (1971)

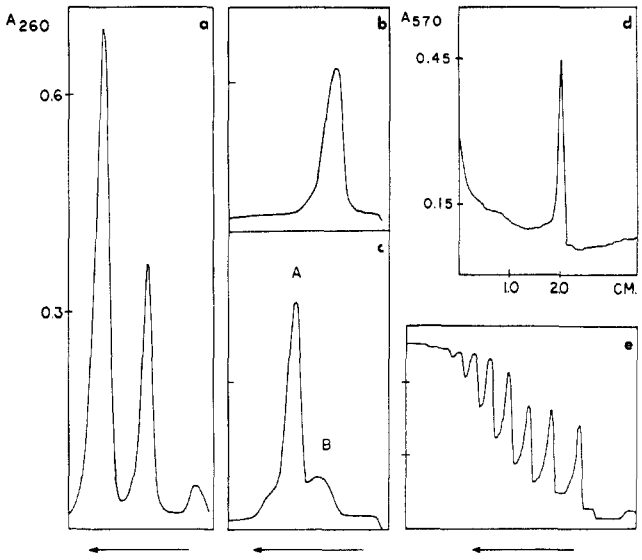

FIGURE 4: Subcellular distribution of minor RNA components. Polysomes were prepared from liver postmitochondrial extracts of four rats by treatment of the extracts with 1% sodium deoxycholate and centrifugation on a discontinuous (1–2 M) sucrose gradient (see Methods). Derived subunits were obtained by resuspending the polysomes in 0.01 M Tris buffer (pH 7.6)–0.01 M NaCl–0.01 M EDTA. The large and small ribosomal subunits were then separated by sucrose gradient centrifugation (15–30% sucrose 70,000*g* for 15 hr). RNA was extracted with phenol from the large and small subunits and analyzed by sucrose gradient centrifugation (5–30% sucrose, 70,000*g* for 15 hr). The RNA peaks were collected separately, concentrated, and analyzed by electrophoresis in 0.5% agarose–3% acrylamide gel slabs. The RNA bands were stained, destained, and scanned at 570 nm as described under Methods: (a) absorbance profile (260 nm) after sucrose gradient centrifugation of ribosomal subunits obtained by dissociation of polysomes; (b) absorbance profile (260 nm) after sucrose gradient centrifugation of RNA extracted from the small ribosomal subunit shown in a; (c) absorbance profile (260 nm) after sucrose gradient centrifugation of the large ribosomal subunit shown in a; (d) gel electrophoresis of RNA prepared from the small subunit and fractionated by sucrose gradient as shown in b (scanning at 570 nm in a Gilford spectrophotometer); (e) absorbance profile (260 nm) of the initial polyribosomal pellet. Centrifugation on a 15–30% sucrose gradient for 3 hr at 75,000*g*. The arrows indicate the direction of the sedimentation.

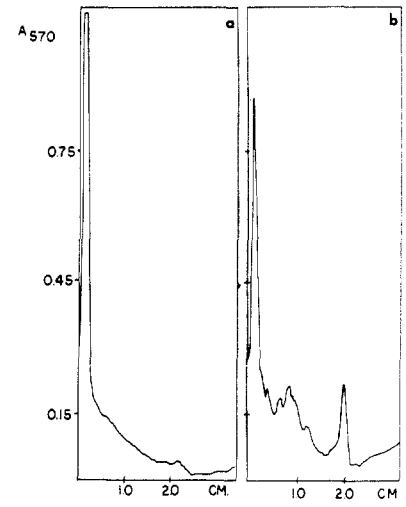

FIGURE 5: Gel electrophoresis of RNA extracted from the large ribosomal subunit. (See the legend of Figure 4 for methods.) RNA was extracted from the large ribosomal subunit and fractionated by sucrose gradient centrifugation as shown in Figure 4c. Fractions corresponding to peaks A and B were collected separately, concentrated, and analyzed by electrophoresis in a 0.5% agarose–3% acrylamide gel slab. The electrophoresis was for 4 hr, at 0° and 200 V. The gels were stained and destained as described under Methods and scanned at 570 nm in a Gilford spectrophotometer: (a) gel electrophoresis of RNA from peak A in Figure 4c; (b) gel electrophoresis of RNA from peak B in Figure 4c. The ordinate shows the absorbance at 570 nm, the abscissa the distance (in centimeters) from the origin

showing that in HeLa cells a 22S RNA component sediments with subribosomal particles containing 28S RNA.

