The Inhibition of Rat Liver Polyribosome Breakdown in the Presence of Liver Supernatant*

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G. Ross Lawford,† Peter Langford, and Harry Schachter
From the Department of Biochemistry, University of Toronto, Toronto 5, Canada

SUMMARY

Rat liver ribosomes prepared from the rapidly sedimenting pellet of a liver homogenate or from the microsomal pellet by the treatment of these pellets with sodium deoxycholate in buffer are deficient in polyribosomes and contain primarily ribosome dimers. Dimer formation can be prevented and a good polyribosome pattern can be obtained from both these pellets provided treatment with sodium deoxycholate takes place in the presence of liver supernatant. It has been shown that the polyribosomes isolated from the rapidly sedimenting pellet incorporate glycine into acid-insoluble material in vivo and are broken down into single ribosomes and ribosome dimers by ribonuclease.

It is generally recognized that growing polypeptide chains are found to be associated with ribosomal aggregates held together by a strand of messenger ribonucleic acid (polyribosomes or ergosomes) rather than with single ribosomes. This concept of protein synthesis is supported by studies of bacteria (1, 2), reticulocytes (3–8), rat liver (9–12), and a variety of other tissues (13–17). A growing polypeptide chain remains attached to one of the ribosomes of a polyribosome while the messenger RNA containing the code for the amino acid sequence moves relative to the ribosome (1, 8, 12, 13, 18).

Liver ribosomes can be prepared by two methods from postmitochondrial supernatant. In one of these methods (19–21), the microsomal pellet is prepared from postmitochondrial supernatant by centrifugation at 144,000 × gmax for 60 min and is then treated with sodium deoxycholate (deoxycholate) to solubilize lipoprotein in the endoplasmic reticulum. The ribosomes are then separated from the solubilized membrane material by centrifugation at 144,000 × gmax for 120 min. In the other method (10, 19), the postmitochondrial supernatant is treated with deoxycholate and the ribosomal pellet is sedimented at 144,000 × gmax for 120 min. The disruption of microsomes by deoxycholate thus occurs in the presence of liver supernatant.

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† The abbreviation used is: gmax, the maximum centrifugal force exerted on the centrifuge tube.

EXPERIMENTAL PROCEDURE

Materials

Male albino rats, Wistar strain, weighing 150 to 200 g were used. Glycine-2-¹⁴C (2.8 mC per mmole) was obtained from New England Nuclear Corporation. Sodium deoxycholate was special

Wettstein, Staehelin, and No1 (10) subjected ribosomes obtained by the second of these methods to zone centrifugation analysis in a sucrose density gradient. A predominance of monomers (single ribosomes) and dimers (aggregates containing two ribosomes) was observed in their early preparations. By maintaining low temperatures throughout their procedure they obtained a larger proportion of polyribosomes. Low temperature probably lowers the activity of ribonuclease released from the liver cell during its disruption and thereby prevents excessive destruction of the messenger RNA responsible for polyribosome structure. Although we obtained good polyribosome patterns by the procedure of Wettstein, Staehelin, and Noll (10), we found that ribosomes prepared by treatment of the microsomal pellet with deoxycholate in buffer yielded mainly dimers on zone centrifugation analysis in a sucrose density gradient. However, if the microsome pellet is treated with deoxycholate in liver supernatant and the ribosomes are then sedimented in the presence of liver supernatant, the breakdown into dimers is prevented and the ribosome preparation is rich in polyribosomes.

Over two-thirds of the ribosomes in rat liver sediment in the centrifugal fields used to prepare postmitochondrial supernatant (22, 23) and are therefore lost in the preparation of ribosomes from this supernatant. Howell, Loeb, and Tomkins (23) have reported a method of purifying ribosomes from these rapidly sedimenting pellets by treating the pellets with deoxycholate in buffer and separating ribosomes from the resulting viscous solution by centrifugation through sucrose layers. Howell, Loeb, and Tomkins (23) found these ribosomes to contain primarily dimers and claimed that this dimer pattern was not due to release of ribonuclease activity during preparation. However, we found that liver supernatant can prevent dimer formation and that the ribosomes in rapidly sedimenting pellets from rat liver homogenates are rich in functional polyribosomes. We attribute the action of liver supernatant to the presence of one or more ribonuclease inhibitors (24–26) which prevent destruction of messenger RNA by nuclease activity released during the disruptive action of deoxycholate on microsomes and other subcellular organelles.
enzyme grade material obtained from Mann Research Laboratories. Crystalline ribonuclease was purchased from Worthington. Buffer A was 0.05 M Tris HCl, pH 7.6, 0.005 M MgSO₄, and 0.025 M KCl. Buffer B was 0.25 M sucrose in Buffer A (10, 27).

