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SUPPRESSION OF SYNTHESIS OF RNA PRESENT IN MICROSOMAL MEMBRANES AFTER ADMINISTRATION OF PHENOBARBITAL*

J. SEIFERT† and H. REMMER
Institut für Toxikologie, Wilhelmstrasse 56, 74 Tübingen, Germany

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Abstract—The synthesis of RNA present in membranes of rat liver endoplasmic reticulum is differently affected by the administration of phenobarbital than that of other cytoplasmic RNA's. Notwithstanding the enhanced proliferation of the microsomal membranes after phenobarbital treatment, the amount of this "minority" RNA component in the endoplasmic reticulum is lower than in control groups. The incorporation of labeled orotic acid is simultaneously reduced. This suggests the inhibition of membrane-RNA synthesis after the application of the drug.

THE MEMBRANES of the endoplasmic reticulum of rat liver contain RNA which differs from all other cytoplasmic RNA's. This mem-RNA differs from t-RNA and r-RNA in its sedimentation coefficient.[1–4] According to other observations, however, it has the same molecular weight as t-RNA,[5,6] but shows a different behavior on hydroxyapatite columns, has little or no acceptor activity, a high content of guanosine + cytidine, a low content of 3′ terminal adenosine groups, of methylated bases and of pseudouridylic acid.[5] No differences in the acceptor activity of mem-RNA and t-RNA have been reported so far.[6] The estimation of template activity showed that RNA isolated from smooth membranes is more active than r-RNA but less active than polyuridylic acid.[2] Mem-RNA differs also metabolically being more rapidly labeled than other cytoplasmic RNA's.[4,7–12]

Since the proliferation of the smooth endoplasmic reticulum is one of the phenomena which accompany the administration of pH,[13–15] we have made an effort to determine to what degree this drug affects the synthesis of this "minority" RNA of liver endoplasmic membranes.

METHODS

Male albino rats (120–130 g) were used. PH (Na-salt, Merck) was applied intraperitoneally in an amount of 80 mg/kg per day. 6-[^{14}C] orotic acid (60 mc/m-mole, Amersham) was injected intraperitoneally in an amount of 2 μc per animal. The rats were starved for 12 hr, decapitated and bled. The liver was excised, weighed and

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† Present address: Institute of Pharmacology, Czechoslovak Academy of Sciences, Prague 2, Albertov 4, Czechoslovakia.

Abbreviations used: mem-RNA = RNA present in microsomal membranes; t-RNA = transfer RNA; r-RNA ribosomal RNA; PH = phenobarbital, Na-salt; DOC = sodium deoxycholate; TCA = trichloracetic acid; RNase = ribonuclease.

homogenized with 6 vol. buffer A (0·25 M sucrose; 0·02 M tris–HCl buffer pH 7·6; 0·01 M magnesium acetate; 0·04 M NaCl; 0·1 M KCl).[16] The homogenate was centrifuged for 10 min at 500 *g* (average), the sediment was discarded and the mitochondrial fraction was removed by centrifugation for 10 min at 10,000 *g* (average) (twice).

The postmitochondrial fraction of the liver was centrifuged at 150,000 *g* (average, titanium rotor 50, Spinco) for 2 hr. The supernatant was used for determination of the relative specific radioactivity of t-RNA. The microsomal pellets were suspended in buffer A and centrifuged once more under the conditions given above to remove the bulk of t-RNA of microsomes. The supernatants were discarded and the washed microsomal pellets were resuspended in buffer A (without sucrose). The solution of DOC was then added (final concentration 1·3%) and after 10 min stirring the ribosomes were sedimented by centrifugation at 150,000 *g* (average) for 3 hr. The ribosomal pellets were suspended in buffer A (without sucrose) and the relative specific radioactivity of the ribosomal RNA's were determined. To the DOC supernatant containing mem-RNA, potassium acetate (final concentration 2%) and 3 vol. of absolute ethanol were added. The precipitate was kept at −10° overnight, then collected by centrifugation and the relative specific radioactivity of mem-RNA was determined as described below.

