

Studies on the Amino Acid-Incorporating Activity of Native Rat Liver Rough Membrane and that Reconstituted *in vitro*

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(Received 7 January 1976)

The amino acid-incorporating activities of free polyribosomes, rough membranes and rough membranes reconstituted *in vitro*, derived from rat liver, were compared. The amino acid-incorporating activity of the two membrane fractions were very similar in their response towards changes in pH, Mg²⁺ concentration and temperature, but differed from the response of the amino acid-incorporating activity of free polyribosomes. Free polyribosomes irreversibly lost part of their amino acid-incorporating capacity after they had become bound to rough membrane, from which the original ribosomes had been removed. Ribonuclease activity present in the membrane fraction may be responsible for this loss.

Membrane-polyribosome interaction has been investigated in several laboratories. The elucidation of this interaction will contribute to our understanding of the regulation of protein synthesis in mammalian cells. One way to study it is the investigation of the reconstitution of rough membranes *in vitro*, from polyribosomes and rough membranes stripped of their ribosomes (Campbell, 1970; Roobol & Rabin, 1971; Rolleston, 1972; Borgese *et al.*, 1974; Shires & Pitot, 1974).

After incubation of stripped rough membranes with polyribosomes, a membrane fraction can be isolated that resembles, in aspects such as RNA/protein ratio, buoyant density (Borgese *et al.*, 1974) and appearance in electron micrographs (Ragland *et al.*, 1971), the rough membrane fraction isolated directly from the tissue. In our laboratory we have isolated, by isopycnic flotation, a rough membrane reconstituted *in vitro*, which is capable of protein synthesis (Hochberg *et al.*, 1975a). The purpose of the present work is to compare some features of the amino acid-incorporating activity of free polyribosomes with that of native rough membrane and of rough membrane reconstituted *in vitro*, to obtain answers to the following questions. Does the membrane-ribosome interaction cause some change in the ribosome or in its close environment that influences its amino acid-incorporating activity, and what is the relationship between amino acid incorporation in reconstituted rough membrane and that in native rough membrane?

The degree of similarity between the biological properties of the native and reconstituted rough membrane may show us how closely the ribosome-membrane interaction in the reconstituted membrane fraction resembles that in the native rough membrane.

Experimental

Chemical and biological materials

L-[4,5-³H]Leucine (specific radioactivity 38 Ci/mmol) and L-phenyl[2,3-³H]alanine (specific radioactivity 20 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. RNAase* inhibitor was obtained from Searle, High Wycombe, Bucks., U.K. Male albino rats (150-180 g) were obtained from Lewenstein, Yokneam, Israel; they were starved overnight (with full access to water) before use.

Preparation of cell fractions

Rat liver free polyribosomes and rough and smooth membranes were prepared as described by Czosnek & Hochberg (1975).

Reconstitution of rough membrane

Rat liver rough membrane was stripped of ribosomes by the KCl/puromycin method (Adelman *et al.*, 1973; Borgese *et al.*, 1974). Binding of free polyribosomes to the stripped rough membrane was done as described by Borgese *et al.* (1974).

* Abbreviation: RNAase, ribonuclease.

The free polyribosome-stripped rough-membrane complex was pelleted at 78000g for 30min at 2°C, and 10–15 mg (protein) of the pellet was suspended in 5ml of 2M-sucrose/TKM buffer [50mM-Tris/HCl (pH 7.6)/25mM-KCl/10mM-MgCl₂]; the suspension was underlaid below 24ml of a linear gradient of 0.9–1.9M-sucrose/TKM buffer. The gradients were spun for 14h at 24000 rev./min in an SW 25.1 Beckman rotor at 2°C. The membrane band was removed by aspiration, pelleted at 78000g for 30min at 2°C and suspended in 1M-sucrose/TKM buffer (Hochberg *et al.*, 1975a). The native and stripped rough-membrane fractions were processed in the same way.

Release of bound polyribosomes from membranes

Bound ribosomes were released from rough or reconstituted rough membrane by incubating these fractions in 0.8% sodium deoxycholate for 5min at 10°C. The mixture was then layered on an 8ml 1.35M-sucrose/TKM buffer cushion and spun for 2h at 78000g. The resulting pellet of ribosomes was suspended in 0.25M-sucrose/TKM buffer and stored in liquid N₂.

