# THE DEGRADATION OF rRNA IN RIBOSOMES BY RIBONUCLEASES PRESENT IN RAT LIVER SUB-CELLULAR FRACTIONS

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Received 28 October 1975

## Introduction

Post-microsomal supernatant (PMicS) is a rich source of a protein which inhibits ribonuclease activity [1], and this presumably accounts for the protection from hydrolysis of polysomes [2–4] and mRNA [5] by PMicS. However, large quantities of PMicS cause a marked increase in the rate at which the endogenous rRNA of various sub-cellular fractions spontaneously degrades to acid-soluble fragments at 37°C [6]. We report here that the autodegradation of rRNA in preparations of total microsomes appears to be brought about mainly by a ribonuclease activity insensitive to inhibitor protein (type RNase-In) and that the stimulation of autodegradation by large quantities of PMicS is due to the presence of RNase-In activity in the PMicS. In contrast, most of the ribonuclease activity displayed by the total microsomes towards an exogenous substrate of yeast RNA is sensitive to the inhibitor (type RNase-Se). We also report that RNase-In activity is inhibited by *p*-chloromercuribenzoic acid (PCMB).

## 2. Materials and methods

Male albino rats (140 g.) of the Sprague–Dawley strain were used. Liver RNA was labelled by injection interperitoneally 48 h before sacrifice of 15 $\mu$Ci. of [6-$^{14}$C]orotic acid (The Radiochemical Centre, Amersham, Bucks., UK) dissolved in 0.5 ml of 0.9% saline. The animals were allowed food and water ad libitum.

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### 2.1. *Preparation of post-mitochondrial supernatant (PMS)*

Death was by cervical dislocation. Livers were excised immediately and rinsed briefly in ice-cold preparative buffer, STKM. The composition of this buffer was 0.25 M sucrose (ribonuclease-free grade from Cambrian Chemicals Ltd., Croydon, UK), 50 mM tris(hydroxymethyl)aminomethane (A.R., from B.D.H. Chemicals Ltd., Poole, UK), 25 mM KCl and 5 mM $MgCl_2$, adjusted to pH 7.5 with A.R. hydrochloric acid at 20°C. After rinsing the excised liver, 2 ml of fresh STKM was added for every 1 g of liver unless otherwise stated. The liver was then chopped finely with scissors and homogenised in a Potter–Elvehjem apparatus using a motor-driven Teflon pestle rotated at 3500 rev/min in a glass vessel with a clearance of 7/1000 inch (0.18 mm.). 20 passes were used to effect homogenisation. Mitochondria, cell debris and nuclei were removed from the preparation by centrifugation of the homogenate in a fixed angle MSE 8 × 50 ml rotor at 12 500 rev/min for 20 min on the MSE Angle 18 centrifuge at 4°C.

### 2.2. *Preparation of $^{14}$C-total microsomes from the PMS*

Unless otherwise stated, each $^{14}$C-PMS preparation was centrifuged for 40 min at 40 000 rev/min (135 000 $g_{av}$) in an MSE Fixed Angle 8 × 50 ml. Rotor to yield a 'total microsome' pellet, containing rough and smooth microsomes together with free polysomes and substantial amounts of glycogen. The pellets were washed twice by resuspension in ice-cold STKM (2 ml per 1 g starting liver) and recentrifugation. The purpose of the washes was to remove contaminating molecular species normally associated with the PMicS, including

soluble $^{14}$C-tRNA. The radioactivity remaining associated with the washed total microsomes is probably almost exclusively in rRNA. For incubations, the total microsomes derived from 4 g of liver were made up with STKM to a thick suspension of vol 1 ml.

2.3. *Preparation of unlabelled post-microsomal supernatant (PMicS)*

Unless otherwise stated, PMS from untreated rats was centrifuged for 2 h at 40 000 rev/min (130 000 $g_{av.}$) in an MSE Fixed Angle 8 × 50 ml rotor and the supernatant collected. The PMicS derived from an initial homogenate in which 2 ml of STKM had been added for every 1 g of liver has been defined as 1 unit·ml$^{-1}$. In this way, a suspension made from 2 g of liver homogenised in 2 ml of buffer is referred to as being 2 units·ml$^{-1}$, etc. This system takes no account of liver volume and refers only to the method of preparation of the PMicS.

