Template and Ribosomal Ribonucleic Acid Components in the Nucleus and the Cytoplasm of Rat Liver*

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ABSTRACT: Both cytoplasm and purified nuclei from rat liver contain ribonucleic acid (RNA) refractory to phenol extraction at pH 7.6, but obtainable by extraction of the phenol residue at pH 8.3. The pH 8.3 RNA fractions have a high template activity. In the nuclear fraction, the template activity sediments as a broad but well-defined peak, around 9–16 S, and the bulk RNA in this zone has a deoxyribonucleic acid (DNA)-like base composition. In the cytoplasmic pH 8.3 fraction, the template material shows sedimentation characteristics similar to those of the nuclear template RNA. The cytoplasmic fraction also contains an 18S RNA component, and a 10S component rich in adenylic acid. After pulse labeling with [^{14}C]orotic acid, the nuclear RNA obtained at pH 8.3 is ten times more radioactive than the nuclear RNA extracted at neutral pH, and about 400 times more labeled than the bulk of the cytoplasmic RNA. In the cytoplasm, the RNA obtained at pH 8.3 is also considerably more radioactive (40 times) than the bulk of the RNA, which is extracted at neutral pH.

Phenol extraction provides a means for the isolation of minor RNA components. It has been shown that extraction of tissue homogenates in the cold at neutral pH removes the bulk of the RNA, but leaves behind a fraction different in base composition and metabolic behavior (Sibatani *et al.*, 1960). The latter can be obtained by reextraction either at elevated temperature or increased pH (Georgiev and Mantieva, 1962; Brawerman *et al.*, 1963). The basis of this fractionation has been assumed to be the nuclear localization of the RNA resistant to phenol extraction (Georgiev and Mantieva, 1962; Hadjivassiliou and Brawerman, 1965). As will be shown in this report, however, RNA fractions refractory to phenol extraction can be obtained both from the nucleus and the cytoplasm of rat liver by extraction at elevated pH. Purified nuclei also contain RNA readily extractable at neutral pH in the cold. It has been proposed in a previous publication from this laboratory that it is the nature of the protein in ribonucleoprotein complexes which determines the ease of extraction of the RNA (Hadjivassiliou and Brawerman, 1965).

In the present study the high pH RNA fractions were found to be heterogeneous. Some of the RNA components in these fractions were investigated with respect to size, nucleotide composition, rate of labeling, and ability to stimulate amino acid incorporation into protein by *Escherichia coli* ribosomes. The latter property will be referred to as template activity.

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Experimental Section

Preparation of RNA Fractions. Adult rats (300-g body weight), fasted for 24 hr, were used in all experiments. The preparation of nuclei and of the *cytoplasmic fraction* was based on the procedure of DiGirolamo *et al.* (1964). The minced livers were disrupted in a glass homogenizer fitted with a Teflon pestle in ten volumes of 0.26 M sucrose, 50 mM Tris-HCl (pH 7.6), and 3 mM $CaCl_2$. The homogenate was filtered through gauze, then centrifuged at 600*g* for 10 min. The supernatant was used directly as the cytoplasmic fraction. The crude nuclear pellet was suspended in four times the original liver volume of 2.2 M sucrose, 10 mM Tris (pH 7.6), and 2mM $CaCl_2$, and the suspension was centrifuged at 45,000*g* for 60 min. The nuclei were obtained as a compact colorless pellet, while a large amount of contaminating material migrated to the top of the centrifuge tubes. The fractionation procedure is outlined in Figure 1.

The *cytoplasmic fraction* was treated with phenol by a procedure used previously with whole liver homogenate (Hadjivassiliou and Brawerman, 1965). After addition of $^1/_{20}$ volume of 1 M Tris (pH 7.6), the cytoplasm was mixed with an equal volume of ice-cold 70% aqueous phenol. The suspension was first stirred for 10 min in the cold. The mixing was next continued for 30 min either in the cold or at 38°. The aqueous phase, separated by centrifugation at 17,000*g* for 20 min, served for the preparation of the *pH 7.6 cytoplasmic RNA*. The phenol residue (interphase plus phenol phase) was reextracted twice by thorough mixing with an equal volume of 0.1 M Tris (pH 7.6) at 33° for 30 min, and centrifuging to separate the aqueous phases. The latter were discarded, and the phenol residue was next extracted with a volume of 0.1 M Tris (pH 8.3) at 38° for 30 min. The resulting aqueous extract served for the preparation of the *pH 8.3 cytoplasmic RNA*.

