Changes in the Free and Membrane-Bound Ribosomes in the Rat Liver with Starvation†

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ABSTRACT: Adult rats starved for 0–5 days showed progressive reduction in the total ribosomal and nonribosomal RNA content of the liver. The decrease in RNA was proportional to the drop in the liver weight, but a rapid preferential loss of free ribosomes occurred during the first day of fasting. The incorporation of [^{14}C]orotic acid into the total RNA of the free ribosomes was exceedingly rapid during the initial 4 hr among 48-hr fasted animals as compared to the fed rats. Only a moderate increase developed in the membrane-bound ribosomes of the fasted rats. The enhanced labeling of the ribosomes among the fasted rats resulted from a decrease in pool size of precursors, specifically UTP. The incorporation of labeled orotic acid into the ribosomal RNA (28S and 18S peaks) showed increasing utilization for 18 hr in both the free and bound ribosomes of the fed rats but only in the free ribosomes of the fasted animals. There was decreased incorporation in the membrane-bound ribosomes of the fasted rats. Experiments using mild ribonuclease preparation (microsomal deoxycholate-soluble fraction) demonstrated that the enhanced labeling of RNA detected in the free ribosomes within 4 hr among the 48-hr starved rats resulted from increased incorporation of both the ribosomal and nonribosomal RNAs, but the greatest increase was in the nonribosomal fraction.

The presence of both the free and the membrane-bound ribosomes in mammalian cells is now well established (Palade and Siekevitz, 1956; Blobel and Potter, 1967b). Reports (Redman, 1968; Hallinan *et al.*, 1968; Hicks *et al.*, 1969) indicate that the free ribosomes in rat liver synthesize protein for intracellular function, whereas the bound ribosomes synthesize serum proteins for extracellular purposes. Thus, both types of ribosomes are concerned with metabolism of hepatic and somatic cells, and the nutritional status of the animal, either fed or fasted, may possibly play a significant role in determining the quantity and the function of both types of ribosomes.

Decreases in ribosomal and nonribosomal RNAs of the rat liver after fasting periods of varying duration have been reported by several investigators (Wilson and Hoagland, 1967; Petermann and Hamilton, 1958). Changes in polysome profiles (Wittman *et al.*, 1969), in the ability to incorporate amino acids (Sox and Hoagland, 1966), as well as variations in DNA-dependent RNA polymerase, acid, and alkaline ribonucleases have also been observed with fasting (Onishi, 1970a,b; Sheppard *et al.*, 1970). Yet a sequential study measuring changes in the amount and the function of free and membrane-bound ribosomes during varying periods of starvation is lacking. The present work represents an effort to measure quantitative changes in the various ribosomal fractions of the rat liver, especially the free and membrane-bound ribosomes, during a fasting period of 0–5 days. Additional experiments include isotopic labeling studies to determine possible changes in metabolic function of the two types of ribosomes with fasting.

Materials and Methods

[6-^{14}C]Orotic acid (36.6 mCi/mmole) was purchased from Schwarz BioResearch, Inc. Sodium deoxycholate, sodium dodecyl sulfate, and poly(vinyl sulfate) were obtained from Fisher Scientific Co.; Dowex 1-X8 (200–400 mesh) was procured from D. & P. Minerals Co.

The studies were performed on male Sprague–Dawley rats weighing between 230 and 250 g. The animals were maintained on Purina Chow diet prior to starvation and were kept in a room with controlled light from 7 to 19 hr; water was available *ad libitum* during periods of starvation from 0 to 5 days. The rats were sacrified by cervical dislocation and the livers immediately placed into 10 ml of cold 0.25 M sucrose in TKM buffer (0.05 M Tris-HCl (pH 7.5)–0.025 M KCl–0.5 mM $MgCl_2$) (Blobel and Potter, 1967a). All procedures were performed at 4°. The liver was minced and homogenized in two volumes of 0.25 M sucrose in TKM buffer containing 0.1 volume of ribonuclease inhibitor prepared from rat liver (Blobel and Potter, 1967b). The homogenate was prepared with 10 strokes of a Potter-Elvehjem homogenizer with a Teflon pestle at 1725 rpm, and filtered through four layers of cheesecloth.

Total Free and Cytoplasmic Ribosomes and Nonsedimentable RNA. The preparation of the total free and cytoplasmic ribosomes including the nonsedimentable RNA fraction followed essentially the procedure described by Blobel and Potter (1967a) using a postnuclear supernatant. The latter was prepared by mixing 1.0 ml of the filtered homogenate with 2.0 ml of 2.3 M sucrose in TKM buffer. This was then layered over 1.0 ml of 2.3 M sucrose in TKM. The sample was centrifuged in a Spinco SW 39 rotor at 39,000 rpm for 30 min. The material adhering to the wall of the centrifuge tube was removed and rehomogenized with the supernatant using five strokes of a Potter-Elvehjem homogenizer.

The total free ribosomes were separated by placing 1.0 ml of the postnuclear supernatant over 3.0 ml of 2.0 M sucrose–TKM buffer. An additional 6.0 ml of 0.25 M sucrose in TKM was added to yield a total volume of 10.0 ml. The preparation was centrifuged in a Spinco 40 rotor at 40,000 rpm for 24 hr. RNA content of the free ribosomes was measured in the pellet.

The total cytoplasmic ribosomes was prepared by mixing

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0.2 ml of 10% sodium deoxycholate with 1.0 ml of postnuclear supernatant. TKM buffer was added to provide a total volume of 10 ml. The sample was mixed and centrifuged using a Spinco 40 rotor for 4 hr at 40,000 rpm. The resulting pellet contained the total cytoplasmic ribosomes. The supernatant was acidified to pH 5.0 with 0.2 M acetic acid and centrifuged in a Spinco rotor 40 at 40,000 rpm for 30 min. The pellet in this case represented the nonsedimentable RNA.

