Ferritin Synthesis by Free and Membrane-Bound (Poly)ribosomes of Rat Liver[1] (35906)

D. G. PURO AND G. W. RICHTER

Department of Pathology, The University of Rochester Medical Center, Rochester, New York 14620

In liver cells (poly)ribosomes[2] are either attached or unattached to the endoplasmic reticulum. Siekevitz and Palade (1) suggested that membrane-bound (poly)ribosomes are involved in the synthesis of proteins to be exported from cells and free (poly)ribosomes in the production of proteins which ordinarily remain within the cells. Ferritin belongs to the latter category. Redman (2), who used *in vivo* and *in vitro* methods, and Hicks *et al.* (3), who used *in vitro* preparations suggested that ferritin is synthesized mainly by free ribosomes (free polyribosomes). Because cell-free mammalian systems that can synthesize specific proteins are still in the developmental stage, comparisons of *in vitro* activities in different cell fractions that contain (poly)ribosomes may be premature. In the two reports just cited, electron micrographs were not included to document the composition of the fractions isolated. Redman (2) estimated the amount of tritium-labeled ferritin in his cell fractions after immunoprecipitation with antibodies, but he had to use a large correction factor because the immunoprecipitates contained labeled contaminants.

Studies done for other purposes led us to investigate independently whether ferritin is synthesized on free (poly)ribosomes or on (poly)ribosomes attached to membranes.

Materials and Methods. Female Sprague-Dawley rats, weighing from 200 to 250 g, were maintained on a Purina Chow diet and injected subcutaneously with 10 mg of iron-dextran biweekly to increase the amount of ferritin in the livers. The rats were fasted overnight and injected intraperitoneally with L-leucine-4,5-^{3}H (0.5 mCi) at the appropriate time before decapitation by guillotine. The livers were promptly removed and chilled in several volumes of 0.25 *M* sucrose in TKC (0.05 *M* Tris HCl, pH 7.5 at 20°, plus 0.025 *M* KCl, and 0.015 *M* CsCl). Fractions containing (poly)ribosomes were isolated by the methods of Blobel and Potter (5), but using cesium chloride rather than magnesium chloride because of Dallner's report (6) that rough, but not smooth, microsomes are aggregated by CsCl. All centrifugations were performed in a Spinco Model L centrifuge with a No. 50 Ti rotor. The livers were minced with scissors and homogenized in a Dounce ball-type homogenizer by 25 strokes of the loose-fitting pestle. The homogenate was filtered twice through Curity No. 60 cheesecloth, then spun for 10 min at 17,000*g*. The flow chart, Fig. 1, shows the cell fractionation scheme.

The pellet containing the membrane-bound (poly)ribosomes was diluted 1:1 (v/v) with 4% Triton N-101 and 1% deoxycholate (DOC) in distilled water to dissolve the membranes and release the contents of the microsomal vesicles. Then ferritin was isolated from the Triton–DOC-treated pellet and from the pooled fractions containing free (poly)ribosomes and other cytoplasmic constituents, by a method based on that of Linder-Horowitz *et al.* (4). Homogenates were heated to 80–85°, kept in this temperature range for 10 min, then chilled for 15 min. A "heat supernatant" was obtained by centrifuging at 15,000*g* for 20 min. The heat supernatant was brought to a pH of 4.8 in the cold by addition of 0.1 vol of 0.2 *M* sodium

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[2] The term (poly)ribosomes, as used in this report, refers to polyribosomes (polysomes) as well as to individual ribosomes (monosomes), and to possible mixtures of polysomes and monosomes.

