Initiation Factors in Protein Synthesis by Free and Membrane-Bound Polyribosomes of Liver and Hepatoma

By CHALLAKONDA N. MURTY, ETHEL VERNEY and HERSCHEL SIDRANSKY
*Department of Pathology, University of South Florida, College of Medicine,
Tampa, Fla. 33620, U.S.A.*

(Received 30 April 1975)

The activity of initiation factors obtained from free and membrane-bound polyribosomes of liver and of transplantable H5123 hepatoma of rats was investigated by using an assay of protein synthesis *in vitro* in which poly(U)-directed polyphenylalanine synthesis was measured. Initiation factors of membrane-bound polyribosomes prepared by using the anionic detergent deoxycholate exhibited less activity in incorporating [^{14}C]phenylalanyl-tRNA into polypeptides than did initiation factors of free polyribosomes. However, when membrane-bound polyribosomes were prepared after using the non-ionic detergent Triton X-100, no significant differences in activities in polyphenylalanine synthesis were observed between the initiation factors of free and membrane-bound polyribosomes. These results suggest that Triton X-100 is preferable to deoxycholate in the isolation of initiation factors from polyribosomes. Initiation factors, prepared by using Triton X-100, of free polyribosomes of hepatoma exhibited greater activity in the stimulation of polyphenylalanine synthesis than did the initiation factors of free or membrane-bound polyribosomes of host livers or of membrane-bound polyribosomes of hepatomas.

Ribosomes of liver and other tissues have been observed by electron microscopy to exist in two distinct topographical forms, those free in the cytoplasm and those associated with intracellular membranes of the rough endoplasmic reticulum (Palade & Siekevitz, 1956). Recent studies in our and other laboratories have indicated that the two polyribosome populations, in addition to being morphologically distinct, appear to have functional and metabolic differences. The two groups of polyribosomes have different rates in their turnover of rRNA (Hallinan & Munro, 1965; Murty & Hallinan, 1968; Blobel & Potter, 1968; Murty & Sidransky, 1974) and of mRNA (Murty & Sidransky, 1972), they respond differently to drugs (Blobel & Potter, 1967; Reid *et al.*, 1971; Pilakis & Korner, 1971; Hill & Saunders, 1971; Glazer & Sartorelli, 1972), to hormones (Pilakis & Korner, 1971) and to physiological and dietary alterations (Sidransky *et al.*, 1969, 1971; Gaetani *et al.*, 1969; Enwonwu, 1972), and they synthesize different proteins (Hallinan *et al.*, 1968; Redman, 1969; Hicks *et al.*, 1969; Puro & Richter, 1971). In view of the biochemical differences, the present study was conducted to examine further the functional properties of free and membrane-bound polyribosomes of normal rat livers and of transplantable hepatomas of Buffalo rats, particularly in relation to cell-free protein synthesis.

Miller & Schweet (1968) first demonstrated the effect of the 0.5M-KCl ribosomal wash fraction on

haemoglobin synthesis *in vitro* by a cell-free system. Subsequently, a number of investigators have reported that the active components of the ribosomal wash in liver are protein factors that are required for the initiation of mRNA translation in eukaryotes (Grummt & Bielka, 1971; Murty *et al.*, 1971; Lynn *et al.*, 1973; Picciano *et al.*, 1973; Schreier & Staehelin, 1973). In a number of reports (Lynn *et al.*, 1973; Lanclos & Bresnick, 1972; Murty *et al.*, 1974a), alterations in the hepatic activity of initiation factors have been observed under certain experimental conditions. Also, the activity of initiation factors of transplantable hepatoma H5123 appeared to be different from that of host liver (Murty *et al.*, 1974b).

Shafritz & Isselbacher (1972), using rabbit liver, and Uenoyama & Ono (1972), using rat liver, have reported that the functional activities of ribosomal washes of free and membrane-bound polyribosomes in cell-free protein synthesis appeared to be different. This finding seemed to be important, since it has been established that hepatic membrane-bound polyribosomes synthesize proteins which are different from those synthesized by free polyribosomes (Hallinan *et al.*, 1968; Redman, 1969; Hicks *et al.*, 1969; Puro & Richter, 1971).