Assay of amino acid incorporation

The standard amino acid-incorporation mixture contained, in a final volume of 110μl: 60mM-Tris/HCl, pH 7.4; 3.5mM-MgCl₂; 50mM-KCl; 0.5mM-ATP; 0.5mM-GTP; 5mM-phosphoenolpyruvate; phosphoenolpyruvate kinase (EC 2.7.1.40) (1μg of protein); 1mM-dithiothreitol; tRNA (rat liver) (10μg); poly(U), when added (20μg); non-radioactive amino acid mixture (minus leucine or minus phenylalanine) (50μM each amino acid); 100μg (protein) of the enzyme fraction [40–70%-satd.-(NH₄)₂SO₄ precipitate of the postmicrosomal supernatant]; [³H]leucine (2μCi) or [³H]phenylalanine (1μCi); free polyribosomes (1.0 E₂₆₀ unit) or membrane fraction (50μg of protein). Samples were withdrawn, and the radioactivity of the material insoluble in hot 5% (w/v) trichloroacetic acid was determined as described by Bollum (1965), being counted for radioactivity in 10ml of toluene containing 0.1g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 3g of 2,5-diphenyloxazole per litre, in a Tri-Carb liquid-scintillation counter.

Use of RNAase inhibitor

RNAase inhibitor (130units/mg) was used in incubation mixtures in a constant ratio of 5units to 100μg of membrane protein or 2E₂₆₀ units of free polyribosomes, where 1 unit of RNAase inhibitor is the amount that gives 50% inhibition of 5ng of crystalline pancreatic RNAase (EC 3.1.4.22) under standard conditions, as described by Shortman (1961).

Determination of RNA and protein

RNA was determined as described by Bloemendal

et al. (1967), and protein as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and Discussion

Fig. 1 shows the kinetics of amino acid incorporation into hot-5%-trichloroacetic acid-insoluble peptides, promoted by free polyribosomes, native rough membrane and rough membrane reconstituted *in vitro*. The initial rate of the amino acid incorporation catalysed by free polyribosomes was nearly equal to that of the rough membrane. In the membrane fractions, the incorporation slowed down after 15min of incubation, whereas that promoted by free polyribosomes continued for at least 30min. Fig. 1 shows that the total amount of amino acids that could be incorporated into the free polyribosomal fraction was two to three times higher than the amount that could be incorporated into the native rough membrane or the rough membrane reconstituted *in vitro* (calculated on the basis of equal RNA content). The

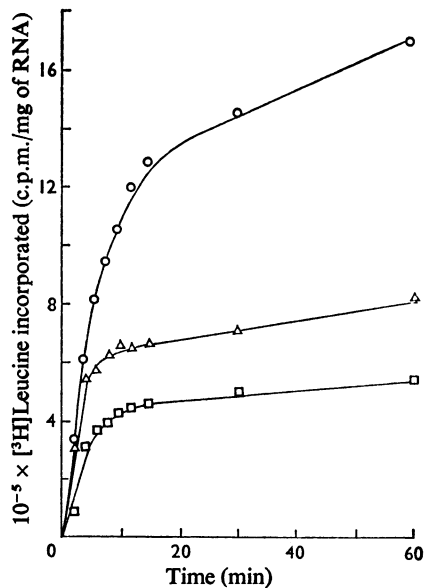


Fig. 1. Kinetics of amino acid incorporation

For incorporation conditions, see the Experimental section. ○, Free polyribosomes; △, native rough membrane; □, reconstituted rough membrane. The same results were obtained when RNAase inhibitor was present during the incubation; reconstituted rough membrane, reconstituted in the presence of RNAase inhibitor, has the same amino acid incorporation as the reconstituted rough membrane in this experiment.

incorporation capacity of the reconstituted rough membrane was 20–50% less than that of the native rough membrane. The reason for this difference is probably the fact that stripping of the rough membrane leaves 30–50% of the RNA but removes most of the ribosomal proteins, as revealed by two-dimensional polyacrylamide-gel electrophoresis (Czosnek *et al.*, 1975), causing the amino acid-incorporating activity to fall to less than 15% of that of the native rough membrane. If one calculates the amino acid-incorporating activity of the reconstituted rough membrane on the basis of the amount of RNA that has been added during reconstitution, one finds approximately equal amino acid-incorporating capacities for native and reconstituted rough membranes.

The amino acid-incorporating activity of the reconstituted rough membrane also resembled that of the native rough membrane in other respects.

Fig. 2 shows the effect of Mg^{2+} concentration on amino acid incorporation by the different fractions. After a short time-interval (in which the initial rate of incorporation was measured), the Mg^{2+} optimum was identical for the three fractions, namely 2.5 mM.