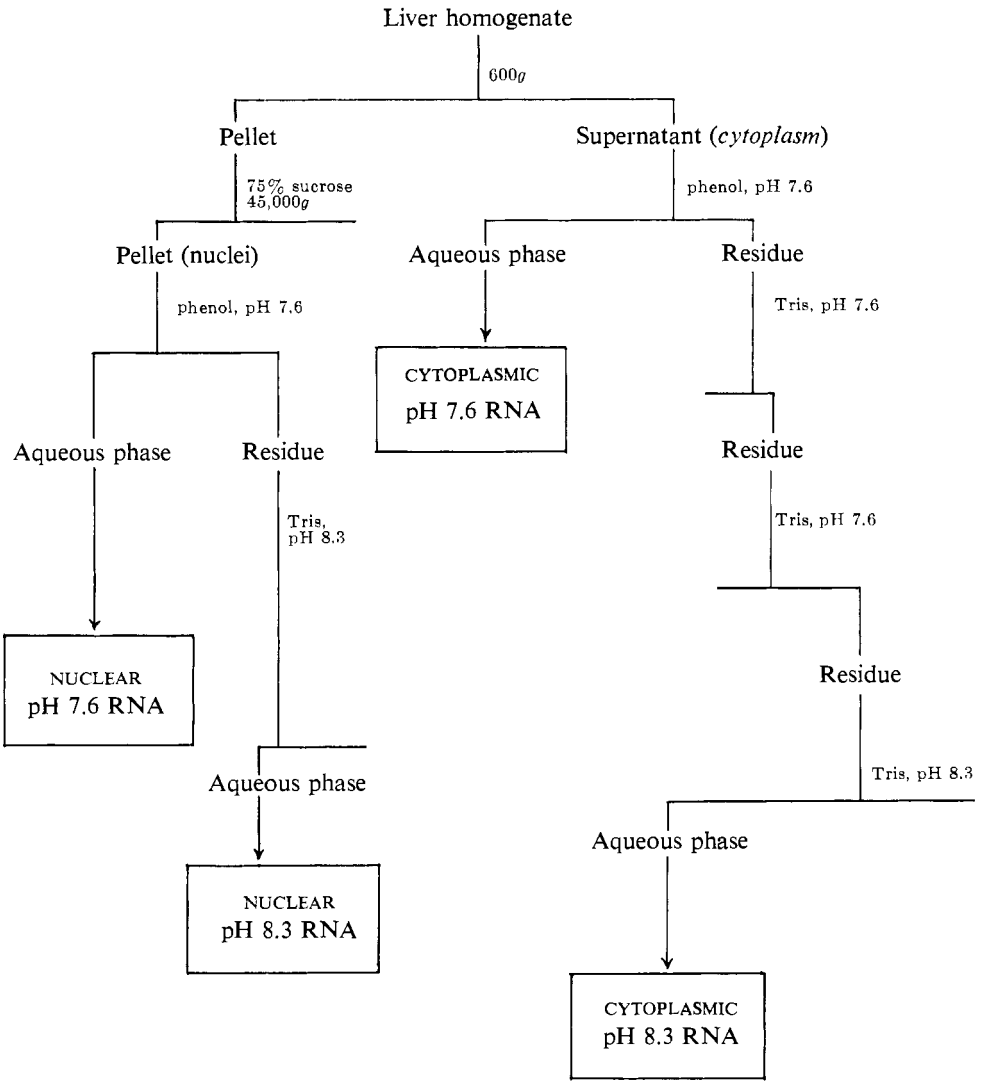

FIGURE 1: Outline of fractionation procedure.

For the nuclei, the procedure had to be modified in order to avoid extraction of the DNA at pH 8.3 (see Results). The nuclei, well suspended in an amount of ice-cold 0.1 M Tris (pH 7.6) equivalent to four times the wet weight of liver used, were mixed with an equal volume of ice-cold aqueous phenol and the suspension was stirred at 0° for 40 min. After centrifugation the resulting aqueous phase served for the preparation of the *pH 7.6 nuclear RNA*. The phenol residue was homogenized in the cold with an equal volume of water and the suspension was stirred at 38° for 5 min. An amount of 1 M Tris (pH 8.3) equivalent to one-tenth the volume of water used was added and the mixing was continued for 30 min at 38°. The resulting aqueous phase served for the preparation of the *pH 8.3 nuclear RNA*.

The RNA was isolated from the aqueous phases as described previously (Hadjivassiliou and Brawerman, 1965). The purification procedure includes a precipitation of the RNA in cold 10% NaCl. This results in the removal of tRNA and of small amounts of DNA which might contaminate the nuclear RNA fractions. The purified RNA preparations were stored as aqueous solutions in the freezer.