2.4. *Pretreatment of yeast RNA*

In order to remove contaminating metal ions, 1 g of yeast RNA (highly polymerised, Na salt, from B.D.H.) was made up to 10 ml with 0.025 M EDTA. It was further dialysed against STKM and the sample removed from the dialysis sack. It was made up to 10 ml with STKM.

2.5. *Measurement of the rate of breakdown of exogenous yeast RNA*

The incubations containing yeast RNA were made up as described in the individual figure legends. Five aliquots of 0.2 ml were removed at regular intervals over a 2-h period of incubation, acidified with 0.5 ml of 7% trichloroacetic acid (TCA) solution and then centrifuged for 2 min on a bench centrifuge. The absorbance of the supernatant was determined at 260 nm. using a Unicam SP-8000 Spectrophotometer. The absorbance at 260 nm was plotted against the time of incubation and the slope of best straight line through the points was taken as the rate of RNA breakdown.

2.6. *Determination of the rates of breakdown of endogenous $^{14}$C-RNA*

Incubations without yeast RNA were made up as described in the figure legends and five 0.2 ml. samples taken at regular time intervals. After acidification with TCA and centrifugation as above, 0.5 ml aliquots of the supernatant were added to 4.5 ml of distilled water, shaken with 5 ml of the commercial scintillation cocktail Instagel (from Packard Instruments International, Zurich) and counted in an Inertechnique Model SL40 Liquid Scintillation Spectrometer. The time course for acid-soluble CPM was plotted and the slope of the best straight line was taken as the rate of breakdown of endogenous RNA.

2.7. *Ribonuclease inhibitor*

Ribonuclease inhibitor was purchased from Searle Biochemicals, High Wycombe UK. The inhibitor had been prepared from rat liver and possessed an activity of 200–400 units·mg$^{-1}$. One unit was defined as that amount of inhibitor which gave a 50% inhibition to 0.005 μg of crystalline pancreatic ribonuclease under standard conditions.

## 3. Results and discussion

As shown in fig.1, there is a biphasic response of the rate of degradation of yeast RNA by total microsomes to increasing concentrations of PMicS. This is consistent with the idea that at least two classes of ribonuclease are involved in the degradation of the yeast RNA. The predominant activity in the total microsomes is of the RNase-Se type, inhibited by some factor in the PMicS, presumably ribonuclease-inhibitor protein, and by the purified inhibitor itself. The PMicS appears to contain RNase-In activity which is progressively expressed with increasing quantities of PMicS. Purified inhibitor has no effect upon the activity expressed by PMicS (fig.1B), indicating that the RNase-Se enzymes present are already fully latent.

As shown in fig.2, the effects of increasing amounts of PMicS on the spontaneous degradation of the endogenous $^{14}$C-RNA of the total microsomes are not the same as the effects on the degradation of the exogenous yeast RNA. The maximal inhibition effected by low concentrations of PMicS is less (5–35% as compared with 60–70%), and the stimulation caused by higher concentrations much greater (500% as compared with 50%). We conclude that most of the spontaneous breakdown of the endogenous RNA of the total microsomes to acid-soluble fragments is catalysed by RNase-In enzymes associated with this

