Alkaline Ribonuclease and Phosphodiesterase Activity in Rat Liver Plasma Membranes

By TERENCE D. PROSPERO, MALCOLM L. E. BURGE,* KENNETH A. NORRIS,†
RICHARD H. HINTON and ERIC REID

Wolfson Bioanalytical Centre, University of Surrey, Guildford, U.K.

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The ribonuclease and phosphodiesterase activities of rat liver plasma membranes, purified from the crude nuclear fraction by centrifugation in an A-XII zonal rotor and flotation, were examined and compared. The plasma membrane is responsible for between 65 and 90% of the phosphodiesterase activity of the cell and between 25 and 30% of the particulate ribonuclease activity measured at pH 8.7 in the presence of 7.5 mM-MgCl₂. Both enzymes were most active between pH 8.5 and 8.9. Close to the pH optimum, both enzymes were more active in Tris buffer than in Bicine or glycine buffer. Both plasma-membrane phosphodiesterase and ribonuclease were strongly activated by Mg²⁺, there being at least a 12-fold difference between the activity in the presence of Mg²⁺ and of EDTA. There is, however, a difference in the response of the enzymes to Mg²⁺ and EDTA in that the phosphodiesterase is fully activated by 1.0 mM-MgCl₂ and fully inhibited by 1.0 mM-EDTA, whereas the ribonuclease requires 7.5 mM-MgCl₂ for full activation and 5 mM-EDTA for full inhibition. Density-gradient centrifugation has indicated that on solubilization in Triton X-100 most of the ribonuclease activity is released into a small fragment of the same size as that containing the phosphodiesterase activity. The relationship between the two activities is discussed in view of these results.

In the past, the distribution of alkaline ribonuclease activity among subcellular particles has been studied by the classical differential centrifugation scheme or by measurements on single 'purified' subcellular fractions. The results thus obtained have indicated that some alkaline ribonuclease activity is present in every subcellular organelle of rat liver (Roth, 1957; Reid & Nodes, 1959; Shugar & Sierakowska, 1967), but the imprecise composition of fractions obtained by differential centrifugation (Leighton *et al.*, 1968) makes it very difficult to estimate the contribution of any particular organelle to the total cellular activity. This information is important because the inevitable cross-contamination between different subcellular fractions means that an activity in some fractions may not, in fact, be due to the organelle that is the principal component of that fraction, but to a contaminant or a mixture of contaminants. This uncertainty can be removed by first identifying the major sites of an activity, then determining whether or not apparently 'minor' activities can be explained by cross-contamination. Zonal rotors (Anderson, 1966; Reid, 1971) provide the requisite tool.

Purified rat liver plasma-membrane fragments have been reported to contain alkaline ribonuclease

(Emmelot *et al.*, 1964) and alkaline phosphodiesterase (Emmelot *et al.*, 1964; Lansing *et al.*, 1967; Coleman *et al.*, 1967; Erecinska *et al.*, 1969; Touster *et al.*, 1970) activities, the latter, especially, comprising a large proportion of the total cellular activity. Alkaline ribonuclease activity has also been reported in Krebs ascites-cell plasma membrane (Stonehill & Huppert, 1968). However, ribonuclease activity is widely distributed within the cell, and it is not clear whether the activity reported in the plasma membrane is due to the membrane fragments themselves or to contaminants. The use of zonal rotors enables the simultaneous separation of big sheets of plasma membrane and of other large organelles such as mitochondria (Hinton *et al.*, 1970, 1971). Scanning for ribonuclease activity, assayed under a variety of conditions, across the fractions thus separated will allow the identification of a distinctive plasma-membrane ribonuclease, if one is indeed present, and will permit an estimation of the contribution made by the plasma membrane to the total amount of ribonuclease in the cell. The results presented below show that there is indeed a distinctive ribonuclease in the plasma membrane. Since a ribonuclease is, formally, only a specific type of phosphodiesterase, it is useful to compare the properties of the ribonuclease activity in the plasma membrane with the properties of the phosphodiesterase activity and to speculate on the possible relationship between the two activities. A preliminary

* Present address: Smiths Food Group, Park Royal Road, London N.W.10, U.K.

† Present address: Vickers Medical, Basingstoke, Hants., U.K.

report of part of these results has been published (Norris *et al.*, 1971), but the purer plasma-membrane preparations used in the experiments now reported have furnished somewhat higher pH optima.