Zone Centrifugations. These were carried out in linear 5–20% sucrose gradients, supplemented with 0.1 M NaCl and 10 mM sodium acetate (pH 5.1). For the isolation of centrifugal fractions, samples containing up to 3 mg of RNA were layered over 29 ml of sucrose gradient solutions and centrifuged in the SW 25 Spinco rotor at 25,000 rpm. Fractions (2 ml) were collected through the bottom of the tubes, 6 ml of ethanol was added to each fraction, and 0.5-ml samples of the ethanol suspensions were diluted with 2 ml of H_2O for absorbancy measurements at 260 mμ. The remainder of the fractions was kept in the cold to allow for complete precipitation of the RNA. The fractions containing the desired RNA components were pooled and collected by centrifugation.

Smaller amounts of RNA (100–300 μg) were centrifuged in the SW 39 Spinco rotor at 35,000 rpm. Fractions of 4 (0.045 ml) and 14 drops (0.16 ml) were collected alternately. To the 14-drop fractions, which were collected in conical centrifuge tubes, three volumes of ethanol was added and the RNA was allowed to precipitate in the cold. They were used either for isolation of centrifugal RNA fractions or for template activity assay as described below. The 4-drop fractions were diluted with 1 ml of H_2O and the absorbancy was measured at 260 mμ. When ^{14}C-labeled RNA was used, 0.7-ml samples of these latter solutions were taken after the ultraviolet measurements for counting as described below. In some experiments where the amount of radioactivity in the RNA was low, 10-drop samples were used for counting. The sedimentation values were rough estimates obtained by comparison with 28S and 18S rRNA.

Assay for Template Activity. The assays were carried out with preincubated 30,000*g* supernatant of *E. coli* (S-30) as described previously (Brawerman *et al.*, 1963). The total volume of the incubation mixtures was 0.15 ml. The composition of the reactions, somewhat modified from that used by Matthaei and Nirenberg (1961), was as follows: Tris buffer (pH 7.8), 100 mM; KCl, 32 mM; NH_4Cl, 20 mM; $Mg(acetate)_2$, 12 mM; phosphoenolpyruvate, Na^+ salt, 4 mM; ATP,[1] Na^+ salt, 1 mM; GTP, 0.6 mM; β-mercaptoethanol, 1.8 mM; mixture of unlabeled amino acids (minus leucine), 0.017 mM each; *E. coli* tRNA, 110 μg/ml; phosphoenolpyruvate kinase (Boehringer Mannheim Corp.), 20 μg/ml; L-[^{14}C]-leucine, uniformly labeled, 40 μc/μmole and 0.8 μc/ml; and *E. coli* S-30 preparation, 0.02 ml; per 0.15 ml. The RNA samples to be tested were included in concentrations usually lower than 100 μg/ml. For the determination of acid-insoluble radioactivity, the procedure described by Mans and Novelli (1961) was used. A *nuclear 8.3* RNA preparation was used as a standard in this study and was included in each series of template activity assays. This was necessary because the extent of stimulation obtained in separate experiments tended to be variable. With the use of this standard the activity values obtained in different experiments could be compared. The template activity was expressed as increase in micromicromoles of leucine incorporated as a result of addition of the RNA.

For the study of sedimentation patterns of material with template activity, the 14-drop fractions obtained by zone centrifugation (see above), to which three volumes of ethanol had been added, were kept in the cold for at least 1 day to allow for precipitation of the RNA. After centrifugation at 2500 rpm for 20 min, the supernatants were removed and the inverted tubes were allowed to drain for about 1 hr in the cold to ensure complete removal of the ethanol. This procedure led to the quantitative precipitation of the RNA, even when only a few micrograms was present. The precipitates were then dissolved in the appropriate amount of water and the components for the template activity assays were added to the tubes.

Analytical Procedures. The purified RNA preparations were analyzed by absorbancy measurements after acid hydrolysis, as described previously (Brawerman *et al.*, 1963). The fractions after zone centrifugation were determined directly in water using a value of 1.0 for the A_{260} of an RNA solution of 45 μg/ml. Nucleotide composition analyses were carried out according to Brawerman and Chargaff (1959). In some of the experiments this procedure was scaled down as follows. Samples (20 μg) of RNA hydrolysate were applied on paper as very compact spots, the chromatography was run for 7 hr, and the separated nucleotides were eluted with 1 ml of buffer. In this manner fully reliable values for nucleotide composition could be obtained with one-quarter the amount of RNA previously required. In some experiments, the orcinol and diphenylamine colorimetric procedures (Mejbaum, 1939; Burton, 1956) were used for the determination of RNA and DNA.