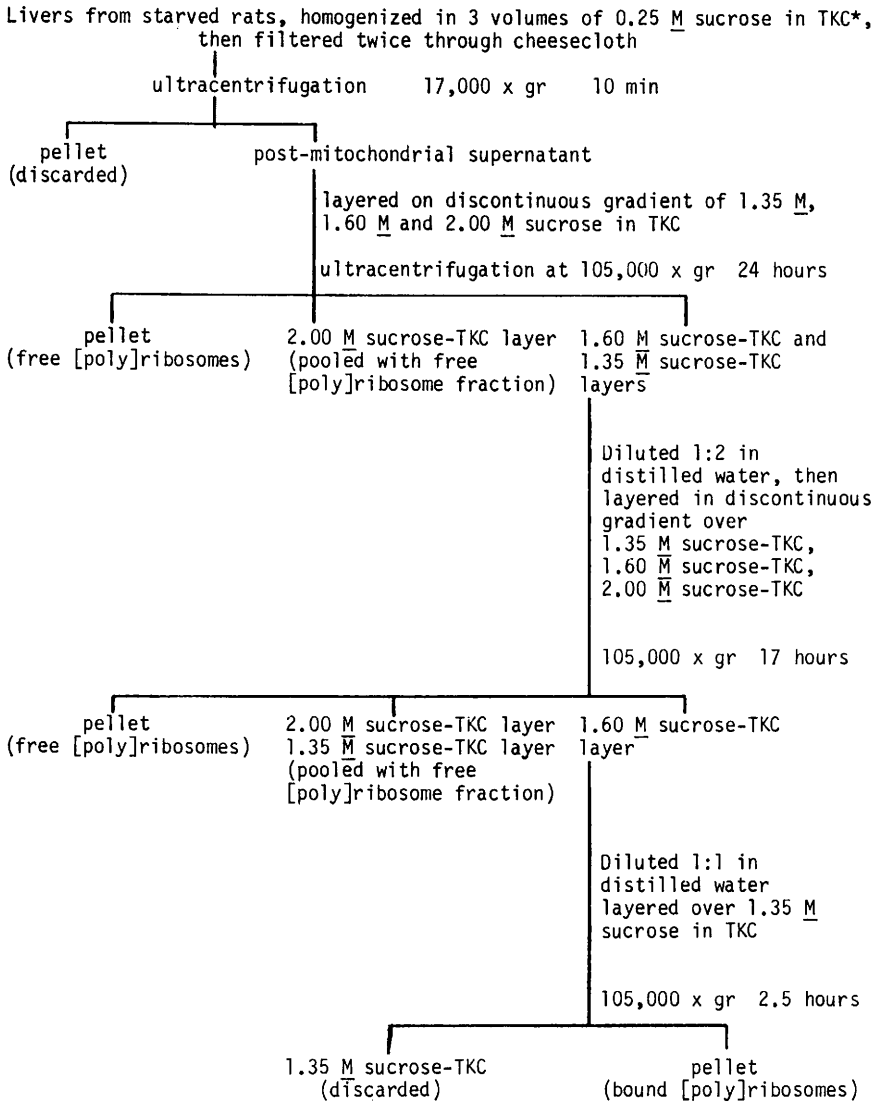

FIG. 1. Procedure for the separation of membrane-bound from free ribosomes.

acetate buffer (pH 4.8), and drop-by-drop addition of 0.2 *M* acetic acid. After 30 min at 0°, the extract was centrifuged for 10 min at 15,000*g* and the supernatant was brought to approximately pH 6.5 by addition of 0.1 vol of 0.2 *M* KH_2PO_4. Then ferritin was precipitated with $(NH_4)_2SO_4$ at 50% saturation and resuspended in 0.9% saline solution. This solution of ferritin was incubated for 60 min at 37° after addition of measured amounts of a solution of rabbit gamma globulin containing antibodies specific for rat liver ferritin, then kept at 4° overnight. Incubation of the ferritin solution without antibody did not result in a precipitate after cooling. The antibodies were raised in rabbits by injecting highly purified rat ferritin prepared according to the procedure of Walker and Richter (10). The specificity of the antibodies was confirmed by immunodiffusion

TABLE I. Incorporation of L-Leucine-4,5-^{3}H into Ferritin and into CCl_3COOH-Insoluble Proteins in Fractions Containing Free and Membrane-Bound (Poly)ribosomes.[a]

(Poly) ribosomal fraction	Total protein (dpm/mg of RNA)	Ferritin (dpm/mg of RNA)	Estimated contamination[b] (dpm/mg of RNA)	Ferritin (%)
Expt. 1				
free	380,000	3500		0.93
bound	1,100,000	3400	210	0.31
Expt. 2				
free	322,000	8300		2.60
bound	450,000	2150	500	0.48
Expt. 3				
free	197,000	6600		3.40
bound	560,000	3570	400	0.64
Expt. 4				
free	151,000	1930		1.28
bound	390,000	1390	150	0.36

[a] Values for each experiment of duplicate determinations, done as described in the text. The rats were injected intraperitoneally with 0.5 mCi of L-Leucine-4,5-^{3}H and decapitated 10 min later.