Experimental

Animals and materials

Male hooded rats of the University of Surrey strain, about 2 months old and weighing 180–230 g, were used in these experiments. Torula yeast RNA type VI was purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and further purified by extraction with phenol and sodium dodecyl sulphate as described elsewhere (Mullock *et al.*, 1971). Low-molecular-weight contaminants were removed by diafiltration by using a Diaflo UM-10 membrane (Amicon Ltd., Oosterhout, The Netherlands). Gel filtration indicated that most of the RNA in this preparation was at least 30 nucleotides long. Some later experiments were made with a commercially available RNA of high molecular weight (*Torula* yeast RNA, B grade; Calbiochem, San Diego, Calif., U.S.A.). There were no significant differences between the results obtained with the two types of RNA. Sodium bis-*p*-nitrophenyl phosphate was purchased from Sigma (London) Chemical Co. Ltd. Gradients in the zonal rotor and for small tubes were prepared from Mineral Water sucrose (Tate and Lyle Ltd., London S.E.6, U.K.). In some experiments the sucrose solutions were pretreated with Norit A-activated charcoal [Sigma (London) Chemical Co. Ltd.] to remove ribonuclease (Steele & Busch, 1967).

Preparation of tissue

The procedures in general were those described by Hinton *et al.* (1970). The livers were perfused *in situ* with 0.25 M-sucrose adjusted to pH 7.5 with 5 mM- NaHCO_3 and were then homogenized with three strokes of a Potter-Elvehjem homogenizer (A. Thomas Inc., Philadelphia, Pa., U.S.A.), with a pestle rotated at 900 rev./min and a diametric clearance of 0.33 mm between pestle and vessel, in ice-cold 0.25 M-sucrose containing 5 mM- NaHCO_3 . Homogenates were filtered through a coarse sieve and centrifuged for 10 min at 400g to collect the crude nuclear fraction. The pellet was resuspended in about 25 ml of the homogenization medium with a hand-operated Teflon-glass homogenizer with a diametric clearance of 0.33 mm.

Zonal centrifugation

A slight modification of Procedure IV described by Hinton *et al.* (1970) was followed. The MSE A-XII zonal rotor was charged with a complex gradient

(A2; see Hinton *et al.*, 1970). The resuspended crude nuclear fraction was loaded on to the zonal rotor after the removal of a sample for analysis and overlaid with 10 ml of 0.25 M-sucrose and 50 ml of 0.08 M-sucrose. All sucrose solutions were adjusted to pH 7.5 by addition of NaHCO_3 to 5 mM immediately before use. Centrifugation was carried out for 50 min at 3700 rev./min. The gradient was then displaced and 34 ml fractions collected. The changes from the previously published method (Hinton *et al.*, 1970), i.e. the use of Norit A-treated sucrose, the use of younger rats and the overlaying of the sample with 0.25 M-sucrose, were made to prevent aggregation, largely caused by nuclear lysis, which sometimes vitiated preparative runs made by the original procedure.

The plasma-membrane fraction collected from the zonal rotor was sometimes further purified by flotation. The density of the fraction was adjusted to 1.19 g/ml with 2 M-sucrose and the fraction was then rehomogenized with three strokes of a Potter-Elvehjem homogenizer with its pestle rotated at 2400 rev./min. Portions (20 ml) were put into the tubes of the 8 × 50 ml rotor of the MSE Super-Speed 65 ultracentrifuge and were overlaid with 15 ml of 0.25 M-sucrose. After centrifugation for 20 min at 133 000g the 'pure' plasma-membrane fraction was collected from the interface between the two layers of sucrose.

Fractionation of solubilized plasma membrane

Plasma membranes purified by flotation were resuspended in 0.1 M-Tris-HCl buffer, pH 8.5, and 'solubilized' by the addition of Triton X-100 to a final concentration of 1% (Steele & Busch, 1967). The solubilized membrane components were separated by centrifugation. Sucrose gradients ranging from 0.1 M to 0.4 M and containing 0.1 M-Tris buffer, pH 8.5, and 1% Triton X-100 were prepared in the tubes of an MSE 3 × 23 ml swing-out rotor. A 'cushion' of 2 ml of 2 M-sucrose was placed in the bottom of the tubes before the preparation of the gradients. A portion (1.0 ml) of the Triton X-100-treated plasma membrane was layered on the gradient and the tube centrifuged for 12.5 h at 93 000g in an MSE Super-Speed 65 ultracentrifuge. After centrifugation the gradients were displaced with 2 M-sucrose by using an apparatus similar to that described by Leighton *et al.* (1968) and 1.5 ml fractions collected into cooled receptacles.