Radioactivity measurements were performed either on samples placed on filter paper disks, or on 0.5–0.7-ml samples mixed with a dioxane scintillation mixture containing 200 g of naphthalene, 10 g of PPO, and 250 mg of POPOP per l. of dioxane (Werbin *et al.*, 1959). The disks containing the RNA samples were either dried directly or first washed with cold 5% trichloroacetic acid and with ether–ethanol (3:1, v/v). The dried disks were placed in toluene scintillation mix (Mans and Novelli, 1961). Washing with cold trichloroacetic acid did not lower the amount of radioactivity on the disks, showing that the purified RNA samples contained no acid-soluble radioactivity.

Results

Fractionation of Rat Liver. The 600*g* supernatant of the liver homogenate, which served for the preparation of the cytoplasmic RNA fractions, contained about 4% of the total DNA (Table I). This provides a maximal value for the extent of contamination of this fraction due to broken nuclei, since mitochondrial DNA must contribute to the DNA value obtained for the cytoplasmic

TABLE I: Nucleic Acid Content of Cytoplasmic and Nuclear Fractions.[a]

	RNA	DNA	RNA: DNA
Cytoplasm[b]	124	1.5	
Crude nuclei[b]	22	38	0.6
Purified nuclei	5.6	38	0.15

[a] Values expressed as mg/20 g of liver (fresh weight). Data represent average values for two experiments. [b] Cytoplasm and crude nuclei represent 600*g* supernatant and sediment of original homogenate, respectively.

[1] Abbreviations used: ATP and GTP, adenosine and guanosine triphosphates; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

fraction. The crude nuclei (600*g* pellet) contained about 15% of the total RNA, but the sedimentation through concentrated sucrose lowered this value to 4% without any measurable loss of DNA. The ratio of RNA to DNA in the purified nuclei was about 0.15 (Table I). This is in good agreement with the value obtained by Sporn *et al.* (1962).

The procedure for the phenol fractionation of the cytoplasm is the same as that used previously for the whole liver homogenate (Hadjivassiliou and Brawerman, 1965). Fractionation of the nuclei presented several problems, and the procedure had to be modified substantially. Phenol treatment of the nuclei at pH 8.3 led to extraction of much of the DNA together with the high pH RNA. The procedure devised previously to prevent extraction of the DNA from the whole liver homogenate consisted of setting the temperature of the pH 7.6 extractions at 38°. After this treatment, extraction of the DNA at pH 8.3 was prevented, but the high pH RNA could still be obtained (Hadjivassiliou and Brawerman, 1965). This procedure could not be applied to the nuclei, however, because with this material the pH 7.6 treatment at 38° tended to remove pH 8.3 RNA as well. Thus the pH 7.6 extraction was carried out at 0°, and only a single treatment was done at this pH. The phenol residue was next suspended in water and brought to 38°. The pH was then raised to 8.3 and the RNA was obtained without DNA. The pretreatment of the phenol residue at 38° had to be done in the presence of added water, otherwise the interphase material became dehydrated and RNA could no longer be extracted from it.

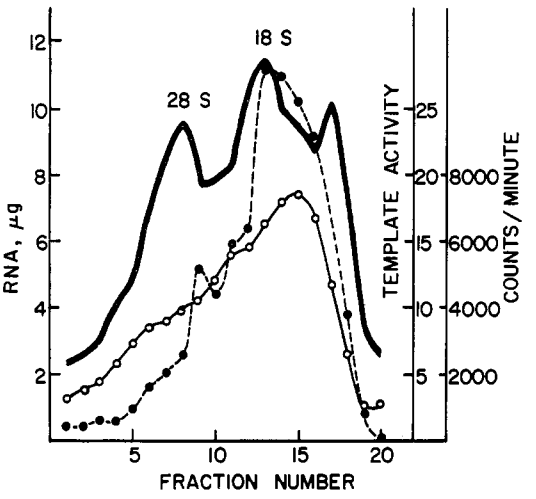

FIGURE 2: Sedimentation characteristics of *nuclear pH 8.3* RNA from rats pulse labeled with orotic acid (for conditions of labeling, see Table II). RNA (200 μg) was centrifuged for 4 hr in SW 39 rotor. All values expressed per 14-drop fractions: (heavy solid line) RNA content as determined by absorbancy at 260 mμ; (thin solid line with open circles) radioactivity; (dashed line with closed circles) template activity. Template activity expressed as total leucine incorporation (in μμmoles)/0.15 ml of reaction; incorporation without RNA was 6 μμmoles.