Distribution of Minor RNA Components and Different Classes of Ribosomal Subunits. If two (or more) classes of large ribosomal subunits exist, one containing only 28S RNA and the other 28S RNA and one or all of the minor RNA bands, it would be expected that these subunits might be at least partially separable by sucrose density gradient centrifugation and gel electrophoresis. The large ribosomal subunit peak from a sucrose gradient centrifugation of polysomes dissociated with EDTA was divided in half and the fractions corresponding to each part of the peak were collected separately, concentrated, and analyzed by gel electrophoresis. The scan of the gel (Figure 6) shows that both halves of the large ribosomal subunit peak contain an identical proportion of minor RNA bands. Attempts to demonstrate heterogeneity of the large ribosomal subunits by direct acrylamide gel electrophoresis of the ribonucleoprotein particles were not successful. The large ribosomal subunits obtained by the dissociation of liver polysomes migrated as a single band under the experimental conditions used.

The proportion of RNA found in the minor bands was determined in three different classes of large ribosomal subunits: (a) subunits obtained from polysomes (derived subunits); (b) subunits found free in the cytosol (native subunits); and (c) subunits derived from monomers. The results are summarized in Table III. The minor components constituted approximately 8% of total ribosomal RNA (18 + 28 S) extracted from postmitochondrial supernatants but only 1.5–2% of the RNA extracted from native subunits. In subunits derived from the dissociation of polysomes the minor components represented approximately 11% of the 28S RNA but in native subunits (ribosomal subunits found free in the cell) the minor components were only about 3% of 28S RNA. Minor components were not found in monomers even when the methods and the quantities of material used permitted the detection of amounts of RNA corresponding to 0.5–3.3% of the total.

Methylation of Ribosomal RNA. The methyl groups present in the 45S ribosomal RNA precursor are retained throughout its maturation and appear in 28S and 18S ribosomal RNAs in the cytoplasm. Since the minor RNA components were found to be a part of the large ribosomal subunit it was important to determine if these RNA species were also methylated. We injected partially hepatectomized rats with [*methyl*-^{3}H]methionine (100 μCi) and isolated liver postmitochondrial RNA. The specific activities of the 28S, 18S, and minor RNA components (molecular weights 8×10^5–1×10^6) after sucrose gradient separation and gel electrophoresis were 27, 26, and 30 dpm/μg of RNA for minor components, 18S, and 28S ribosomal RNAs, respectively.

Labeling Kinetics of 28S, 18S, and Minor RNA Bands in Normal and Regenerating Livers. The kinetics of [^{14}C]orotic acid incorporation and decay from 28S, 18S, and minor RNA components were studied in intact, sham-operated, and

TABLE III: Distribution of Minor RNA Components.[a]

Source	Amt of Minor Components as % of Total Ribosomal RNA	Amt of Minor Components as % of 28S RNA
RNA from postmitochondrial extracts	8	12
Derived ribosomal subunits		11.4
Native subunits[b]	1.5	2.5
Native subunits	1.9	3.5
Free monomers[b]	Undetectable	Undetectable
Free monomers	Undetectable	Undetectable

[a] RNA was prepared from postmitochondrial extracts, derived ribosomal subunits, native subunits, and free monomers. Derived subunits were obtained by dissociation of polysomes with EDTA; native subunits and free monomers refer to ribosomal subunits or monomers found free in the cell (not associated with polysomes). The extracted RNA was fractionated by sucrose gradient centrifugation and separated by electrophoresis in 0.5% agarose–3% acrylamide for 4 hr at 0°. The bands were stained and destained as scanned at 570 nm and the amounts of RNA present in each RNA species were calculated as described under Methods. The limits of detectability for the minor RNA components in the monomer experiments were between 0.5 and 1% of the total. [b] These samples were separated by electrophoresis without prior sucrose gradient fractionation.