The ribosomal pellets obtained from the preparation of free and total ribosomes and nonsedimentable RNA were each suspended in 1.0 ml of Tris buffer (0.005 M Tris-HCl, pH 7.5); the nuclear pellet was suspended in 1.0 ml of Tris buffer.

RNA and DNA Measurements. To each of the ribosomal or nuclear suspensions, alkaline hydrolysis was undertaken by adding 1.0 ml of 0.3 N KOH and incubating the mixture for 1 hr at 37°. RNA was determined using a modified Schmidt–Tannhauser technique by neutralizing the sample with 0.2 N cold perchloric acid, acidifying with an equal volume of 1.0 N perchloric acid and centrifuging at 17,000*g* for 20 min. The supernatant was analyzed spectrophotometrically for RNA using 32 μg/ml = 1.00 absorbancy unit (Fleck and Munro, 1962; Munro and Fleck, 1966). The pellet obtained from alkaline hydrolysis described above was dissolved in 5.0 ml of 0.3 N KOH and the DNA was measured according to the method of Ceriotti as modified by Blobel and Potter (1967a).

The membrane-bound ribosomes were determined by measuring the difference between the total cytoplasmic ribosomes and the total free ribosomes in the present quantitative assay and in the isotopic labeling experiments described later. The values for membrane-bound ribosomes were more consistent and reproducible using this method rather than as a separate determination employing a different technique because of the technical difficulties encountered in completely removing the bound ribosomal particles in the interphase (Blobel and Potter, 1967a). In addition the latter method utilized postmitochondrial supernatant rather than a postnuclear supernatant; such a change in the original supernatant preparation will alter the quantitative recoveries of ribosomes (Blobel and Potter, 1967b).

Isotopic Labeling of the Free and Membrane-Bound Ribosomes. [^{14}C]Orotic acid (25 μCi) was injected intraperitoneally into each rat of two groups of 12 rats, fed or 48-hr fasted. The starved group was provided water *ad libitum*. The animals were killed by cervical dislocation at specified times after injection and a 33% w/v liver homogenate was prepared with 0.25 M sucrose in TKM buffer. All procedures were conducted at 4°. From the homogenate the following studies were done.

1. The specific activity of the free and membrane-bound ribosomes from total cytoplasmic and total free ribosomes using a post nuclear supernatant was determined as described earlier.

2. The total radioactivity in the free and membrane-bound ribosomes was determined.

3. The total radioactivity in the whole homogenate was determined by precipitating RNA directly from the 33% w/v homogenate.

4. The acid-soluble and acid-insoluble counts in the two groups of animals were obtained after precipitation with 0.1 volume of 50% trichloroacetic acid.

Incorporation of [^{14}C]Orotic Acid into Liver UTP. To a different set of fed and 48-hr fasted animals, numbering eight in each group, 25 μCi of [^{14}C]orotic acid was injected intraperitoneally into each rat. The rats were sacrificed at specified times postinjection and a 33% w/v liver homogenate was prepared with 0.25 M sucrose in TKM buffer.

The homogenate was treated with an equal volume of 10% perchloric acid, the supernatant neutralized, and the nucleotides were separated on Dowex 1X-8Cl columns. The nucleotides (UTP and GTP) were hydrolyzed to their respective mononucleotides and finally separated through a second Dowex 1X-8Cl column. The absorbancy at 260 mμ and the radioactivity measured as UMP represented the incorporation of [^{14}C]orotic acid into liver UTP.

Isotopic Labeling of the Ribosomal RNA in the Free and the Membrane-Bound Ribosomes. Using the same 33% w/v homogenate from the previous experiments to measure labeled UTP, total free and total cytoplasmic ribosomes were prepared from a postnuclear supernatant fraction as described earlier. RNA was extracted from the ribosomal pellets using the cold phenol technique and layered on a 5–20% sucrose gradient for ultracentrifugal analysis. The total absorbancy and the total radioactivity corresponding to the 28S and 18S peaks were determined to measure the specific activity of the ribosomal RNAs.

Isotopic Labeling of the Ribosomal and Nonribosomal RNAs in the Total Free and Total Cytoplasmic Ribosomes. [^{14}C]-Orotic acid (25 μCi) was injected intraperitoneally into each of two rats weighing 190 g (fed) and 170 g (48-hr fasted). The animals were sacrificed 4 hr postinjection. Total free and total cytoplasmic ribosomes were prepared from a postnuclear supernatant fraction of 33% w/v homogenate. One-half of the ribosomal suspension was treated with a microsomal deoxycholate-soluble fraction possessing mild ribonuclease (Tanaka *et al.*, 1970) to remove mRNA. RNA was extracted separately from the treated and nontreated ribosomal suspensions with cold phenol and analyzed on a 5–20% sucrose gradient. The changes in the radioactivity and absorbancy profiles were utilized to denote changes in the ribosomal RNA and the nonribosomal RNA including mRNA.

Preparation of Microsomal Deoxycholate-Soluble Fraction (Tanaka et al., 1970). From the 33% w/v homogenate from 5.0 g of an adult rat liver, the postmitochondrial supernatant centrifuged at 105,000*g* for 1 hr to obtain the microsomal pellet. The latter was suspended in 2.0 ml of 0.25 M sucrose in TKM buffer and treated with 10% sodium deoxycholate to yield a final concentration of 1.3%. The preparation was centrifuged at 59,300*g* for 1 hr and the clear upper half of the resulting supernatant was used for analysis. The protein content was determined using the method of Lowry *et al.* (1951).