TABLE II: Template Activity and Specific Radioactivity of Phenol Fractions.[a]

RNA Fraction	Yields (mg of RNA/ 20 g of liver)	Radioactivity (cpm/mg of RNA)	Template Act.[b] (μμ-moles of leucine incorp/mg of RNA)
	Nuclear		
pH 8.3	1.4	490,000	1,930
pH 7.6	1.5	46,700	360
	Cytoplasmic		
pH 8.3	1.6	50,400	1,150
pH 7.6	106	1,340	170

[a] Phenol fractionation carried out on livers from rats injected intraperitoneally 40 min prior to sacrifice with 10 μc/100 g of body weight of [2-^{14}C]orotic acid (25 μc/μmole). *Cytoplasmic 7.6* RNA obtained by extraction at 0°. [b] Incorporation without added RNA was 6 μμmoles of leucine/0.15 ml of reaction. Incorporations with RNA ranged from 8.5 (for *cytoplasmic pH 7.6* RNA) to 30 μμmoles (for *nuclear pH 8.3* RNA).

The two nuclear RNA fractions and the *cytoplasmic pH 8.3* fraction were each obtained in yields of about 1% of the total liver RNA (Table II). The purified nuclear fractions account for approximately 50% of the total nuclear RNA (Tables I and II). The phenol residue after the pH 8.3 extraction was found to contain additional RNA (Drews and Brawerman, 1967). The amount of DNA extracted together with the nuclear RNA was about 0.5 mg/20 g of liver, and much of it was removed after precipitation of the RNA with cold 10% NaCl.

RNA Fractions from Rat Liver Cytoplasm and Nuclei. It has been shown previously that a fraction containing RNA rich in adenylic acid as well as material with high template activity can be obtained from whole liver homogenate by pH phenol fractionation (Hadjivassiliou and Brawerman, 1965). When this fractionation is applied to cytoplasm and to purified nuclei, *pH 8.3* RNA with high template activity is obtained from both cell components (Table II). After incorporation of [^{14}C]-orotic acid for 40 min, the *nuclear pH 8.3* RNA shows the highest rate of labeling, and is about ten times more radioactive than the corresponding cytoplasmic fraction. The latter is far more labeled than the bulk of the cellular RNA (*pH 7.6 cytoplasmic*). The two nuclear fractions also differ sharply in this respect. Thus the pH phenol fractionation yields four fractions with distinct biochemical and metabolic properties.

The nucleotide composition of the two pH 8.3 RNA fractions is listed in Tables III and IV. The *cytoplasmic*

TABLE III: Nucleotide Composition of RNA Components from *Nuclear pH 8.3* Fraction.[a]

	n	A	G	C	U(T)	A:U
Nuclear 8.3						
Total	1	22.1	28.3	27.0	22.6	0.98
9–16 S	4	26.0 (0.8)	24.3 (0.5)	23.8 (0.2)	25.9 (0.7)	1.00
18 S	2	23.2 (1.2)	26.9 (0.9)	25.8 (0.5)	24.1 (0.2)	0.96
28 S	1	21.1	29.0	28.0	21.9	0.96
DNA[b]		28.6	21.4	21.5	28.4	1.00
rRNA[c]						
18 S	1	22.5	31.8	26.2	19.5	1.15
28 S	1	18.0	34.8	30.8	16.4	1.10

[a] RNA obtained from zone sedimentation fractions as described in the Experimental Section. Values in parentheses represent standard deviation in the case of 9–16S component, and deviation from mean in the case of 18S RNA; *n* represents the number of preparations analyzed. [b] Data from Wyatt (1951). [c] Components isolated from *cytoplasmic 7.6* RNA by zone centrifugation.

8.3 RNA shows the low G, C, and U content previously described for the *pH 8.3* RNA obtained from whole liver homogenate. This RNA was previously believed to originate from nuclei (Georgiev and Mantieva, 1962; Hadjivassiliou and Brawerman, 1965), but the present results indicate a cytoplasmic origin for this material. The *nuclear pH 8.3* RNA shows a G and C content lower than that of rRNA, but here the amounts of A and U are equal. The *nuclear pH 7.6* values were close to those of rRNA.

Characteristics of the Nuclear 8.3 Fraction. Figure 2 shows the zone sedimentation pattern of *nuclear 8.3* RNA from rats pulse labeled with orotic acid. Three peaks with sedimentation values of approximately 28, 18, and 8 S are evident, together with a shoulder around 9–16 S and some material heavier than 28 S. The radioactivity pattern is rather heterogeneous, but shows a major peak which appears to coincide with the 9–16S shoulder. Most of the template activity sediments as a well-defined peak, also in the region of the shoulder. The bulk RNA isolated from the 9–16S region has a nucleotide composition close to that of DNA, with equal amounts of A and U (Table III). RNA from the 18S peak shows a somewhat higher GC content, and the 28S component has a composition approaching that of rRNA. The material in the leading edge of the 28S peak is nearly as radioactive as the 9–16S component, but has little template activity.