**Injection of Isotope**

³⁵C-Glycine, 50 μCi in 1.0 ml of 0.9% NaCl was injected into the portal vein over a period of about 30 sec while the rat was under ether anesthesia. The liver was removed 5 min after the start of the injection while the rat was still under anesthesia.

**Preparation of Ribosomes**

All of the rats were fasted for 20 hours prior to the experiment to remove liver glycogen which interferes with the preparation of ribosomes (19). Livers, removed either under ether anesthesia or after stunning and decapitation, were washed in ice-cold Buffer B. All of the remaining procedures were performed at 0–4°C; rotors and centrifuges were cooled to −4°C prior to runs. The washed liver was cut into small pieces and was homogenized in Buffer B with 6 strokes of a motor-driven glass-Teflon Potter-Elvehjem homogenizer (Arthur H. Thomas Company, size C).

**DR and RP Ribosomes**—About 2 g of washed liver were homogenized in 15 ml of Buffer B and centrifuged at 20,000 × gₘₐₓ for 20 min to yield a pellet (D) containing nuclei, mitochondria, and other heavy material. The postmicrosomal supernatant was recentrifuged at 20,000 × gₘₐₓ for 15 min to ensure complete absence of heavy particles. Pellet D was homogenized as above in 15 ml of Buffer B after which 1.5 ml of 14.3% deoxycholate were added with stirring to give a final deoxycholate concentration of 1.3%. The solution was centrifuged at 20,000 × gₘₐₓ for 20 min but frequently a firm pellet failed to form. The peloton supernatant was layered in 5.5-ml aliquots over 4.0 ml of 0.5 M sucrose in Buffer A and 3.0 ml of 1.8 M sucrose in Buffer A in a 13 ml centrifuge tube as described by Wettstein, Stachelin, and Noll (10). The tubes were centrifuged at 144,000 × gₘₐₓ for 120 min, the supernatant layers were removed by suction, and the inside walls of the tubes were wiped dry with absorbent paper. The transparent pellet was suspended in 1 ml of Buffer A, centrifuged at 2,000 rpm for 5 min in a clinical centrifuge to remove a small amount of aggregated material, and then appropriate amounts were layered on a sucrose density gradient for zone centrifugation analysis as described below. This ribosome suspension is called “DR ribosomes” and is similar to the preparation described by Howell, Loeb, and Tomkis (23). A similar purification by centrifugation through layers of sucrose solutions was applied to the deoxycholate-containing solutions obtained in the various procedures for preparing ribosomes described below.

The postmicrosomal supernatant obtained above was made 1.3% in deoxycholate by adding 14.3% deoxycholate with stirring, and a ribosomal pellet was prepared by centrifugation through sucrose layers and resuspended, as described above. These ribosomes are called “RP ribosomes” and are almost the same as the “C ribosomes” described by Wettstein, Stachelin, and Noll (10).

**MPS Ribosomes**—About 2 g of washed liver were homogenized in 10 ml of Buffer B. Nuclei, mitochondria, and other heavy particles were removed by centrifugation at 20,000 × gₘₐₓ for 20 min, repeated twice. The postmicrosomal supernatant was centrifuged at 320,000 × gₘₐₓ for 30 min in a T-60 centrifuge (International) equipped with a 965 rotor. The resulting microsomal pellet was suspended in 10.0 ml of Buffer B and then 1.0 ml of 14.3% deoxycholate was added. The solution was centrifuged through sucrose layers, and a ribosome suspension was prepared as described above. These ribosomes are called “MPS ribosomes.”

**DRS Ribosomes**—About 2 g of washed liver were left undisturbed in ice-cold Buffer B while 10.0 ml of postmitochondrial supernatant were prepared from another 2 g of liver as described above. Microsomes were removed from the postmitochondrial supernatant by centrifugation at 320,000 × gₘₐₓ for 30 min. The other piece of liver was then homogenized in 10.0 ml of Buffer B, and Pellet D was obtained by centrifugation at 20,000 × gₘₐₓ for 20 min. Pellet D was homogenized in 10.0 ml of postmicrosomal supernatant and then 1.0 ml of 14.3% deoxycholate was added with stirring. Ribosomes were sedimented through sucrose layers and resuspended as described above. This preparation is called DRS-1.