Chemical and enzyme assays

Protein was measured by an automated adaptation (Hinton & Norris, 1972) of the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Glucose 6-phosphatase, succinate dehydrogenase and acid β -glycerophosphatase acti-

vities were assayed as described by Hinton *et al.* (1970). 5'-Nucleotidase activity was also assayed as described by these authors except for the inclusion of 0.01M-sodium (+)-tartrate in the assay medium to prevent interference owing to non-specific phosphatase (El-Aaser & Reid, 1969).

Acid and alkaline phosphodiesterase activities were generally assayed by the automated method described by Hinton & Norris (1972) with bis-*p*-nitrophenyl phosphate as substrate, but in some experiments an equivalent manual assay was used. In this case 1 ml of 0.25M-Tris, -Bicine [*NN*-bis(2-hydroxyethyl)glycine] or -glycine or 0.3M-dimethylglutarate buffer of the appropriate pH (measured at 37°C) was mixed with 0.2 ml of MgCl₂ or EDTA and 0.5 ml of an appropriate tissue suspension. The reaction was started by the addition of 0.3 ml of 0.01M-bis-*p*-nitrophenyl phosphate. After incubation for between 30 and 60 min at 37°C the reaction was stopped by the addition of 2 ml of 0.25M-NaOH, precipitated protein was centrifuged off, and the *E*₄₀₀ of the supernatant measured in a Pye Unicam SP.500 spectrophotometer. For the purpose of routine measurements, the assay was performed in Tris buffer, pH 8.7; 0.2 ml of 0.05M-MgCl₂ or of EDTA being added as activator.

Ribonuclease activity was assayed by an adaption of the method used by de Duve *et al.* (1955) for acid ribonuclease. A sample (0.5 ml) of the appropriate tissue suspension was mixed with 0.4 ml of a mixture containing buffer and activator and the reaction started by the addition of 0.1 ml of a 10 mg/ml solution of RNA. The tube was vigorously shaken and then incubated for 61 min at 37°C. After incubation the reaction was stopped by the addition of 1.5 ml of 10% HClO₄ containing 0.25% uranyl acetate. After standing for 20 min at 0°C, the precipitated protein and RNA were removed by centrifugation. The supernatant was diluted with at least an equal volume of water before measurement of the *E*₂₆₀ on a Pye Unicam SP.500 spectrophotometer. Assays were always performed at least in duplicate. The results were corrected for acid-soluble material in the sample or substrate by subtraction of the extinction obtained when the assay mixture was incubated for only 1 min before the addition of the HClO₄-uranyl acetate reagent. A blank with water instead of tissue was also incubated for 61 min to check for any breakdown caused by traces of ribonuclease in the buffer or substrate. The assay of acid ribonuclease activity was performed in 0.09M-dimethylglutarate buffer, pH 5.0. Alkaline ribonuclease activity was measured at pH 7.8 or 8.7 in 0.075M-Tris buffer. MgCl₂ or EDTA were added to give the concentrations mentioned in the Results section.

Where structural latency existed, as with the lysosomal enzymes, acid phosphatase, acid phosphodiesterase and acid ribonuclease, the bound activity was liberated by freezing and thawing the fractions

eight times before assay. Protein values and enzyme activities were corrected for sucrose inhibition (Hinton *et al.*, 1969) by using the computer program described by Hinton (1971).

Results

Centrifugation in an A-XII zonal rotor of a crude nuclear fraction, under the conditions described in the Experimental section, results in the separation of four distinct peaks of material (Fig. 1), corresponding to microsomes (i.e. small membrane fragments) (on the left-hand side), mitochondria, plasma-membrane sheets, and a band of material, lying against the 'cushion', which contains nuclei together with whole cells and aggregated material (Hinton *et al.*, 1970). Lysosomes, indicated by acid phosphatase activity, sediment between the microsomal and the mitochondrial peaks. Examination of the distribution of alkaline ribonuclease activity assayed in the presence of Mg²⁺ (Fig. 1*d*) shows a complex spread of activity across the slowly sedimenting material in the first half of the gradient, with a distinct peak in the plasma-membrane region and at the end of the gradient. Acid ribonuclease activity was distributed through the gradient similarly to that of acid phosphatase.