Nuclear 7.6 RNA Fraction. The zone centrifugation pattern in Figure 3 shows two well-defined peaks which correspond probably to the 18S and 28S rRNA components. A light peak is also evident, but is not likely to represent tRNA, since the latter is removed from the preparation during the NaCl precipitation step. The distribution of radioactivity in the heavy region indicates

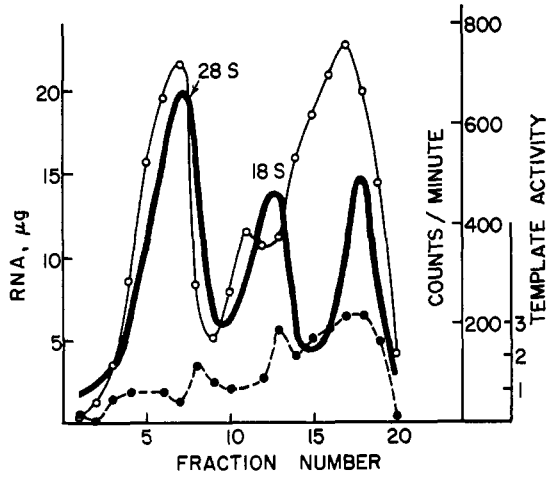

FIGURE 3: Sedimentation characteristics of *nuclear pH 7.6* RNA (for details see Figure 2).

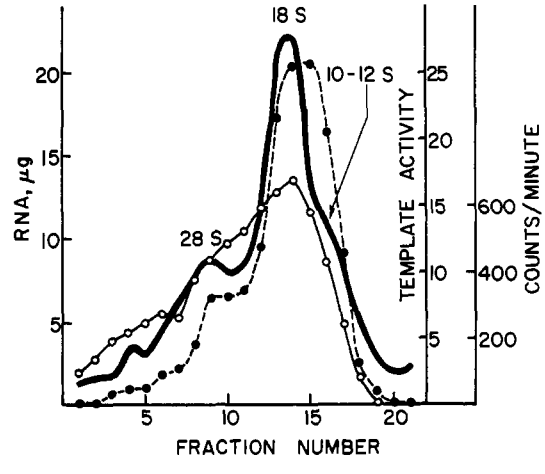

FIGURE 4: Sedimentation characteristics of *cytoplasmic pH 8.3* RNA (for details see Figure 2).

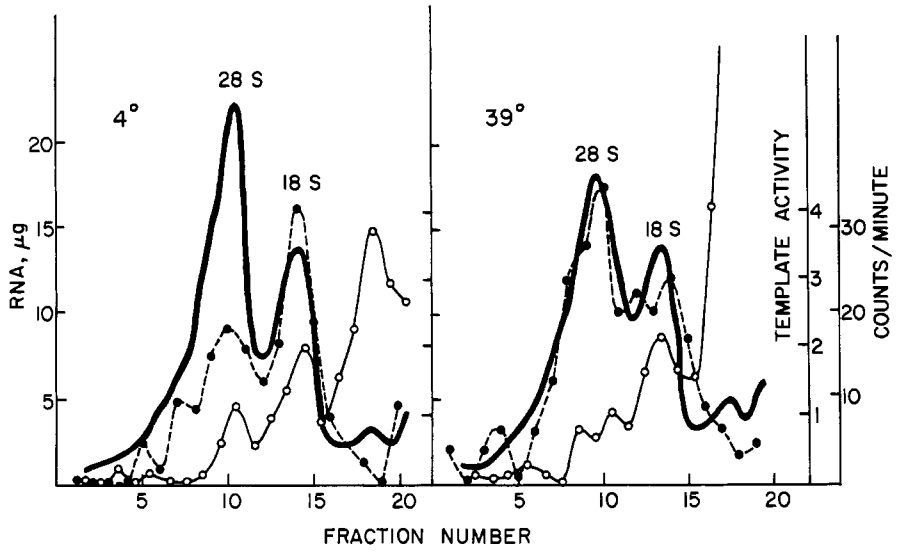

FIGURE 5: Sedimentation characteristics of *cytoplasmic 7.6* RNA extracted at 4° (a) and 39° (b). Alternate 10-drop ractions were collected for analysis and for template assays (for other details see Figure 2).

that the labeled material is not 28S rRNA, but a component heavier than 28 S. It probably corresponds to the 30–35S precursor rRNA described by various investigators (Perry, 1962; Scherrer *et al.*, 1963). The radioactivity profile in the 18S region bears no resemblance to the RNA profile. It is doubtful, therefore, that the 18S component is labeled to any significant extent. The large radioactivity peak in the light region appears to be associated with some template RNA. As in the *nuclear 8.3* fraction, the heavy radioactive RNA contains relatively little template activity.