Results

The results in Table I indicate a definite decrease in the liver RNA fraction with increasing number of days of starvation in accordance with previous studies. Reductions have been observed previously in total cytoplasmic RNA, homogenate RNA, soluble RNA, and nuclear RNA (Sox and Hoagland, 1966; Fukuda and Sibatani, 1953); however, the most unusual change noted in the present study was the preferential loss of free ribosomes during the first 24 hr of fasting. There is approximately 45% loss during this short period reducing the per cent ratio of free to total ribosomes from 37 to 28. Blobel and Potter (1967b) reported an average per cent ratio of 25 in their studies, but the present work indicates wide variations in the free to total ribosome ratios during periods of fasting. After an initial rapid decline, there is a gradual but continued loss of free ribosomes. In contrast, the

TABLE I: Changes in RNA Fractions of the Total Liver with Starvation.

No. of Days Rat Starved	Nuclear Pellet (mg)	Total Ribosomes (mg)	Free Ribosomes (mg)	Bound[a] Ribosomes (mg)	Free/Total Ribosomes × 100	Nonsedimentable RNA[b]	
						Precipitate (mg)	Supernatant (mg)
0	2.37	42.22	15.64	26.58	37.0	5.49	2.59
1	1.96	30.70	8.64	22.06	28.1	3.31	3.09
2	1.41	22.49	7.10	15.39	31.6	2.89	1.55
3	1.50	22.15	6.91	15.24	31.2	2.45	1.15
4	1.23	19.87	5.69	14.19	28.6	2.73	1.21
5	1.27	15.60	5.51	10.09	35.3	2.16	0.99

[a] Membrane-bound ribosomes = total ribosomes − free ribosomes. [b] 1.0 ml of supernatant resulting from acidification and centrifugation was hydrolyzed and the RNA determined using Schmidt–Tannhauser method. The results listed represent an average of two to three separate determinations.

bound ribosomes show a steady and consistent decrease throughout the entire duration of fasting. At the end of the fifth day, however, there is a 65% decrease in the free ribosomes *vs.* 62% in the membrane-bound ribosomes indicating approximately the same total loss after an extended starvation period.

The RNA content in the supernatant representing the nonsedimentable RNA is elevated during the period of rapid loss of free ribosomes; otherwise, there is a decrease in RNA content in this particular fraction with starvation. The per cent recovery of the various RNA fractions was not determined since the values obtained for total homogenate RNA were inordinately low and inconsistent even after several separate determinations.

The results shown in Table II indicate that there is a rapid decline in the weight of the liver within the first 48 hr of starvation, but the total amount of DNA remains constant. Thus, if the RNA fractions are expressed as per weight of the liver (33% w/v homogenate) in the manner previously described by Blobel and Potter (1967a), the decrease in the RNA fractions are minimized because of the proportionate drop in liver weight. Previous studies have shown that the amount of RNA does correlate with the total liver weight (Petermann and Hamilton, 1958); recently Onishi (1970a) has observed that DNA-dependent RNA polymerase falls in proportion to the drop in liver weight. Table II also indicates that there is a marked drop in the body weights. At the end of 5 days of starvation there was 36% body weight loss as compared to 45% loss in the weight of the liver. Onishi (1970a) has reported that free and latent alkaline ribonuclease is maintained up to 20–25% body weight loss, but is changed when the weight loss reaches 40–45% of the initial weight.

There is no consistent change in the total DNA in the liver with fasting. Although reports (Fukuda and Sibatani, 1953;

TABLE II: DNA Determination and Changes in Body and Liver Weight with Fasting.

No. of Days Rat Starved	Total Weight of Rat (g)	Weight of Liver (g)	Total DNA in Liver (mg)
0	250	11.30	31.37
1	220	8.77	38.97
2	200	6.73	29.37
3	175	6.46	36.35
4	170	6.26	34.85
5	160	6.20	38.40

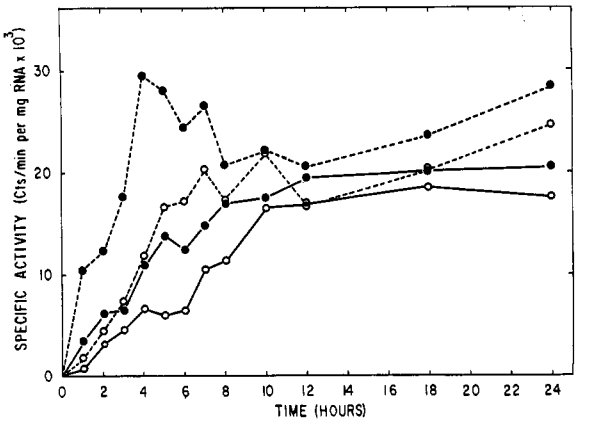

FIGURE 1: Specific activity of the free and membrane-bound ribosomes in rat liver after intraperitoneal injection of 25 μCi of [^{14}C]-orotic acid. Fed, free ribosomes ●——●; fed, membrane-bound ribosomes ○——○; 48-hr starved, free ribosomes ●— —●; 48-hr starved, membrane bound ○- - - -○. A postnuclear supernatant was obtained from the 33% w/v homogenate. The total free and cytoplasmic ribosomes were separated as described in the text and the resulting ribosomal pellets were dissolved in 1.0 ml of Tris buffer (pH 7.5). A 0.1-ml aliquot of the ribosomal solution was diluted to 1.0 ml with TKM buffer (pH 7.6). The RNA was precipitated with 0.1 ml of 50% trichloroacetic acid using 0.03 ml of 5% bovine serum albumin as carrier. The RNA preparation was placed in a Millipore filter, washed two times with 5% trichloroacetic acid, and dried, and the radioactivity measured using a thin window gas flow counter. The remaining 0.9 ml of the ribosomal solution was hydrolyzed and the RNA content determined according to the modified Schmidt–Tannhauser procedure described in the text. The specific activity was then calculated as radioactive counts per minute per mg of RNA. The membrane-bound ribosomal content and radioactivity represented the difference between total cytoplasmic ribosomes and free ribosomes. Every paired specific activity of the free and the membrane-bound ribosomes represents the results from a single injection of 25 μCi of [^{14}C]orotic acid with the animals sacrificed at varying times postintraperitoneal injection. Every point recorded in our isotope studies represents an average of two or three separate determinations. Experiments were actually repeated using different sets of rats to confirm the initial readings where definite changes in specific activities were recorded at 2, 4, 6, and 12 hr postinjection.