The peak of alkaline ribonuclease activity in the plasma-membrane region is enhanced if the assay is carried out at pH 8.7 instead of pH 7.8, the conventional pH for ribonuclease assays, but is almost totally absent if the ribonuclease is assayed in the presence of EDTA instead of Mg²⁺. The distribution of alkaline phosphodiesterase activity assayed with Mg²⁺ present and with bis-*p*-nitrophenyl phosphate as substrate parallels the distribution of the plasma-membrane marker 5'-nucleotidase. When the 'crude' plasma-membrane preparation obtained by zonal centrifugation is further purified by flotation, most of the alkaline ribonuclease and phosphodiesterase activities remain associated with the membrane fragments and are purified in parallel with 5'-nucleotidase activity (Table 1). There is, however, some indication of a small selective loss, especially of the phosphodiesterase activity. A large part of the EDTA-insensitive ribonuclease activity is also removed at this stage. Subsequent work on the properties of the enzymes was carried out on membrane fragments purified by the flotation procedure.

The ribonuclease and phosphodiesterase activities shown by plasma-membrane fragments have very similar pH optima, i.e. 8.8 and 8.5 respectively (Fig. 2). Both enzymes give the same, rather complex, pattern with the various buffers used to determine the pH curve. Close to the pH optimum the highest activity is obtained with Tris buffer, slightly lower activity with Bicine buffer and lower activity still with glycine buffer. The buffer used to determine the shape of the

Fig. 1(a) and 1(c)

pH curve on the acid side of neutrality, $\beta\beta$ -dimethylglutarate, gave markedly lower activities than Tris buffer at the overlap pH values 7.2 and 7.5. Thus at pH 7.2, a plasma-membrane preparation released 0.119 E_{260} unit of acid-soluble material from RNA in 1 h if dimethylglutarate buffer was used but 0.142 units with Tris buffer. At pH 7.5, the corresponding values were 0.138 and 0.187 unit.

Both the ribonuclease and the phosphodiesterase activities depend on the presence of Mg^{2+} (Fig. 3).

The phosphodiesterase activity responds sharply to changes in the Mg^{2+} concentration, being almost fully activated by 1 mM- Mg^{2+} and almost fully inhibited by 1 mM-EDTA. The response of the ribonuclease is quantitatively different, the concentration of Mg^{2+} or EDTA required to give full activation or inhibition being 7.5 mM. The ribonuclease is 15 times more active in the presence of 7.5 mM- Mg^{2+} than in the presence of 7.5 mM-EDTA; the phosphodiesterase shows a 14–16-fold activation. The difference in the

Fig. 1. Distribution of marker enzymes, phosphodiesterase and ribonuclease activities after centrifugation for 50 min at 3700 rev./min in an A-XII zonal rotor of a crude nuclear fraction prepared from rat liver perfused with 0.25M-sucrose-5 mM- NaHCO_3 .

For details see the Experimental section. (a) ----, Density; —, protein. (b) $\circ$, 5'-Nucleotidase; $\square$, glucose 6-phosphatase; $\bullet$, acid β -glycerophosphatase; +, succinate dehydrogenase activities. (c) $\circ$, Phosphodiesterase activity assayed at pH 8.7 in the presence of 5 mM- MgCl_2 ; $\bullet$, phosphodiesterase activity assayed at pH 8.7 in the presence of 5 mM-EDTA. (d) Ribonuclease activity assayed at: $\circ$, pH 8.7 in the presence of 7.5 mM- MgCl_2 ; $\bullet$, pH 8.7 in the presence of 7.5 mM-EDTA; Δ , pH 7.8 in the presence of 5 mM- MgCl_2 . Values refer to the total amount per fraction.

Table 1. Purity and yield of marker enzymes, phosphodiesterase and ribonuclease at successive stages in the purification of plasma-membrane fragments

The results are given as means $\pm$ s.e.m., with numbers of plasma-membrane preparations examined (in parentheses). Specific activities are given as $\mu\text{mol}/\text{min}$ per g of protein except for ribonuclease where the values represent E_{260} for released nucleotides (notionally in 1 ml)/min per g of protein. Recovery and relative specific activity values refer to the homogenate taken as 100% and unity respectively.

Enzyme	Homogenate			Nuclear fraction			'Zonal' membrane fraction			'Purified' plasma membrane		
	Specific activity	Recovery (%)	Relative specific activity	Specific activity	Recovery (%)	Relative specific activity	Specific activity	Recovery (%)	Relative specific activity	Specific activity	Recovery (%)	Relative specific activity
5'-Nucleotidase	62 $\pm$ 2 (7)	121 $\pm$ 12 (7)	28.4 $\pm$ 1.9 (7)	1.97	7.65 $\pm$ 0.84 (7)	14.6	897 $\pm$ 78 (7)	0.34 $\pm$ 0.06 (7)	0.72	1710 $\pm$ 151 (6)	6.92 $\pm$ 0.80 (6)	27.8
Glucose 6-phosphatase	65 $\pm$ 4 (7)	50 $\pm$ 7 (7)	10.00 $\pm$ 1.08 (7)	0.77	42 $\pm$ 6 (7)	0.30 $\pm$ 0.06 (5)	13.4 $\pm$ 2.8 (5)	0.30 $\pm$ 0.06 (5)	0.62	27 $\pm$ 4 (6)	0.11 $\pm$ 0.02 (6)	0.41
Succinate dehydrogenase	21.5 $\pm$ 2.1 (6)	21.9 $\pm$ 2.1 (6)	13.83 $\pm$ 1.67 (6)	1.02	5950 $\pm$ 660 (4)	7.87 $\pm$ 0.84 (4)	15.65	2.0 $\pm$ 0.9 (5)	0.02 $\pm$ 0.01 (5)	8860 $\pm$ 71 (4)	4.25 $\pm$ 0.65 (4)	23.3
Phosphodiesterase assayed at pH 8.7 with 7.5 mM-MgCl ₂	380 $\pm$ 26 (6)	929 $\pm$ 77 (5)	29.7 $\pm$ 1.8 (5)	2.44								
Ribonuclease assayed at:												
(a) pH 8.7 with 7.5 mM-MgCl ₂	182 $\pm$ 25 (6)	248 $\pm$ 59 (6)	17.1 $\pm$ 2.1 (6)	1.36	881 $\pm$ 94 (5)	2.30 $\pm$ 0.12 (5)	4.84	1340 $\pm$ 150 (4)	1.91 $\pm$ 0.25 (4)	7.35		
(b) pH 8.7 with 7.5 mM-EDTA	53 $\pm$ 15 (4)	127 $\pm$ 49 (4)	28.5 $\pm$ 2.4 (4)	2.39	259 $\pm$ 123 (4)	1.94 $\pm$ 0.47 (4)	4.88	93 $\pm$ 18 (3)	0.30 (2)	1.76		
(c) pH 7.8 with 5 mM-MgCl ₂	207 $\pm$ 38 (4)	260 $\pm$ 39 (4)	19.2 $\pm$ 2.3 (4)	1.26	744 $\pm$ 142 (4)	1.77 $\pm$ 0.46 (4)	3.59	1090 (2)	0.96 (2)	5.24		

sharpness of the effect of change in Mg^{2+} concentration may simply be due to the ability of RNA to bind Mg^{2+} .

It has been suggested (Dickman *et al.*, 1956) that Mg^{2+} may decrease the solubility of small oligonucleotides. We were unable to demonstrate this effect. Mg^{2+} had no significant effect on the blank assays. Moreover, assays in which Mg^{2+} at various concentrations was added at the end of the incubation period immediately before stopping the reaction with the HClO_4 -uranyl acetate reagent gave values identical with control assays.

When the plasma membranes are treated with 1% Triton X-100 between 60 and 70% of the membrane protein is solubilized in agreement with the results of Dulaney & Touster (1970). Both the ribonuclease and the phosphodiesterase are activated about 20% by this process. Proteins solubilized from the membrane may be separated by centrifugation on sucrose gradients in the presence of Triton X-100. Details of the procedure are given in the Experimental section. After centrifugation, enzyme assays show that the phosphodiesterase activity and about 70% of the ribonuclease activity are in a single peak sedimenting in the central part of the gradient (Fig. 4). The precision limits of the ribonuclease assay are such that one cannot be confident about the significance of the small peak of ribonuclease activity at the end of the gradient; yet it was noticed in other runs similar to the one illustrated.

Discussion

The results presented in this paper clearly demonstrate that the plasma membrane of rat liver cells possesses ribonuclease activity that makes a significant contribution to the total ribonuclease activity of the rat liver cell. If it is assumed that 5'-nucleotidase activity is a perfect marker for the plasma membrane [the activity in the endoplasmic reticulum described by Widnell (1972) can only be a small proportion of the total activity in the cell], and that the ribonuclease and the nucleotidase activities are distributed in the same way over the surface of the cell, then one can calculate that the plasma membrane contributes between 25 and 30% of the particulate ribonuclease activity assayed at pH 8.7 in the presence of Mg^{2+} .

This calculation is performed by dividing the 'relative' specific activity of the ribonuclease in 'zonal' membrane fraction and the 'purified' membrane fraction by the relative specific activity of 5'-nucleotidase in the same fraction. The lower value, which is obtained from the 'purified' membrane fraction is probably the more accurate because the 'zonal' fraction is, as will be discussed below, probably contaminated by supernatant ribonuclease. However, complete confidence cannot be placed in values from the 'purified' fraction because of the risk that some

Fig. 2. Activity of (a) ribonuclease and (b) phosphodiesterase of a purified plasma-membrane fraction as a function of the pH of the assay medium

Ribonuclease activity was assayed in the presence of 7.5mM-MgCl₂, and that of phosphodiesterase in the presence of 5mM-MgCl₂. The buffers employed were: ○, 0.09M-dimethylglutarate; ×, 0.075M-Tris; ●, 0.075M-Bicine; △, 0.075M-glycine. The inset in (a) shows the relationship between the activities given by the last three buffers close to the pH optimum and derives from a different experiment from that shown in the main part of the figure.

intrinsic plasma-membrane components may be lost in the rehomogenization and flotation. It should be noted that calculation on this simple basis is only valid if the fractions are essentially pure. Thus calculations based on the crude nuclear fraction are meaningless. A similar calculation may be carried out for phosphodiesterase activity. If this calculation is based on the 'purified' plasma-membrane fraction, then 65% of the phosphodiesterase activity must be in the plasma membrane; if the calculation is based on the 'zonal' plasma-membrane fraction then the figure is 95%. Therefore up to 30% of the phosphodiesterase of the cell is associated with the membrane sheets during sedimentation but is separated on flotation. This is much too large a value to be explained by

contaminants in the plasma-membrane fraction and suggests that phosphodiesterase or phosphodiesterase-rich membrane fragments are specifically eluted from the membranes during rehomogenization. Some doubt must, however, attach to the assumptions on which these calculations are based, for the plasma-membrane fragments recovered from the crude nuclear fraction show many bile-canalicular membranes (Hinton *et al.*, 1970) and it is distinctly possible that the sinusoidal membranes are fragmented to microsomes, as at least one-half of the 5'-nucleotidase of the cell is recovered in this fraction. *p*-Chloromercuribenzoate was not included in the assay medium, so that little of the supernatant ribonuclease activity will have been detected (Roth, 1958).

Fig. 3. Activity of (a) ribonuclease and (b) phosphodiesterase in purified plasma membrane as a function of the concentration of $MgCl_2$ or of EDTA

The assays were carried out at pH 8.7 in 0.075 M-Tris buffer.

The uncertainty in the proportion of the enzymes in the plasma-membrane fraction is due to the calculation based on the 'zonal' plasma membrane giving consistently high results than calculations based on the 'purified' plasma membrane. This cannot be due to the membrane being partly split by the rehomogenization into the 'light' and 'heavy' subfractions described by Evans (1970) as both subfractions would float at density 1.19. One possibility for the loss of ribonuclease activity at this stage is that it is due to the removal of contaminants. The EDTA-resistant ribonuclease, which is lost on flotation, is probably largely due to the alkaline ribonucleases of mitochondria and the supernatant, which are trapped inside the membrane bags (Hinton *et al.*, 1970)

and which are released on rehomogenization. Emmelot *et al.* (1964) have shown that part of the ribonuclease activity of their plasma-membrane preparations is removed by washing with 0.15 M-NaCl, and is therefore probably supernatant ribonuclease adsorbed on to the membrane. Emmelot *et al.* (1964) showed that this adsorbed enzyme is distinguished from the bound enzyme by being more active at pH 7.6 than at pH 8.9. They also showed that the pH 7.6 activity of the unwashed membranes is distinguished from the pH 8.9 activity in being partially resistant to treatment with dilute H_2SO_4 . This also indicates the presence of two distinct enzymes.

The ribonuclease of the plasma membrane is clearly distinguished from the other 'large particulate' ribonucleases, which are responsible for the activity in the early part of the zonal gradient, by its response to EDTA and to changes in the pH of the assay medium. The ribonucleases responsible for the activity in the early part of the gradient include the 5'-endonuclease of mitochondria (Morais, 1969) and a lysosomal alkaline ribonuclease, which is possibly located on the lysosomal membrane (M. L. E. Burge, unpublished work). It is not clear why the plasma membrane possesses so much ribonuclease activity, but it is interesting to speculate that it may be there to act as a barrier against invading RNA-containing viruses.