Cytoplasmic 8.3 RNA Fraction. Zone centrifugation of the *cytoplasmic 8.3* fractions reveals one major component with a sedimentation value close to that of 18S rRNA (Figure 4). A shoulder around 10–12 S is evident and some heavy material is also present. This pattern resembles closely that of the pH 8.3 fraction obtained from whole liver homogenate (Hadjivassiliou and Brawerman, 1965). As in the previous study, the material rich in adenylic acid is localized in the 10S region. This is evident from the data on the nucleotide composition of the 10–12S and 18S components (Table IV).

TABLE IV: Nucleotide Composition of RNA Components from the *Cytoplasmic pH 8.3* Fraction.[a]

	A	G	C	U	A:U
Total	29.8	24.5	24.5	21.2	1.40
10–12 S	32.2	23.0	22.4	22.4	1.45
18 S	26.3	27.0	25.8	20.6	1.28

[a] Fractions of 10–12S and 18S zones were recentrifuged in a SW-39 rotor; RNA from peaks was precipitated with ethanol and analyzed. One preparation of each component was used for analysis.

The distribution of template activity resembles rather closely that of the nuclear fraction. A broad, but well-defined 9–16S peak is evident. There is no obvious correlation between the template peak and the ultraviolet absorption profile in this region. The template activity covers both the 10–12S shoulder and the 18S peak.

We interpret the diagram in Figure 4 as indicating the presence in the 9–18S region of three distinct RNA components: an 18S RNA, a 10–12S RNA rich in A, and template RNA. The radioactivity profile does not show any obvious relation to either of the three components. Thus the extent of labeling of any of these components cannot be deduced from these results.

Cytoplasmic 7.6 RNA Fraction. The sedimentation pattern of this material shows the two components characteristic for rRNA (Figure 5). Preparations obtained by extraction at 4° and at 39° show similar ultraviolet absorption patterns. The patterns for template activity, however, differ markedly in the two preparations. In the 4° RNA the major template component is localized in the 18S region, while in the 39° RNA most of the template activity is in the 28S region. This dependence of the sedimentation behavior of template RNA on the temperature during phenol extraction has been reported previously from this laboratory (Brawerman *et al.*, 1965). In the present study the behavior of the pulse-labeled RNA was compared to that of the template material. As can be seen in Figure 5, the sedimentation pattern of the labeled RNA in the 18S and 28S regions is essentially the same in the two preparations. These results indicate that the pulse-labeled RNA and the template material represent different components in this preparation.

Discussion

The phenol extraction procedure used here distinguishes between RNA readily extracted at neutral pH and minor components which can be obtained after raising the pH. The latter could be either localized in structures which render them inaccessible to the phenol treatment, or complexed with basic proteins, as discussed previously (Hadjivassiliou and Brawerman, 1965). These RNA fractions, both from the nucleus and the cytoplasm, contain rapidly labeled components. They are also highly active in stimulating polypeptide synthesis by *E. coli* ribosomes, an indication of the possible presence of mRNA.

In the nuclear fraction, most of the material with template activity sediments as a 9–16S component. The bulk RNA in this sedimentation zone has a nucleotide composition quite close to that of DNA. The RNA with higher sedimentation values has a nucleotide composition closer to that of rRNA, and also a far lower template activity. Thus the template activity of liver nuclear RNA can be attributed to the DNA-like RNA component.

Most of the pulse-labeled RNA of the nuclear fraction also sediments around 9–16 S. It was shown by Drews and Brawerman (1967) that the light pulse-labeled RNA has a DNA-like base composition, and that the labeled material with higher sedimentation values has a composition approaching that of rRNA. It appears, therefore, that the *nuclear 8.3* fraction of normal rat liver contains primarily DNA-like pulse-labeled RNA, with sedimentation characteristics similar to that of the bulk DNA-like RNA. In regenerating liver, the heavy pulse-labeled RNA with the ribosomal base composition was shown to be the most abundant species (Drews and Brawerman, 1967).

The sedimentation characteristics of the DNA-like RNA described in this report are considerably lower than those reported recently from several laboratories for the pulse-labeled DNA-like RNA of some mammalian systems (Soeiro *et al.*, 1966; Attardi *et al.*, 1966; Scherrer *et al.*, 1966). It is possible, therefore, that the present material is fragmented. A fragmentation process leading to the preparations described here, however, would have to be highly selective. It would appear to apply only to the DNA-like RNA, leading to a population of molecules with a well-defined range of sedimentation values and nearly free of ribosomal components. Experiments in progress in this laboratory suggest that a fragmentation of pulse-labeled DNA-like RNA may occur during the purification of the nuclei.