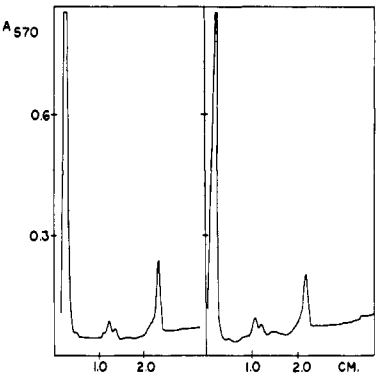

FIGURE 6: Distribution of minor RNA components in RNA obtained from the large ribosomal subunit. Rat liver polysomes were dissociated with EDTA and separated by sucrose gradient centrifugation. The "light" and "heavy" fractions of the large ribosomal subunit peak were collected separately. RNA was extracted from the light and heavy fractions, concentrated, and analyzed by electrophoresis in a 0.5% agarose–3.0% acrylamide gel slab for 4 hr at 0°. At the end of the electrophoretic run the gels were stained and destained as described under Methods. The figure shows the scan at 570 nm of RNA obtained from the light (left) and heavy (right) parts of the large ribosomal subunit peak. The ordinate shows the absorbance and the abscissa the distance from the origin.

partially hepatectomized rats. Liver regeneration is a coordinated response in which the main biochemical events follow a defined time sequence. An important aspect of this response is a marked increase in the incorporation of precursors into ribosomal RNA and in the production of ribosomes (Lieberman and Kane, 1965; Fausto and Van Lancker, 1969). All partially hepatectomized or sham-operated rats were killed 18 hr after the operation. [^{14}C]Orotic acid was injected intraperitoneally at various times before killing the animals. Table IV shows the specific activity of 28S, 18S, and minor RNA bands obtained after sucrose gradient fractionation and gel electrophoresis separation of liver cytoplasmic RNA from sham-operated and partially hepatectomized rats. In regenerating livers, the specific radioactivity of all RNA species increases sharply between 20 min and 1 hr after the injection of label. Between 1 and 18 hr after orotic acid injection the specific radioactivity of all three classes of RNA continues to rise but at a slower rate than initially. In sham-operated rats the initial rate of increase in specific radioactivity of 28S, 18S, and minor RNA bands is approximately ten times slower than that of partially hepatectomized animals. Howeve, in both partially hepatectomized and sham-operated rats the specific radioactivity of the minor RNA components increases at approximately the same rate as that of 18S and 28S cytoplasmic RNA. The kinetics of labeling and radioactivity loss of 28S, 18S, and RNA from minor bands was investigated in long-term experiments in intact rats. These animals were given a single injection of [^{14}C]orotic acid and killed 13 hr, 5 days, and 10 days after (Figure 7). The specific radioactivity of all three classes of RNA rises between 13 hr and 5 days and then decreases between 5 and 10 days. The results do not show any delayed rise in specific activity of minor band RNA which would be expected if the minor RNA components were derived from the breakdown of "old" 28S cytoplasmic RNA.

TABLE IV: Specific Radioactivity of Cytoplasmic RNA in Normal and Regenerating Liver.[a]

Treatment	Labeling Time (hr)	28S (dpm/μg)	Minor Bands (dpm/μg)	18S (dpm/μg)
Sham operation	1	11	8	10
	3	56	68	60
	5	74	85	95
	13	120	87	182
	18	440	153	260
Partial hepatectomy	20 (min)	5	55	43
	1	160	120	140
	5	*b*	107	150
	13	*b*	118	190
	18	620	250	325

[a] Sham-operated or partially hepatectomized rats were injected intraperitoneally with 50 μCi of [^{14}C]orotic acid. All animals were killed 18 hr after partial hepatectomy or sham operation. RNA was extracted from postmitochondrial supernatants, fractionated by sucrose gradient centrifugation, concentrated, and separated by electrophoresis in 0.5% agarose–3% acrylamide gel slabs (4 hr at 0°). The gels were stained, destained, scanned at 570 nm, and sliced for radioactivity determination. The specific radioactivity for each RNA species was calculated as dpm/μg of RNA. The amount of RNA under each peak was calculated by weighing the area under the peak after scanning of the gels. A conversion factor was used as described in Methods. Three rats were used for each labeling time under study. [b] Not measured.