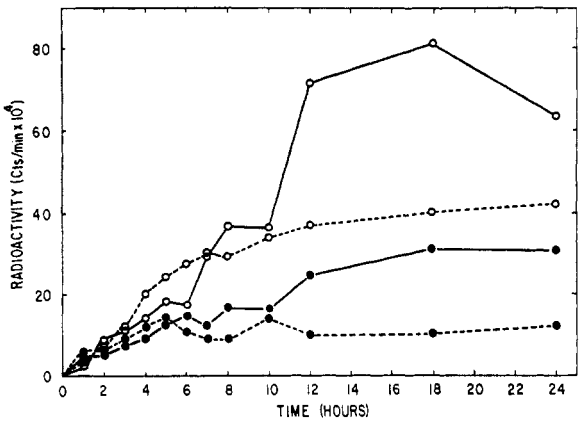

FIGURE 2: The total radioactivity in the ribosomal fractions of the whole rat liver. Fed, free ribosomes ●——●; fed, membrane-bound ribosomes ○——○; 4-hr starved, free ribosomes ●— —●; 48-hr starved, membrane-bound ribosomes ○- - - -○. The total radioactive counts were calculated from the results obtained from experiments shown in Figure 1.

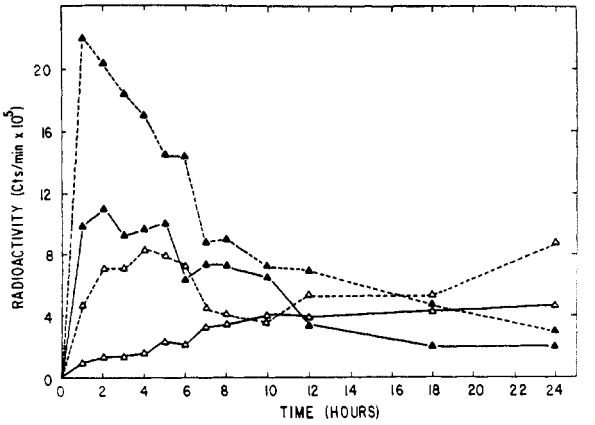

FIGURE 4: Acid-soluble and acid-insoluble counts per 1.0 ml of homogenate (33% w/v). Fed, acid-soluble counts ▲——▲; fed, acid-insoluble counts △——△; 48-hr starved, acid-soluble counts ▲- - - -▲; 48-hr starved, acid-insoluble counts △- - - -△. To 2.0 ml of the 33% w/v homogenate, 0.1 volume of 50% trichloroacetic acid was added, mixed, and centrifuged at 10,000*g* for 10 min. The precipitate was washed two times with 5.0 ml of 5% trichloroacetic acid. The supernatant and the washings were pooled and the radioactivity was measured by placing a 0.5-ml aliquot in 8.0 ml of Bray's solution and using a liquid scintillation counter. The precipitate was suspended in 10.0 ml of 10% trichloroacetic acid and placed in a boiling water bath for 15 min. The preparation was cooled and a 0.5-ml aliquot placed in 8.0 ml of Bray's solution for radioactivity measurement.

Conrad and Bass, 1957; Harrison, 1953) conflict concerning the effect of starvation upon the amount of DNA per liver cell, there are changes in the amount of DNA per weight of liver according to the shifts in extracellular and intracellular water volume reported by Harrison (1953).

The results of the isotopic labeling of the free and the membrane-bound ribosomes are illustrated in Figure 1. There is a striking difference in the pattern of incorporation of ^{14}C-labeled orotic acid between the fed and the 48-hr starved animals. The fed rats show a slow and progressive increase in the specific activity of both the free and the membrane-bound ribosomes throughout the 24-hr period of study; the starved rats display a marked rapid rise in the specific activity of the free ribosomes within 4 hr followed by a decrease and a gradual leveling pattern. The radioactive incorporation of the starved membrane-bound ribosomes is not as dramatic but still remains higher than the specific activities measured among the fed animals.

The total radioactive counts in the free and the membrane-bound ribosomes from the same series of animals display a different distribution as depicted in Figure 2. The greater total radioactivity in the bound ribosomes represents the larger amount of membrane-bound ribosomes throughout the period

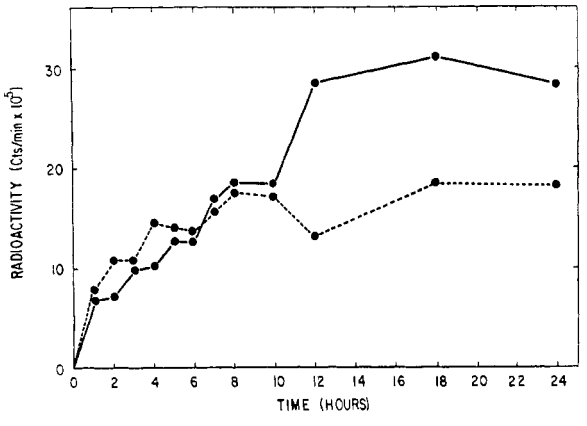

FIGURE 3: The total radioactive counts in the RNA of the liver homogenate. Fed animals ●——●; 48-hr starved ●- - - -●. From the same rats studied in Figures 1 and 2, a 0.1-ml aliquot of the filtered 33% w/v liver homogenate was diluted to 1.0 ml with TKM buffer (pH 7.6). The RNA was precipitated with 0.1 ml of 50% trichloroacetic acid, collected on Millipore filters, dried, and plated on planchets, and the radioactivity determined using a thin window gas flow counter.