In order to increase the relative amount of supernatant used in the preparation of DRS ribosomes, postmicrosomal supernatant was prepared by homogenizing the livers from 2 rats in 24.0 ml of Buffer B and removing particulate matter by successive centrifugations at 20,000 × gₘₐₓ for 20 min (performed twice) and at 320,000 × gₘₐₓ for 30 min. Liver, 2 g, from a third rat was then homogenized in 10.0 ml of Buffer B, and Pellet D was obtained and homogenized in 24.0 ml of postmicrosomal supernatant. The homogenate was made 1.3% in deoxycholate, and ribosomes were purified and resuspended as described previously. This ribosome preparation is called DRS-2.

**MPS Ribosomes**—About 2 g of washed liver were used to prepare 10.0 ml of postmicrosomal supernatant and a microsome pellet by successive centrifugation of the liver homogenate at 20,000 × gₘₐₓ for 20 min (performed twice) and 320,000 × gₘₐₓ for 30 min. The microsome pellet was resuspended in the postmicrosomal supernatant and then 1.0 ml of 14.3% deoxycholate was added. Ribosomes were purified and resuspended as described above. The preparation is called MPS.

**Zone Centrifugation in Sucrose Density Gradient and Measurement of Radioactivity**

Linear sucrose density gradients, 0.3 M to 1.0 M sucrose in Buffer A, 27 ml total volume, were prepared over a period of 30 min each, with a Buchler gradient-forming device and precision pump (28, 29). The gradients were prepared at 4°C and remained at that temperature for 24 hours prior to use. The absorbances at 260 μm of the various ribosomal suspensions were measured and aliquots were layered on the gradients such that the input on every gradient was 20 absorbance units for Preparations RP, MPS, and DRS, and 12 units for Preparations DR and MP. The gradients were centrifuged in a SW25.1 rotor at 25,000 rpm for either 2 or 3 hours in a Spinco model L centrifuge; rotor and centrifuge were cooled to −4°C prior to the run. Fractions of 1 ml each were collected from the bottom of the centrifuge tube, and absorbances at 260 μm were measured on a Beckman model DB spectrophotometer; concentrated samples were diluted with water to give an absorbance of 0.7 or less prior to taking the reading. Bovine serum albumin (0.25 mg) was added to every fraction as a carrier followed by 1.0 ml of 12% trichloracetic acid. The samples were left at 4°C overnight and were filtered two at a time on Millipore type HA membranes (0.45-μ pore size, 25-mm disk diameter). The membranes were washed with 6% trichloracetic acid and with chloroform-ether-methanol (2:1:1, v/v),
dried at 50°, and placed in a counting vial to which was added 15 ml of a solution containing 3 g of p-terphenyl and 30 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per liter of toluene. Radioactivity was determined in a Nuclear-Chicago model 6725 liquid scintillation counter at an efficiency of 72 to 75% calculated by the channels ratio method.

RESULTS

Fig. 1 shows the pattern obtained on zone centrifugation analysis of RP ribosomes. It is apparent that there is a large proportion of polyribosomes (the fraction between 1.0 and 15.0 ml of effluent) according to criteria put forth by Wettstein, Stachelin, and Noll (10). Further, 5 min after the injection of 14C-glycine, acid-insoluble radioactivity is found primarily over this polyribosome region indicating these ribosomes to be active in protein synthesis (1, 5, 23). Fig. 2 confirms the findings of Howell, Loeb, and Tomkins (23) that DR ribosomes contain primarily dimers (15 to 18 ml of effluent) and that following intravenous injection of 14C-amino acid, the acid-insoluble radioactivity corresponds exactly to the absorbance pattern of these ribosomes.

Fig. 3 shows that MP ribosomes give a pattern indistinguishable from that of DR ribosomes (Fig. 2). The dimer peak is predominant and the polyribosome region is relatively insignificant. However, the characteristic polyribosome pattern can be obtained from both the microsome pellet and Pellet D (defined under "Experimental Procedure") if ribosomes are prepared in the presence of liver supernatant. Fig. 4 shows that MPS ribosomes have the same pattern as RP ribosomes. Figs. 5 and 6 show that liver supernatant prevents excessive dimer formation when ribosomes are prepared from Pellet D. Preparation DRS-2 shows a somewhat better polyribosome pattern than Preparation DRS-1 since a relatively larger amount of supernatant was used in preparing DRS-2 ribosomes.

Fig. 6 shows the pattern of acid-insoluble radioactivity in DRS ribosomes when Pellet D is obtained from a rat previously treated by injection with 14C-glycine. Incorporation is highest in the polyribosome region (1.0 to 15.0 ml of effluent) indicating that the ribosomes in Pellet D which are active in protein synthesis are polyribosomes and not dimers as suggested by Howell, Loeb, and Tomkins (23).

