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EFFECTS OF MEMBRANE RIBONUCLEASE AND 3′-NUCLEOTIDASE ON THE DIGESTION OF POLYURIDYLIC ACID BY RAT LIVER PLASMA MEMBRANE

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SUMMARY

1. Fragments of isolated rat liver plasma membrane possess a ribonuclease activity which at pH 7.8 in the presence of 10 mM EDTA can digest polyuridylic acid (poly(U)) and polycytidylic acid (poly(C)) but not polyadenylic acid (poly(A)) and polyguanylic acid (poly(G)). Under these conditions, the membrane preparation does not degrade native or denatured DNA.

2. The products of the reaction with poly(U) (10 mM EDTA present) can be separated on DEAE-Sephadex into oligonucleotides of increasing chain length. Most of the products are di- to hexa-nucleotides which contain terminal 3′-phosphate groups.

3. When EDTA is not present (pH 7.8 or 8.8) the plasma membrane preparation degrades both poly(A) and poly(U). With poly(A) the product is all nucleoside while with poly(U) as substrate most of the product is nucleoside, but also some oligonucleotides are produced.

4. The ribonuclease releases acid soluble products very slowly from high concentrations of poly(U) (4 mg/ml).

5. Uridine trinucleotide with and without a terminal 3′-phosphate group is degraded by rat liver plasma membrane. The trinucleotide diphosphate is rapidly hydrolyzed to nucleoside while the trinucleotide itself is slowly digested and yields intermediate products, including nucleoside.

INTRODUCTION

Rat liver plasma membrane has been shown to contain ribonuclease activity [1–4]. We have previously suggested that a metabolic pathway for RNA catabolism functions on the liver surface membrane and that this system of enzymes is composed of an endonuclease, 3′-nucleotidase, phosphodiesterase I, and 5′-nucleotidase. These enzymes would act sequentially to degrade RNA and yield nucleosides and inorganic phosphate as products [2, 5]. All of the previous studies on membrane ribonuclease

have used yeast RNA as a substrate and have measured the course of the reaction by the appearance of acid-soluble products. Using this method it is difficult to characterize the contribution of the endonuclease to the overall reaction sequence, since there is present a very active membrane phosphodiesterase I which is also capable of hydrolyzing RNA [6]. This last enzyme releases an acid-soluble product with each cleavage, while this is not the case for an endonuclease In order to delineate better this endonucleolytic activity on the membrane which is not inhibited by EDTA, we have studied the degradation of high molecular weight polyuridylic acid ($M_r >$ 100 000) by rat liver plasma membrane The products from this RNA homopolymer are more easily separated by column chromatography. In addition, studies with uridine trinucleotides as substrates for the liver membrane preparation indicate a very important function for 3'-nucleotidase in this catabolic processing of RNA

MATERIALS AND METHODS

Polyuridylic acid, Type II (poly(U)) polyadenylic acid (poly(A)), polycytidylic acid (poly(C)), polyguanylic acid (poly(G)) alkaline phosphatase (*Escherichia coli*, Type III) and pancreatic RNAase were purchased from Sigma Chemical Co Yeast RNA was obtained from Plenum Scientific Research, Inc. All other materials were commercial products of reagent grade.

Plasma membrane fragments (fraction P_2) were isolated as previously from the microsomal fraction of a liver homogenate prepared in isotonic sucrose [8, 9]. In fraction P_2, the specific activity of plasma membrane marker enzymes (5'-nucleotidase and phosphodiesterase I) is at least 25-fold increased over the homogenate specific activity.

Both RNA and poly(U) hydrolyses were measured as previously described with a slight modification [2]. 0.1 ml of each reaction mixture was added to 0.9 ml of acidic butanol (*t*-butanol/glacial acetic acid 2 : 1 v/v) in order to precipitate unreacted high molecular weight RNA or poly(U) After centrifugation, 0 5 ml of the supernatant was diluted to 1 5 ml with water and the absorbance at 260 nm was measured. For calculating the specific activities of poly(U) hydrolyses, a molar extinction coefficient for uridylic acid of $1 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ was used.

Poly(U) hydrolyses were performed in 0 1 M Tris buffer pH 7.8 or 8.8. The remaining conditions of poly(U), protein and EDTA concentration are indicated in figure and table legends for each group of assays. To measure endonuclease activity alone, EDTA (10–50 mM) was included in the reaction mixture, since EDTA at a 10 mM concentration or greater totally inactivates phosphodiesterase I [2]. In order to observe the combined effect of all membrane enzyme activities on poly(U), EDTA was omitted from the reaction A drop of toluene was added to all reactions to prevent microbial growth

To calculate the chain-length of released oligonucleotides, the ratio of their terminal 3'-phosphate groups to total organic phosphate was determined. The total and inorganic phosphate assays were done according to the methods of Ames and Dubin [10].

Poly(U) digests were separated on a DEAE-Sephadex A-25 column (10 mm × 24 cm). The fraction size and flow rate were 5.5 ml and 33 ml/h respectively. The experimental detail for this separation is described in Results

Descending paper chromatography for separating degradation products from trinucleotides was performed on Whatman 3MM paper. Chromatograms were developed for approximately 10 h in 95 % ethanol/1M ammonium acetate (1 : 1). Location of ultraviolet-absorbing components was then determined by observing the chromatogram under an ultraviolet lamp.

RESULTS

Effect of reaction conditions on poly(U) hydrolysis

Poly(U) hydrolyses by plasma membrane fraction P_2 were performed in 0.1 M Tris buffer since the reaction was inhibited at higher buffer concentrations. During a 4 h poly(U) digestion in the absence of EDTA, the hydrolytic activity increased with increasing poly(U) substrate concentration (Fig. 1). However, in the presence of 10 mM EDTA, the amount of acid-soluble product increased only up to a poly(U) concentration of 1 mg/ml, beyond which there was a steady decline in the reaction, and no measurable acid-soluble products were produced from an incubation mixture containing 4 mg/ml poly(U). After eight hours the hydrolysis rate at a poly(U) concentration of 1 mg/ml had leveled off, while at the 2 mg/ml poly(U) concentration, the hydrolysis rate had exceeded that at the 1 mg/ml poly(U) concentration.

At pH 7.8 in the presence of 5–20 mM EDTA the hydrolysis of poly(U) by the plasma membrane fraction is decreased by a constant 60 % and the remaining 40 % of the activity represents endonuclease action. A number of reactions in the presence and absence of EDTA were performed to study the effect of pH on the hydrolysis of poly(U) by the plasma membrane fraction. Differences are seen between the hydrolysis of poly(U) and yeast RNA by liver plasma membrane. In the absence of EDTA

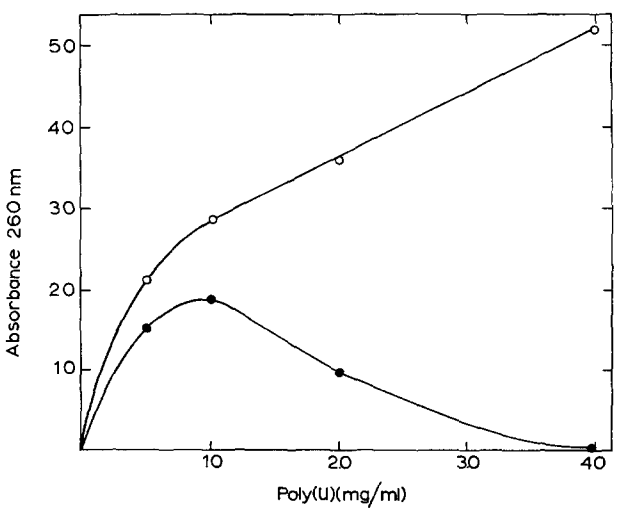

Fig 1 The effect of substrate concentration on the release of acid-soluble products from poly(U) by plasma membrane Poly(U) hydrolyses were performed for 4 h in 100 mM Tris/HCl buffer either in the absence of EDTA at pH 8 8, or in the presence of 10 mM EDTA at pH 7.8. At pH 7 8 the P_2 plasma membrane concentration was 0 28 mg/ml and at pH 8.8 the concentration was 0 07 mg/ml. ○, pH 8.8, ●, pH 7 8+10 mM EDTA.

TABLE I

EFFECT OF pH ON THE HYDROLYSIS OF RNA AND POLY(U) BY PLASMA MEMBRANE IN THE PRESENCE AND ABSENCE OF EDTA

Specific activities were determined from the acid-soluble product formation as described in Methods Plasma membrane protein concentration was approximately 0 25 mg/ml and each reaction was in 0 1 M Tris/HCl buffer at the designated pH RNA and Poly(U) concentrations were 10 mg/ml and 1 mg/ml, respectively

pH	Specific activity (μmol/min per mg protein)	
	Substrate RNA	Poly(U)
7 8 (− EDTA)	0 055	0 041
8 8 (− EDTA)	0 17	0 041
7 8 (+ 10 mM EDTA)	0 018	0 012
8 8 (+ 10 mM EDTA)	0 009	0 006

the ratio of the activities at pH 8 8 to 7 8 for RNA hydrolysis is about 3, while for poly(U) hydrolysis this ratio is 1 (Table I) In the presence of EDTA the hydrolysis rate of both RNA and poly(U) is twice as great at pH 7 8 as at pH 8 8 In all cases acid-soluble products are produced more rapidly from yeast RNA than from poly(U).

Separation and characterization of poly(U) hydrolysis products

A plasma membrane digest of poly(U) was analyzed by chromatography on DEAE-Sephadex (Fig. 2) The incubation was performed at pH 7 8 in the presence of 10 mM EDTA for 24 h On chromatography of one portion of the original digest a pattern of distinctive peaks eluting at fractions 41–42, 46–48, 51–52 and 55–56 was found The chain-length analyses of these fractions indicated they are tri-, tetra-, penta- and hexanucleotides, respectively (Table II) Prior to fraction 40 the elution profile was very complex suggesting the possible presence of a mixture of terminals at the 3′-ends of these oligonucleotides. A second portion of the digest was first treated with acid (pH 1 0, 20 min, 22 °C) in order to cleave any terminal cyclic phosphate groups that may have been present. The completeness of this treatment was confirmed using cytidine 2′ 3′-cyclic monophosphate as a standard. Poly(U) was incubated under these same conditions and no hydrolysis was found The acid treatment was followed by incubation with alkaline phosphatase (0 1 unit/ml, 2 h, 37 °C) to release terminal 3′-phosphates A reduction in the complexity of the DEAE-Sephadex elution profile upon acid and alkaline phosphatase treatments of the digest was observed (Fig 2) The changes suggested the presence of cyclic 2′ 3′-terminal phosphate groups, as well as a mixture of oligonucleotides with and without terminal phosphate groups A comparison of the elution profiles from the digest indicated that the second part of the doublet at fraction 20, the small peak at fraction 31, and the shoulder initiating at fraction 40 were probably oligonucleotides with cyclic terminal phosphate groups based on the observed loss of these fractions which occurred after treatment only with acid (not shown). The second part of the doublet at fraction 36 also appeared to contain some oligonucleotide with cyclic terminal phosphate groups A comparison of the elutions of the two parts of the digest also indicated the large amount of 3′-phosphate terminals on the original digestion products This is to be expected since

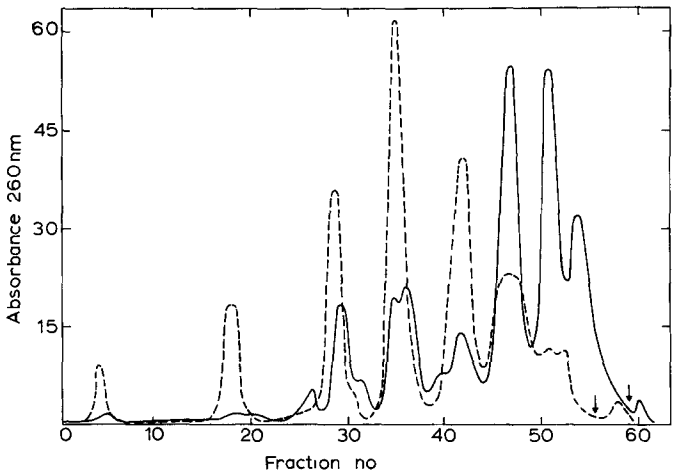

Fig. 2. Chromatographic separation of oligonucleotide products of plasma membrane digest of poly(U) in the presence of 10 mM EDTA Poly(U) (2 mg/ml) was incubated for 24 h at 37 °C with fraction P_2 (0 26 mg/ml) at pH 7 8 in the presence of 10 mM EDTA The pH of the digest was lowered to 7.0, and the mixture was placed in boiling water for 5 min to denature the membranes, which were removed from solution by centrifugation at $8000 \times g$ As described in the text, the digest was divided into two parts. One half was treated with acid and alkaline phosphatase to remove terminal 3'-phosphate groups, and each of these samples was diluted 1 1 with water and then chromatographed on a DEAE-Sephadex column (Cl^--form) eluted with a linear gradient of NaCl from 0 to 0 5 M in 25 mM Tris/HCl, pH 7 5 At arrows, 2 M NaCl was added to remove any remaining pieces of substrate from the column ——, untreated portion of digest; – – –, acid and alkaline phosphatase-treated portion of the digest

10 mM EDTA has been shown to inhibit more than 75 % of the 3'-nucleotidase activity of the membranes [2].

Poly(U) hydrolysis by P_2 membranes was performed at pH 7.8 for 4 h in the absence of 10 mM EDTA. The elution profile (not shown) for this digest showed

TABLE II

CHAIN-LENGTH DETERMINATION OF POLY(U) OLIGONUCLEOTIDES RELEASED BY PLASMA MEMBRANE ENDONUCLEASE

DEAE-Sephadex fractions (see Fig 2) from a 24-h digest of poly(U) by plasma membrane (with 10 mM EDTA present) were dialyzed against water for 24 h with two changes in order to remove most of the chloride and Tris. The dialysis tubing was pretreated with 20% acetic anhydride in pyridine followed by boiling in distilled water in order to decrease the pore size These fractions were then analyzed for total and terminal phosphate as described in Methods The terminal phosphate was determined as inorganic phosphate after treatment of the oligonucleotides with 0.05 units of alkaline phosphatase The samples had been first treated with acid to open cyclic phosphates

Major peak fraction	Ratio. $\frac{\text{total phosphate}}{\text{terminal phosphate}}$	Eluted in fractions
3	3 04	41–42
4	4 18	46–48
5	4 92	51–52
6	6 47	55–56

almost all products to be nucleoside, but small amounts of intermediate-sized oligonucleotides were still present This result is similar to that found for yeast RNA digestion by the non-EDTA-treated plasma membrane preparation [2]

The ability of plasma membrane to degrade poly(A) was also tested In the presence of 10 mM EDTA there was no release of acid-soluble products from this polynucleotide However, in the absence of EDTA at pH 8 8, there was substantial cleavage of this homopolymer (4 h incubation, 2 mg/ml poly(A) and 0 28 mg/ml fraction P_2) The rate of hydrolysis of poly(A) by P_2 membranes was about 40 % of the rate for poly(U) hydrolysis by non-EDTA-treated membranes Separation of the poly(A) reaction on DEAE-Sephadex yielded only nucleoside and very large chains of poly(A) which were eluted from DEAE-Sephadex by 2 M NaCl No intermediate-size oligonucleotides were observed Poly(G) was not hydrolyzed by the plasma membrane endonuclease, and although poly(C) was degraded, the rate of acid-soluble product formation was slow In the presence of EDTA (pH 7.8) poly(U) was digested seven times faster than poly(C), while in the absence of EDTA the rate was only 1 4 fold greater.

EDTA-treated plasma membrane fragments were unable to hydrolyze either native or denatured DNA Since phosphodiesterase I has been shown to degrade denatured or single-stranded DNA [6], the fact that the endonuclease cannot do so further distinguishes the latter as a separate membrane activity involved in overall RNA catabolism

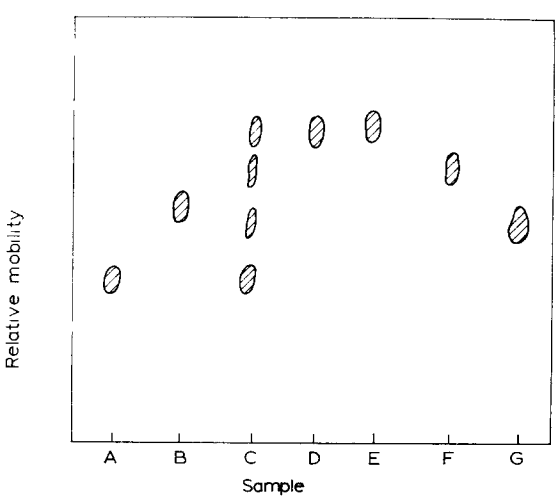

Fig 3 Paper-chromatographic separation of plasma membrane digests of uridine trinucleotides with and without terminal 3′-phosphate groups Digestions of uridine trinucleotide and uridine trinucleotide diphosphate were performed at 37 °C for 30 min at pH 7 8 The substrate concentrations were approximately 5 absorbance units (260 nm)/ml, and P_2 membrane concentration was 0 28 mg/ml The procedure for chromatography on Whatman 3MM paper is described in Methods A, uridine trinucleotide, B, uridine trinucleotide diphosphate, C, digest of uridine trinucleotide, D, digest of uridine trinucleotide diphosphate, E, uridine, F, uridine dinucleotide monophosphate, G, uridine 5′-phosphoric acid

Digestion of poly(U) trinucleotides

By using small oligonucleotides as substrate, the effect of membrane phosphodiesterase I and 3′- and 5′-nucleotidase activities on poly(U) digestion could be examined without interference from the endoribonuclease activity. Poly(U) trinucleotides both with free 3′-hydroxyl groups and 3′-terminal phosphate groups were isolated from pancreatic RNAase digests. The isolated poly(U) trinucleotides were reacted with P_2 membranes in the absence of EDTA (30 min at pH 7.8). The digests were adjusted to approximately pH 7 0, placed in a boiling water bath for 5 min to stop the reaction, and then concentrated and spotted on Whatman 3MM paper. As can be seen in Fig. 3, the digestion of the trinucleotide with a free 3′-hydroxyl group went to completion. Using the trinucleotide with a 3′-terminal phosphate group present as a substrate, the reaction showed the intermediate products of the digestion: dinucleotide monophosphate, 5′-UMP and undegraded trinucleotide.

DISCUSSION

5′-Nucleotidase [1, 11] and phosphodiesterase I [2] are excellent marker enzymes for liver plasma membrane; however, the physiological functions of these two membrane enzymes have not been clearly deduced. Suggestions have been made that these enzymes may be involved in transporting nucleotides across the cell membrane [12] or that they participate in the degradation of coenzyme A and other nucleotides [13, 14] The results of this study substantiate our earlier proposal [2, 5] that these two enzymes, along with 3′-nucleotidase and an EDTA-resistant endonuclease, provide the plasma membrane with a physiological capacity to hydrolyze RNA to nucleosides and inorganic phosphate. Based on the separation and characterization of the nucleotide products from membrane digests of poly(U), poly(A) and uridine trinucleotides with and without terminal 3′-phosphate, all four of these enzyme activities are involved in this catabolic process (Figs 2, 3).

The liver plasma membrane endoribonuclease, in contrast to pancreatic RNAase, was not inhibited by either iodoacetate or cytidine 2′-phosphate. On the basis of using polynucleotides as substrates, the enzyme appears to be specific for cleaving adjacent to pyrimidine bases, with highest activity for nucleotide linkages containing uridine. Changes in the chromatographic separation of poly(U) oligonucleotide products occurred after mild acid treatment which was shown to open 2′ : 3′-cyclic phosphates but whether such an intermediate is part of the mechanism of the enzyme reaction is uncertain. The membrane nuclease released acid-soluble products from yeast RNA of molecular weight approx. 25 000 more rapidly than from poly(U) of molecular weight greater than 100 000 (Table I). However, due to the nature of the assay (measurement of acid-soluble products), the rates of nucleotide cleavage with each of the RNA substrates may not be accurately reflected. The fact that poly(U) at high concentrations (4 mg/ml) does not yield any acid-soluble products except after long periods of incubation suggests that the ribonuclease preferentially cleaves the longest chains of the substrate, and therefore the initial products would not be soluble in acid. The endonuclease appears to become more critical for the membrane degradation of the very high molecular weight poly(U) as compared to degradation of yeast RNA, since the rate of cleavage of the former polymer at pH 7.8 (optimum of endonuclease) is the same as that at pH 8.8 (close to the optimum of phosphodiesterase I

[2]). The hydrolysis of RNA by membranes is 3 times faster at pH 8.8 than at pH 7.8, because phosphodiesterase would have a sufficient number of 3′-polynucleotide ends to begin its catalysis early in the reaction with this lower molecular weight substrate. Gavard et al. [4] have reported an acid ribonuclease (pH 6 3) present in rat liver plasma membrane However, this acid activity has been reported to be solubilized in hypotonic medium and we feel that this enzyme does not contribute to the degradation of poly(U) observed in our study, since fraction P_2 was always washed and suspended in 5 mM Tris buffer, pH 8.0

The presence of 3′-nucleotidase activity on rat liver plasma membrane is still disputed [2, 3, 13]. Our study clearly indicates an important participation of such an activity in the overall degradation of poly(U) The presence of a 3′-phosphate on the terminus of uridine trinucleotide greatly decreased the hydrolysis of the oligonucleotide (Fig. 3); however, digestion still occurred

Poly(A) was not digested by membranes treated with EDTA and yielded only nucleosides as product after incubation with untreated membranes Others have reported that purified liver phosphodiesterase I degrades RNA, and this enzyme (in combination with 5′-nucleotidase) must have been responsible for poly(A) hydrolysis to nucleoside by the membranes. Recently, Bischoff et al. [7] have claimed that purified liver nucleotide pyrophosphatase (phosphodiesterase) does not degrade RNA, although their data does indicate a slow cleavage of the polymer when compared to hydrolysis of small nucleotides.

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