The similarity in the properties of the plasma-membrane ribonuclease and phosphodiesterase activities suggests that both activities may be due to the same enzyme. This supposition is strengthened by the observation that the two activities sediment together after dissolution of the membranes in Triton X-100. Touster *et al.* (1970) have argued that the non-specific phosphodiesterase that breaks down bis-*p*-nitrophenyl phosphate is the same as the 5'-phosphodiesterase that breaks down *p*-nitrophenyl 5'-thymidylate and a variety of synthetic oligonucleotides and can also break the pyrophosphate bond in NADP. The results of Futai & Mizuno (1967), who state that purified 5'-phosphodiesterase does not attack bis-*p*-nitrophenyl phosphate, argue against the identity of the two activities, but it is difficult to reconcile their stated findings with those of Touster *et al.* (1970).

The properties of both non-specific phosphodiesterase and the plasma-membrane ribonuclease are similar to the properties of the glycerolphosphorylcholine phosphohydrolase studied by Lloyd-Davies *et al.* (1972). Although it is argued, on the basis of differences in the behaviour of the enzymes during purification, that the glycerolphosphorylcholine phosphohydrolase and 5'-phosphodiesterase cannot be the same enzyme, the results presented could possibly be due to the adhesion of the soluble form of the former enzyme to material in the initial pellet, and its elution later in the purification. If this were so, then one may postulate that the plasma membrane contains a single enzyme that is capable of attacking both pyro-

Fig. 4. Distribution of ribonuclease and phosphodiesterase activity after centrifugation for 12.5 h at 30000 rev./min in a 3×23 ml swing-out rotor of a preparation of 'purified' plasma membrane treated with 1% Triton X-100

The sample was layered on a 0.1–0.4 M-sucrose gradient containing 0.1 M-Tris buffer, pH 8.5, and 1% Triton X-100. ○, Ribonuclease activity assayed at pH 8.7 in the presence of 7.5 mM-MgCl₂; ●, phosphodiesterase activity assayed at pH 8.7 in the presence of 5 mM-MgCl₂. Values refer to the total amount per fraction.

phosphate and a variety of phosphodiester bonds. If the various activities are due to different enzymes, then one must conclude that their characteristics are remarkably similar.

The properties of the plasma-membrane phosphodiesterase described in the present paper are similar to the properties of the plasma-membrane (Touster *et al.*, 1970) and microsomal (Futai & Mizuno, 1967; de Lamirande *et al.*, 1966) 5'-phosphodiesterase described by other authors. The pH optimum is somewhat lower than is reported for the microsomal enzyme, but the extremely sharp response to the addition or removal of Mg²⁺ ions is similar. It is less easy, however, to identify the plasma-membrane ribonuclease activity with activities reported by other authors. Although the enzyme resembles 'ribonuclease III' as described by Rahman (1966) in its high pH optimum, it is very sharply differentiated by its response to Mg²⁺ ions, for ribonuclease III is inhibited at Mg²⁺ concentrations greater than 1 mM. Morais & de Lamirande (1965) showed that an exonuclease was responsible for some of the breakdown of endogenous RNA in microsomes. Their results suggest that at pH 8.0 in the presence of Mg²⁺ up to one-third of the observed ribonuclease activity may be due to the exonuclease. As up to 40% of the plasma membrane is fragmented to microsomal size on homogenization (Hinton *et al.*, 1970), it is reasonable to identify this exonuclease activity with the phosphodiesterase of the plasma-membrane fraction, especially

as both activities are activated by Mg²⁺. Other workers have already shown that alkaline phosphodiesterase activity remains associated with 5'-nucleotidase activity during the sub-fractionation of microsomes (Thinès-Sempoux *et al.*, 1969).

Since the completion of our experiments a brief report has appeared which suggests that plasma membranes degrade RNA by a series of reactions involving an endonuclease, 3'- and 5'-nucleotidases and a Zn²⁺-dependent phosphodiesterase (Yannarell & Aronson, 1972). In the absence of full experimental details it is difficult to discuss these results, but the very low activity of ribonuclease in our plasma-membrane preparations in the presence of EDTA argues against there being any distinctive endonuclease of the type required by these authors, and it is difficult to reconcile their absolute Zn²⁺ requirement with our demonstration of strong Mg²⁺ activation. Previous work by our group (El-Aaser & Reid, 1969) has indicated that plasma membranes lack a distinctive 3'-nucleotidase.