Isolation and fractionation of polyribosomes associated with or bound to membranes require that a detergent be used to release the polyribosomes from the membranes. Several studies have indicated that treatment of polyribosomes with non-ionic or anionic

detergents may have different effects on the properties of the polyribosomes. Hunter & Korner (1966) found that polyribosomes isolated after using Triton X-100 had a consistently higher ability to incorporate amino acids into protein in a cell-free system than did polyribosomes prepared after using sodium deoxycholate. Olsnes (1971) reported that treatment with sodium deoxycholate removed some protein from the polyribosomes and also disaggregated polyribosomes, whereas treatment with Triton X-100 did not. Therefore in the present study initiation factors were isolated from free and membrane-bound polyribosomes of rat liver in two different ways and their activities in poly(U)-directed polyphenylalanine synthesis were compared. The results indicated that the initiation factors of both classes of ribosomes exhibited different activities in polypeptide synthesis when the membrane-bound polyribosomes were treated with the anionic detergent deoxycholate. However, no differences were observed when membrane-bound polyribosomes were prepared after using the non-ionic detergent Triton X-100 in place of sodium deoxycholate. By using Triton X-100, initiation factors were prepared from free and membrane-bound polyribosomes of host livers and of intrahepatically transplanted H5123 hepatomas and their activities in protein synthesis *in vitro* were compared. The results revealed that the initiation factors of free polyribosomes of hepatoma exhibited greater activity in the stimulation of polyphenylalanine synthesis than that of initiation factors of either free of membrane-bound polyribosomes of host liver or of membrane-bound polyribosomes of hepatoma.

Methods

Materials

L-[U-¹⁴C]Phenylalanine (492 Ci/mol) was obtained from The Radiochemical Centre, Amersham/Searle, Des Plaines, Ill., U.S.A. Poly(U) (ammonium salt) was obtained from Miles Research Laboratories, Kankakee, Ill., U.S.A. ATP and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. GTP was obtained from General Biochemical Corp., Chagrin Falls, Ohio, U.S.A. Sodium deoxycholate was obtained from Schwarz-Mann, Orangeburg, N.Y., U.S.A. Aurintricarboxylic acid was obtained from Fisher Chemicals, Fair Lawn, N.J., U.S.A.

Tumour tissue and animals

Hepatoma H5123 was maintained by serial subcutaneous transplantations by using inbred female Buffalo rats (obtained from Simonsen Laboratories,

Gilroy, Calif., U.S.A.). In the present study hepatomas were transplanted intrahepatically into the left lobes of the livers of animals 20–30 days before they were killed. Tumour tissue was dissected free of surrounding host liver and of any necrotic areas, and was then homogenized in a suitable buffer as described below. Host livers were obtained from the same animals bearing hepatomas. In other experiments livers were obtained from female rats of the Sprague-Dawley strain (obtained from Sprague-Dawley, Madison, Wis., U.S.A.) maintained on stock diet and weighing 100–120 g.

Preparation of components

Animals were starved overnight but had free access to water until death. Free and membrane-bound polyribosomes were isolated by using the discontinuous-sucrose-gradient procedure described by Reid *et al.* (1971). Postmitochondrial supernatant (15 ml) buffered with Tris-KCl-MgCl₂ buffer (50 mM-Tris-HCl, pH 7.5, 25 mM-KCl, 5 mM-MgCl₂) was layered over a two-step discontinuous sucrose gradient of 7 ml each of 1.38 M- and 2.0 M-sucrose containing Tris-KCl-MgCl₂ buffer. The gradients were centrifuged at 78000g at 2°C for 24 h in a 30 rotor in a Beckman L3-40 ultracentrifuge. Under these conditions free polyribosomes were obtained as a pellet. The clear supernatant at the top of the gradient was removed and discarded. After removal of the 1.38 M-sucrose layer, which contained membrane-bound polyribosomes, it was treated with either sodium deoxycholate or Triton X-100 to a final concentration of 1%, layered over 7 ml of 2.0 M-sucrose in Tris-KCl-MgCl₂ buffer, and centrifuged at 78000g for 24 h in a 30 rotor. Membrane-bound polyribosomes sedimented as a pellet. Total polyribosomes were obtained by centrifuging detergent-treated post-mitochondrial supernatant over a similar two-step sucrose gradient for 24 h.