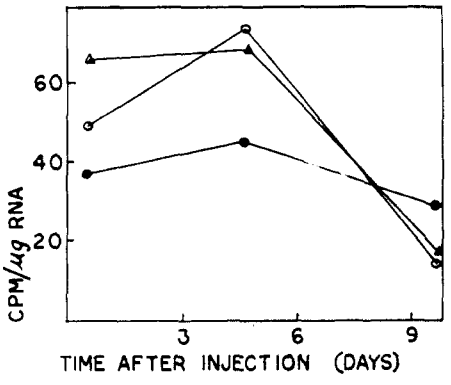

FIGURE 7: Specific radioactivity of minor RNA components after long incorporation times. Intact rats were injected with 40 μCi of [^{14}C]orotic acid and killed 13 hr, 4.5 days, and 9.5 days after the injection. Liver RNA was extracted from postmitochondrial supernatants and fractionated by sucrose gradient centrifugation. Fractions corresponding to the 18S and 28 SRNA peaks were concentrated and analyzed by electrophoresis in 0.5% agarose–3% acrylamide gels for 4 hr at 0°. The gels were stained, destained, and sliced for radioactivity determination. The specific radioactivity is expressed as counts per minute per microgram of RNA for each RNA species: (Δ) 28S RNA; (○) 18S RNA; (●) minor RNA components. The amounts of RNA were calculated by weighing the area corresponding to each peak after scanning of the gels at 570 nm. Each point represents the specific radioactivity of RNA extracted from three livers.

TABLE V: Effect of Starvation on Minor RNA Components.[a]

		28S	Minor Bands	18S
RNA (% of total recovered from gel)	Starved	63	11	26
	Fed	55	9.0	36
Specific radioactivity (dpm/μg)	Fed	523	252	364
	Starved	174	224	203

[a] Intact rats received an intraperitoneal injection of 100 μCi of [^{14}C]orotic acid and were fed *ad libitum* for 4 days. The animals were then separated into two groups of three rats. One group of rats (fed) continued to receive food while the other group (starved) was given water but no food. Seven days after the orotic acid injection all animals were killed and the RNA was extracted from the postmitochondrial supernatants of liver homogenates. The RNA was fractionated by sucrose gradient centrifugation, concentrated, and separated by electrophoresis in a 0.5% agarose–3% acrylamide gel slab for 4 hr at 0°. At the end of the run the gels were stained, destained, and scanned at 570 nm and the radioactivity was determined as described under Methods. Amounts of RNA were calculated by weighting the area of paper under the peak of each RNA species after gel scanning. A standard factor for conversion into micrograms was used as described under Methods.

Effect of Starvation on Minor RNA Components. Starvation of rats causes disaggregation of liver polysomes and an accelerated breakdown of existing ribosomal RNA (Hirsch and Hiatt, 1966; Enwonwu *et al.*, 1971). If the minor RNA components are breakdown products of 28S ribosomal RNA, it would be expected that their amount would increase under conditions that accelerate ribosomal breakdown. Intact rats received an injection of [^{14}C]orotic acid and were fed *ad libitum*. Four days after the injection, when ribosomal RNA labeling was maximal, the animals were separated into two groups. Group 1 animals received water but no food and group 2 animals were continued to be fed *ad libitum*. Rats from groups 1 and 2 were killed 7 days after orotic acid injection. In fed groups the minor RNA components constituted approximately 9% of the amount of ribosomal RNA while in the starved animals the proportion of minor RNA bands was approximately 11%. There was also no increase in the specific activity of the minor RNA components with starvation (Table V).

Discussion

This study shows that rat liver cytoplasm contains four minor RNA components with molecular weights ranging from 8×10^5 to 1×10^6 which sediment between 18S and 28S RNA after sucrose gradient centrifugation. These minor components represent only approximately 5% of all (excluding mitochondrial) cytoplasmic RNA from rat liver and are generally found as a part of the "18S peak," under the commonly used conditions of centrifugation. The technique that gives the clearest resolution of these RNAs is electrophoresis in 3.0% acrylamide–0.5% agarose gels of cytoplasmic RNA previously fractionated on a sucrose density gradient.

