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Inhibition of Rat Liver Isoleucyl-tRNA Formation by Microsomal Membrane and the Partial Prevention of This Inhibition by Spermine[1]

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Rat liver isoleucyl-tRNA formation in the presence of more than 0.6 mM Mg^{2+} was inhibited by smooth and rough endoplasmic reticula. The inhibition was partially prevented by ATP, spermine, oligomycin, and Triton X-100. This suggests that microsomal ATPases are responsible for the inhibition of isoleucyl-tRNA formation.

Among the microsomal ATPases, an oligomycin-sensitive ATPase was found to be inhibited by spermine. The inhibition of the oligomycin-sensitive ATPase by spermine explains well its partial prevention of the inhibition by endoplasmic reticulum of isoleucyl-*t*RNA formation.

Keywords——isoleucyl-tRNA; microsomal membrane; endoplasmic reticulum; rat liver; polyamines; spermine; ATPase; oligomycin

It is well known that polypeptide synthetic activity in a rat or rabbit liver cell-free system is inhibited by endoplasmic reticulum.[3] Although the main reason for this phenomenon is thought to be the existence of RNases in the membrane, a detailed study of this inhibition has not been performed.

Recently, we reported that polyamines can stimulate rat liver Ile-tRNA formation even in the presence of physiological concentrations of Mg^{2+}, and that the stimulatory effect of spermine functions mainly by preventing the tRNA inhibition of the isoleucyl-AMP·enzyme complex formation.[4] We have also reported that polyamines prevented the inhibition of rat liver Ile-tRNA formation by poly(G), poly(I), and ribosomes.[5]

In the present paper, we report the inhibition of rat liver Ile-tRNA formation by ER and the partial prevention of this inhibition by spermine. Evidence is also described which suggests that spermine decreases the inhibitory effect of ER on Ile-tRNA formation by inhibiting an oligomycin-sensitive ATPase in ER.

Materials and Methods

Materials——Purified Ile-tRNA synthetase was prepared from rat liver S100 fraction, as described previously.[4a] The purified enzyme was a complex of Ile-tRNA, Gln-tRNA, Leu-tRNA, Lys-tRNA, and Met-tRNA synthetases, as reported by Vennegoor and Bloemendal.[6] Rat liver tRNA was prepared from S100 fraction according to the procedure of Zubay,[7] except for the addition of 0.1% macaloid to the S100 fraction, the omission of the 2-propanol treatment, and the insertion, before use, of successive dialyses of the preparation against the following buffers: 10 mM Tris-HCl (pH 7.5), 2 M NaCl, and 1 mM EDTA; 10 mM Tris-

1) Abbreviations: ER, endoplasmic reticulum; SER, smooth ER; RER, rough ER; Ile-tRNA, isoleucyl-tRNA.

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HCl (pH 7.5) and 1 mM EDTA; and 10 mM Tris-HCl (pH 7.5). Rat liver SER, RER, and mitochondria were prepared by the method of Adelman *et al.*[3b]

Assay for Ile-tRNA Formation——The standard reaction mixture (0.05 ml) contained the following components: 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 2 mM ATP, 50 μg of rat liver tRNA, 0.05 μCi of [^{14}C]isoleucine (specific activity: 354 mCi/mmol) (Radiochemical Centre, Amersham), 2 μg of Ile-tRNA synthetase, and SER, magnesium acetate and spermine at the specified concentrations. After incubating the reaction mixture at 30° for 10 min, a 0.04 ml aliquot of each mixture was placed on a paper disc (25 mm diameter) and the cold trichloroacetic acid-insoluble radioactivity was counted using a liquid scintillation spectrometer.