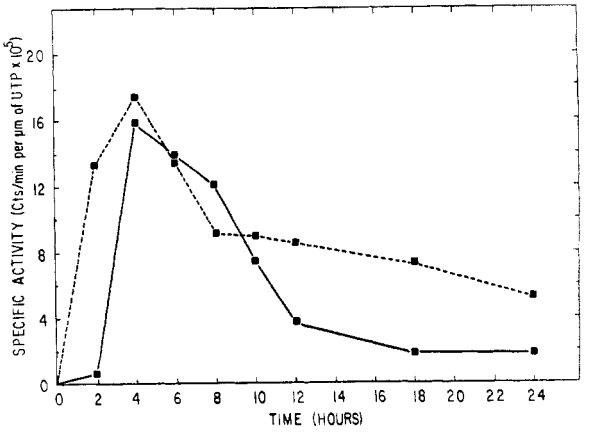

FIGURE 5: The incorporation of [^{14}C]orotic acid into liver UTP after intraperitoneal injection of 25 μCi of orotic acid. Specific activity of fed rats ■——■; of 48-hr starved rats ■- - - -■. To 5.0 ml of filtered 33% w/v homogenate, 5.0 ml of 10% $HClO_4$ was added and centrifuged. The method of measuring UTP as UMP was modified from the procedure described by Ove *et al.* (1966). The pellet was washed once with 5.0 ml of 5% $HClO_4$. The pooled supernatant was neutralized with 5.0 M KOH using Phenol Red indicator and $KClO_4$ was removed by centrifugation. The sample was placed on Dowex 1X-8Cl (1 × 10 cm) column and washed with 200 ml of 0.01 M HCl–0.1 M KCl followed by 50 ml of 0.05 N HCl. The mixture of UTP and GTP was eluted with 30 ml of 1 N HCl and hydrolyzed in a boiling water bath for 15 min. The resulting solution was cooled and placed on Dowex 1X-8Cl (1 × 10 cm) column and UMP eluted with 100 ml of 0.045 N HCl collecting 5.0-ml fractions. The absorbancy at 260 mμ was measured to calculate the UMP concentration and radioactivity determined by placing a 0.5-ml aliquot in 8.0 ml of Bray's solution using a liquid scintillation counter.

TABLE III: Amount of UTP in the Fed and Fasted Rats.[a]

No. of Hours Post-injection (Rats)	Fed		Starved	
	UTP (μmole per gram of Wet Liver)	UTP Total (μmole per Liver)	UTP (μmole per gram of Wet Liver)	UTP Total (μmole per Liver)
2	0.043	0.369	0.025	0.152
4	0.048	0.435	0.031	0.193
6	0.034	0.299	0.023	0.133
8	0.047	0.320	0.026	0.149
10	0.043	0.331	0.031	0.194
12	0.038	0.348	0.031	0.168
18	0.031	0.326	0.037	0.196
24	0.034	0.312	0.041	0.220
Average	0.040	0.343	0.031	0.175
S.D.	(±0.006)	(±0.043)	(±0.006)	(±0.030)

[a] Recovery of UTP (measured as UMP). The animals shown in Figure 5 were utilized to calculate the amount of UTP in the fed and fasted rats.

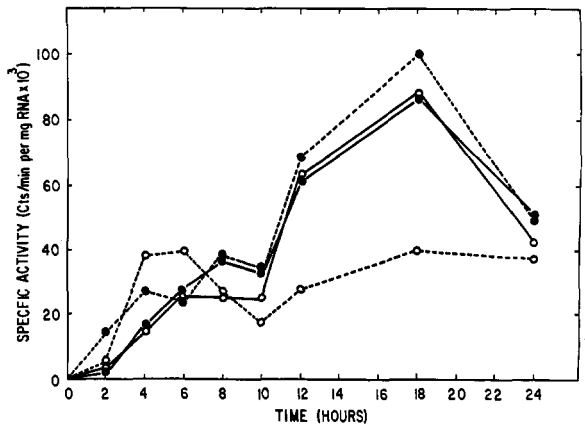

FIGURE 6: Incorporation of [^{14}C]orotic acid into the rRNA (28S and 18S peaks) of the free and membrane-bound ribosomes. Specific activity of the fed, free ribosomes ●——●; fed, membrane-bound ribosomes ○——○; 48-hr starved, free ribosomes ●- - - -●; 48-hr starved, membrane-bound ribosomes ○- - - -○. Five ribosomal pellets obtained from either the total free or the total cytoplasmic ribosomal preparation described earlier were pooled and suspended in 5.0 ml of 0.005 M Tris buffer (pH 7.5). Poly(vinyl sulfate) (0.025 ml, 0.1%), 0.25 ml of 10% sodium dodecyl sulfate, and an equal volume of water-saturated phenol was added and mixed for 3 min, incubated for 20 min at 4° and centrifuged at 17,000*g* for 10 min. The phenol layer was reextracted with 5.0 ml of 0.1 M sodium acetate buffer, mixed, incubated, and centrifuged. The aqueous layers from both phenol extractions were pooled and treated again with 5.0 ml of cold water-saturated phenol and centrifuged. Ethanol (95%, 2.5 volume) and 0.1 volume of 1.0 M K acetate buffer (pH 5.0) were added to the resulting aqueous preparation and the RNA was allowed to precipitate overnight. RNA pellets were obtained by centrifugation at 20,000*g* for 15 min, dissolved in 1.0 ml of cold water, and centrifuged at 30,000*g* for 15 min. For sucrose gradient analysis 0.61 mg of RNA (absorbance at 260 mμ × 40 = μg of RNA) was placed on a 5–20% sucrose gradient in 0.1 M KOAc (pH 5.0) buffer. After centrifugation at 22,500 rpm for 17 hr in a Spinco S.W. 25.1 rotor, the absorbancy was recorded using a Gilford automatic spectrophotometer. The amount of ribosomal RNA was determined by calculating the total absorbance corresponding to the 28S and 18S peaks (1 absorbancy unit = 40 μg/ml of RNA). The membrane-bound ribosomes represented the difference between the total cytoplasmic ribosomes and the free ribosomes. Radioactivity was determined by placing 0.8-ml fractions in 8.0 ml of Bray's solution and counting in a liquid scintillation spectrometer. The radioactivity of the membrane-bound ribosomes was taken as the difference between the radioactivity in the total cytoplasmic ribosomes minus the radioactivity in the free ribosomes. The specific activity was then calculated as radioactive counts per minute per mg of RNA. Each point recorded in the figure represents an average of 2–3 separate determinations. At 2, 4; 8, 10; and 18, 24 hr, the experiments were repeated with a different group of animals to confirm the original results.