These minor RNA components of rat liver cytoplasm do not appear to be the result of RNA digestion during the extraction procedure. They have been detected by Dingman *et al.* (1970) in preparations of liver cytoplasm and ribosomes in which RNA digestion has been carefully monitored. Takagi *et al.* (1971), using a somewhat different extraction procedure than the one used in the present work, and Aaij *et al.* (1971), employing several alternative isolation procedures including Macaloid and diethyl pyrocarbonate as ribonuclease inhibitors, have also reported the presence of the minor RNA components. We found that with our extraction procedures which included the presence of bentonite as ribonuclease inhibitor (Petermann and Pavlovec, 1963a; Payne and Loening, 1970), the four minor RNA components constituted a fixed proportion of total ribosomal RNA and could be detected only in some subcellular particles in a consistent pattern not compatible with a random digestion process. Petermann and Pavlovec (1963b, 1964), in studies of ribosomal RNA from rat livers, found a 22S component that was clearly resolvable from the other RNA species by schlieren optics. Under conditions that lead to destruction of 28S RNA or RNA aggregation, the proportion of 22S RNA remained constant, representing approximately 10% of total ribosomal RNA (Petermann and Pavlovec, 1963b, 1964), a value very close to what we obtained in the present experiments. Nair and Knight (1971) found a class of RNA in HeLa cell cytoplasm with a sedimentation value of 22S which was not an artifact caused by the extraction procedure, had similar base composition, and competed effectively with 28S RNA. They did not analyze this RNA species by electrophoresis but it would appear that the 22S cytoplasmic RNA from HeLa corresponds to the minor RNA components in rat liver cytoplasm. In rat liver the small ribosomal subunit containing 18S RNA is much more sensitive to ribonuclease than the large subunit (Hüvos *et al.*, 1972; Krechetova *et al.*, 1972). The presence of small RNA fragments sedimenting slower than 18S RNA can be taken as an indication of degradation occurring during the

TABLE VI

	RNA Present in a Single 60S Subunit	RNA (Mol wt $\times 10^{-6}$)	Subunit (Mol wt $\times 10^{-6}$)
1.	28S	1.6	3.0
2.	Minor band a + minor band d	1.8	3.33
3.	Minor band b + minor band c	1.8	3.33
4.	One species minor band RNA	0.9 (av)	1.66 (av)
5.	28S + one minor band RNA	2.5 (av)	4.62 (av)
6.	4 species minor band RNA	3.6	6.66
7.	28S + 4 species minor band RNA	5.2	9.62

extraction procedure. In our experiments we only detected such fragments after self-digestion of polyribosomal pellets.

Takagi *et al.* (1971) and Nair and Knight (1971) found that the minor RNA components sediment with the 60S ribosomal subunit and conclude that these RNA species are structural components of the ribosome. Our observations are in agreement with this conclusion and indicate that the minor RNA components cannot be dissociated from the large ribosomal subunit, implying that their structural position in the ribosome is similar to that of 28S ribosomal RNA. Since with the methods used we did not find any obvious heterogeneity among the large subunits isolated from liver polysomes, an analysis of the molecular weights of the minor components suggests that in some ribosomal particles 28S RNA has been replaced by either bands a + d or b + c as shown in Table VI. We assume that in all particles RNA corresponds to 54% of the weight and use 3.0×10^{-6} as the molecular weight of the large ribosomal subunit from rat liver (Hamilton *et al.*, 1971). Alternatives 2 and 3 seem to be the only plausible ones. This replacement is found in the large subunit derived from polysomes, to a much smaller extent in the native subunits, and is not observed in monomers.

The labeling kinetics of the minor RNA components in partially hepatectomized and sham-operated rats and also in fed and starved intact rats does not suggest that the minor components are precursors to 18S ribosomal RNA as appears to be the case for 23S nucleolar RNA (Egawa *et al.*, 1971), nor are they the result of the breakdown of "old" 28S ribosomal RNA molecules *in vivo*. The data indicate that the minor RNA components are labeled in parallel with 28S and 18S RNAs (and even at a slightly higher rate for short incorporation times) and decay at the same or slower rate as 28S ribosomal RNA. It appears that both Aaij *et al.* (1971) and Nair and Knight's (1971) long term labeling data can be interpreted as showing only that the minor RNA components turn over more slowly than either 28S or 18S ribosomal RNA.

We found minor RNA bands between 28S and 18S RNA after agarose–acrylamide gel electrophoresis of nucleolar RNA (D. Pierce, unpublished results). The small proportion of these RNA components makes it difficult to determine if they represent 22–24S precursors to 18S RNA (Egawa *et al.*, 1971) or the minor components that we described in the cytoplasm.

Our interpretation is that the minor RNA components do not result from degradation of "old" 28S ribosomal RNA but rather are formed by cleavage of 28S ribosomal RNA present in the 60S subunits of ribosomes that are a part of polysomes. It is possible that the particular conformation assumed by 28S ribosomal RNA in polysomes determines preferential breaking points on the molecule (Spirin, 1969) and that a certain percentage of these breaks can yield minor RNA components. Applebaum *et al.* (1966) suggest that in silkmoth pupae the 26S rRNA of active ribosomes may have breaks produced by specific enzyme action while the ribosome was still intact and Stevens and Pachler (1972) found discontinuities in the 26S rRNA in a free-living amoeba. However in silkmoth, amoeba, and Jensen Sarcoma ribosomes (Applebaum *et al.*, 1966; Stevens and Pachler, 1972; Petermann and Pavlovec, 1966), the RNA components are obtained by heating or treating 28S RNA with formamide or dimethyl sulfoxide while in rat liver these treatments do not increase the proportion of the minor components.