To the postmicrosomal supernatant, ribosomal suspension and ethanol precipitate of the DOC fraction, cold TCA was added (final concentration 5%). After centrifugation the sediment was washed twice with cold 5% TCA. The sediment was then extracted twice with a mixture of alcohol and ether (3:1) at 70° for 3 min. After centrifugation the sediment was hydrolysed with 5% TCA at 90° for 30 min. The precipitate was separated and TCA removed from the supernatant by extraction with ether until the reaction of the supernatant was neutral. The aqueous phase was heated in a water bath at 50° for 10 min to remove dissolved ether. Aliquots of the clear hydrolysate of RNA's were applied on paper discs (Schleicher & Schüll, No. 598) and the radioactivity was measured in the toluene scintillator fluid using the Packard liquid scintillation counter. In aliquots the absorbance of hydrolysed

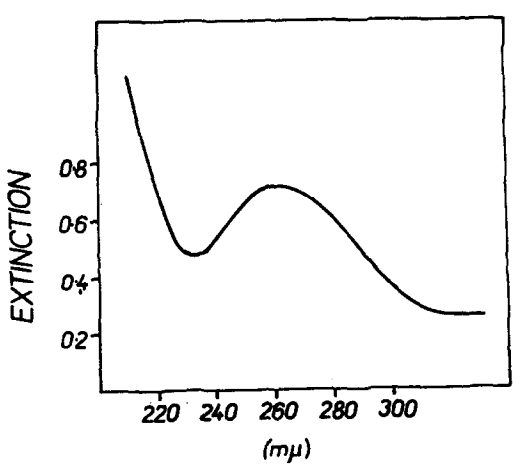

FIG. 1. Ultraviolet spectrum of TCA hydrolysate of microsomal membrane-RNA.

TABLE 1. INCORPORATION OF 6-[^{14}C] OROTIC ACID INTO CYTOPLASMIC RNA'S OF RAT LIVER

Expt.	Time after application of orotic acid (hr)	t-RNA		r-RNA		mem-RNA		Liver weight (g)		Liver weight increase (%)
		C	PH	C	PH	C	PH	C	PH	
a	96	680 ± 58	620 ± 64 92%	625 ± 70	575 ± 71 91%	460 ± 60	470 ± 75 102%	5·2 ± 0·5	6·6 ± 0·6	+26
b	132	480 ± 40	440 ± 34 92%	460 ± 44	420 ± 56 91%	400 ± 52	320 ± 44 80%	4·7 ± 0·4	6·1 ± 0·5	+30

The values represent the relative specific radioactivity (counts/min/mg RNA), including the standard deviation of the mean for six determinations. 6-[^{14}C] orotic acid was applied intraperitoneally in an amount of 3 μc per animal. The rats were given phenobarbital five times.

Expt. a: labeled orotic acid was injected 12 hr after the first dose of phenobarbital.

b: 24 hr before the first dose of the drug.

C = controls, PH = phenobarbital-treated animals.

RNA's at 260 nm was determined. Figure 1 shows the spectrum of hydrolysed mem-RNA which indicates that the absorbing material is of nucleotide character.

Mem-RNA for density gradient centrifugation was isolated as follows. To the DOC supernatant sodium dodecylsulphate (Serva Heidelberg) and sodium acetate at pH 5·0 (final concentration 0·5% and 0·1 M, respectively) were added. Mem-RNA and proteins were precipitated in the presence of 2% potassium acetate with 3 vol. absolute ethanol. The mixture was centrifuged at 24,000 *g* (average) for 20 min. The sediment was suspended in 0·1 M NaCl and 0·05 M tris–HCl buffer, pH 7·0, and digested 1 hr with pronase (5 mg/ml, Serva Heidelberg) at 37°. The enzyme was pre-incubated 1 hr at the same temperature. The precipitate was removed by centrifugation at 24,000 *g* (average) for 20 min and t-RNA (*E. coli* t-RNA, 1 mg/ml, General Biochemicals) was added to the supernatant. The RNA's were precipitated as described above. The precipitate was dried, dissolved in a small volume of water and the RNA's were examined by sucrose density gradient centrifugation. The possible contamination of sucrose with RNAse was prevented by the procedure of Fraenkel-Conrat *et al.*[17]

RESULTS

The relative specific radioactivity of cytoplasmic t-RNA, r-RNA, and mem-RNA 12 hr after the administration of labeled orotic acid to rats which had been treated with PH for 5 days is shown in Fig. 2. The relative specific radioactivity of mem-RNA is markedly higher than the specific radioactivity of t-RNA and r-RNA. The application of five doses of PH decreased the specific radioactivity of mem-RNA by 50 per cent. After the correction of the relative specific radioactivity for the increased liver

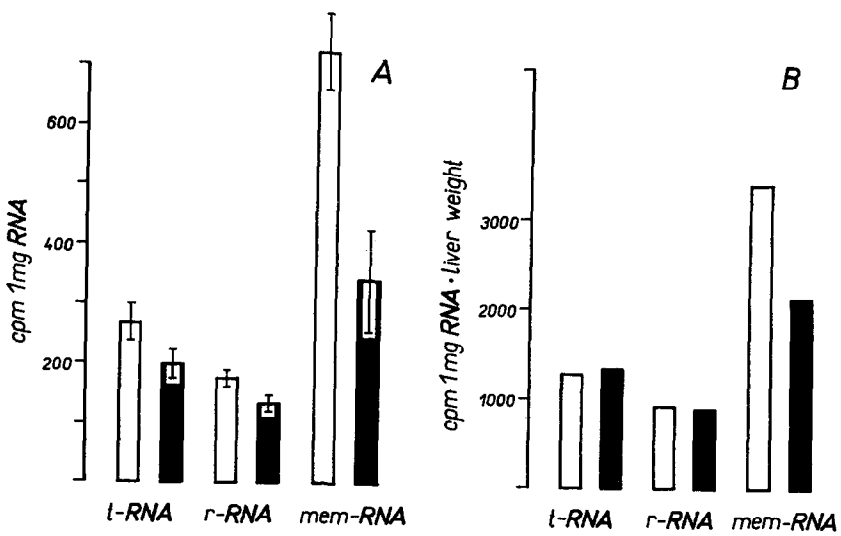

FIG. 2. Incorporation of labeled orotic acid into cytoplasmic RNA's of rat liver. 6-[^{14}C] orotic acid was injected intraperitoneally 12 hr before decapitation. A = relative specific radioactivity, B = relative specific radioactivity corrected per liver weight. The rats were given phenobarbital five times. The values represent the mean of six determinations including standard deviation. Empty columns—controls. Full columns—phenobarbital treated animals.

weight the differences between t-RNA and r-RNA are eliminated and the specific radioactivity of mem-RNA remains decreased.

The values of relative specific radioactivity after long term contact with labeled orotic acid in the PH treated animals are the same (expt. a) or only a little lower (expt. b) than the values of specific radioactivity of mem-RNA in the control group (Table 1).

The quantitative determination of mem-RNA of the endoplasmic reticulum showed that the administration of PH results in a decrease of the mem-RNA content of these membranes. The amount of mem-RNA in the endoplasmic reticulum isolated from the liver of control animals as calculated from the sum of extinction values at 260 nm[18] of the TCA hydrolysate is 25 μg/g liver. The corresponding value for animals treated with five doses of PH is about one half of this value (Table 2).

TABLE 2. AMOUNT OF mem-RNA IN RAT LIVER MICROSOMAL MEMBRANES

μg of mem-RNA per 1 g of liver		μg of mem-RNA per whole liver		Liver weight (g)	
C	PH	C	PH	C	PH
25 ± 6	13 ± 4	100	69	4·0 ± 0·4	5·3 ± 0·5

The rats were given phenobarbital five times. The values are the mean of six determinations (12 animals) including the standard deviation. The amount of mem-RNA was calculated from the sum of extinction of TCA hydrolysate at 260 nm. The extinction of 1 mg of RNA hydrolysate was taken to represent 34,2 (ref. 18).
C = controls, PH = phenobarbital-treated rats.

The density gradient centrifugation pattern shows that the radioactivity of RNA isolated from DOC supernatant of liver cells of control animals coincides with the absorbance of t-RNA added, and that its sedimentation coefficient is therefore about 4 S (Fig. 3).

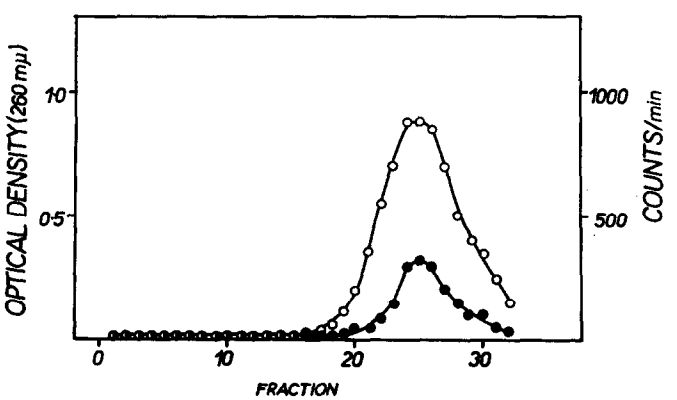

FIG. 3. Density gradient centrifugation of isolated mem-RNA of microsomes. The labeled mem-RNA from control animals and the unlabeled t-RNA were centrifuged in a 5–20% sucrose gradient at 32,000 rev./min for 14 hr in Spinco SW 39 rotor. Optical density (○——○) and radioactivity (●——●) were determined on eight drop fractions taken from the bottom of the gradient tube. The radioactivity was estimated using the scintillation fluid according to Bray.[19]

DISCUSSION

Experiments with the incorporation of labeled orotic acid into the mem-RNA have shown that this RNA has a higher relative specific radioactivity than the other cytoplasmic RNA's, i.e. t-RNA and r-RNA. Similar results were obtained by other authors using different isolation techniques.[4,7–12] The higher relative specific radioactivity of mem-RNA isolated from DOC supernatant indicated that (disregarding a certain degree of contamination with other RNA's) the bulk of the RNA represents mem-RNA and that this approach can be used for the study of the turnover of mem-RNA of microsomes.

PH belongs to substances which induce the microsomal enzymes of drug metabolism.[20,21] The repeated application of the drug causes the increase in liver weight. Although after application of PH one may observe the activation of DNA synthesis,[22,23] the growth of liver mass is caused by the cell hypertrophy[15,24] associated with a practically parallel increase of the cell proteins, lipids, glycogen[23] and ribonucleic acid.[25]

The lower value of the relative specific radioactivity of r-RNA after PH application is caused by the dilution of labeled ribosomes by unlabeled ribosomes, which are present in hypertrophic liver cell after administration of the drug in an increased amount. The loss of the relative specific radioactivity after 20 hr exposure of labeled orotic acid varies inversely with the increase in liver mass. When this value is corrected for liver weight it is approximately the same as in the control group.[26] The same holds probably true for t-RNA. The relative specific radioactivity of RNA of microsomal membranes is also lower than in the control groups. When corrected for increased liver weight, however, the values remain decreased. Even though the employed isolation method eliminated the presence of intact cytoplasmic ribosomes and their subunits, it is quite possible that a part of the radioactivity can be derived from the degradation products of ribosomal precursors. The RNA's of ribosomal precursors, however, are synthetized in the nucleus and their synthesis is activated after the administration of PH rather than inhibited.[27]

When labeled orotic acid was injected before the first dose of PH, the values of relative specific radioactivity were only a little lower than in the control groups even though one would expect that the increased synthesis of smooth membranes of the endoplasmic reticulum during the 5-day administration of PH[13–15] would be accompanied also by increased mem-RNA synthesis. The relative specific radioactivity of mem-RNA should therefore be decreased due to the "dilution" of labeled mem-RNA with newly synthetized unlabeled mem-RNA. The quantitative determination of mem-RNA showed that the application of PH leads to a decrease not only in the amount per gram of liver but also per whole organ. In spite of the fact that the amount of mem-RNA can be influenced by a number of rather uncontrollable factors, the simultaneous decrease of the relative specific radioactivity of mem-RNA indicates that the administration of PH inhibits its synthesis. This inhibition might be, to a smaller extent, accompanied with simultaneous inhibitions of mem-RNA degradation after the administration of PH, which has been observed with cytoplasmic ribosomes,[26] microsomal phospholipids[28] as well as with microsomal proteins.[29–31] The changes in the amount of mem-RNA cannot be caused by the variations in the level of ribonucleases, because there is a dramatic depression of microsomal RNase[32] as well as of other liver RNases after the application of PH.[33]

During the induction of liver growth by hormones and in the process of liver regeneration the labeling of mem-RNA is more stimulated than the labeling of r-RNA. The opposite effect of PH indicates a different mechanism of growth activation.[9]

The density gradient analysis showed that mem-RNA has the same molecular weight as t-RNA. Although the same results were obtained also by other authors,[5,6] the possibility cannot be excluded that a certain amount of high molecular weight RNA had been destroyed during the isolation procedure.

At present we are lacking clear-cut evidence showing that membrane bound RNA is either a functional part of the microsomal membrane of an artefact formed during the isolation procedure. Our present knowledge of its properties, quantity and function is incomplete and extremely controversial. The differences in the results can obviously be accounted for by variations in the employed isolation procedures which may give rise to varying contamination by the remaining cytoplasmic RNA's or by their degradation products. The most common feature of mem-RNA seems to be its rapid turnover as compared to cytoplasmic t-RNA and r-RNA.[4,7-12] Its faster turnover and template activity[2] have led some authors to the assumption that this RNA component of the endoplasmic reticulum could represent a stable messenger RNA and could thus play a role in the induction of microsomal enzymes participating in the drug metabolism.[1] Recently Pitot *et al.*[34] denotes this RNA as the "membron" which might represent the membrane associated RNA operon. Its other function could be the attachment of the ribosomes to membranes of the endoplasmic reticulum.[35] The investigations of mem-RNA, its turnover, content in the smooth and rough membranes of endoplasmic reticulum of the liver, after the administration of PH will be the subject of our future studies.

REFERENCES

1. V. Shapot and H. C. Pitot, *Biochim. biophys. Acta* **119**, 37 (1966).
2. H. W. S. King and W. Fitschen, *Biochim. biophys. Acta* **155**, 32 (1968).
3. N. P. Rodionova and V. S. Shapot, *Biochim. biophys. Acta* **129**, 206 (1966).
4. V. S. Shapot and N. P. Rodionova, *Molek. Biologija* **3**, 785 (1969).
5. J. A. A. Gardner and M. B. Hoagland, *J. biol. Chem.* **243**, 10 (1968).
6. T. Scott-Burdon and A. O. Hawtrey, *Biochem. J.* **115**, 1063 (1969).
7. J. R. Tata, *Nature, Lond.* **213**, 566 (1967).
8. J. R. Tata, *Biochem. J.* **105**, 47P (1967).
9. J. R. Tata, *Biochem. J.* **104**, 1 (1967).
10. P. Goswami, G. C. Barr and H. N. Munro, *Biochim. biophys. Acta* **55**, 408 (1962).
11. S. Petrovič, J. A. Bacarevič and J. Petrovič, *Biochim. biophys. Acta* **91**, 118 (1965).
12. C. Bergeron-Bouvet and Y. Moulé, *Biochim. biophys. Acta* **123**, 617 (1966).
13. H. Remmer and H. J. Merker, *Klin. Woch.* **41**, 276 (1963).
14. S. Orrenius and J. L. E. Ericsson, *J. cell. Biol.* **28**, 181 (1966).
15. W. Stäubli, R. Hess and E. R. Weibel, *J. cell. Biol.* **42**, 92 (1969).
16. A. J. Munro, R. J. Jackson and A. Korner, *Biochem. J.* **92**, 289 (1964).
17. A. Fraenkel-Conrat, B. Singer and A. Tsugita, *Virology* **14**, 54 (1961).
18. H. S. Wilson and M. B. Hoagland, *Biochem. J.* **103**, 556 (1967).
19. G. A. Bray, *Anal. Biochem.* **1**, 270 (1960).
20. H. Remmer, *Naturwiss.* **45**, 1989 (1958).
21. A. H. Conney and J. J. Burns, *Nature, Lond.* **184**, 365 (1959).
22. T. S. Argyris, *Develop. Biol.* **17**, 187 (1968).
23. I. Schlicht, W. Koransky, S. Magour and R. Schulte-Hermann, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **261**, 26 (1968).
24. W. Kunz, G. Schaude, W. Schmid and M. Siess, *Proc. Europ. Soc. for the Study of Drug Toxicity, Rome*, Vol. VII, 113 (1966).

25. W. KUNZ, G. SCHAUDE, H. SCHIMASSEK, W. SCHMID and M. SIESS, *Proc. Europ. Soc. for the Study of Drug Toxicity, Rome*, Vol. VII, 138 (1966).
26. J. SEIFERT and H. REMMER, in press.
27. W. J. STEELE, *Intern. Symp. on Microsomes and Drug Oxidations, Tübingen* (1969).
28. J. L. HOLTZMAN and J. R. GILLETTE, *J. biol. Chem.* **243**, 3020 (1968).
29. H. JICK and L. SHUSTER, *J. biol. Chem.* **241**, 5366 (1966).
30. L. SHUSTER and H. JICK, *J. biol. Chem.* **241**, 5361 (1966).
31. Y. KURIYAMA, T. OMURA, P. SIEKEVITZ and G. E. PALADE, *J. biol. Chem.* **244**, 2017 (1969).
32. R. T. LOUIS-FERDINAND and G. C. FULLER, *Biochem. biophys. Res. Commun.* **38**, 811 (1970).
33. J. SEIFERT and J. VÁCHA, *Chem.-Biol. Interactions*, in press.
34. H. C. PITOT, N. SLADEK, W. RAGLAND, R. K. MURRAY, G. MOYER, H. D. SOLIG and J. P. JOST, in *Microsomes and Drug Oxidations* (Eds. J. R. GILLETTE *et al.*) p. 59, Academic Press, New York (1969).
35. Y. MOULÉ, in *Cellular Membranes and Development* (Ed. M. LOCHE) p. 97, Academic Press, New York (1964).