Initiation factors were prepared from total, free and membrane-bound polyribosomes as described previously (Lynn *et al.*, 1973; Murty *et al.*, 1974b). After preparation of the ribosomal pellets as described above, they were rinsed and resuspended in 2.5 ml of buffer A [containing 20 mM-Tris-HCl (pH 7.6), 5 mM-MgCl₂, 100 mM-KCl, 1 mM-dithiothreitol, 0.25 mM-EDTA and 10% (v/v) glycerol]. The ribosomal suspension was then treated with a high-salt buffer (20 mM-Tris-HCl, pH 7.6, 15 mM-MgCl₂, 2 M-KCl, 1 mM-dithiothreitol, 0.25 mM-EDTA and 10% glycerol) to a final concentration of 1 M-KCl. The suspension was maintained at 0°C for 1 h with gentle stirring and then the ribosomes were pelleted at 213000g for 2 h in a Spinco 50.1 rotor. The supernatant, which contained initiation factors, was removed and the excess of KCl was dialysed out against buffer containing 20 mM-Tris-HCl, pH 7.4, 5 mM-MgCl₂,

100mM-KCl, 1mM-dithiothreitol, 0.25mM-EDTA and 10% glycerol. The preparation was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The precipitate was dissolved in a buffer containing 20mM-Tris-HCl, pH7.4, 5mM-MgCl₂, 200mM-KCl, 1mM-dithiothreitol, 0.25mM-EDTA and 15% glycerol and dialysed against this same buffer, and then stored at -20°C. Salt-washed ribosomes were prepared from the pellets, obtained above after centrifugation of 1M-KCl-treated ribosomes, which were resuspended in a buffer (containing 20mM-Tris-HCl, pH7.4, 10mM-MgCl₂, 500mM-KCl, 1mM-dithiothreitol, 0.25mM-EDTA and 10% glycerol) and then centrifuged through 5ml of 25% sucrose containing the same buffer at 105000g for 5h. The pellets were resuspended and dialysed overnight against buffer A.

Preparation of [¹⁴C]phenylalanyl-tRNA and elongation factors

Labelled rat hepatic phenylalanyl-tRNA was prepared by the method of Moldave (1963) with some modifications described by Pilkis & Korner (1971). pH5 enzyme was prepared from rat liver and was used as a source of tRNA and of aminoacyl-tRNA synthetases. tRNA was charged with [U-¹⁴C]-phenylalanine (513Ci/mol) along with 19 unlabelled amino acids. A mixture of elongation factors I and II was prepared from pH5 supernatant of rat liver by the method of Moldave (1963).

Polymerization assay

Incorporation of phenylalanine into polypeptide from [¹⁴C]phenylalanyl-tRNA, under the direction of poly(U), was performed by the standard procedure described by Murty *et al.* (1974b). Incubation conditions are given in the appropriate Table legends.

Chemical determinations

Protein was determined by the method of Lowry *et al.* (1951), with purified serum albumin as a standard. RNA was determined by the method of Fleck & Munro (1962).

Results

Assay for initiation of protein synthesis in vitro

Poly(U)-directed polyphenylalanine synthesis with 1M-KCl-washed ribosomes at low Mg²⁺ concentrations (3–5mM) has been shown to require several protein factors which are recovered from the 0.5–1.0M-KCl washings (Grummt & Bielka, 1971; Murty *et al.*, 1971, 1974b; Lynn *et al.*, 1973; Picciano *et al.*, 1973; Schreir & Staehelin, 1973). In the present study, ribosomal wash fraction and salt-washed ribo-

somes prepared from free polyribosomes of normal rat liver were tested for poly(U)-directed polyphenylalanine synthesis. The results of a typical assay presented in Table 1 show that, under the conditions used, polymerization of [¹⁴C]phenylalanyl-tRNA is dependent on the presence of washed ribosomes, ribosomal wash factors, elongation factors, poly(U) and GTP. Addition of aurintricarboxylic acid, an inhibitor of initiation of protein synthesis (Stewart *et al.*, 1971), to the complete system markedly decreased the binding of [¹⁴C]phenylalanyl-tRNA to ribosomes.