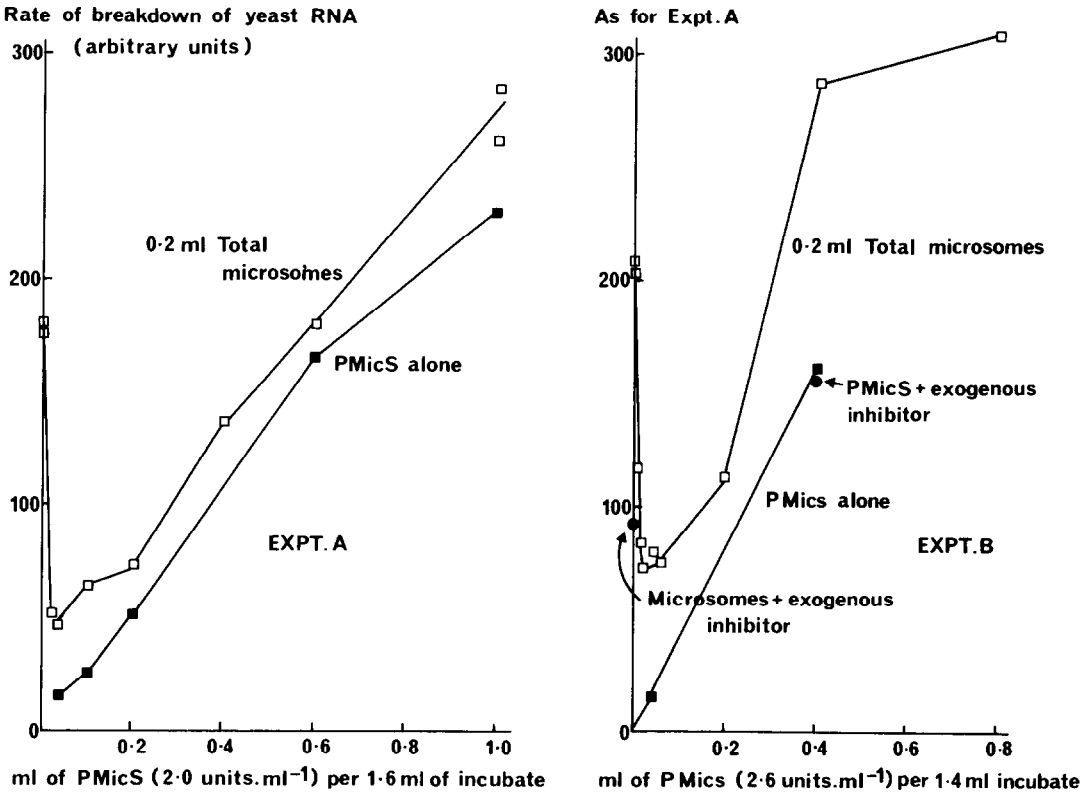


Fig.1. Effect of increasing quantities of PMicS on the rate of degradation of yeast RNA to acid-soluble fragments by total microsomes at 37°C. (Expt.A) 0.2 ml of suspension of total microsomes, PMicS as indicated, 0.2 ml of yeast RNA solution, made up to 1.6 ml with STKM. (Expt.B) as for Expt.A except 0.2 ml of ribonuclease inhibitor (2 mg·ml$^{-1}$) added where indicated. Made up to 1.4 ml.

fraction and that the addition of PMicS results in an increase in RNase-In activity. The addition of PMicS to fractions containing ribosomes is therefore unlikely to prevent deterioration of rRNA and large quantities of PMicS will increase the rate of breakdown of the rRNA.

From a comparison of figs.1 and 2, it is clear that the RNase-In enzymes present in total microsomes act more efficiently on the endogenous substrate (rRNA in situ) than RNase-Se enzymes, which prefer exogenous substrate (yeast RNA). This suggests that the enzymes responsible for the degradation of rRNA in vivo may be of the RNase-In type.

PCMB is known to activate latent RNase-Se enzymes in biological systems [1], but when PCMB is added to a suspension of total microsomes in PMicS to a final concentration of 1.4 mM, an inhibition in the rate of breakdown of *endogenous* $^{14}$C-RNA is observed (fig.3). This contrasts most strikingly with the large stimulation of the rate of degradation of exogenous yeast RNA that PCMB causes in the same system (fig.4). A reasonable explanation is that the RNase-In enzymes present in the PMicS and mainly responsible for the degradation of the endogenous $^{14}$C-RNA are inactivated by PCMB. Thus the response of any system to PCMB as regards the degradation of endogenous RNA is a balance between the release from the latent form of less-effective RNase-Se activity and the inhibition of the more effective RNase-In enzymes. The response of the same system to PCMB towards yeast RNA is different because of the particular substrate preferences of the enzymes involved, with the RNase-Se enzymes acting preferentially towards exogenous substrate.