After longer time-intervals there was an apparent drift of the Mg^{2+} optimum for the two membrane fractions to a higher concentration. These results can be explained by taking into account the differences between the kinetics of amino acid incorporation by membrane fractions and free polyribosomes. At 2.5 mM- Mg^{2+} , the rate of amino acid-incorporation catalysed by the membrane fractions fell sharply after only 5 min of incubation, whereas at higher Mg^{2+} concentration the initial rate of the incorporation was lower than at 2.5 mM- Mg^{2+} , but fell more slowly with time. Vernie *et al.* (1972) also compared the Mg^{2+} optima of the amino acid incorporating activity of free and bound polyribosomes and found different Mg^{2+} optima for the two fractions. Their results and ours are difficult to compare because, first, the rough-membrane fraction used by those authors was isolated in a different way, and, secondly, they determined the incorporation only at one time-interval, namely after 30 min of incubation.

Fig. 3 shows the pH dependence of the amino acid incorporation of the three fractions. The two membrane fractions differed in this respect from

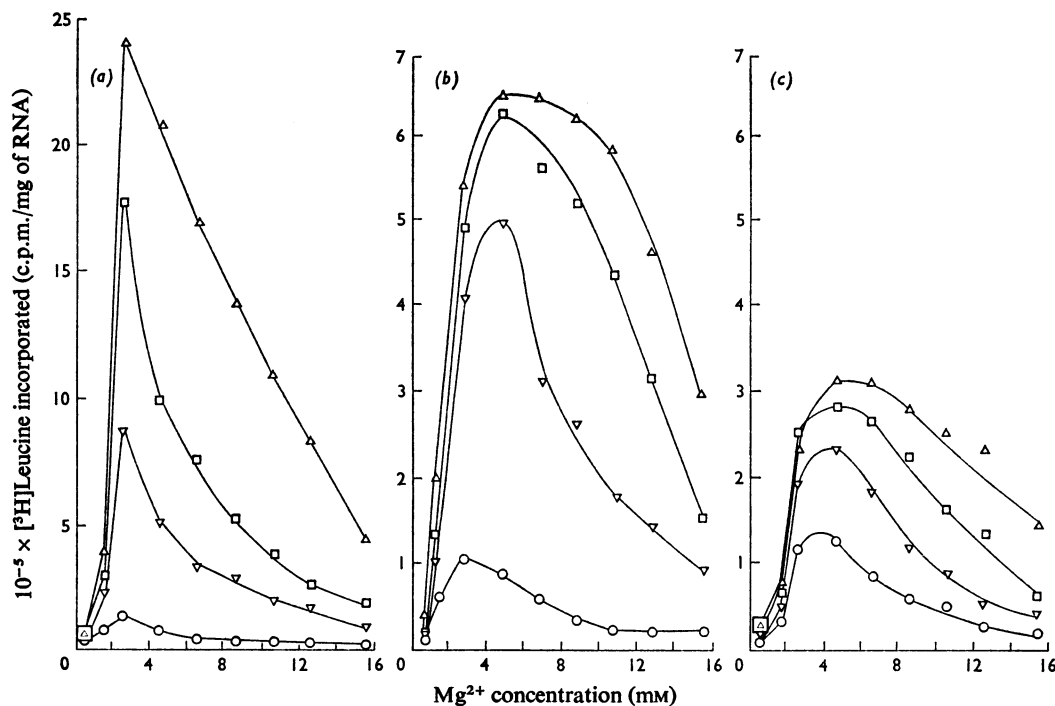


Fig. 2. Effect of Mg^{2+} concentration on amino acid incorporation

For incorporation conditions, see the Experimental section. The effect of Mg^{2+} concentration on amino acid incorporation promoted by the free polyribosomes (a), native rough membrane (b) and reconstituted rough membrane (c) was measured after 2 min (O), 5 min (V), 10 min (□) and 30 min (Δ).