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References

- Anderson, N. G. (ed.) (1966) *Nat. Cancer Inst. Monogr.* **21**
- Coleman, R., Michell, R. H., Finean, J. B. & Hawthorne, J. N. (1967) *Biochim. Biophys. Acta* **135**, 573–579

- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- de Lamirande, G., Bioleau, S. & Morais, R. (1966) *Can. J. Biochem.* **44**, 273–279
- Dickman, S. R., Aroskar, J. P. & Kropf, R. B. (1956) *Biochim. Biophys. Acta* **21**, 539–545
- Dulaney, J. T. & Touster, O. (1970) *Biochim. Biophys. Acta* **196**, 29–34
- El-Aaser, A. A. & Reid, E. (1969) *Histochem. J.* **1**, 417–439
- Emmelot, P., Bos, C. J., Benedetti, E. L. & Rumke, Ph. (1964) *Biochim. Biophys. Acta* **90**, 126–145
- Erecinska, M., Sierakowska, H. & Shugar, D. (1969) *Eur. J. Biochem.* **11**, 465–471
- Evans, W. H. (1970) *Biochem. J.* **116**, 833–842
- Futai, M. & Mizuno, O. (1967) *J. Biol. Chem.* **242**, 5301–5307
- Hinton, R. H. (1971) in *Separations with Zonal Rotors* (Reid, E., ed.), pp. Z-5.1–Z-5.10, Wolfson Bioanalytical Centre, University of Surrey, Guildford
- Hinton, R. H. & Norris, K. A. (1972) *Anal. Biochem.* **47**, 247–258
- Hinton, R. H., Burge, M. L. E. & Hartman, G. C. (1969) *Anal. Biochem.* **29**, 248–256
- Hinton, R. H., Fitzsimons, J. T. R., Dobrota, M. & Reid, E. (1970) *Eur. J. Biochem.* **12**, 349–359
- Hinton, R. H., Norris, K. A. & Reid, E. (1971) in *Separations with Zonal Rotors* (Reid, E., ed.), pp. S-2.1–S-2.16, Wolfson Bioanalytical Centre, University of Surrey, Guildford
- Lansing, A. I., Belkhome, M. L., Lynch, W. E. & Lieberman, I. (1967) *J. Biol. Chem.* **242**, 1772–1775
- Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482–513
- Lloyd-Davies, K. A., Michell, R. H. & Coleman, R. (1972) *Biochem. J.* **127**, 357–368
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Morais, R. (1969) *Biochim. Biophys. Acta* **189**, 38–45
- Morais, R. & de Lamirande, G. (1965) *Biochim. Biophys. Acta* **95**, 40–47
- Mullock, B. M., Hinton, R. H., Dobrota, M., Froomburg, D. & Reid, E. (1971) *Eur. J. Biochem.* **18**, 485–495
- Norris, K. A., Burge, M. L. E. & Hinton, R. H. (1971) *Biochem. J.* **122**, 53P
- Rahman, Y. E. (1966) *Biochim. Biophys. Acta* **119**, 470–479
- Reid, E. (ed.) (1971) *Separations with Zonal Rotors*, Wolfson Bioanalytical Centre, University of Surrey, Guildford
- Reid, E. & Nodes, J. T. (1959) *Ann. N.Y. Acad. Sci.* **81**, 618–630
- Roth, J. S. (1957) *J. Biol. Chem.* **227**, 591–604
- Roth, J. S. (1958) *J. Biol. Chem.* **231**, 1097–1105
- Shugar, D. & Sierakowska, H. (1967) *Progr. Nucl. Acid Res. Mol. Biol.* **7**, 369–429
- Steele, W. J. & Busch, H. (1967) *Methods Cancer Res.* **13**, 61–152
- Stonehill, E. H. & Huppert, E. (1968) *Biochim. Biophys. Acta* **155**, 353–359
- Thinès-Sempoux, D., Amar-Costesec, A., Beaufay, H. & Berthet, J. (1969) *J. Cell Biol.* **43**, 189–192
- Touster, O., Aronson, N. W., Jr., Dulaney, J. T. & Hendrickson, H. (1970) *J. Cell Biol.* **47**, 604–618
- Widnell, C. (1972) *J. Cell Biol.* **42**, 542–558
- Yannarell, A. & Aronson, N. W. (1972) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **31**, 411