In a previous report (Murty *et al.*, 1974b), we have demonstrated that the ribosomal wash fraction

Table 1. *Requirements for initiation of poly(U)-dependent polyphenylalanine synthesis by hepatic free ribosomes at low Mg²⁺ concentration*

Salt-washed ribosomes, initiation factors and elongation factors were prepared from the livers of rats starved overnight. The complete system was a reaction mixture of 600μl containing: salt-washed ribosomes (120μg of RNA); initiation factors (200μg of protein); elongation factors I and II (62.5μg); poly(U) (100μg); [¹⁴C]phenylalanyl-tRNA (7800 c.p.m.); Tris-HCl, pH7.4 (12μmol); Mg²⁺ (3μmol); K⁺ (60μmol); dithiothreitol (0.6μmol); GTP (0.4μmol); glycerol (10%, v/v, final concentration). Where indicated, the reaction mixture contained 0.12μmol of aurintricarboxylic acid. The reaction mixtures were incubated at 37°C for 30min and the reactions were stopped by the addition of an equal volume of ice-cold 10% (w/v) trichloroacetic acid. The mixtures were hydrolysed at 90°C for 15min and then filtered through a glass-fibre filter (type A25 millimetres; Gelman). The filters were washed three times with 10ml of ice-cold 5% trichloroacetic acid, dried in the air, and after addition of 15ml of toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazol-2-yl)benzene (3316:17:1, v/w/w) scintillation liquid were counted for radioactivity in a Packard liquid-scintillation spectrometer (model 3375) at a counting efficiency of 90% for ¹⁴C. Values represent means of three experiments. In each experiment, tissue from eight to ten animals was pooled.

System	[¹⁴ C]Phenylalanine incorporated into protein from [¹⁴ C]phenylalanyl-tRNA	
	(c.p.m.*)	(% of activity)
Complete	2604	100
Complete-ribosomes	144	6
Complete-initiation factors	676	26
Complete-elongation factors	1464	56
Complete-poly(U)	321	12
Complete-GTP	62	2
Complete+aurintricarboxylic acid	67	3

* 830 c.p.m. = 1pmol of phenylalanine.

contains activities of initiation factors by its ability to lower the Mg^{2+} concentration optimum for poly(U)-directed polyphenylalanine synthesis from 12 to 5 mM- Mg^{2+} and also by its ability to bind initiator tRNA, methionyl-tRNA, to the small ribosomal subunit directed by the synthetic template AUG. These results indicate that the conditions used to investigate the initiation of protein synthesis with free and membrane-bound polyribosomes are valid.

Initiation factors-dependent stimulation of polyphenylalanine synthesis by free and membrane-bound polyribosomes of normal rat liver

Initiation factors and salt-washed ribosomes were prepared from free polyribosomes and from membrane-bound polyribosomes either before or after solubilization of membranes with deoxycholate. The results in Table 2(a) indicate that the initiation factors from free polyribosomes stimulated polyphenylalanine synthesis by both types of ribosomes to a greater degree than did initiation factors prepared from membrane-bound polyribosomal fractions containing membranes. Similar findings were reported by Shafritz & Isselbacher (1972) for rabbit liver. However, the validity of such a comparison between the activities of initiation factors of free and membrane-bound polyribosomes is open to question, since the initiation factors prepared from the membrane-bound polyribosomes were diluted severalfold by membrane protein. Therefore this would account for the lower activity in polypeptide synthesis when compared with that of initiation factors of free polyribosomes, since the activities in each case were expressed on the basis of protein. Further, the results in Table 2(a) reveal that the initiation factors prepared from the deoxycholate-released membrane-bound polyribosomes also exhibited lower activity in the stimulation of polyphenylalanine synthesis when compared with that of initiation factors of free polyribosomes. Thus it appears that deoxycholate, used to free or liberate polyribosomes from membranes, is probably in some way affecting the activity of initiation factors.

Sodium deoxycholate, an anionic detergent, has been widely used in solubilizing membranes and in purifying ribosomes from protein contaminants. However, some reports have indicated that treatment with deoxycholate may affect the properties of polyribosomes. For example, studies by Murty & Hallinan (1969) and by Olsnes *et al.* (1972) have shown that deoxycholate disaggregates polyribosomes from heavier to lighter aggregates and that it removes protein that seems to be bound to the mRNA in polyribosomes. To test whether deoxycholate itself might affect the activity of initiation factors in the stimulation of polypeptide synthesis, we treated isolated free

polyribosomal fractions with sodium deoxycholate and then prepared initiation factors. In the same experiments we also treated some of the free polyribosomal fractions with another detergent, Triton X-100, and studied the activity of initiation factors in polypeptide synthesis. The results in Table 3 show that the initiation factors obtained from the deoxycholate-treated free polyribosomal fraction stimulated polypeptide synthesis to a much smaller extent than did the initiation factors prepared from either control untreated free polyribosomes or Triton X-100-treated free polyribosomes. These studies indicate that deoxycholate treatment causes a loss of activity of initiation factors, whereas Triton X-100 treatment does not significantly affect the activity of initiation factors in the stimulation of polyphenylalanine synthesis.

Having found that deoxycholate but not Triton X-100 affects the activity of initiation factors of free polyribosomes, we prepared membrane-bound polyribosomes after using either Triton X-100 or deoxycholate. Then initiation factors were prepared and their activities were compared with those of the initiation factors of free polyribosomes. Table 2(b) summarizes the results of these experiments and indicates that the initiation factors obtained from Triton X-100-treated membrane-bound polyribosomes exhibited similar activities in polypeptide synthesis to those of initiation factors of free polyribosomes. In contrast, initiation factors obtained from deoxycholate-treated membrane-bound polyribosomes revealed significantly lower activities in the polymerization of phenylalanine.

Effect of deoxycholate or Triton X-100 on the activities of initiation factors of total polyribosomes of normal rat liver

To evaluate further the effects of deoxycholate treatment on the activity of initiation factors in polypeptide synthesis, initiation factors were obtained from the total hepatic polyribosomes (free plus membrane-bound) prepared after using each of the detergents and their activities in the stimulation of polyphenylalanine synthesis were compared. The results in Table 4 show that the initiation factors of total polyribosomes obtained after deoxycholate treatment had significantly lower activities than that of initiation factors of total polyribosomes obtained after Triton X-100 treatment.

Effect of deoxycholate or Triton X-100 on the recovery of initiation factors from free and membrane-bound polyribosomes of normal rat liver

During the course of the preceding studies on the isolation of initiation factors, differences in the

Table 2. *Initiation factors-dependent stimulation of polyphenylalanine synthesis by ribosomes and initiation factors of free and membrane-bound polyribosomes of rat liver*

Initiation factors and salt-washed ribosomes were prepared from free polyribosomes and from membrane-bound polyribosomes, untreated or after solubilization of membranes by deoxycholate or Triton X-100 treatment. Assay for the synthesis of polyphenylalanine from [^{14}C]phenylalanyl-tRNA was carried out by using initiation factors (200 μg of protein) as described in the legend to Table 1. Values represent means of three to five experiments. In each experiment livers (30–40 g) were pooled. When initiation factors were not added, 586, 867, 621 and 677 c.p.m. respectively were obtained with free, membrane-bound, membrane-bound (deoxycholate-released) and membrane-bound (Triton X-100-released) ribosomes. These values were subtracted from the other values. In each experiment the controls were arbitrarily set at 100% activity and the other groups were expressed as percentages relative to controls, and average % activity values ($\pm$ S.E.M.) were calculated from these values. * $P < 0.05$; ** $P < 0.02$; † $P < 0.001$; according to Student's t test.

Ribosome type	Initiation factors added	[^{14}C]Phenylalanine incorporated into protein from [^{14}C]phenylalanyl-tRNA	
		(c.p.m.)	(% activity)
(a) Free	Free ribosomes	2084	100
	Membrane-bound ribosomes	1025	49 $\pm$ 8.6*
	Membrane-bound ribosomes (deoxycholate-released)	839	40 $\pm$ 7.3*
Membrane-bound	Free ribosomes	2121	100
	Membrane-bound ribosomes	615	29
	Membrane-bound ribosomes (deoxycholate-released)	415	20
Membrane-bound (deoxycholate-released)	Free ribosomes	2896	100
	Membrane-bound ribosomes	1371	47 $\pm$ 6.2**
	Membrane-bound ribosomes (deoxycholate-released)	1447	50 $\pm$ 0.1†
(b) Free	Free ribosomes	2159	100
	Membrane-bound ribosomes (deoxycholate-released)	920	43 $\pm$ 3.2†
	Membrane-bound ribosomes (Triton X-100-released)	2274	105 $\pm$ 5.8
Membrane-bound (deoxycholate-released)	Free ribosomes	2451	100
	Membrane-bound ribosomes (deoxycholate-released)	1100	45 $\pm$ 3.7†
	Membrane-bound ribosomes (Triton X-100-released)	2598	106 $\pm$ 8.3
Membrane-bound (Triton X-100-released)	Free ribosomes	2177	100
	Membrane-bound ribosomes (deoxycholate-released)	739	34 $\pm$ 2.3**
	Membrane-bound ribosomes (Triton X-100-released)	2162	99 $\pm$ 3.7

recoveries of protein were noticed in the 1 M-KCl washes of polyribosomes obtained from similar portions of livers after deoxycholate or Triton X-100 treatments. Therefore the possibility was considered that the decreased activity of the initiation factors of polyribosomes obtained after deoxycholate treatment could be due to less recovery of protein. Therefore in several experiments we studied quantitatively the yield of initiation-factor proteins (expressed as mg of protein in the ribosomal wash/mg of ribosomes) of polyribosomes isolated after either deoxycholate

or Triton X-100 treatment. The results in Table 5 show that there was indeed significantly less recovery of protein related to initiation factors of either membrane-bound or total polyribosomes isolated after deoxycholate treatment compared with that of membrane-bound or total polyribosomes isolated after Triton X-100 treatment. On the other hand, no significant differences were observed in the recovery of RNA from the free and the membrane-bound polyribosomal fractions prepared after using either deoxycholate or Triton X-100.

Table 3. *Effect of treating hepatic free polyribosomes with deoxycholate or Triton X-100 on the activity of initiation factors in polyphenylalanine synthesis*

Free polyribosomes were prepared from normal livers as described in the Methods section. When detergents were used, free polyribosomes were treated with either deoxycholate or Triton X-100 to a final concentration of 1.0% of the detergent and the ribosomes were pelleted by centrifugation at 40000g for 2h. Initiation factors and salt-washed ribosomes were isolated from deoxycholate-treated, Triton X-100-treated and untreated free polyribosome fractions as described in the Methods section. The assay for polyphenylalanine synthesis from [14 C]phenylalanyl-tRNA was carried out as described in the legend to Table 1, by using initiation factors (200 μ g of protein). Values represent means of three or four experiments. In each experiment livers (30–40g) were pooled. When initiation factors were not added, 605, 791 and 531 c.p.m. respectively were obtained with free, free (deoxycholate-treated) or free (Triton X-100-treated) ribosomes. These values were subtracted from the other values. In each experiment the controls were arbitrarily set at 100% activity and the other groups were expressed as percentages relative to controls, and average % activity values $\pm$ s.e.m. were calculated from these values. * $P < 0.05$; ** $P < 0.02$; according to Student's t test.

Ribosome type	Initiation factors added	[14 C]Phenylalanine incorporated into protein from [14 C]phenylalanyl-tRNA	
		(c.p.m.)	(% activity)
Free	Free ribosomes	2444	100
	Free ribosomes (deoxycholate-treated)	368	15 $\pm$ 3.2*
	Free ribosomes (Triton X-100-treated)	2251	92 $\pm$ 10.1
	Free ribosomes	2595	100
Free (deoxycholate-treated)	Free ribosomes (deoxycholate-treated)	675	26 $\pm$ 3.2**
Free (Triton X-100-treated)	Free ribosomes	2768	100
	Free ribosomes (Triton X-100-treated)	2793	101 $\pm$ 9.2

Table 4. *Effect of treating total hepatic polyribosomes with deoxycholate or Triton X-100 on the activities of initiation factors in polyphenylalanine synthesis*

Initiation factors and salt-washed ribosomes were isolated from deoxycholate-released and Triton X-100-released total polyribosomes of normal livers as described in the Methods section. Assay for polyphenylalanine synthesis from [14 C]-phenylalanyl-tRNA was carried out as described in the legend to Table 1 by using initiation factors (200 μ g of protein). Values represent means of three experiments. In each experiment livers (15–20g) were pooled. When initiation factors were not added, 707 and 656 c.p.m. were obtained with total ribosomes, Triton X-100-released and deoxycholate-released respectively. These values were subtracted from the other values. In each experiment the controls were arbitrarily set at 100% activity and the other groups were expressed as percentage of controls, and average % activity values $\pm$ s.e.m. were calculated from these values. * $P < 0.02$; ** $P < 0.01$; according to Student's t test.