[b] The degree of contamination in the membrane-bound ribosome fraction was determined by adding ^{3}H-ferritin to the initial homogenate, obtained from rats that had not been injected with ^{3}H-leucine before sacrifice, and then proceeding with the isolation. Any labeled ferritin in the membrane-bound ribosome fraction was considered to be due to contamination. The results indicate that 6% (av of two isolations) of the labeled ferritin originally in the free (poly)ribosome fraction can be recovered in the membrane-bound pellet.

(Ouchterlony method). The use of specific antibodies after the indicated isolation procedure, makes it likely that only ferritin was precipitated with the antibody. The other fractions obtained during isolation of ferritin were pooled, diluted 1:1 with 10% CCl_3COOH, heated in a boiling water bath for 15 min, then centrifuged 15 min at 3000*g*. The resulting pellet, which was washed with 5% CCl_3COOH (1:1, v/v), contained protein; whereas the supernatant contained RNA. The amount of RNA was determined by a modified Schmidt-Tannhauser procedure (7) using rat liver RNA as standard.[3] The protein fractions (ferritin, CCl_3COOH-insoluble proteins) were dissolved in 1 ml of NCS solubilizer (Amersham/Searle Corp.), and the solution was mixed with 10 ml of toluene phosphor. The mixture was counted in a Tri-Carb liquid scintillation counter with internal quenching.

Portions of pellets taken for electron microscopy were fixed in 2% glutaraldehyde at 4° for 3 hr and post-fixed in 1% OsO_4 at 4° for 1 hr. The samples were dehydrated, and embedded in Epon 812 epoxy resin by standard procedures (9). Thin sections cut on an ultramicrotome were stained with uranyl acetate and lead citrate by well-known procedures.

Results and Discussion. Our data on incorporation *in vivo* of L-leucine-4,5-^{3}H into ferritin show that labeled ferritin was present in both, cell fractions containing free (poly)ribosomes and cell fractions containing (poly)ribosomes attached to membranes (rough ER) (Table I). The specific radioactivity in the pellets containing attached (poly)ribosomes was significant because this radioactivity was approximately 10 times the expected level of contamination (Table I).

The micrograph (Fig. 2) indicates qualita-

[3] We thank Dr. Alexander Dounce, Dept. of Biochemistry, University of Rochester, for the gift of this reference material.