It is possible that the DRS pattern may have been partly due to contamination of postmicrosomal supernatant with micro-
region (Fig. 6) than over the dimer and monomer regions (the fractions from 17 to 19 ml and 20 to 22 ml, respectively); the polyribosomes cannot therefore be solely due to aggregation of dimers and monomers during the preparative procedure.

When a suspension of DRS ribosomes is treated with ribonuclease (1 μg in 0.5 ml of suspension) for 15 min at 4° prior to zone centrifugation, the polyribosomes disappear and are replaced by a predominant monomer peak and a smaller dimer peak. This indicates that the polyribosomes in Pellet D are probably held together by a strand of messenger RNA (8, 10).

**DISCUSSION**

It has been shown that when ribosomes are prepared by treating either the microsomal pellet or the rapidly sedimenting Pellet D from a liver homogenate with deoxycholate in buffer, the content of polyribosomes is low and the predominant species is a ribosome dimer. However, if these pellets are treated with deoxycholate in liver supernatant, dimer formation is prevented and a characteristic polyribosome pattern is obtained. Liver supernatant is known to contain a very potent ribonuclease inhibitor (24–26) and the most likely explanation for the protective action of liver supernatant toward polyribosomes is its content of this inhibitor. Microsomes and other subcellular components contain various ribonucleases which become active during cell rupture and can thus destroy the messenger RNA which holds the polyribosomes together; the predominance of dimers rather than monomers is probably due to Mg++ binding since dialysis of DR ribosomes against 0.0001 M Mg++ results in complete disaggregation to single ribosomes (23).

The role of the ribonuclease inhibitor in liver is a matter for speculation. The turnover rate of messenger RNA has been suggested as an important control mechanism for the process of protein synthesis (2, 9, 11, 14, 30, 31). This turnover rate can be affected either by changes in the rate of DNA-dependent messenger RNA synthesis or by changes in the rate of messenger RNA breakdown; the latter may be controlled partially at least by a ribonuclease inhibitor. It is interesting that Webb, Blobel, and Potter (31) have reported that the C ribosomes prepared from the postmitochondrial supernatant of several hepatomas are characterized by a markedly high proportion of dimers and monomers. Although Webb, Blobel, and Potter (31) have ruled out the presence of excessive ribonuclease activity in hepatoma homogenates, the level of ribonuclease inhibitor in hepatoma was not determined and, in fact, a low level of this inhibitor in the tumor tissue could explain the ribosome patterns obtained.

As Howell, Loeb, and Tomkins (23) have pointed out, the rapidly sedimenting pellet from rat liver homogenate (Pellet D) contains a large proportion of the liver's ribosomes. They claim that deoxycholate detaches the ribosomes in Pellet D from messenger RNA and results in Mg++-dependent dimerization. They have shown that these DR ribosomes are capable of protein synthesis in vitro only when synthetic messenger RNA (polyuridylic acid) is added. We have shown that deoxycholate treatment of Pellet D can result in the preparation of ribosomes rich in polyribosomes, provided liver supernatant is present during deoxycholate treatment. The polyribosomes of these DRS ribosomes are active in protein synthesis in vitro and are degraded by ribonuclease. Therefore, the only difference between the polyribosomes in Pellet D and those in the postmitochondrial supernatant appears to be that Pellet D ribosomes may be attached to large pieces of endoplasmic reticulum, possibly connected quite tightly to cell nuclei and other subcellular organelles, and thus sediment in low centrifugal fields. Pellet D ribosomes also contain intranuclear ribosomes (32). In other words, although our work and that of Howell, Loeb, and Tomkins (23) indicate no qualitative differences between DRS and RP ribosomes, it is likely that there may be a difference in the loca-
tion of these ribosomes within the cell. The DRS ribosomes may be attached to a more central and more extensive membrane network and may thus be involved in the biosynthesis of different proteins than the more peripherally located RP ribosomes. The difference in the \(^{14}C\)-glycine labeling patterns of RP ribosomes (Fig. 1) and DRS ribosomes (Fig. 6) may indicate a difference in the functions of these two types of ribosomes. However, the most likely explanation for the relatively greater labeling of monomers and dimers in the DRS preparation than in the RP preparation is that there is greater breakdown of radioactive polysomes into monomers and dimers in the DRS ribosomes than in the RP preparation. The latter explanation is compatible with the finding that larger amounts of supernatant are required to prevent polysome breakdown during the preparation of DRS ribosomes than during the preparation of an equivalent amount of MPS ribosomes (compare Figs. 4, 5, and 6), presumably because Pellet D has more nuclease activity than is present in the corresponding microsomal pellet.

REFERENCES
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