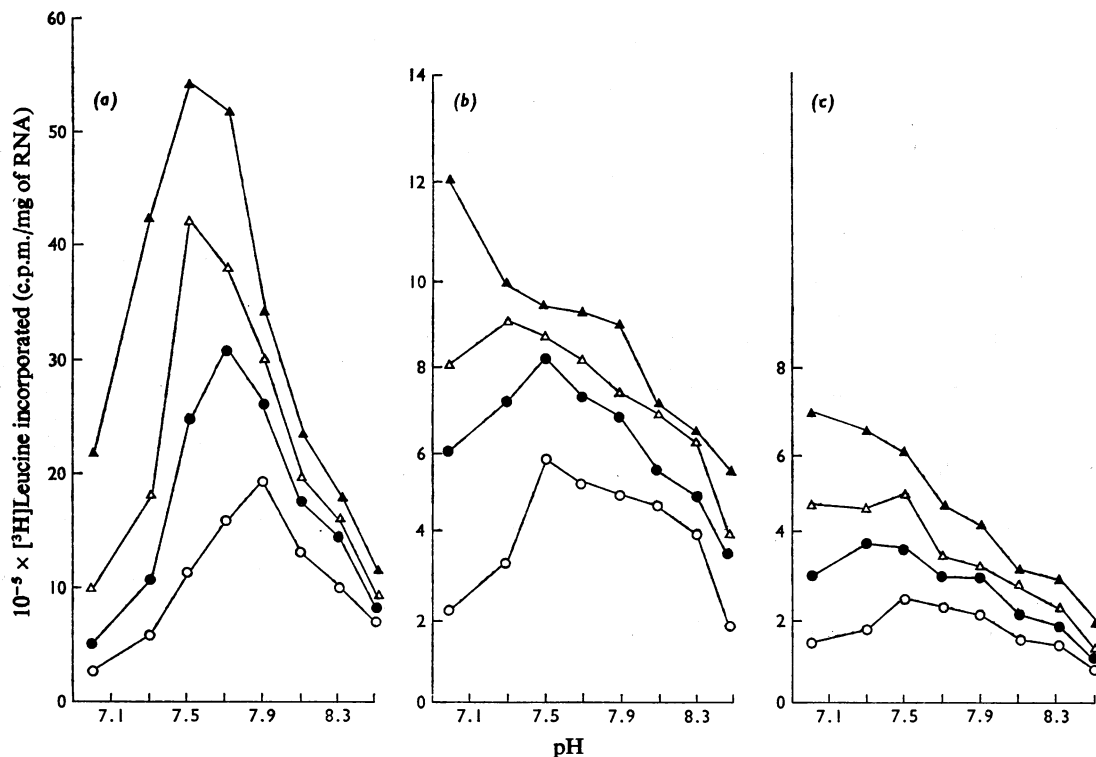


Fig. 3. Dependence of the amino acid-incorporating activity of the different fractions on the pH

For incorporation conditions, see the Experimental section. The pH was measured at the start and at the end of the incorporation. Incorporation promoted by free polyribosomes (a), native rough membrane (b) and reconstituted rough membrane (c) was measured after 7 min (○), 11 min (●), 16 min (△) and 30 min (▲).

the behaviour of the free polyribosomes. At all time-intervals measured, the pH optimum for incorporation promoted by free polyribosomes was higher than for that catalysed by membrane-bound ribosomes (with both native and reconstituted rough membranes); the native and the reconstituted rough membrane behaved very similarly.

In Fig. 4 we compare the effect of temperature on amino acid incorporation of the three fractions. Again, the two membrane fractions responded similarly to temperature changes, but differently from the polyribosomes. The rate of amino acid incorporation of the membrane fractions increased to a much greater extent between 10° and 20°C than that of free polyribosomes. At temperatures below 18°C, the absolute rate of incorporation catalysed by the membrane fractions was equal to or higher than that catalysed by the free polyribosomes. From these observations it can be concluded that the presence of the membrane in both native and reconstituted rough membrane directly influences

the kinetics of at least one of the intermediate steps in protein synthesis.

We also measured the stability of the ribosomes in the three fractions at different temperatures. The fractions were preincubated at temperatures over the range 18–60°C, and the amino acid-incorporating activity was then measured at 37°C. No significant difference was observed in the thermal stability of the three fractions (results not shown).

The reconstituted rough-membrane fractions used in all the experiments described were obtained by incubating at 0°C stripped rough membrane with polyribosomes from the free polyribosome fraction. Several possibilities exist to explain the difference between the amino acid-incorporating capacity of the free polyribosomes and that of the reconstituted rough membrane. One possible explanation is the accumulation of an amino acid-incorporation inhibitor in the incubation mixtures of the membrane fractions or the destruction of one or more of the factors obligatory for the incorporation reaction by

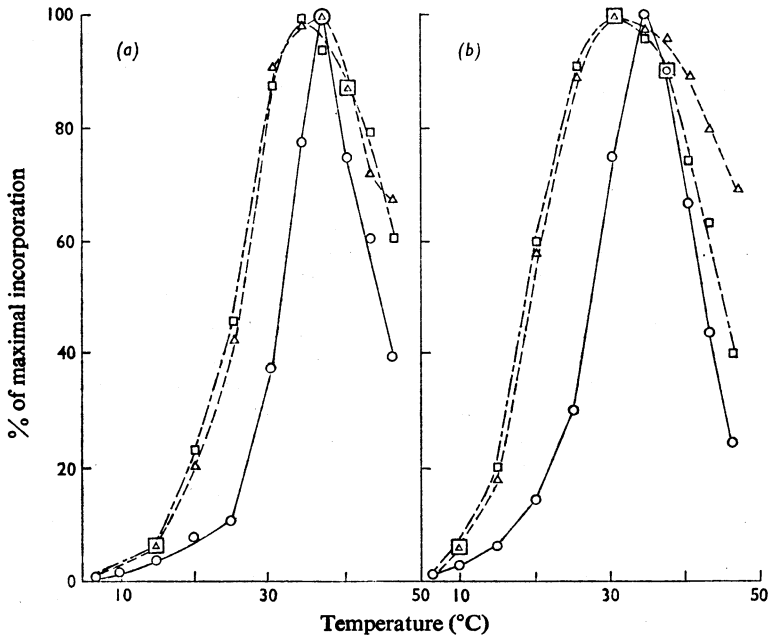


Fig. 4. Effect of temperature on the amino acid-incorporating activity of the different fractions at (a) 10 min and (b) 30 min of incorporation

The amino acid incorporation of each fraction at the optimal temperature was arbitrarily set at 100. O, Free polyribosomes; Δ, native rough membrane; □, reconstituted rough membrane.

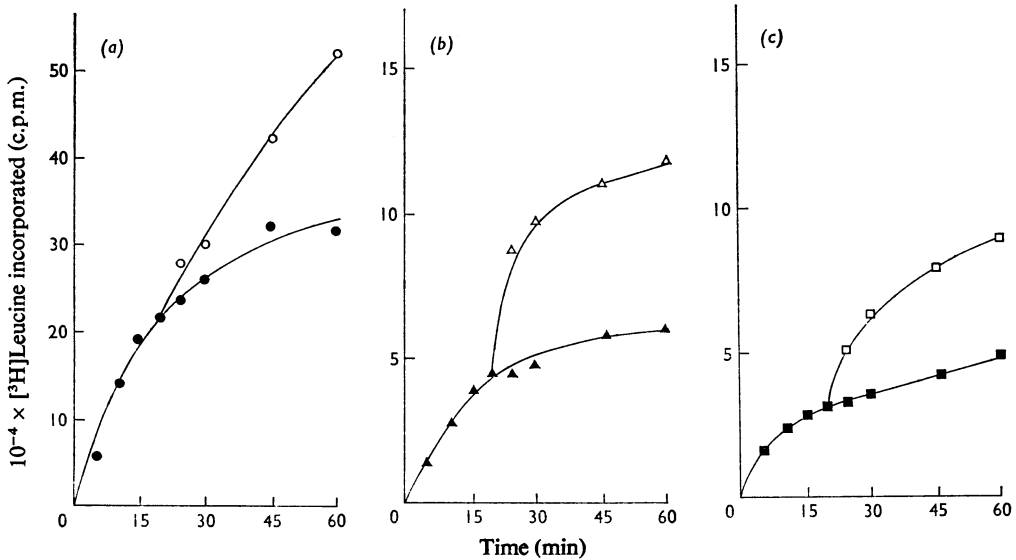


Fig. 5. Effect of ribosomal fractions, added 20 min after the start of the incorporation reaction, on amino acid (leucine) incorporation

For incorporation conditions, see the Experimental section. After 20 min of incubation, a fresh portion of free polyribosomes (O), native rough membrane (Δ) or reconstituted rough membrane (□) respectively was added to the incorporation medium containing free polyribosomes (●), native rough membrane (▲) or reconstituted rough membrane (■).

the membrane. To investigate these possibilities we carried out the following experiments.

To an amino acid-incorporation mixture with the native or the reconstituted rough-membrane fractions was added (after 20 min of incubation when the amino acid-incorporating rate had slowed down markedly) a fresh portion of the membrane fraction. The added membrane fractions had the same amino acid-incorporating activity as the original portion; the same was true for a similar experiment with free polyribosomes (Fig. 5). From these results it may be concluded that no amino acid-incorporation inhibitor accumulated during the incubation period before the second portion of ribosome fraction was added, nor had any of the non-ribosomal components obligatory for amino acid incorporation been exhausted or destroyed during the first incubation step. Additional support for this conclusion comes from the results described in Fig. 6. Free polyribosomes and native and reconstituted rough membranes were allowed to catalyse incorporation of amino acids. After 20 min, when the incorporation rate had fallen to nearly zero, poly(U) was added; the poly(U) was translated with an efficiency of at least 60% of that for poly(U) added to membrane fractions at zero time. Therefore it is clear that the ribosomes were still active in all steps of the elongation cycle at the time

when the translation rate for endogenous mRNA had fallen to nearly zero. Preincubation of free polyribosomes (Fig. 7a), native (Fig. 7b) and reconstituted rough membrane (Fig. 7c), together with the enzyme fraction, caused a much smaller decrease in the amino acid-incorporating capacity than a similar preincubation in the presence of all cofactors necessary for amino acid incorporation, such as ATP, GTP and the amino acid mixture (except for radioactive leucine). These results can be explained by assuming that amino acid incorporation takes place during the preincubation with all the cofactors, because of the presence of amino acids in the enzyme fraction and/or in the membrane fraction (their actual presence was proved by isotopic-dilution measurements).

From these results, it is clear that preincubation of the ribosomal fractions with the enzyme involved in the incorporation reaction leaves the incorporation potential of the ribosomes (the bound and the free) nearly intact. Moreover, the free polyribosomes do not differ in this respect from the two membrane fractions.

Another explanation of the low amino acid-incorporating capacity of the native and reconstituted rough-membrane fractions is the existence of an active RNAase in the membrane fractions (Hochberg *et al.*, 1975b). This activity is shown

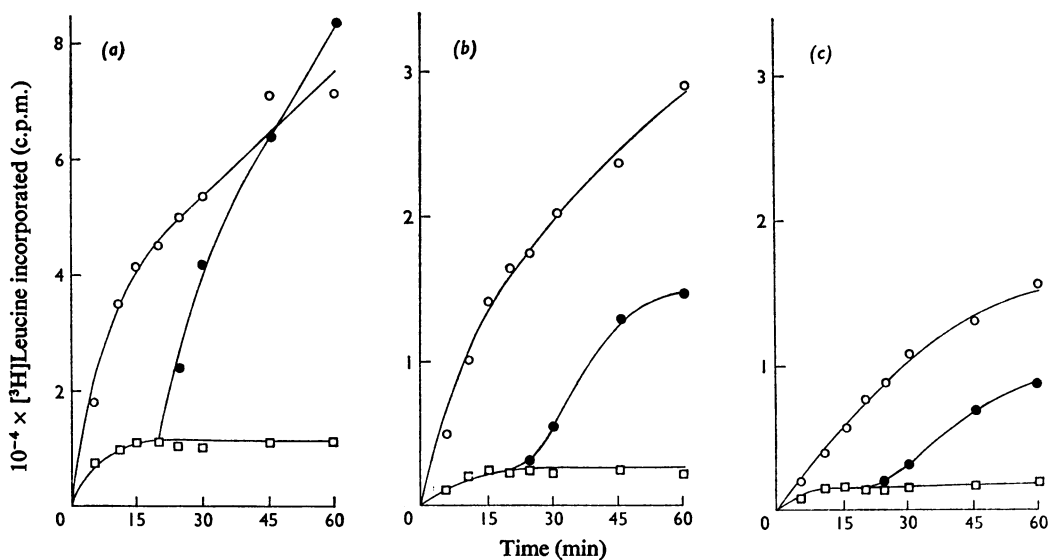


Fig. 6. Effect of poly(U), added 20 min after the start of the amino acid-incorporation reaction, on the incorporation of phenylalanine into hot-5%-trichloroacetic acid-insoluble material

For incorporation conditions, see the Experimental section. After 20 min of incubation, poly(U) and Mg^{2+} to a final concentration of 10 mM were added to the standard amino acid-incorporation mixture, containing free polyribosomes (a), native rough membrane (b) and reconstituted rough membrane (c). ○, Poly(U) added at zero time; ●, poly(U) added after 20 min of incorporation; □, no addition of poly(U).

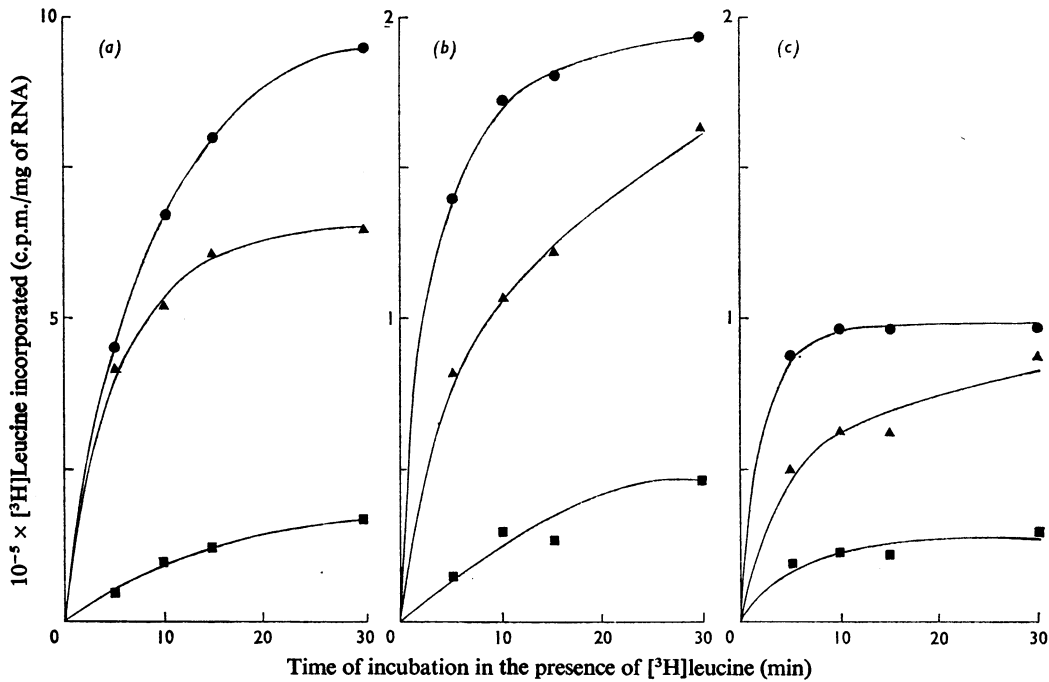


Fig. 7. Effect of preincubation of the different fractions on endogenous mRNA translation

For incorporation conditions, see the Experimental section. Free polyribosomes (a), native rough membrane (b) and reconstituted rough membrane (c) were preincubated for 10min at 37°C with the enzyme fraction alone (▲) or with the whole incorporation mixture without radioactive amino acid (■); after 10min at 37°C all the other components needed for incorporation were added. ●, No preincubation.

Table 1. RNAase activity of the different rat liver fractions

The incubation mixture (37°C) contains in a final volume of 200μl: [³H]leucyl-tRNA [3300c.p.m./μg of RNA (prepared as described by Ziv *et al.*, 1971), 20μg]; 60mM-Tris/HCl, pH 7.5; 50mM-KCl; 1 mM-dithiothreitol; 10mM-MgCl₂; free polyribosomes (2.0E₂₆₀ units) or membrane fraction (100μg of protein); RNAase inhibitor, when used (5 units). Samples (30μl) were withdrawn and placed on Whatman 3MM filter-paper discs, washed three times with cold 5% trichloroacetic acid, once with acetone/ether (1:1, v/v) and finally with ether; they were dried and the radioactivity was counted.

Fraction	RNAase inhibitor	Degradation (%)		
		10min	15min	30min
Rough membrane	-	66	76	89
	+	3	15	11
Stripped rough membrane	-	67	80	90
	+	5	10	18
Reconstituted rough membrane	-	27	37	67
	+	4	5	13
Free polyribosomes	-	8	12	20
	+	2	2	3

in Table 1. We therefore added RNAase inhibitor to the amino acid-incorporation mixture at the same concentration as that used in the experiment described in Table 1, which inhibited 80% or more of RNAase activity; however, it changed neither the kinetics nor the final amount of amino acid incorporation of the three fractions concerned. Also, the presence of RNAase inhibitor during the reconstitution step (at the same concentration as in Table 1) caused no change in the amino acid-incorporating activity of the reconstituted rough membrane. However, as shown in Table 1, it did not inhibit all the RNAase activity of the membrane fractions, and therefore the possibility still exists that this residual activity is the cause of the limited amino acid-incorporating capacity of the rough membrane reconstituted *in vitro*, by causing partial hydrolysis of its mRNA. If this is the case, then free polyribosomes, after they have been incorporated into the reconstituted rough-membrane complex, have permanently lost part of their amino acid-incorporating activity, which will not be restored to the original value if the ribosomes are detached

from the membrane. That this is so is shown by the following experiments (Table 2).