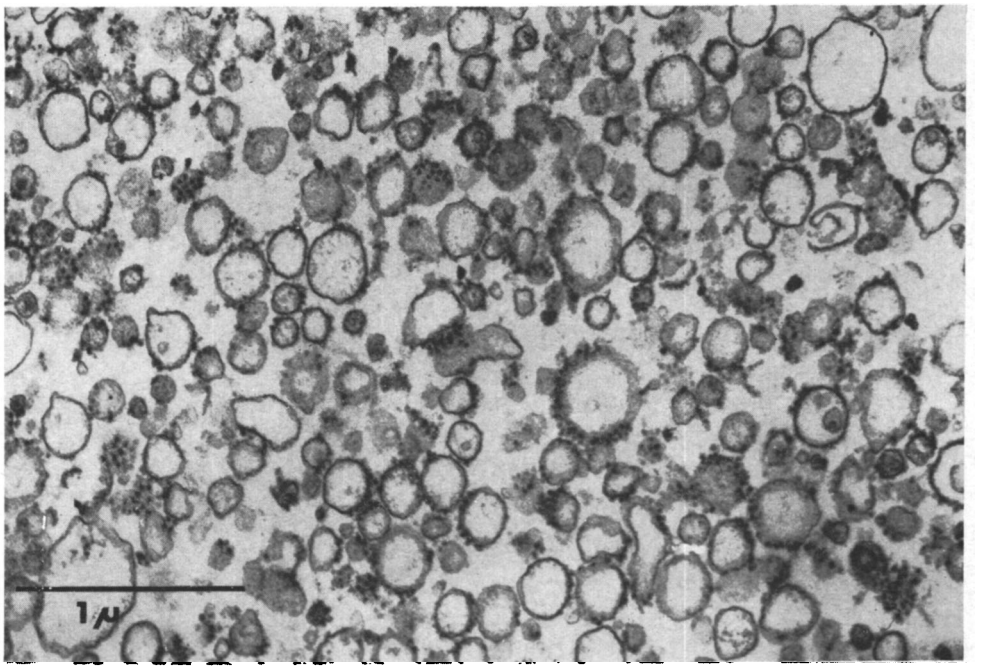

FIG. 2. Electron micrograph of microsomal pellet showing vesicles of rough-surfaced endoplasmic reticulum. Note attachment of (poly)ribosomes to vesicles, some of which are seen *en face*. Section stained with uranyl acetate and lead citrate; ×30,000.

tively the degree of separation of membrane-bound (poly)ribosomes from free polysomes (or ribosomes). The quantity of free (poly)ribosomes in the membrane-bound fractions was estimated by a modification of Redman's procedure (2) as follows. Aliquots of pellets of membrane-bound polysomes were diluted with a 1:1 (v/v) mixture of 4% Triton-101 and 1% DOC (in distilled water). Other aliquots of the pellets were suspended in 0.9% aqueous NaCl solution. These two kinds of suspensions were layered over sucrose–TKC zones of 1.35 *M* and 2.00 *M*, and centrifuged at 105,000*g* for 24 hr. RNA was determined in material that had sedimented through the 2.00 *M* sucrose–TKC layer. Membrane-bound (poly)ribosomes cannot pass through the 2.00 *M* sucrose–TKC layer (8). Any RNA-containing pellet obtained from material *not* treated with Triton–DOC represents contamination by free (poly)ribosomes. In this way, we found that 4 to 8% of the RNA in the membranous pellet was due to RNA of free ribosomes (or polyribosomes).

Contamination of the membrane-bound (poly)ribosome fraction with ferritin from outside the endoplasmic reticulum (ER) was estimated by adding aliquots of ^{3}H-ferritin to the initial liver homogenates of rats, which had not been given any L-leucine-4,5-^{3}H. The membrane-bound (poly)ribosomes were separated from the free polysomes (or ribosomes) and cytoplasm. Any labeled ferritin in the bound fraction was considered to be contaminant. This control check indicated that from 3.5 to 9.5% of ferritin outside the ER may have been isolated together with the membrane-bound (poly)ribosome fraction in the course of the fractionation procedure we used.

Rats loaded with iron by repeated injections of iron-dextran synthesize and store large amounts of ferritin in their livers. Electron micrographs of the livers revealed huge quantities of ferritin molecules in the cytoplasmic matrix, in siderosomes or in compound lysosomes, but not in the cisternae of the rough or smooth ER. If (poly)ribosomes attached to the ER do have a role in ferritin synthesis, it would have to be in the synthesis of apoferritin which cannot be seen in

embedded, sectioned tissue in the electron microscope. In that case, insertion of iron would have to occur outside the rough ER.

As shown by the checks for efficiency of cell fractionation, the method of isolation separated free (poly)ribosomes from bound (poly)ribosomes with relatively little contamination, and it is particularly noteworthy that the membranous pellets revealed no ferritin molecules inside rough ER vesicles.

Thus, both, membrane-bound (poly)ribosomes and free (poly)ribosomes, participate in the synthesis of the protein moiety of ferritin, but free (poly)ribosomes synthesize substantially more of the protein per milligram of RNA.

Summary. To learn where ferritin is synthesized—on free and/or membrane-bound (poly)ribosomes—fractions rich in (poly)ribosomes were isolated from rat livers by the method of Blobel and Potter (5), with modifications. The degree of separation of membrane-bound (poly)ribosomes from free (poly)ribosomes was checked by electron microscopy, biochemically, and with a radioactive tracer. Results of experiments on the incorporation of labeled amino acids into the protein of ferritin (apoferritin) indicate that both, membrane-bound (poly)ribosomes and free (poly)ribosomes are implicated in the synthesis of apoferritin, and that free (poly)ribosomes synthesize substantially more apoferritin per milligram RNA than bound (poly)ribosomes.

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