Assay for ATPase Activity——The ATPase activity was measured by the colorimetric determination of liberated inorganic phosphate, by the method of Fiske and SubbaRow,[8] as modified by Kobayashi and Anraku.[9] The standard reaction mixture (0.3 ml) contained the following components: 50 mM Tris-HCl (pH 7.5), 2.5 mM ATP, 5 μg of bovine serum albumin, 30 μg protein of SER, and magnesium acetate and spermine at the specified concentrations. After incubating the reaction mixture at 30° for 10 min, the reaction was stopped by the addition of 0.15 ml of 0.1 N HCl and 1.05 ml of a solution containing 0.15 ml of 5 N H_2SO_4, 0.15 ml of 2.5% ammonium molybdate, 0.15 ml of 3% $NaHSO_3$-1% *p*-methylaminophenol sulfate, and 0.6 ml of water. After 10 min at 18°, the absorbance at 660 nm was measured. When the reaction mixture contained Triton X-100, 0.05 ml of 10% $NaHSO_3$ solution containing 4% Triton X-100 was added after the addition of 0.15 ml of 0.1 N HCl according to the method of Nakao *et al.*[10] The inorganic phosphate was then measured as described above. Since a slightly higher value was always obtained in the presence of Triton X-100, this was corrected by measuring the inorganic phosphate in the presence and absence of Triton X-100.

Under our experimental conditions, only ADP was identified as a hydrolysis product of ATP by polyethyleneimine-cellulose thin-layer chromatography.[11]

Assay for Cytochrome Oxidase Activity——Cytochrome oxidase was assayed essentially by the method of Smith,[12] with cytochrome c being reduced according to the method of Martin *et al.*[13] The reaction mixture (1.5 ml) contained 20 mM Na,K-phosphate buffer (pH 7.4), 15 μM reduced cytochrome c, and an appropriate amount of SER or RER. The rate of decrease in absorbance at 550 nm was measured with a Jasco UVIDEC-2 spectrophotometer. The optical density of completely oxidized cytochrome c was determined by the addition of a small drop of saturated $K_3Fe(CN)_6$.

Determination of Protein and RNA Contents——Protein content was determined by the method of Lowry *et al.*[14] RNA content was measured by the method of Schneider[15] after fractionation of the RNA according to Schmidt and Thannhauser.[16]

Results

Effect of ER and Polyamines on Ile-tRNA Formation

As shown in Fig. 1, SER inhibited Ile-tRNA formation in the presence and absence of spermine at Mg^{2+} concentrations of 0.6 mM or more. The percentage inhibition of Ile-tRNA formation in the presence of spermine was less than that in its absence. At 1.1 mM Mg^{2+}, for example, Ile-tRNA formation was inhibited 70% by SER (30 μg protein) in the absence of spermine, while it was inhibited 37% by the SER in the presence of 2 mM spermine. Since the reaction proceeded linearly under our experimental conditions, the differences between the percentage inhibitions by SER in the presence and absence of spermine show that spermine partially prevented SER inhibition of Ile-tRNA formation. Spermidine had an effect similar to that of spermine; however, the effective concentration was somewhat higher.

Next, the effects of SER and RER were compared. As shown in Fig. 2, RER inhibited Ile-tRNA formation to the same degree as did SER, although it is known that ribosomes inhibit Ile-tRNA formation.[5] Our RER preparation contained 130 μg of RNA per mg of protein.

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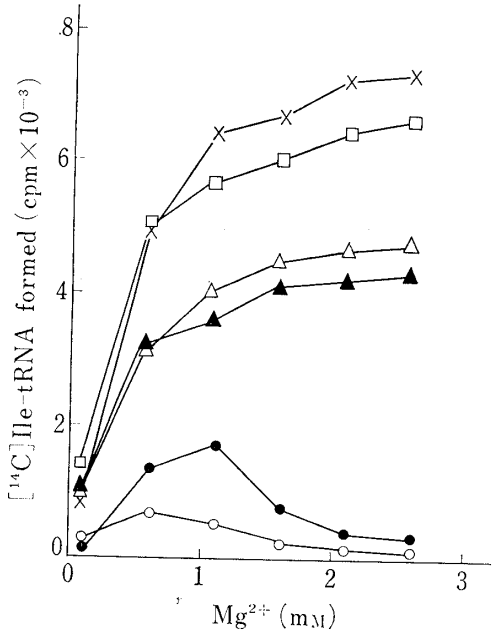

Fig. 1. Effects of Smooth Endoplasmic Reticulum and Spermine on Isoleucyl-tRNA Formtion

The assays were carried out under the standard conditions with SER, Mg^{2+}, and spermine as indicated. —●—, no addition; —○—, SER (30 μg protein); —×—, 2 mM spermine; —□—, 4 mM spermine; —△—, 2 mM spermine and SER (30 μg protein); —▲—, 4 mM spermine and SER (30 μg protein).

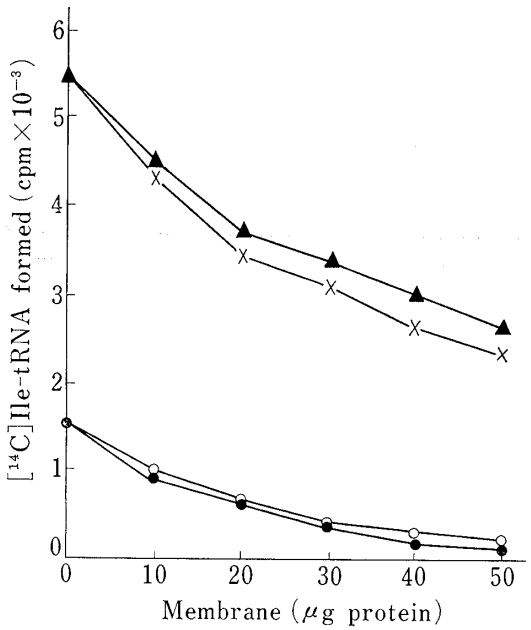

Fig. 2. Effects of Smooth and Rough Endoplasmic Reticula on Isoleucyl-tRNA Formation

The assays were carried out under the standard conditions with SER (or RER) and spermine as indicated. The Mg^{2+} concentration was 1.1 mM. —●—, SER; —○—, RER; —▲—, SER and 2 mM spermine; —×—, RER and 2 mM spermine.

These results suggest that this quantity of ribosomes present in RER is not responsible for the RER inhibition of Ile-tRNA formation.

Since functional similarities of polyamines and NH_4 (or K^+) ions have been observed in the binding of these ions to single-stranded synthetic polynucleotides,[17] the breakdown of synthetic polynucleotides by *Escherichia coli* RNase II[18] and the amino acid-dependent pyrophosphate ATP exchange catalyzed by aminoacyl-tRNA synthetase,[19] the sensitivity of Ile-tRNA formation to SER inhibition in the presence of K^+ was also examined (Fig. 3). The percentage inhibition of Ile-tRNA formation in the presence of 100 mM K^+ was less than that in the absence of K^+. At 1.1 mM Mg^{2+}, for example, Ile-tRNA formation was inhibited 68% by SER (30 μg protein) in the absence of K^+, while the inhibition was only 31% in the presence of 100 mM K^+.

Mechanism of Inhibition of Ile-tRNA Formation by ER

As shown in Fig. 4, the concentration of ATP required for maximum Ile-tRNA formation increased when SER was added to the reaction mixture. The addition of spermine caused a decrease in the optimal ATP concentration. These results suggest that microsomal ATPase(s) may be involved in the inhibition of Ile-tRNA formation and in the partial prevention by spermine of the SER inhibition of Ile-tRNA formation.

The involvement of ATPase(s) of the membrane in the inhibition of Ile-tRNA formation was also suggested by the results shown in Fig. 5. As shown in the figure, oligomycin and

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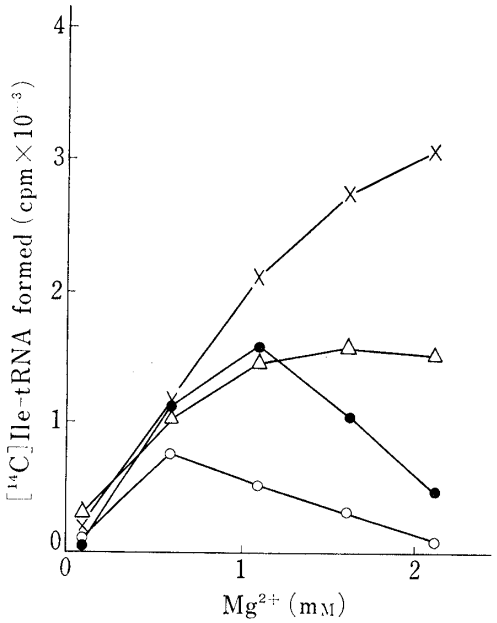

Fig. 3. Effects of Smooth Endoplasmic Reticulum and K^+ on Isoleucyl-tRNA Formation

The assays were carried out under the standard conditions with SER, Mg^{2+}, and K^+ as indicated. —●—, no addition; —○—, SER (30 μg protein); —×—, 100 mg K^+; —△—, SER (30 μg protein) and 100 mM K^+.

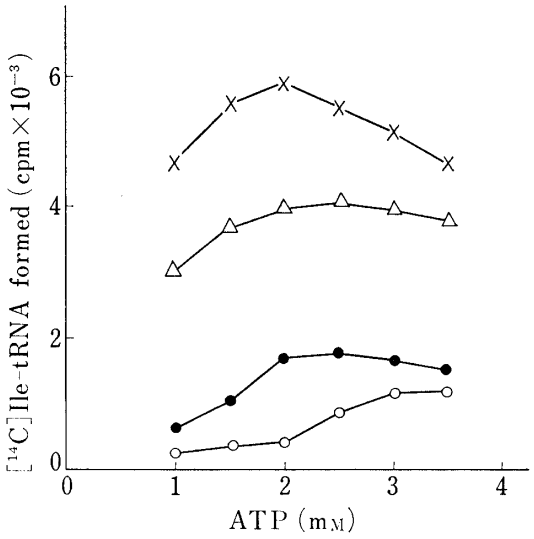

Fig. 4. Effects of ATP and Spermine on Isoleucyl-tRNA Formation in the Presence or Absence of Smooth Endoplasmic Reticulum

The assays were carried out under the standard conditions with SER, spermine, and ATP as indicated. The Mg^{2+} concentration was 1.1 mM. —●—, no addition; —○—, SER (30 μg protein); —×—, 2 mM spermine; —△—, SER (30 μg protein) and 2 mM spermine.

Triton X-100, which are inhibitors of ATPase, increased the Ile-tRNA formation in the presence of SER with slight decreases in the optimal ATP concentrations. In the absence of SER, however, oligomycin did not affect the degree of Ile-tRNA formation or the optimal ATP concentration. The addition of an energy supplying system (creatine phosphate plus creatine kinase) in the presence of SER produced a clear shift of the optimal ATP concentration. The addition of ouabain did not have any effect in the presence and absence of SER (data not shown).

Effect of Spermine on the Activities of Microsomal ATPases

Since ATPase(s) were implied to be responsible for the inhibition of Ile-tRNA formation by the membrane, the properties of ATPases in SER were examined. As shown in Fig. 6, oligomycin and Triton X-100 were found to inhibit ATPase activity in SER partially, but ouabain was ineffective. Fig. 7 shows the effects of various concentrations of oligomycin on the ATPase activity in SER and on Ile-tRNA formation in the presence of SER. The degree of inhibition by oligomycin of the ATPase activity in SER reached a plateau at about 10 μM oligomycin. At just the same concentration of oligomycin, the increase in Ile-tRNA formation in the presence of SER reached a plateau. These results suggest that oligomycin may prevent the SER inhibition of Ile-tRNA formation by inhibiting the ATPase activity in SER.

Next, the effects of spermine and K^+ on oligomycin-sensitive and oligomycin-insensitive ATPases in SER were examined. As shown in Table I, oligomycin-sensitive ATPase activity was inhibited by spermine and K^+. However, no significant inhibition of an oligomycin-insensitive ATPase was observed upon the addition of spermine or K^+. Oligomycin-sensitive ATPase was also inhibited by $LaCl_3$. The activities of oligomycin-sensitive and oligomycin-insensitive ATPases of RER were slightly less than those of SER. The activity of an oligomycin-sensitive ATPase in RER was also inhibited by spermine (data not shown). These results suggest that the inhibition by spermine of an oligomycin-sensitive ATPase may be responsible for its partial prevention of the inhibition by ER of Ile-tRNA formation.

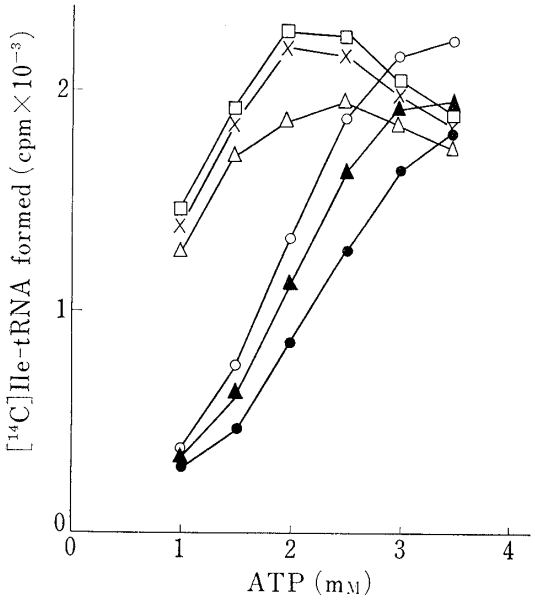

Fig. 5. Effects of Various Factors known to influence ATP Concentration on Isoleucyl-tRNA Formation in the Presence of Smooth Endoplasmic Reticulum

The assays were carried out under the standard conditions with SER, ATP, and various chemicals as indicated. The Mg^{2+} concentration was 1.1 mM. —×—, no addition; —●—, SER (30 μg protein); —□—, 10 μM oligomycin; —○—, SER (30 μg protein) and 10 μM oligomycin; —▲—, SER (30 μg protein) and 0.05% Triton X-100; —△—, SER (30 μg protein), 5 mM creatine phosphate, and creatine kinase (5 μg).

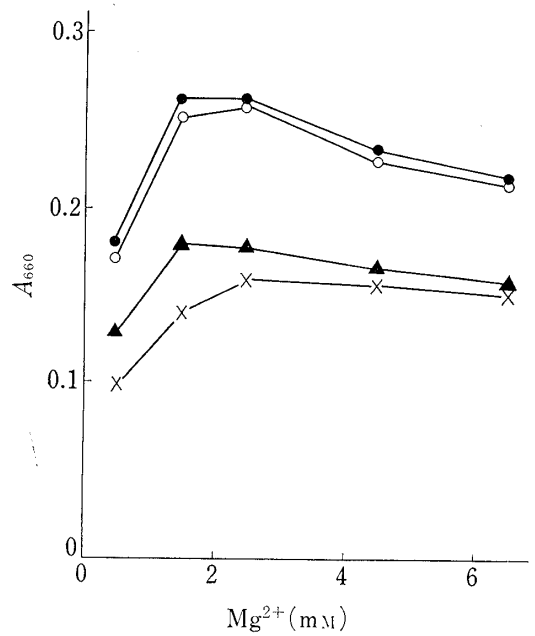

Fig. 6. Effects of Ouabain, Oligomycin, and Triton X-100 on ATPase Activity in Smooth Endoplasmic Reticulum

The assays were carried out under the standard conditions with Mg^{2+} and various chemicals as indicated. —●—, no addition; —○—, 100 μM ouabain; —×—, 10 μM oligomycin; —▲—, 0.05% Triton X-100.

Table I. Effects of Spermine and K^+ on the Activities of Oligomycin-Sensitive and Oligomycin-Insensitive ATPases in Smooth Endoplasmic Reticulum

Cation (mM)	Absorbance at 660 nm: Oligomycin-sensitive ATPase	Absorbance at 660 nm: Oligomycin-insensitive ATPase
—	0.130	0.145
Spermine (1)	0.118	0.148
Spermine (2)	0.092	0.143
Spermine (3)	0.054	0.150
Spermine (4)	0.032	0.147
K^+ (25)	0.125	0.142
K^+ (50)	0.097	0.149
K^+ (75)	0.072	0.141
K^+ (100)	0.050	0.145

The assays were carried out under the standard conditions with spermine and K^+ as indicated. The Mg^{2+} concentration was 1.1 mM. Oligomycin-insensitive ATPase activity was measured in the presence of 10 μM oligomycin. Oligomycin-sensitive ATPase activity was defined as the total ATPase activity minus oligomycin-insensitive ATPase activity.

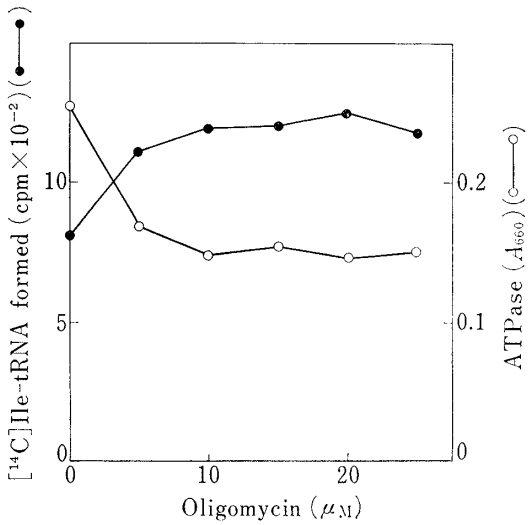

Fig. 7. Effects of Oligomycin on ATPase Activity in Smooth Endoplasmic Reticulum (SER) and on Ile-tRNA Formation in the Presence of SER

ATPase activity was assayed by the standard method with the addition of various amounts of oligomycin. Ile-tRNA formation was assayed by the standard method with 1.1 mM Mg^{2+}, 30 μg protein of SER, and various amounts of oligomycin. The amount of [14C]-Ile-tRNA formed in the absence of SER and oligomycin was 1950 cpm.

Discussion

It is inferred from the data presented here that not only RNases but also ATPases in the membrane influence the polypeptide synthetic activity in a rat liver cell-free system. Therefore, the inhibition of ATPase as well as RNase activities should be taken into consideration for the establishment of a good polypeptide synthetic system containing ER.

Results indicating that the bulk of our microsomal oligomycin-sensitive ATPase was not due to contamination by mitochondrial ATPase are as follows: (1) the cytochrome oxidase activity in SER was about 8% of that in the mitochondrial fraction, while the oligomycin-sensitive ATPase activity in SER was about 30% of that in the mitochondrial fraction; (2) the ATPase activity in SER was nearly equal to that in RER, the latter being more contaminated with mitochondria; and (3) the spermine inhibition of the microsomal oligomycin-sensitive ATPase was greater than the spermine inhibition of the mitochondrial oligomycin-sensitive ATPase.

Our preparation of microsomes did not contain (Na^+-K^+)-ATPase, although the existence of this enzyme in microsomes has been reported previously.[20] This discrepancy may be due to the differences in the sources and methods of preparation of microsomes.

It has been reported that spermine inhibits (Na^+-K^+)-ATPase in the presence of high concentrations of K^+, Na^+, Mg^{2+}, and ATP.[21] However, spermine was found to stimulate (Na^+-K^+)-ATPase in the presence of low concentrations of K^+ and ATP.[22] In the present studies we have shown an oligomycin-sensitive ATPase is inhibited by spermine and K^+. The inhibition of a microsomal ouabain-insensitive ATPase by monovalent cations has been reported by Izutsu *et al.*[20b] This ATPase may be the same as our oligomycin-sensitive ATPase.

The degree of stimulation of Ile-tRNA formation by spermine without ER in the present paper was much greater than that reported previously.[4,5] This difference may be due to the use of macaloid as the RNase inhibitor when tRNA was prepared from S100 fraction.

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