Acknowledgment

We thank Drs. E. Henshaw and A. Dahlberg for their valuable suggestions.

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Interaction of an Apolipoprotein (ApoLP-Alanine) with Phosphatidylcholine†

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ABSTRACT: The apolipoprotein containing C-terminal alanine (apoLP-Ala) from very low density lipoprotein and phosphatidylcholine were used as a prototype to study lipid–protein interactions in human plasma lipoproteins. ApoLP-Ala strongly inhibited the reactivation of delipidated mitochondrial β-hydroxybutyrate dehydrogenase, an enzyme which requires phosphatidylcholine for biological activity. When apoLP-Ala was sonicated with a 100-fold molar excess of phosphatidylcholine, a lipid–protein complex resulted which could be isolated free of excess lipid by ultracentrifugal flotation in potassium bromide solution at a density of 1.063–1.21 g/ml. The complex contained an average of 38 ± 5 phosphatidylcholine molecules for every 1 apoLP-Ala molecule. Further experiments showed that ultracentrifugation on a sucrose density gradient afforded a heterogeneous population of complexes whose average stoichiometry was 46 phosphatidylcholine molecules per apoLP-Ala molecule. When a saline gradient was used, the average stoichiometry was reduced to 30:1. When the titration of apoLP-Ala with a sonicated dispersion of phosphatidylcholine was observed by circular dichroism, the calculated α-helical content of the protein increased from 22 to 54%. At maximal helicity, the average stoichiometry of the complex was about 50 phosphatidylcholine molecules to 1 apoLP-Ala molecule. When the titration experiment was monitored by intrinsic fluorescence of the tryptophan residues, the maximum at 352 nm was gradually blue-shifted until a level of about 80 phosphatidylcholine molecules to 1 apoLP-Ala molecule was reached. These studies indicate that apoLP-Ala can bind up to a saturating level of 50–80 phosphatidylcholine molecules. The binding of phosphatidylcholine induces a shift from a disordered to a helical secondary structure and shifts one or more of the three tryptophan residues from a more polar to a more hydrophobic environment. These results show that highly lipidated species of apoLP-Ala may be formed which can be partially dissociated at high salt concentrations and suggest that ionic associations of lipid and apolipoproteins may play at least a minor role in the formation of plasma lipoprotein complexes. Our experiments are discussed in terms of their relationship to possible lipid–protein interactions in membranes.

The very low density lipoproteins (VLDL)[1] of human plasma represent the major vehicle for the transport of endogenously synthesized triglycerides in blood. By weight, the VLDL particles have an approximate composition of 51% triglyceride, 20% cholesterol, 19% phospholipid, and 8% protein (Oncley and Harvie, 1969). The protein constituents of VLDL are heterogeneous (Brown *et al.*, 1969; Shore and Shore, 1969; Pearlstein and Aladjem, 1972; Albers and Scanu, 1972). There are several relatively small protein components comprising 40–50% of the total protein which have been referred to collectively as the "D peptides" (Brown *et al.*, 1970b), the "C proteins" (Alaupovic, 1971), or "fraction V" (Scanu *et al.*, 1969). The other major protein component of VLDL, comprising 40–50% by weight, is the β protein or apoLDL (Brown *et al.*, 1970b). This protein component(s) has been recently shown by Gotto *et al.* (1972) to be immunochemically identical with and indistinguishable in amino acid composition and circular dichroism (CD) from the major protein constituent of LDL. Bilheimer *et al.* (1972) have shown that the larger, triglyceride-rich VLDL particles have a relatively high ratio of "C proteins" to apoLDL. The ratio is reversed in the smaller VLDL particles. Furthermore, the "C proteins" have been found to exchange rapidly *in vitro* and *in vivo* between VLDL and HDL (Eisenberg *et al.*, 1972). It is not known at this time whether the exchange involves the transfer of phospholipid and possibly other lipids as well.

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[1] Abbreviations used are: apoLP-Ala, alanine apolipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide.