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LOCALIZATION OF AN RNA-DEGRADING SYSTEM OF ENZYMES ON THE RAT LIVER PLASMA MEMBRANE

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SUMMARY

In comparison to other subcellular fractions, isolated rat liver plasma membrane has been shown to have a high capacity to degrade RNA at pH 8.8.

1. Upon incubation of RNA with these membranes 0.056 μmoles of acid-soluble products and 0.023 μmole of inorganic phosphate are released per min per mg of membrane protein. This rate of RNA hydrolysis is comparable to that catalyzed by rat liver lysosomes.

2. The activities of four enzymes, phosphodiesterase I (EC 3.1.4.1), 3′-nucleotidase (EC 3.1.3.6), 5′-nucleotidase (EC 3.1.3.5) and an endonuclease, are involved in this degradation of RNA.

3. All four of these activities are localized with the plasma membrane fraction of liver.

4. When phosphodiesterase I and 3′- and 5′-nucleotidases are inhibited by the the addition of EDTA, the hydrolysis of RNA still occurs. This implies that another enzyme capable of degrading RNA, yet not inhibited by EDTA, must be present on liver plasma membrane.

5. When EDTA is absent from the reaction medium a large quantity of nucleosides is produced, but when EDTA is present, no significant amount of nucleosides or mononucleotides is released. This indicates that the non-EDTA inhibited activity is an endonuclease.

6. The pH optimum of the endonuclease is 7.7, while the optimum of overall RNA hydrolysis by plasma membrane is 9.3.

INTRODUCTION

The plasma membrane of rat liver is known to possess a number of hydrolytic enzymes, most of which have not been assigned a definitive physiological role. In this paper, we will attempt to establish a function of RNA degradation for four of the enzymatic activities which reside on the plasma membrane of liver tissue. These four activities are; phosphodiesterase I (EC 3.1.4.1), 3′-nucleotidase (EC 3.1.3.6), 5′-nucleotidase (EC 3.1.3.5) and an endonuclease. Phosphodiesterase I is an exonuclease which liberates only 5′-mononucleotides from RNA and requires a free 3′-hydroxyl group for activity[1,2], while 3′- and 5′-nucleotidases liberate inorganic

phosphate from the 3′ and 5′ termini of their respective nucleotide substrates. The specificity of the endonuclease is currently under study.

Several investigators have reported the localization of phosphodiesterase I on the plasma membrane of rat liver[3,4], and this enzyme, along with 5′-nucleotidase, is generally used as a marker enzyme for the plasma membrane. A 3′-nucleotidase activity has also been reported to be present on rat liver plasma membrane[5]. Ribonuclease activity has been reported to be found on HeLa cell surfaces[6], on the cell surface of Krebs II mouse ascites tumor cells[7], and on isolated rat liver plasma membrane[8,9].

In this paper, we show that phosphodiesterase I, 3′-nucleotidase, 5′-nucleotidase, and endonuclease activities are indeed localized on rat liver plasma membrane. A scheme for the degradation of RNA by these membranes is proposed and the possible physiological significance of an RNA-degrading system of enzymes on the surface membrane is discussed. This work has previously been presented in preliminary form[10].

MATERIALS AND METHODS

Yeast RNA was purchased from Plenum Scientific Research Inc. 3′-AMP, 5′-AMP and *p*-nitrophenylthymidine 5′-phosphate were purchased from Sigma Chemical Company. DEAE-cellulose (DE-52) anion exchanger was purchased from Reeve Angel. 1-Amino-2-naphthol-4-sulfonic acid, dry mixture, was purchased from Fisher Scientific Co. All other materials were commercial products of reagent grade.

Plasma membranes were isolated by the procedure of Touster *et al.*[4]. In this scheme the rat livers are homogenized in isotonic sucrose, and plasma membrane vesicles are isolated by means of a number of differential centrifugation steps followed by a final purification of the membranes in a discontinuous sucrose gradient. This procedure allows for plasma membranes to be isolated from the nuclear as well as the microsomal fraction. The membranes from the nuclear fraction are designated as Fraction N_2, while those from the microsomal fraction are designated as Fraction P_2. Both these membrane fractions appear identical in electron micrographs[4]. Specific activities of plasma membrane marker enzymes in Fractions N_2 and P_2 are increased at least 25-fold over the homogenate, while the specific activities in Fractions N_2 and P_2 for marker enzymes of other cell organelles are less than homogenate specific activities. Plasma membrane fragments isolated from the microsomal fraction were used for all assays and hydrolyses unless otherwise indicated.

Golgi membranes were isolated by the procedure of Morre *et al.*[11].

A purified lysosomal fraction was isolated by the procedure of Leighton *et al.*[12]. The lysosomes were disrupted in 5 mM sodium bicarbonate and the lysosomal membranes were removed by centrifugation. The supernatant, which contains the majority of the lysosomal nuclease activity[13], was the lysosomal fraction used for degrading RNA.

In order to separate and identify the products of the RNA hydrolysis by plasma membranes, a procedure described by Tomlinson and Tenner[14] was used. The procedure involves chromatography of the RNA digest on a DEAE-cellulose column with a linear gradient of NaCl in 7 M urea. The urea eliminates all secondary binding forces between the nucleic acid degradation products and the cellulose,

and therefore this procedure separates mixtures of polynucleotides on the basis of their net negative charge. The hydrolysis mixture therefore is separated into fractions containing mononucleotides, dinucleotides, trinucleotides, etc.

Phosphodiesterase I and 5′-nucleotidase were assayed by the procedures described by Touster *et al.*[4].

The activity of 3′-nucleotidase was assayed in the same manner as 5′-nucleotidase, except that 3′-AMP was used as the substrate, and the reaction was carried out for 5 h as compared to 20 min for the 5′-nucleotidase assay. In order to prevent bacterial growth, a drop of toluene was added to all assays which had incubation times longer than two hours.

RNA degradation was followed by the formation of acid-soluble products and by the release of inorganic phosphate.

In the assay for acid soluble products, a solution of *t*-butanol–glacial acetic acid (2:1, v/v) was used as the precipitating agent. At various time points, 0.2 ml of the reaction mixture was added to 1.8 ml of the precipitating agent, and the resulting solution was cooled for 1 h at 4 °C. The precipitate was removed by centrifugation for 20 min at $6000 \times g$ in a Sorvall RC-2 centrifuge, using an SS-34 rotor. 1 ml of the supernatant was removed and diluted to 3.5 ml with water. The absorbance was then read at 260 nm. Controls containing substrate with no enzyme and enzyme with no substrate were run and all reaction samples were corrected for these values. A nomograph based on extinction coefficients determined by Warburg and Christian[15] was used to convert absorbance at 260 nm to mg/ml of RNA. A protein concentration of zero was used on the nomograph, since any 260 nm absorbance due to protein would be corrected for by the enzyme control. Based on an average molecular weight of a mononucleotide (340 g/mole), the results are reported in terms of μmoles of acid-soluble products released.

Inorganic phosphate release was measured by the following procedure. At various time points 0.2 ml of the reaction mixture was added to 0.8 ml of 2.5% ammonium molybdate in 2.5 M H_2SO_4 and cooled for 1 h at 4 °C. The precipitate was removed by centrifugation for 20 min at $6000 \times g$, 0.5 ml of the supernatant was removed and added to 1.4 ml of water, and 0.1 ml of 1-amino-2-naphthol-4-sulfonic acid was then added. 10 min were required for color development, and the absorbance was then read at 660 nm. Absorbance of each sample was converted into μmoles/ml by comparison with a standard solution of inorganic phosphate.

RNA hydrolyses were carried out either in 0.1 M Tris–HCl, pH 7.8, or 0.1 M ammonium carbonate, pH 8.8. RNA and protein concentrations are indicated for each group of assays.

When assaying for the endonuclease, EDTA (10–50 mM) was added to the reaction mixture. EDTA at a 10 mM concentration or greater totally inactivates phosphodiesterase I (see Table II). In order to observe the combined action of all four enzyme activities on RNA, EDTA was omitted from the reaction mixture.

RESULTS

The digestion of macromolecules, including that of nucleic acids, is a physiological function of lysosomes[13]. Therefore, in order to determine if degradation of RNA is also a normal metabolic function of rat liver plasma membrane, we compared

the capacity of these membranes to degrade RNA at alkaline pH to that of lysosomes at acid pH (Table I). Even though the lysosomal fraction has a higher specific activity than the plasma membrane fraction, both fractions are able to effect extensive degradation of RNA. After a 72-h incubation the plasma membrane fraction converts 90% of the RNA to acid-soluble products and releases 45% of the phosphate as inorganic phosphate, while the lysosomal fraction converts 98% of the RNA to acid-soluble products and releases 40% of the phosphate as inorganic phosphate. These results clearly indicate that plasma membranes break down RNA to a degree comparable to the lysosomal fraction. Isolated rat liver plasma membrane therefore has a physiological capacity to degrade RNA.

TABLE I

COMPARISON OF THE ABILITY OF LYSOSOMES AND PLASMA MEMBRANES TO DEGRADE RNA

RNA hydrolyses were performed at 37 °C for 2 h with an RNA concentration of 3.2 mg/ml. The hydrolysis by plasma membranes was in 0.1 M ammonium bicarbonate, pH 8.8, with a plasma membrane protein concentration of 0.5 mg/ml. The hydrolysis by lysosomes was in 0.1 M sodium acetate, pH 5.0, with a lysosomal protein concentration of 0.18 mg/ml. Specific activities are an average of the values obtained from two experiments, and each experiment was performed in duplicate. Variation in the results was less than 10%.

Cell fraction	*Specific activity (μmoles/min per mg protein)*	
	Acid-soluble product formation	*Inorganic phosphate release*
Plasma membranes	0.056	0.023
Lysosomes	0.39	0.047

Phosphodiesterase I has been found to be inhibited by metal chelating agents such as EDTA[1,2,16]. This fact has been confirmed in our laboratory. We also found that upon removal of the EDTA by dialysis, 60% of the original activity could be restored by addition of Zn^{2+} to a concentration of 0.1 mM. This level of restoration of activity could not be equaled by any other metal tested, although other divalent metals such as Co^{2+}, Ca^{2+}, Mn^{2+} and Mg^{2+} were partially effective. The fact that only 60% of the original activity was restored can be explained by the fact that upon dialysis of plasma membranes in the absence of EDTA for 12 h against 5 mM Tris–HCl, pH 8.0, 40% of the phosphodiesterase activity is lost and this cannot be restored by addition of Zn^{2+}. Therefore we have concluded that phosphodiesterase I of rat liver plasma membrane requires Zn^{2+} for activity.

Phosphodiesterase I has been shown to be able to degrade RNA[2,16]. It was important therefore to see if another enzyme capable of degrading RNA were present on the plasma membrane. We therefore compared the hydrolysis of RNA by plasma membranes in the presence and absence of EDTA. These results are shown in Fig. 1. With no EDTA present, there is a delay in inorganic phosphate release, which is probably due to the fact that in the early part of the reaction the substrates for the nucleotidase activities are in very low concentrations. In the absence of EDTA,

after a 10-h incubation, approximately 40% of the RNA was converted to acid-soluble products and 20% of the phosphate was released as inorganic phosphate. When 50 mM EDTA was included in the reaction mixture, 5% of the RNA was converted to acid-soluble products and no inorganic phosphate was released. As mentioned above, 50 mM EDTA totally inactivates phosphodiesterase I, and in addition it inactivates practically all of the 3'-and 5'-nucleotidase activities (see Table II). This would account for the fact that no inorganic phosphate is released when 50 mM EDTA is present in the reaction mixture.

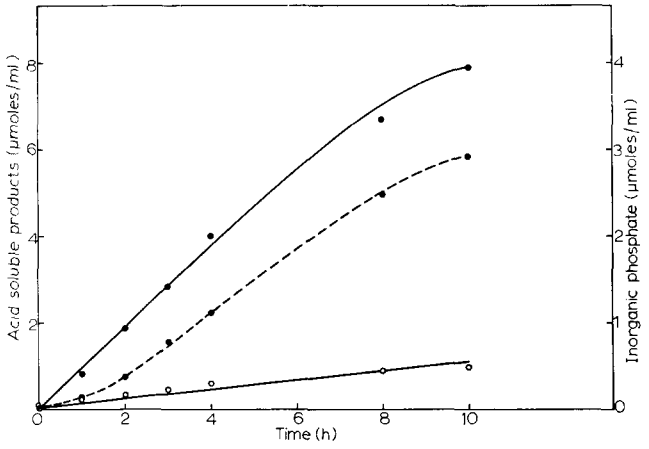

Fig. 1. RNA digestion by rat liver plasma membrane in the presence and absence of EDTA. RNA hydrolyses were performed in 0.1 M ammonium carbonate, pH 8.8, at 37 °C for 10 h. The RNA and plasma membrane protein concentrations in the reaction vessels were 5.6 mg/ml and 0.1 mg/ml, respectively. EDTA was added to one reaction vessel, which was then incubated for 5 min at 37 °C before RNA was added to start the reaction. Duplicate samples were taken at each time point. ——, acid-soluble products; - - - -, inorganic phosphate; ●, no EDTA; ○, *plus* 50 mM EDTA.

TABLE II

EFFECT OF EDTA CONCENTRATION ON ENZYME ACTIVITIES

RNA hydrolysis was measured by acid-soluble product formation. The conditions for RNA hydrolysis were the same as in Fig. 1, except that here the EDTA concentration was varied and the reaction time was 8 h. Values are for one experiment and are the average of duplicate determinations.

EDTA concn (mM)	*Percent inhibition*			
	RNA hydrolysis	*Phosphodiesterase I*	*5'-Nucleotidase*	*3'-Nucleotidase*
0	0	0	0	0
1	69	90	13	22
2	76	—	—	—
5	91	96	30	75
10	92	97	58	—
20	91	—	—	—
50	92	—	96	88
100	—	—	97	—

Even upon inhibition of phosphodiesterase I and 3′-and 5′-nucleotidases, acid-soluble products are still released. This indicates that the plasma membrane possesses another activity, not inactivated by EDTA, which will degrade RNA.

Table II shows the effects of varying EDTA concentration on the RNA hydrolysis and on phosphodiesterase I, 3′-nucleotidase and 5′-nucleotidase activities. The inhibition of RNA hydrolysis reached a maximum of about 90% at a concentration of 5 mM EDTA. This is due to the fact that phosphodiesterase I is almost completely inhibited at an EDTA concentration of 5 mM. The data indicate that about 10% of the acid-soluble products are released by an activity other than phosphodiesterase I. The release of acid-soluble products due to the non-EDTA inhibited activity would be slow if it were an endoribonuclease, since such an enzyme would not yield an acid-soluble fragment upon every cleavage. In the case of phosphodiesterase I, only 5′-mononucleotides are liberated, and therefore every cleavage would yield an acid-soluble fragment. Stonehill and Huppert[7] found a similar situation in their work on Krebs II mouse ascites tumor cells. They located an endonuclease on the plasma membrane of these cells which, in an infectivity assay using viral RNA and protoplasts from *Escherichia coli* 200 PSF^-, gave identical analytical results as a given concentration of pancreatic ribonuclease. However, when the enzymes at the same concentrations as in the infectivity assay were examined for their ability to release acid-soluble products from RNA, the membrane enzyme was much less effective than pancreatic ribonuclease. They explained their results by suggesting that the endonuclease on the cell membrane split the RNA at only a few sites. Table II also shows that both nucleotidases are strongly inhibited at an EDTA concentration of 50 mM.

The pH optimum of phosphodiesterase I has been reported to be in the range from pH 9.0–10.0[2,16], while 5′-nucleotidase is reported to have two optima of activity; one at pH 7.0, which is insensitive to EDTA, and the other at pH 9.1, which is inhibited by EDTA[17]. Knowing these facts, we determined the pH optimum of the hydrolysis of RNA by plasma membranes in the absence and presence of EDTA. The latter experiment should indicate the pH optimum of the endonuclease (Figs 2 and 3).

Fig. 2 shows the pH curve of the hydrolysis of RNA without EDTA present in the reaction mixture. The pH optimum is 9.3 for both acid-soluble product formation and release of inorganic phosphate. Fig. 3 indicates the pH curve of the hydrolysis of RNA by membranes preincubated with EDTA. Here the pH optimum is 7.7. This is much different from the pH optimum of RNA hydrolysis without EDTA and that of phosphodiesterase I. This is further proof that there is an activity on the plasma membrane, other than phosphodiesterase I, which is capable of degrading RNA.

The pH optimum for acid-soluble product formation in the absence of EDTA is near the pH optimum of phosphodiesterase I, because, as previously stated, most of the acid-soluble products come from the action of phosphodiesterase I.

The pH optimum of the 3′-nucleotidase has been reported to be between 8.9 and 9.5[5]. Since both nucleotidases have a pH optimum near 9.0, the pH optimum of inorganic phosphate release would be expected to be around 9.0.

Since the *in vitro* 3′-nucleotidase activity is very slow, this enzymatic step may be rate limiting in the hydrolysis, and therefore it would determine the pH

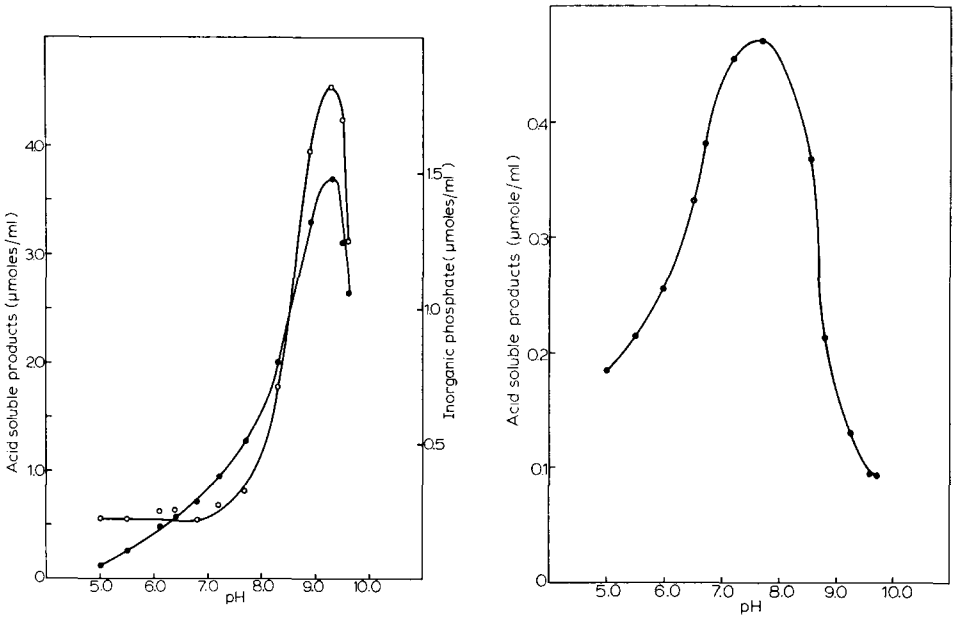

Fig. 2. The pH dependence of the hydrolysis of RNA by plasma membranes in the absence of EDTA. Reactions were performed in 0.1 M Tris–acetate buffer at 37 °C for 4 h. The RNA and plasma membrane protein concentrations in the reaction vessels were 10 mg/ml and 0.1 mg/ml, respectively. The indicated pH was determined in the reaction vessel itself and did not vary during the reaction time. ●—●, acid-soluble products; ○—○, inorganic phosphate.

Fig. 3. The pH dependence of the hydrolysis of RNA by plasma membranes which were pre-incubated with EDTA. The pH measurements and reaction conditions were the same as in Fig. 2, except that the plasma membranes were incubated with 20 mM EDTA for 15 min at 37 °C, after which the EDTA was removed by dialysis. The plasma membrane protein concentration in the reaction vessel was 0.05 mg/ml.

optimum of the overall reaction. This is another possible reason for the pH optimum of the overall hydrolysis being above 9.0.

In order to help elucidate the mechanism of the overall reaction, we tried to separate and identify the products of the RNA hydrolysis by column chromatography[14]. Fig. 4 shows a comparison between three RNA digests; one without EDTA, one containing EDTA, and a substrate control. Fig. 4A shows the elution profile for RNA digested by plasma membranes at pH 8.8 for 10 h. The first fraction emerging with the void volume comprises about 10% of the RNA substrate and 40% of the acid-soluble products. This fraction has been characterized as nucleosides by the fact that it has a standard nucleic acid absorbancy with a maximum at 258 nm and it contains no phosphate. In Fig. 4B the same type of column was run but using an EDTA-inhibited RNA digest as a sample. In this experiment no nucleosides or large fraction of mononucleotides were found, but many of the remaining fractions of RNA degradation products were still present. Since three of the four enzyme activities involved are inhibited by EDTA, the appearance of the degradation products present in Fig. 4b can be attributed to the endonuclease. The fact that no nucleosides or large fraction of mononucleotides are obtained when phosphodiesterase I

is inhibited, indicates that the non-EDTA inhibited activity is indeed an endonuclease. The remaining fractions, eluted later from the column, are likely to contain larger oligonucleotides, but they have not yet been chemically characterized. Fig. 4C shows the elution profile of the substrate control. Only one very small absorption peak was observed, therefore eliminating the possibility of any appreciable non-enzymatic hydrolysis of the RNA.

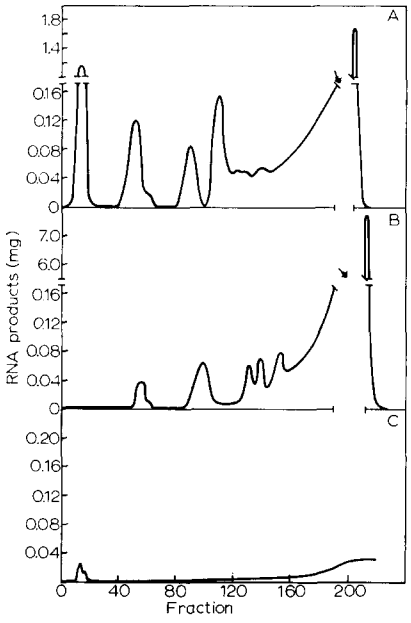

Fig. 4. The fractionation of RNA digests on DEAE-cellulose columns. RNA hydrolyses were performed at 37 °C for 10 h in 0.1 M ammonium carbonate, pH 8.8. The RNA and plasma membrane protein concentrations in the reaction vessels were 11.2 mg/ml and 0.1 mg/ml, respectively. In (A) the reaction mixture contained no EDTA, in (B) 10 mM EDTA was present, and (C) was a substrate control containing RNA and 10 mM EDTA but no plasma membranes. Reactions were stopped by placing the reaction vessels in boiling water for 5 min. The denatured membranes were removed by centrifugation. Ammonium carbonate was removed by 4–5 evaporations on a Buchler Evapo-Mix. The samples were then dissolved in distilled water and applied to the columns. The fractionation was performed using the method of Tomlinson and Tenner[14], with the exception that when the gradient was completed as indicated by the arrows, 4 M NaCl was added to the column in order to elute the remaining RNA fragments. Column size, 2.5 cm × 40 cm; fraction size, 10 ml; flow rate, 60 ml/h.

Table III shows the subcellular distribution of phosphodiesterase I, 3′-nucleotidase, 5′-nucleotidase, ribonuclease activity with EDTA present, and ribonuclease activity without EDTA in the assay medium. For the plasma membrane fractions, the ribonuclease activity with EDTA present is considered to be a measure of the endonuclease activity. RNA hydrolysis with no EDTA present represents total ribonuclease activity. All five enzymatic activities have their highest relative specific activities in the plasma membrane fractions. The results show phosphodiesterase I and 5′-nucleotidase are both localized on the plasma membrane. The 3′-nucleotidase activity is higher in the plasma membrane fractions than in the other fractions, but

TABLE III

SUBCELLULAR DISTRIBUTION OF PHOSPHODIESTERASE I, 3′-AND 5′-NUCLEOTIDASES, AND RNA HYDROLYSIS IN THE PRESENCE AND ABSENCE OF EDTA

RNA hydrolysis in the presence and absence of EDTA was measured by acid-soluble product formation. RNA hydrolysis in the presence of 10 mM EDTA was performed in 0.1 M Tris–HCl, pH 7.8. RNA hydrolysis with no EDTA present was performed in 0.1 M ammonium carbonate, pH 8.8. Both RNA hydrolyses were carried out at 37 °C for 4 h with an RNA concentration of 10 mg/ml. The protein concentration varied with each fraction. The fraction designations are those used by Touster *et al.*[4]. Fraction E represents the supernatant above the nuclear pellet and contains the bulk of all of the cellular organelles except the nuclei. Fraction N represents the nuclear fraction. Fraction ML is the mitochondrial *plus* lysosomal fraction. Fraction P is the microsomal fraction which contains rough and smooth endoplasmic reticulum, plasma membranes, and probably some Golgi membranes. Fraction S contains the soluble enzymes. Fraction P_2 represents plasma membrane fragments isolated from the microsomal fraction, while Fraction N_2 represents plasma membrane fragments isolated from the nuclear fraction. The values which are given in the table are in terms of relative specific activity which is defined as the specific activity of a particular fraction divided by the specific activity of the homogenate. The specific activity of the homogenate was calculated by dividing the total number of enzyme units in Fraction E *plus* Fraction N by the total number of mg of protein in Fraction E *plus* Fraction N. A unit is defined as μmoles of product formed per min. The numbers in parentheses indicate the number of separate experiments which were performed and each separate experiment was carried out in duplicate. Variation in the results was less than 15%, except for RNA hydrolysis in the presence of 10 mM EDTA (see Discussion).

Enzyme	*Relative specific activity*						
Fraction:	*E*	*N*	*ML*	*P*	*S*	P_2	N_2
Phosphodiesterase I	0.89 (3)	1.3 (3)	0.17 (3)	2.6 (3)	0.18 (3)	35.8 (3)	38.2 (3)
3′-Nucleotidase	0.92 (3)	1.3 (3)	0.65 (3)	1.4 (3)	0.82 (3)	12.0 (3)	15.4 (3)
5′-Nucleotidase	0.78 (3)	1.6 (3)	0.21 (3)	2.7 (3)	0.30 (3)	32.3 (3)	40.7 (3)
RNA hydrolysis (10 mM EDTA)	0.85 (2)	1.5 (2)	1.7 (2)	1.3 (2)	0.13 (2)	7.7 (2)	4.1 (2)
RNA hydrolysis (no EDTA)	0.88 (2)	1.3 (2)	0.89 (2)	1.6 (2)	0.75 (2)	11.7 (2)	13.5 (2)

its relative specific activity in the plasma membranes is not as high as either phosphodiesterase I or 5′-nucleotidase. This could be due to two factors. One is that the 3′-nucleotidase is not bound as tightly to the membrane as the other enzymes and some of it is released during the isolation procedure. The fact that its relative specific activity is higher in the soluble fraction than 5′-nucleotidase and phosphodiesterase I agrees with this proposal. Another possibility is that more than one cellular organelle contains 3′-nucleotidase activity. Since there is such an abundance of ribonuclease in the cell, it is difficult to compare the relative specific activities for the RNA hydrolyses to those for the other enzymatic activities. However, in the case of the RNA hydrolysis in the presence of 10mM EDTA, the relative activities of the plasma membrane fractions are 2–4-fold higher than for any of the other fractions, therefore indicating that a particular ribonuclease, namely the endonuclease is localized on the plasma membranes. The relative specific activity of total RNA hydrolysis by the plasma membrane (performed in the absence of EDTA) is 8–10-fold higher than for any other fraction. This indicates that the plasma membrane of rat liver possesses a highly active system of enzymes capable of degrading RNA.

Of the total enzyme units found in the homogenate (Fractions E+N of Ta-

ble III), the combined plasma membrane Fractions P_2 and N_2 contained 10% of total RNA hydrolysis activity at pH 8.8, 3.4% of RNA hydrolysis activity in the presence of 10 mM EDTA (endonuclease activity), 7% of 3′-nucleotidase activity, 25% of phosphodiesterase activity and 20% of 5′-nucleotidase activity. This is a further indication that 3′-nucleotidase and alkaline and non-EDTA-inhibited ribonuclease enzymes are located on more than one cellular structure. When Fraction P_2 was washed with 0.15 M NaCl, the specific activity of RNA hydrolysis in the presence of EDTA decreased 25% while that of total RNA hydrolysis at pH 8.8 decreased only 5%. The latter result agrees with the data reported by Emmelot[8]. The fact that more endo-ribonuclease activity is removed by washing may indicate that the saline-soluble activity is non-membrane enzyme adsorbed from the supernatant fraction, or possibly this represents a portion of an acid ribonuclease activity that Emmelot[8] found to be washed easily from liver plasma membranes by physiological saline solution. In either case, the washed Fraction P_2 membrane fraction has a relative specific activity greater than 5 and this still represents the highest specific activity of any subcellular fraction described in Table III.

Compared with the plasma membrane Fractions P_2 and N_2, the specific activity of the endonuclease is 4–6-fold lower in Fraction P_4, a fraction enriched with the endoplasmic reticulum marker enzyme, glucose-6-phosphatase[4]. This excludes the possibility that the endonuclease activity in the plasma membrane fractions is due to their contamination by fragments of endoplasmic reticulum.

Thus, the results in Table III show that the ability of plasma membranes to degrade RNA is not due to contamination by any of the subcellular fractions which were examined. However, one cellular organelle which had not been checked and which we thought could have been contaminating our plasma membrane preparations was the Golgi apparatus. Golgi membranes were therefore isolated by the procedure of Morre *et al.*[11] in order to examine this possibility. We also wanted to compare the enzymatic content of our plasma membrane fractions to plasma membranes isolated by another procedure. Therefore, plasma membranes were isolated by the method of Ray[18], in which the liver is homogenized in dilute bicarbonate buffer. The specific activity of phosphodiesterase I, 3′-nucleotidase, 5′-nucleotidase and RNA hydrolysis in the presence of 10 mM EDTA were determined for our plasma membrane fractions, plasma membranes prepared according to Ray[18] and Golgi membranes (Table IV). The specific activity of phosphodiesterase I, 3′-nucleotidase and 5′-nucleotidase are very similar for Fractions P_2 and N_2, while the specific activity of RNA hydrolysis in the presence of 10 mM EDTA is twice as great for Fraction P_2 as it is for Fraction N_2. This same pattern was also observed in Table III. For plasma membranes obtained by the procedure of Ray[18], the specific activities for all four enzymes were much lower than for Fractions P_2 and N_2. The Golgi fraction has approximately a 10-fold lower specific activity for phosphodiesterase I and 5′-nucleotidase than did Fractions P_2 and N_2. This could either be due to contamination of the Golgi fraction by plasma membranes, or Golgi membranes may contain a lower quantity of phosphodiesterase I and 5′-nucleotidase. The 3′-nucleotidase activity is approximately 5 times greater in Fractions P_2 and N_2 than it is in Golgi. This again could be caused either by contamination of the Golgi fraction, or Golgi may contain a lesser amount of 3′-nucleotidase. Indeed, Cheetham *et al.*[19] have reported that the Golgi apparatus has enzyme content that is intermediate

TABLE IV

COMPARISON OF THE ENZYMATIC CONTENT OF PLASMA MEMBRANES AND GOLGI MEMBRANES

RNA hydrolyses were determined by acid-soluble product formation. P_2 and N_2 membrane fractions are defined in Table III, and Golgi membranes were isolated as described in Materials and Methods. RNA hydrolyses were carried out in 0.1 M Tris–HCl, pH 7.8, at 37 °C for 4 h with an RNA concentration of 10 mg/ml. The protein concentration varied with each cell fraction. The numbers in parentheses indicate the number of separate experiments which were performed and each experiment was carried out in duplicate. Variation in the results was less than 15%.

Enzyme	*Specific activity (μmoles/min per mg protein)*			
Fraction:	P_2	N_2	*Plasma membranes**	*Golgi membranes*
Phosphodiesterase I	0.80 (3)	0.74 (3)	0.31 (2)	0.086 (3)
3′-Nucleotidase	0.010 (3)	0.013 (3)	0.0053 (1)	0.0023 (2)
5′-Nucleotidase	1.8 (3)	2.2 (3)	0.73 (2)	0.16 (3)
RNA hydrolysis (10 mM EDTA)	0.020 (3)	0.010 (3)	0.0023 (1)	0.011 (3)

* Isolated by the method of Ray[18].

between endoplasmic reticulum and plasma membrane. In the case of RNA hydrolysis in the presence of 10 mM EDTA, the specific activities of Golgi and Fraction N_2 are equal whereas the specific activity of Fraction P_2 is twice that of Golgi and Fraction N_2. Therefore we feel that the EDTA-resistant ribonuclease activity of Golgi is not due to plasma membrane contamination and also that the EDTA-resistant ribonuclease activity of plasma membranes is not caused by Golgi contamination. It seems likely that both the Golgi and plasma membrane contain a ribonuclease that is not inhibited by EDTA.

The one large discrepancy between Fractions P_2 and N_2 is that Fraction P_2 contains twice as much endonuclease activity as does Fraction N_2. Data obtained by Tan and Aronson (Tan, L. and Aronson, N. N., unpublished) on sialyl transferase and by Dewald and Touster (Dewald, B. and Touster, O., personal communication) concerning galactosyl transferase indicate that Fractions P_2 and N_2 contain both of these enzymes. Fraction P_2, however, has a much higher specific activity for both enzymes than does Fraction N_2. Both of these glycosyl transferases are reported to be marker enzymes for the Golgi apparatus[20], although some sialyl transferase has been found to be present on the plasma membrane[21]. These data appear to indicate that Fraction P_2 is contaminated with Golgi fragments while Fraction N_2 is either contaminated to a much lesser extent or not contaminated at all. Since Golgi appears to have an EDTA-resistant ribonuclease, Fraction P_2 would have its own EDTA-resistant ribonuclease activity *plus* that of Golgi. This could explain why Fraction P_2 has a higher specific activity than Fraction N_2 for RNA hydrolysis in the presence of 10 mM EDTA.

DISCUSSION

We have shown that rat liver plasma membrane has a physiological capacity to degrade RNA by the fact that these membranes can extensively hydrolyze RNA

to inorganic phosphate and nucleosides and to a degree comparable to lysosomal hydrolysis of RNA. In addition to the well characterized plasma membrane marker-enzyme, phosphodiesterase I, the membrane contains another enzymic activity which is capable of degrading RNA. This second enzyme activity has been characterized as an endonuclease, since the principal products of its action are oligonucleotides. The endonuclease is not inhibited by EDTA and has a pH optimum of 7.7. The four activities, phosphodiesterase I, 3′-nucleotidase, 5′-nucleotidase and the endonuclease, are all involved in catabolic metabolism of RNA and are localized on the plasma membrane. Total RNA hydrolysis, performed in the absence of EDTA at pH 8.8, has its highest activity in the plasma membrane fractions. These results show that the plasma membrane of rat liver possesses a highly active enzymatic system for RNA degradation.

Fig. 5 shows a possible mechanism for the degradation of RNA by rat liver plasma membrane. First, attack on the RNA molecule by the endonuclease occurs liberating two oligonucleotide fragments, one with a 3′-phosphate group and the other with a free 5′-hydroxyl group. Second, there is removal of the 3′-phosphate by 3′-nucleotidase, liberating inorganic phosphate. Third, the oligonucleotide with a free 3′-hydroxyl group is attacked by phosphodiesterase I, liberating a 5′-mononucleotide. Fourth, the removal of the 5′-phosphate by 5′-nucleotidase liberates inorganic phosphate and a nucleoside. The mechanism agrees with the evidence that we have thus far obtained concerning the degradation of RNA by rat liver plasma membrane.

Since our work on this system of enzymes has just begun, we have been assuming the presence of only one ribonuclease, the endonuclease, which is not inhibited by EDTA and also the presence of only one RNA-degrading enzyme

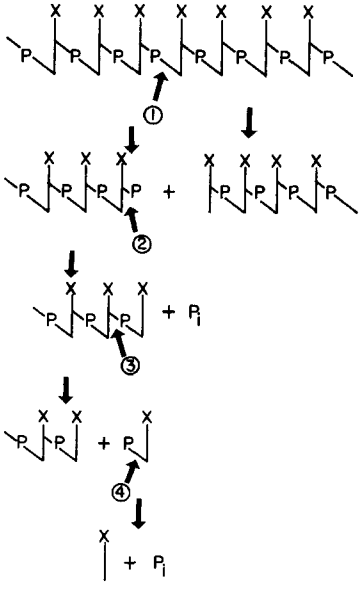

Fig. 5. 1, endonuclease; 2, 3′-nucleotidase; 3, phosphodiesterase I; 4, 5′-nucleotidase.

phosphodiesterase I, which is inhibited by EDTA. Rahman[22] has reported the presence of a ribonuclease in the particulate fraction of rat liver, which has a pH optimum of 9.5 and is inhibited by EDTA. Evidence was also presented that this enzyme is not phosphodiesterase I. If this enzyme is localized on the plasma membrane, it could be involved in the RNA-degrading system reported here. This enzyme would not be observed in our endonuclease assay since both it and phosphodiesterase I would be inhibited in the presence of EDTA.

There have been many reports of an RNAase inhibitor being present in the soluble fraction of liver homogenates[23–25], and this presented a definite problem when trying to follow quantitatively the subcellular distribution of liver ribonuclease activity. In Table III the relative specific activity for the endonuclease, ribonuclease activity in the presence of 10 mM EDTA, varied up to 40%. However, the variation in the relative specific activity was not due to a variation in the absolute specific activity of the endonuclease in the plasma membrane fractions (Table IV), but was caused by a variation of the absolute enzyme specific activity in Fraction E. This variation was probably caused by the presence of such a soluble ribonuclease inhibitor in the E fraction. Roth reported that the inhibitor activity decreases rapidly upon storage at 0 °C (ref. 23). Indeed, we observed this phenomenon when our E fraction was stored at 0 °C. Upon assaying Fraction E for ribonuclease activity both in the presence and absence of EDTA, the activity was found to increase upon storage at 0 °C for one week, after which it remained constant. The ribonuclease activities of our plasma membrane fractions, however, were not affected by storage at 0 °C.

The biological significance of RNA degradation by rat liver plasma membrane is unknown. The enzymes could participate in the degradation of intracellular RNA, or the degradative system may be a defense mechanism to protect the cell from foreign RNA. Further characterization of this system of RNA-degrading enzymes should lead to the determination of its physiological role on the plasma membrane.

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