The finding of a cytoplasmic RNA fraction refractory to phenol extraction at neutral pH was rather unexpected. It is not likely that this material is derived from disrupted nuclei, since the amount of DNA in the cytoplasmic fraction is very low. The sedimentation characteristics of the template RNA component are close to those of the corresponding nuclear component, and it presumably represents mRNA. It could be either the mRNA of the polysomes, or a form of mRNA in transit from the nucleus to the polysomes (Spirin *et al.*, 1964; Nemer and Infante, 1965). The latter possibility would account for the relatively high amount of radioactivity in this fraction after pulse labeling. The nature of this radioactive RNA, however, is not certain. The pulse-labeled material appears to cosediment with the template RNA component, but part of it could be associated with the 18S RNA component also present in this fraction. A 45S cytoplasmic particle which contains rapidly labeled 18S RNA has been described (Girard *et al.*, 1965; Henshaw *et al.*, 1965). The 18S component of the *cytoplasmic 8.3* fraction could possibly represent the same material. The high adenylic acid content of the cytoplasmic fraction appears to be due to polyadenylic acid, probably associated with microsomal membranes (Hadjivassiliou and Brawerman, 1966).

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Attachment of Glucosamine to Protein at the Ribosomal Site of Rat Liver*

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ABSTRACT: Rats were treated first with glucosamine-^{14}C and then with puromycin and the effects on the bound glucosamine-^{14}C and sialic acid-^{14}C of microsomal subfractions of liver and plasma proteins were measured. A rapid decrease of protein-bound glucosamine-^{14}C in ribosomes, rough microsomal membranes, and smooth microsomes occurred and the appearance of glucosamine-^{14}C-labeled plasma protein was inhibited. The incorporation of sialic acid-^{14}C, derived from glucosamine-^{14}C *in vivo*, was inhibited only in the smooth microsomal fraction and plasma proteins. Ribosomes labeled with glucosamine-^{14}C *in vivo* were isolated and treated with puromycin; 18–34% of the ribosomal radioactivity was released due to puromycin. Most of the released material could be precipitated by 5% trichloroacetic acid. Factors (salts, temperature, and mercaptoethanol and puromycin concentration) enhancing the puromycin-dependent release of nascent polypeptides had parallel effects on the release of glucosamine-^{14}C-labeled proteins. The specific activity (disintegrations per minute per micromole of glucosamine) of the released polypeptides was ten times higher than that of the residual ribosomes. The experiments suggest that glucosamine attachment to glycoproteins begins while the polypeptides are still associated with tRNA at the ribosomal site.

Some of the basic problems in the synthesis of glycoproteins are the course and subcellular site of attachment of the connecting monosaccharide to the polypeptide backbone and the assembly of the other carbohydrates forming an oligosaccharide unit. A number of studies led to the conclusion that the oligosaccharide chains are put together on the polypeptide within the membranes of the endoplasmic reticulum after the polypeptide has been completed and separated from the ribosomes (Sarcione, 1964; Sarcione *et al.*, 1964; Molnar *et al.*, 1965a; Cook *et al.*, 1965; Spiro and Spiro, 1966; Bouchilloux and Cheftel, 1966; Lawford and Schachter, 1966). The appearance of protein-bound labeling of ribosomes isolated from livers of glucosamine-^{14}C-treated rats (Helgeland, 1965) and a kinetic analysis of this labeling (Molnar *et al.*, 1965a) have implicated, however, the ribosomes as a site of attachment of glucosamine to protein. This suggestion has been supported recently by Lawford and Schachter (1966) who observed that puromycin treatment of glucosamine-^{14}C-labeled liver homogenate yielded ribosomes with diminished radioactivity. In this paper we present further evidences for the involvement of ribosomes in the attachment of glucosamine to polypeptide.

Experimental Procedure

Materials and Methods

Holtzman strain rats weighing 300–400 g were starved overnight. Each rat received 4 μc of glucosamine-1-^{14}C (12 mc/mmole, New England Nuclear Corp.). After 30 min, 15 mg of puromycin dihydrochloride (Nutritional Biochemicals Corp.) in 0.2 ml of distilled water was also injected by the same route (all injections were made intraperitoneally). Control animals were treated in the same manner except puromycin was omitted. At given time intervals the animals were killed by decapitation; plasma proteins, liver ribosomes, rough microsomal membranes, and smooth microsomes were isolated as described previously (Molnar *et al.*, 1965a).

Isolation of Labeled Ribosomes. Rats were given 4 μc of glucosamine-^{14}C or 4 μc of L-leucine-U-^{14}C (210 mc/mmole, Volk Radiochemical Co.). The glucosamine-treated rats were killed 40 min later, while the leucine-^{14}C-treated rats were killed 20 min after injection. The

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