of study, and considerably more radioactivity is present in the fed than starved animals. The total radioactivity in the homogenate RNA (Figure 3) further confirms the increased amount of labeled RNA in the fed animals indicating greater synthesis of RNA although there is an increased labeling of the homogenate RNA during the first 4–6 hr among the fasted rat.

The 1-hr, acid-soluble (nonprecipitable) radioactive counts of the starved animals designated as counts per minute per 1.0 ml of homogenate exceed the radioactivity of the fed animals by twofold indicating a severely contracted pool size of precursors (Figure 4). The acid-soluble radioactive counts decline sharply in both groups of rats as the labeled precursors are utilized, but the incorporation of labeled precursors is greater in the fed animals after the initial rapid phase since the fed rats continue to produce more RNA.

The acid-insoluble counts, representing incorporation of [^{14}C]orotic acid into RNA, show a gradual increase throughout the study among the fed rats, although the starved rats display an early increase.

The results in Figure 5 indicate that the maximum incorporation of [^{14}C]orotic acid into the liver UTP occurs within the first 4 hr in both the fed and fasted animals. There is a measurable delay in the rise of specific activity among the fed rats in contrast to the rapid rise displayed in the starved rats. These observations denote a marked reduction in the pool size of unlabeled UTP among the starved rats as denoted in Table III. There is a decrease in both the concentration and the total amount of UTP among the starved rats. Since the fed rats continue to synthesize more RNA and utilize UTP, the decline in the specific activities of UTP is steeper than the decrease exhibited by the fasted animals.

Figure 6 illustrates the results of determining the incorporation of [^{14}C]orotic acid specifically into ribosomal RNA by measuring the radioactivity in the 28S and 18S peaks after sucrose gradient analysis. Among the fed animals both the free and the bound ribosomes display an uniform and parallel rise in specific activities throughout the first 18 hr of study followed by a decline. This radioactivity pattern is similar to that reported by Moule and Delhumeau de Orgay (1964) studying incorporation of [^{14}C]orotic acid into ribosomal RNA of rat liver from partially hepatectomized but fed animals even though a different technique was employed to extract RNA. The 48-hr fasted animals in our studies, however, display a wide divergence of specific activities between the free and the membrane-bound ribosomes. The free ribosomes have an initial sharp rise due to the decreased pool size of UTP, but the remainder of the radioactive incorporation is identical with the pattern already observed in the free and the bound ribosomes of the fed animals. The bound ribosomes also display an initial rapid rise but it is followed by a low level of activity indicating decreased synthesis of RNA. Again the high radioactivity values observed during the first 4–6 hr result from the markedly contracted UTP pool.

The effects of microsomal deoxycholate-soluble fraction

TABLE IV: Free Ribosomes.[a]

		Total Radioactive Counts	% of Total Radioactivity	% of Radioactivity Lost with Treatment
Fed Rats Untreated	28S and 18S	2.985	53.5	
	Nonribosomal RNA	2.594	46.5	
Fed Rats Treated	28S and 18S	2.722	67.6	8.8
	Nonribosomal RNA	1.304	32.4	49.7
Fasted Rats Untreated	28S and 18S	11.371	45.2	
	Nonribosomal RNA	13.785	54.8	
Fasted Rats Treated	28S and 18S	10.275	66.5	9.6
	Nonribosomal RNA	5.180	33.5	62.4

[a] The effect of the microsomal deoxycholate-soluble fraction on the radioactivity in the ribosomal RNA (28S and 18S peaks) and nonribosomal RNA of the free ribosomes. Ten ribosomal pellets obtained from the total free ribosomal preparation described in the text were pooled and suspended in 10.0 ml of TKM buffer (pH 7.5); 5.0 ml of the ribosomal suspension was treated with 2.0 ml (2.5 mg of protein) of microsomal deoxycholate-soluble fraction for 5 min at 0°. Both the treated and nontreated ribosomal preparations were centrifuged at 59,300*g* for 90 min and suspended in 5.0 ml of TKM buffer. RNA was extracted from both ribosomal preparations using cold phenol method and subjected to sucrose gradient analysis as described in Figure 6. The radioactivity corresponding to 28 and 18S peaks were measured as ribosomal RNA incorporation.

TABLE V: Total Ribosomes.[a]

		Total Radioactive Counts	% of Total Radioactivity	% of Radioactivity Lost with Treatment
Fed Rats Untreated	28S and 18S	4.696	65.1	
	Nonribosomal RNA	2.513	34.9	
Fed Rats Treated	28S and 18S	4.495	71.9	4.2
	Nonribosomal RNA	1.754	28.1	30.2
Fasted Rats Untreated	28S and 18S	21.604	55.3	
	Nonribosomal RNA	17.480	44.7	
Fasted Rats Treated	28S and 18S	20.139	65.5	6.8
	Nonribosomal RNA	10.599	34.5	39.4

[a] The effect of the microsomal deoxycholate-soluble fraction on the radioactivity in the ribosomal and nonribosomal RNA of the total cytoplasmic ribosomes. The sedimentation profiles and experiments were detailed in Table IV. Ten ribosomal pellets obtained from the total cytoplasmic ribosomal preparation were treated in the manner described in Table IV.

upon the sedimentation profiles of fed and 48-hr fasted rats 4 hr after injection of [^{14}C]orotic acid are summarized in Tables IV and V. The 4-hr point was specifically selected because the highest specific activity of the free ribosomes among fasted rats occurred at this period (Figure 1); in addition, Tanaka *et al.* (1970) observed that the greatest incorporation of labeled precursors into mRNA of free ribosomes developed at the fourth hour postintraperitoneal injection. With fasting there is a marked increase in radioactive incorporation associated with a proportionately greater amount of radioactivity in the nonribosomal RNA. A modest decrease is observed in the radioactivity of each ribosomal RNA with ribonuclease treatment, but every nonribosomal RNA preparation displays from 30 to 62% loss. The greatest effect is upon the nonribosomal RNA in the free ribosomes of the fasted rat; these results were interpreted by Tanaka *et al.* (1970) to represent loss of mRNA of the free ribosomes. However, the amount of radioactivity lost by the total ribosomes indicates that the nonribosomal RNA of the membrane-bound ribosomes is also affected to some degree.

Discussion

Investigators have utilized both postmitochondrial and postnuclear supernatant for the fractionation of the free and membrane-bound ribosomes. Our initial studies using postmitochondrial supernatant yielded inordinately low and variable values for the preparation of total ribosomes, especially among the fasted animals. A similar problem of low recovery with total ribosomes with postmitochondrial supernatant was reported earlier by Blobel and Potter (1967a) even though the values for the free ribosomes were comparable from both the postmitochondrial and postnuclear supernatant. However, the use of the postnuclear supernatant does impose the additional problem of contamination by the "nonhepatocyte" nuclei (labeled NII nuclei) reported by Bushnell *et al.* (1969). Additional experiments were conducted in this laboratory using 20% Triton X-100 to isolate and to measure the radioactivity of the nonhepatocyte nuclei from the postnuclear supernatant among the fed and 48-hr fasted animals at 2, 4, and 7 hr postinjection of 25 μCi of [^{14}C]orotic acid. The total radioactivity in the NII nuclear preparation ranged between 0.3 and 3.6% of the corrected total radioactivity determined in the total ribosomes. In general the NII nuclear radioactivity was similar in both groups of animals; the radioactivity counts were slightly higher during the earlier postinjection period. Nevertheless, the presence of NII nuclei did not interfere with the overall results observed in our isotope labeling experiments displayed in Figure 1, except for a minimal but similar decrease in the specific activities of the free ribosomes of both the fed and 48-hr fasted animals. The low incorporation of isotopically labeled orotic acid into the nonhepatocyte nuclei has been observed previously by Albrecht (1968) and by Johnston *et al.* (1968).

The extensive studies of Blobel and Potter (1967a) provided detailed information concerning the preparation of the various

ribosomal fractions and compared the ribosomal RNA content of 12-hr fed rats *vs.* 36-hr fasted animals. The objective of this present work differs in that experiments were designed to determine overall trends or changes in ribosomal fractions that may develop during an extended period of starvation rather than a direct comparison between fed and starved rats. The results tabulated in Table I clearly indicate continued and measurable decrease in ribosomal fractions in both the free and membrane-bound ribosomes throughout the 5 days of starvation. The most striking change was observed in the rapid and preferential loss of free ribosomal RNA within the first 24 hr of fasting. Thus, any study involving the measurement of free ribosomes in rat liver must be cognizant of a rapid drop in free ribosomal RNA within a short period of fasting, and such experiments should preferably include animals fed *ad libitum* for comparison. The practice of fasting experimental animals overnight will produce marked reduction in free ribosomes since rats are predominantly night feeders; our current studies do not indicate in terms of hours how rapidly this loss of free ribosomes may develop with starvation.

In addition to quantitative changes in ribosomal fractions there were associated metabolic changes in both the free and the membrane-bound ribosomes with fasting as shown in our isotopic labeling experiments. Figure 1 indicates an enhanced and rapid labeling of the free ribosomal RNA among the 48-hr starved rats with the highest specific activity occurring at 4 hr after intraperitoneal injection. Although the animals were fasted for a relatively short period, Blobel and Potter (1968) have observed an increased specific activity of the free ribosomes during the early postinjection period with isotopically labeled orotic acid. Tanaka *et al.* (1970) reported that the highest specific activity of the messenger RNA of the free ribosomes developed at exactly the same 4-hr postintraperitoneal injection with animals starved for 18 hr. The fasted state of their experimental rats enabled the investigators to achieve such marked labeling of the free ribosomal mRNA since fed rats in the present study did not display such increased incorporation of ^{14}C-labeled orotic acid.

Comparison of the results of Figure 6 with Figure 1 indicates that the early enhanced labeling of the free ribosomes with fasting involves predominantly the nonribosomal RNAs of the free ribosomes rather than the ribosomal RNA (28S and 18S peaks). Tanaka *et al.* (1970) employing microsomal deoxycholate-soluble fraction stated that the mRNA of the free ribosomes was involved in the rapid incorporation of labeled orotic acid. The effects of microsomal deoxycholate-soluble fraction upon the ribosomal RNA tabulated in Tables IV and V indicate that the mild ribonuclease activity is upon the nonribosomal fraction of the free ribosomes, presumably mRNA. Neither the studies of Tanaka *et al.* (1970) nor the present experiments have proven the functional aspects of such mRNA or the possibility of involvement of other nonribosomal RNAs. The decrease in the total ribosomes of the starved rats with ribonuclease treatment summarized in Table V further suggests that the nonribosomal RNA of the membrane-bound ribosomes is similarly involved to a lesser degree with the rapid incorporation of labeled orotic acid. Finally, the marked increase in the specific radioactivity of the free ribosomes 4 hr postinjection shown in Figure 1 must therefore result from the combined effect of the measurable decrease in free ribosomes (Table I), the increased incorporation of the ribosomal RNA and especially the nonribosomal RNA (Tables IV, V; Figures 1, 6) and the marked reduction in the pool size of precursor UTP (Figure 5).

The 48-hr fasted animals, displayed in Figure 6, in sharp contrast to the fed group, show definite depressed metabolic activity in the ribosomal RNA of the bound ribosomes beyond the initial few hours. The modest increase in the specific activity observed during the early postinjection period probably results from the decreased pool size of precursor UTP, but the continued decrease in specific activity indicates that there is a definite reduction in the extracellular function of the membrane-bound ribosomes with fasting, whereas the intracellular metabolic activity of the free ribosomes is maintained at a level of activity corresponding to the free and the bound ribosomal RNAs of the fed animals.

The quantitative analyses of ribosomal RNA fractions and the radioisotope labeling experiments in the present study have both indicated significant changes in RNA content and metabolic activity with food deprivation. Even a relatively short fasting period does impose profound quantitative and functional alterations upon both the free and membrane-bound ribosomes of the rat liver. Thus, any experiment concerned with the measurement of half-lives or the differential labeling of the free and the membrane-bound ribosomes should be interpreted with proper recognition of the effect of fasting alone upon such studies.

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A Monomeric, Allosteric Enzyme with a Single Polypeptide Chain. Ribonucleotide Reductase of *Lactobacillus leichmannii*†

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ABSTRACT: Ribonucleoside triphosphate reductase has been purified from *Lactobacillus leichmannii* on a large scale as well as on the conventional scale. The final preparation has been shown homogeneous by behavior in plateau gel filtration, velocity sedimentation as examined by several criteria, equilibrium sedimentation, and polyacrylamide gel electrophoresis. The mean value of the molecular weight determined by all methods is 76,000. Equilibrium sedimentation under various denaturing conditions and polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate indicated that the protein cannot be disaggregated into subunits. The amino acid analysis is consistent with a molecular weight of 76,000 and indicates the presence of eight methionine residues. Cleavage with cyanogen bromide gave the expected nine peptides as shown by polyacrylamide gel electrophoresis and by gel filtration. The only detectable N-terminal residue is serine and the only C-terminus lysine. No aggregation of the enzyme occurred in the absence or presence of allosteric modifiers under the experimental conditions employed, except under denaturing conditions.

The ribonucleotide reductase of *Lactobacillus leichmannii* catalyzes reduction of GTP, ATP, CTP, ITP, and to a much smaller extent, UTP by dithiols such as dihydrolipoate or by reduced thioredoxin, a low molecular weight protein with two sulfhydryls (Blakley and Vitols, 1968). This reduction is absolutely dependent on the presence of deoxyadenosylcobalamin (coenzyme B_{12}). Several of the deoxynucleoside triphosphate products specifically activate reduction of particular ribonucleotides. For example, dGTP specifically activates ATP reduction and dATP specifically activates CTP reduction. On the basis of kinetic studies it has been suggested (Goulian and Beck, 1966; Beck, 1967; Vitols *et al.*, 1967a) that the deoxynucleotides produce such activation by binding at a site other than the catalytic site, that is at an allosteric or regulatory site. Kinetic evidence has also been obtained (Vitols *et al.*, 1967a) which suggests that ribonucleoside triphosphates also bind at such a site.

Binding of deoxynucleotide modifiers at the regulatory site has a profound effect on the interaction of cobamides with the enzyme. Thus, binding of cob(II)alamin (B_{12r}) to the active site is greatly enhanced by such modifiers, each of which probably also determines a specific conformation of the enzyme–cobamide complex (Hamilton *et al.*, 1971; Yamada *et al.*, 1971). Exchange of hydrogen between water and the cobalt-bound methylene group of 5′-deoxyadenosylcobalamin is dependent on a modifier nucleotide (Hogenkamp *et al.*, 1968), and degradation of deoxyadenosylcobalamin in presence of enzyme and dithiol to 5′-deoxyadenosine and cob(II)alamin, presumably *via* an active intermediate, occurs only in presence of a modifier (Hamilton *et al.*, 1971; Yamada *et al.*, 1971).

Many enzymes subject to regulatory control, including most of those studied intensively, have proven to be oligomeric proteins. However, estimates of the molecular weight of ribonucleotide reductase of *L. leichmannii* (Goulian and Beck, 1966; Vitols *et al.*, 1967b) indicate that this enzyme is unlikely to contain many subunits, and indeed suggest that it might be monomeric. It was therefore of interest to redetermine the molecular weight of the lactobacillus reductase, to determine whether any aggregation or gross conformational changes occur in the presence of modifiers and to establish the number of polypeptide chains constituting the catalytically active enzyme.

Materials

Materials were obtained commercially as follows: sodium dodecyl sulfate and urea, Fisher (both chemicals were recrystallized from 95% ethanol); guanidine hydrochloride (enzyme grade), ammonium sulfate, imidazole, and diisopropyl fluorophosphate (DFP) treated bovine pancreatic carboxypeptidase A, Mann Research Laboratories; 3,3-dimethylglutaric acid, Tris, serononin creatinine sulfate, didimethylaminonaphthalenesulfonyl chloride (dansyl chlo-

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