Source of total ribosomes	Source of initiation factors	[14 C]Phenylalanine incorporated into protein from [14 C]phenylalanyl-tRNA	
		(c.p.m.)	(% activity)
Total ribosomes (Triton X-100-released)	Total ribosomes	5306	100
	Total ribosomes (Triton X-100-released)	1969	37 $\pm$ 6.3*
	Total ribosomes (deoxycholate-released)	5640	100
Total ribosomes (deoxycholate-released)	Total ribosomes (Triton X-100-released)	2496	44 $\pm$ 2.0**
	Total ribosomes (deoxycholate-released)		

Table 5. *Effect of treating free, membrane-bound and total polyribosomes with deoxycholate or Triton X-100 on the recovery of protein in the ribosomal wash fractions*

Free polyribosomes, deoxycholate-released and Triton X-100-released membrane-bound and total polyribosome fractions were prepared as described in the Methods section. The free polyribosome fraction was treated with either deoxycholate or Triton X-100 and the ribosomes were pelleted by centrifugation at 40000g for 2 h. Initiation factors were isolated from the polyribosome fractions, protein was measured and the recovery of protein in the initiation factors was expressed as mg of protein/mg of polyribosomes. The values represent means of three to five experiments. In each experiment livers (20–30 g) were pooled. * $P < 0.02$; ** $P < 0.01$; according to Student's t test.

Ribosome type	Detergent used	Recovery of protein in the ribosomal wash	
		(mg of protein/mg of polyribosomes)	(% recovery)
Free		0.380	100
Free	Deoxycholate	0.020	6 $\pm$ 4.3*
Free	Triton X-100	0.280	78 $\pm$ 11.5
Membrane-bound	Triton X-100	0.320	100
Membrane-bound	Deoxycholate	0.060	26 $\pm$ 6.0**
Total	Triton X-100	0.195	100
Total	Deoxycholate	0.062	31 $\pm$ 3.2**

Initiation factor-dependent stimulation of polyphenylalanine synthesis by free and membrane-bound polyribosomes of host liver and hepatoma

In a previous study (Murty *et al.*, 1974b) we observed that the initiation factors isolated from total polyribosomes of hepatoma H5123 exhibited significantly greater activity than did the initiation factors of host liver in the stimulation of polyphenylalanine synthesis as well as in the formation of 40 S ribosomal subunit–phenylalanyl-tRNA–poly(U) and 40 S ribosomal subunit–methionyl-tRNA–AUG complexes. Therefore we decided to investigate whether the greater activity of initiation factors of hepatoma was due to increased activity of free, membrane-bound or both fractions. The results presented in Table 6 show that the initiation factors from free polyribosomes of hepatoma exhibited more (210%) activity in the stimulation of polyphenylalanine synthesis than did the initiation factors of free or membrane-bound polyribosomal fractions of host liver or membrane-bound polyribosomes of hepatoma. Further, the results indicate that there were no alterations in the activity of membrane-bound polyribosomes of hepatoma compared with initiation factors of either free or membrane-bound polyribosomes of host liver.

Discussion

The results in the present paper show that there were no significant differences between the free and the membrane-bound ribosomal wash fractions of normal rat liver in the stimulation of polyphenylalanine synthesis when the non-ionic detergent

Triton X-100 was used to prepare the membrane-bound polyribosomes. The results also demonstrate that, when sodium deoxycholate, the most commonly used anionic detergent, was used to release ribosomes from membranes of the endoplasmic reticulum, the ribosomal wash fraction of the unattached membrane-bound polyribosomes showed considerably less activity in the stimulation of polyphenylalanine synthesis than when Triton X-100 was used to free the ribosomes from membranes. The differences in the activities of initiation factors obtained from deoxycholate-released and from Triton X-100-released membrane-bound polyribosomes of livers of normal rats suggest that one should be cautious in interpreting results when deoxycholate is used in the preparation and purification of polyribosomes.

Initiation factors, which are recovered by washing ribosomes with high salt concentration, are required for the initiation of protein synthesis and conceivably they may play an important role in the regulation of protein synthesis. Although several studies have been concerned with the metabolic and functional properties of hepatic free and membrane-bound polyribosomes, little is now known about the role of initiation factors of the two different populations of polyribosomes. Shafritz & Isselbacher (1972) reported that, when endogenous and exogenous messengers were used, the ribosomal wash from free polyribosomes of livers of normal rabbits stimulated translation to a greater degree than did the ribosomal wash of membrane-bound polyribosomes. They claimed that, for translation from endogenous messenger, each ribosomal system exhibited a partial specificity for the homologous wash fraction.

Table 6. *Initiation factors-dependent stimulation of polyphenylalanine synthesis by free (F) and membrane-bound (MB) polyribosomes of host liver and hepatoma*

All animals were starved overnight. Morris hepatoma H5123 was transplanted intrahepatically as described in the Methods section. Three experiments were carried out. In each experiment livers and tumours (30–35 g of tissue) were pooled separately. In all experiments preparations of 1 M-KCl-washed, free and membrane-bound ribosomes (prepared after using Triton X-100) and initiation factors were freshly prepared from host livers and hepatoma tissues as described in the Methods sections. Elongation factors were prepared from host livers only. Assay for the synthesis of polyphenylalanine from [¹⁴C]phenylalanyl-tRNA with salt-washed ribosomes and initiation factors was carried out by using initiation factors (200 µg of protein) as described in the legend in Table 1. The values are from one representative experiment. In the summary, the amount of [¹⁴C]phenylalanyl-tRNA polymerized with initiation factors and salt-washed ribosomes from membrane-bound polyribosomes of host liver was taken as 100% activity. The other groups were expressed as percentage of controls, and average % activity values ± S.E.M. were calculated from these values. The values in the summary represent means of three experiments. **P* < 0.02, according to Student's *t* test.

Tissue and type of ribosomes	Source of initiation factors	[¹⁴ C]Phenylalanine incorporated into protein from [¹⁴ C]-phenylalanyl-tRNA (c.p.m.)
Host liver MB	Host liver MB	1574
Host liver MB	Host liver F	1721
Host liver MB	Hepatoma MB	1653
Host liver MB	Hepatoma F	5327
Host liver F	Host liver MB	1374
Host liver F	Host liver F	1498
Host liver F	Hepatoma MB	1775
Host liver F	Hepatoma F	4635
Hepatoma MB	Host liver MB	1466
Hepatoma MB	Host liver F	1615
Hepatoma MB	Hepatoma MB	1859
Hepatoma MB	Hepatoma F	4968
Hepatoma F	Host liver MB	1521
Hepatoma F	Host liver F	1803
Hepatoma F	Hepatoma MB	1939
Hepatoma F	Hepatoma F	4957

Summary		Activity	
Tissue	Ribosome fraction	Initiation factors	Ribosomes
Host liver	MB	100	100
	F	115 ± 8.01	95 ± 4.2
Hepatoma	MB	107 ± 6.0	98 ± 2.9
	F	310 ± 22.9*	101 ± 4.3

However, in their studies, the ribosomal wash from the hepatic membrane-bound polyribosomes was prepared without removing membrane fragments

from the ribosomes. Also, Uenoyama & Ono (1972), using endogenous mRNA, reported that protein synthesis proceeded at maximal rates only if ribosomes, mRNA and high-KCl wash were all from the same source.

In our experiments with livers from normal rats, the initiation factors prepared from membrane-bound polyribosomes while still attached to membranes (without detergent treatment) exhibited less activity in translating poly(U) than did the initiation factors prepared from membrane-bound polyribosomes released by Triton X-100 treatment. The lower activity of this ribosomal fraction in polypeptide synthesis could be due to either the dilution by membrane proteins or inhibition caused by the membrane fragments when isolated together with the polyribosomes.

Olsnes (1971) has reported that there was more protein associated with Triton X-100-treated polyribosomes than there was with deoxycholate-treated polyribosomes. These findings are in agreement with our results that the amount of protein in the initial polyribosomal pellet was significantly higher after Triton X-100 treatment than after deoxycholate treatment. Studies concerned with the recoveries of initiation factors suggest that the larger amount of protein associated with the Triton X-100-treated polyribosomes may be related at least in part to protein from initiation factors. Further studies are needed to develop techniques for the isolation and purification of initiation factors, especially from membrane-bound polyribosomes, without the use of detergents.

The present findings also indicate no specificity in each ribosomal system for the homologous wash fraction. Initiation factors of free or membrane-bound polyribosomes were able to stimulate polyphenylalanine synthesis with ribosomal species of normal liver or of host liver or hepatoma.

The present results for hepatoma indicate that the earlier findings of increased activity of initiation factors of hepatoma (Murty *et al.*, 1974b) appear to be due to increased activity of initiation factors of free polyribosomes rather than to initiation factors of membrane-bound polyribosomes. This increased activity of initiation factors in hepatoma due to the free polyribosome fraction is of special interest, since in the hepatoma nearly 90% of the polyribosomes are present free in the cytoplasm. Further studies are needed to determine whether there is a relationship between the observed high activity of initiation factors of the free polyribosomes in hepatoma and the enhanced rapid growth of the neoplastic cells.

This investigation was supported by U.S.P.H.S. Research Grants AM 16530 from the National Institute of Arthritis and Metabolic Diseases and CA 14156 from the National Cancer Institute.

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