In agreement with our findings, Roth [1] noted

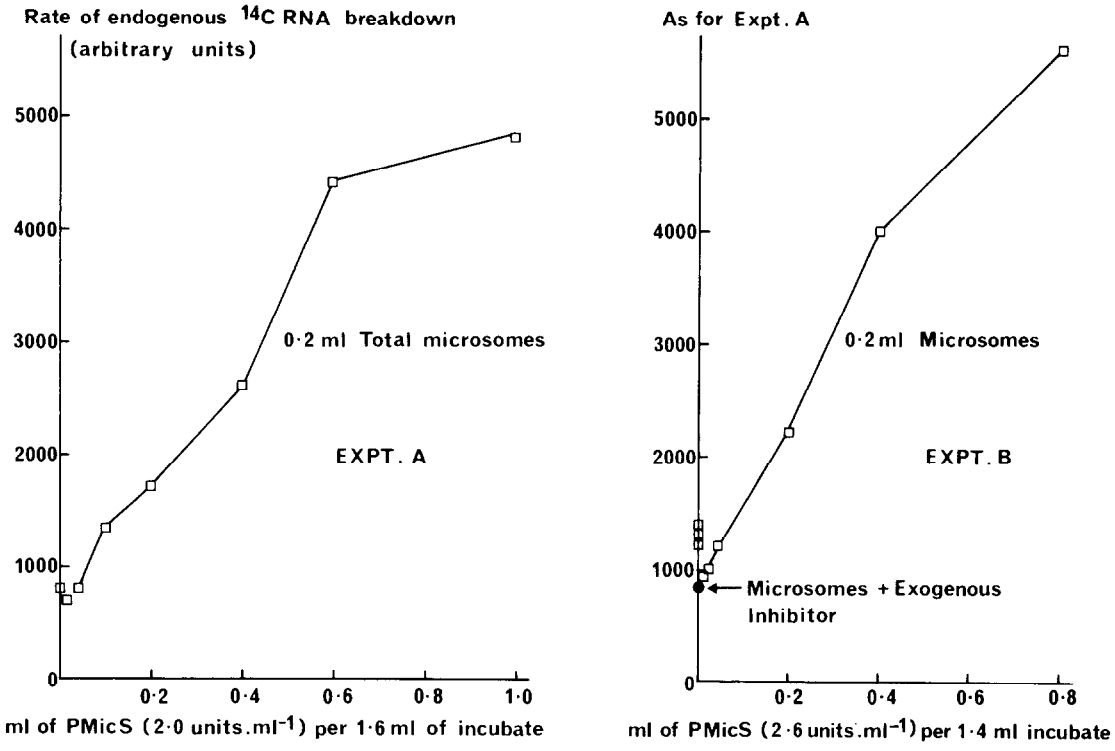


Fig.2. Effect of increasing quantities of PMicS on the rate of degradation of endogenous $^{14}$C-RNA to acid-soluble fragments by total microsomes at 37°C. (Except.A) 0.2 ml of suspension of total microsomes, PMicS as indicated, made up to 1.6 ml with STKM. (Except.B) As for Except.B except 0.2 ml of ribonuclease inhibitor (2 mg·ml$^{-1}$) added where indicated. Made up to 1.4 ml.

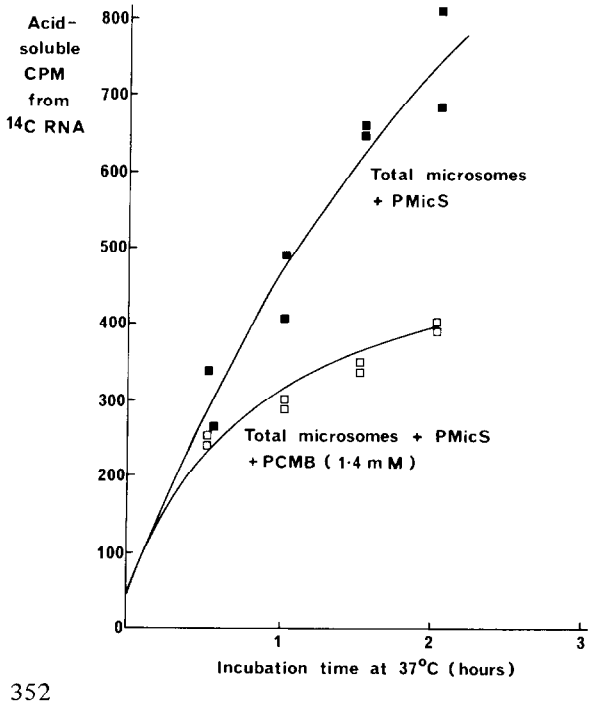


Fig.3. Inhibition of the degradation of endogenous $^{14}$C-RNA in a mixed system of total microsomes and PMicS by 1.4 mM PCMB. The $^{14}$C-total microsomes were prepared as a pellet from $^{14}$C-PMS centrifuged for 1 h at 30 000 rev/min (120 000 $g_{av.}$) in an MSE Swing-Out 40Ti Rotor (6 × 14 ml) at 4°C. The pellet was resuspended in unlabelled PMicS and recentrifuged identically. The final pellet was resuspended in 25 ml of further unlabelled PMicS. The unlabeled PMicS was prepared 1 unit·ml$^{-1}$ in strength by centrifugation as above. The mixture of $^{14}$C-total microsomes and unlabelled PMicS was incubated at 37°C with or without 1.4 mM PCMB. Acid-soluble CPM were estimated as in Materials and methods. Each incubation also contained 0.25% w/v dimethylsulphoxide and 1.5 mg·ml$^{-1}$ NADPH for reasons unconnected with this communication.

that enzymes present in PMicS could account for up to 10% of the total alkaline ribonuclease activity expressed by fractionated rat liver. Hunter and Korner [7] also observed such an activity, and ascribed it to an exonuclease capable of attacking polyribonucleotides from the 5′-end, for example the phospho-

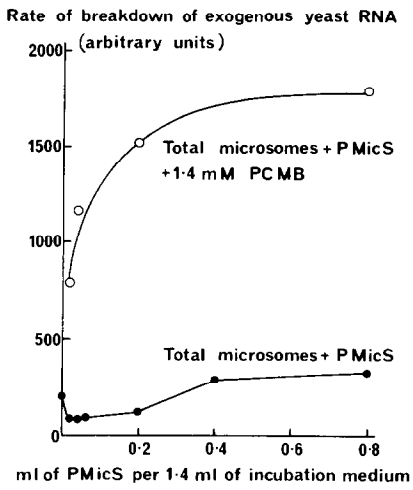


Fig.4. Effect of PCMB on the degradation of exogenous yeast RNA by total microsomes + PMicS (2.6 units·ml$^{-1}$). Experimental details as in fig.1 (Expt.B).

diesterase II of Razzell [8]. The relationship of such a 5′-phosphodiesterase to the RNase-In activity of the PMicS is not established, although the observation that phosphodiesterases are not inhibited by the inhibitor present in PMicS [9] is consistent with a common identity. However, the possible involvement of acid ribonuclease cannot be discounted since this enzyme, which possesses some activity in the pH range 7.0–7.5 [10], is also not suppressed by the inhibitor [10].

The results presented in this communication clearly demonstrate that the addition of PMicS to preparations containing ribosomes is unlikely to protect ribosomal RNA from degradation and, indeed, can be deleterious in large quantities. It is also clear that rRNA is more susceptible to the action of enzymes of the type RNase-In, which suggests that these enzymes may be important in the turnover of rRNA in vivo. The common use of yeast RNA to assess the ribonuclease activity of sub-cellular fractions does not take into account the hydrolytic capacity for endogenous RNA expressed by RNase-In enzymes.

## Acknowledgements

This work was supported by grants from the Cancer Research Campaign and the Medical Research Council, and carried out in part in laboratories provided by The Nuffield Foundation. We appreciate the skilled assistance of Ms Rosemary Clark in preparing the figures for this publication.

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