Ribosomes were released from the reconstituted rough membrane by treating the membrane fraction with 0.8% sodium deoxycholate. The amino acid-incorporating capacity of the released ribosomes was measured (Table 2, line 2) and was 10% less than that of the same ribosomes when still part of the reconstituted rough membrane (Table 2, line 1), and much less than that of the free polyribosomes, which remained unbound in the reconstitution mixture and were separated from the reconstituted rough membrane by isopycnic flotation (Table 2, line 3). Also, when ribosomes were released from native rough membrane by the same method, the same results were obtained for their acid-incorporating capacity (Table 2, lines 4 and 5). The difference between the amino acid-incorporating activities of the unbound and sodium deoxycholate-released ribosomes was shown to be not due to the use of sodium deoxycholate in the releasing process as follows. Free polyribosomes were pelleted and suspended in 5ml of 2M-sucrose/TKM buffer; the suspension was underlaid below the linear sucrose gradient used in the reconstitution experiment and spun as described in the Experimental section. The resultant ribosomal pellet was suspended in 0.25M-sucrose/TKM buffer, and the amino acid-incorporating capacity of this fraction was compared with that of the same fraction treated with 0.8% sodium deoxycholate (Table 2, lines 6 and 7). Sodium deoxycholate treatment decreased the amino acid-incorporating capacity of the free polyribosomes only to a small extent, this decrease being much too small to account for the great difference in amino acid-incorporating

capacity between the unbound and released ribosomes.

From the results described in this paper, one may conclude that the free polyribosomes lose part of their amino acid-incorporating capacity the moment they become part of the reconstituted ribosome-membrane complex. It is reasonable to assume that the RNAase activity of the membrane is responsible for this loss of activity, but it is not clear why this RNAase activity does not harm the protein-synthesizing capacity of the rough membrane *in vivo*.

Changes in the concentration of ions, such as Mg^{2+} , and in temperature and pH influence the amino acid-incorporating activity of the native and reconstituted rough membranes differently from the way they influence amino acid incorporation in free polyribosomes. In all these respects, the rough membrane reconstituted *in vitro* behaves in a similar way to the native rough membrane.

It should be pointed out that in the reconstitution experiments described, we have added polyribosomes from the free polyribosome fraction to stripped rough membranes. The question arises whether we can expect functional reconstitution by using this fraction. To answer this question, one has to consider the following. Electron micrographs of freeze-fractured liver tissue show that at least 80% of ribosomes are membrane-bound (Hochberg *et al.*, 1975c), but in our preparations, 50% of the ribosomes were in the free polyribosomal fraction. It seems therefore that a major part of our free polyribosomes are ribosomes that were part of the rough endoplasmic reticulum and were detached from the membranes during the isolation procedure. We found that on prolonging the centrifugation time, the amount of free polyribosomes increased and this supports the above-mentioned suggestion. We used a large excess of free polyribosomes in our reconstitution experiments, therefore the possibility exists that ribosomes that were part of the rough endoplasmic reticulum will preferentially bind to the stripped rough membrane.

Whether or not the ribosomes in the reconstituted rough membrane are bound in such a way that the nascent peptide chain is vectorially transported into the intravesicular space is still an open question and needs further investigation. However, the finding that for maximal stripping of our reconstituted rough membrane we need both puromycin and a high KCl concentration indicates that peptidyl-tRNA plays an important role in the ribosome-membrane interaction (Hochberg *et al.*, 1975a). The RNAase present in both native and stripped rough membrane could easily cause cleavage of the mRNA and therefore may prevent the normal termination process and the release of the completed protein into the cisternae.

Table 2. *Amino acid incorporation with free, bound and released polyribosomes*

Results are given as c.p.m. of [3H]leucine incorporated after 30 min incubation/mg of RNA.

Reconstituted rough membrane	416000
Free polyribosomes released from reconstituted rough membrane	370000
Free polyribosomes unbound during the reconstitution process	660000
Rough membrane after isopycnic flotation	540000
Bound polyribosomes released from the rough membrane	460000
Free polyribosomes suspended in 2M-sucrose/TKM buffer and spun in a continuous sucrose gradient	620000
Same free polyribosomes treated with 0.8% sodium deoxycholate	580000

This work was supported by grant no. 173 from the United States-Israel Binational Science Foundation. We thank Mrs. Tamar Schneider and Miss Batya Gottlieb for their excellent technical assistance.

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