Purification of Rat Liver Particulate Neutral Ribonuclease and Comparison of Properties with Pancreas and Serum Ribonucleases

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Rat liver particulate neutral ribonuclease (EC 3.1.4.22) was extensively purified (up to 40 000-fold). It is shown to be an endonuclease, specific for pyrimidine bases, hydrolysing 5'-phosphate ester bonds. The enzyme specificity, K_m , pH optimum, stability in acid medium and thermal stability at high temperature are the same as those of rat pancreatic and serum ribonucleases. Like pancreatic and serum neutral ribonucleases, the hepatic enzyme is sensitive to the liver natural inhibitor. This inhibitor was purified 8000-fold; its association with ribonuclease follows zero-order kinetics. These identical properties for ribonuclease of rat liver, pancreas and serum support the hypothesis [Bartholeyns, Peeters-Joris & Baudhuin (1975) *Eur. J. Biochem.* **60**, 385–393] of an extrahepatic origin for the liver enzyme, the plasma ribonuclease of pancreatic origin being taken up by endocytosis in the liver. Neutral ribonuclease activity was detected in all rat organs investigated; its distribution among tissues is different from the distribution of the natural ribonuclease inhibitor.

In rat liver, neutral ribonuclease activity (EC 3.1.4.22; pH 7.5) can be differentiated from the lysosomal acid ribonuclease (pH 5) and from the alkaline ribonuclease (pH 9.1) associated with mitochondria by its sensitivity to the natural inhibitor of the cytosol (Baudhuin *et al.*, 1975; Bartholeyns *et al.*, 1975a). Sometimes referred as an alkaline ribonuclease, the enzyme has been studied by Roth (1954, 1958, 1967), Siebert *et al.* (1966), De Lamirande & Allard (1959), Reid & Nodes (1959) and Rahman-Li (1967). It has been purified by Roth (1957) and Gordon (1965a). In a previous study from our laboratory (Bartholeyns *et al.*, 1975b), we showed that particulate neutral ribonuclease is associated with the lysosomal system and that partial pancreatectomy results in a significant decrease of the hepatic neutral ribonuclease activity; other lysosomal nucleases are not affected. On the basis of these observations, it was proposed that the liver enzyme is mainly of extrahepatic origin, being taken up from the plasma by endocytosis. The plasma neutral ribonuclease would be itself of pancreatic origin.

Although a similar hypothesis has been proposed for the kidney enzyme (Zan-Kowalczevska *et al.*, 1974), neutral ribonuclease is to our knowledge the only case of a liver lysosomal enzyme acquired by endocytosis. Further, it is the most active ribonuclease found in the liver (16 units/g wet wt., compared with 7 units/g for the acid ribonuclease and 6.8 units/g for the alkaline ribonuclease, pH 9.1). Hence it was worth while to substantiate the hypothesis of the pancreatic origin of the liver enzyme by

comparing some properties of neutral ribonuclease from liver and pancreas. Purification of the liver particulate enzyme has been achieved and the characteristics of ribonucleases of liver, serum, and pancreas have been investigated. The relative activities of neutral ribonuclease in various rat organs have also been studied.

Experimental

Biochemical determinations

Fractionation procedures, methods for nuclease determinations and for the assay of the natural ribonuclease inhibitor were as described previously (Bartholeyns *et al.*, 1975a,b; Baudhuin *et al.*, 1975). The method of Lowry *et al.* (1951) was used for protein determination, except for preparations with low protein content, for which the assay described by Hiraoka & Glick (1963) was used: quenching of fluorescence by protein was determined by using a 10 μ M-Eosin solution at pH 3.1. Emission at 550 nm was measured with a Zeiss fluorimeter, with excitation at 519 nm. This method was found adequate to measure protein concentration down to 0.05 μ g/ml. Bovine serum albumin was used as standard for both methods. Units of enzyme activity are defined as the amount of enzyme yielding 1 μ mol of reaction product/min, under the standard assay conditions. Acid-soluble oligonucleotides are exposed as equivalents of mononucleotides; an average absorption coefficient of $10.8 \times 10^3 \text{ litre}^{-1} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ was as-

sumed for mononucleotides (Beaven *et al.*, 1955). For the natural inhibitor, 1 unit is defined as the amount inhibiting 50% of the activity of 5 ng of

bovine pancreatic ribonuclease A, incubated for 10 min at 37°C and pH 7.5, in the presence of 2 mg of RNA/ml in a volume of 0.5 ml.

Table 1. *Purification scheme of neutral hepatic ribonuclease*

		Neutral ribonuclease	
		Relative sp. activity	Yield (%)
Rat liver homogenate in 0.25 M-sucrose		1	100
↓			
Cytoplasmic extract			
↓			
Large-granule fraction		1.8	42
↓			
Suspended in 10 g of Triton X-100/l, then centrifuged at $15 \times 10^6 g$ -min			
S1	P1	3.2	35
↓			
Suspended in 0.1 M-pyrophosphate buffer, pH 9, 0.15 M-NaCl, 10 g of Triton X-100/l, then centrifuged at $15 \times 10^6 g$ -min			
P2	S2	7.6	31
↓			
Incubated for 40 min at 50°C, pH 5, then centrifuged at 8500 g-min			
P3	S3		
↓			
27.5% satn. with $(NH_4)_2SO_4$, then centrifuged at $15 \times 10^6 g$ -min			
P4	S4	34	18
↓			
55% satn. with $(NH_4)_2SO_4$ then centrifuged at $15 \times 10^6 g$ -min			
S5	P5		
↓			
Incubated for 48 h at 0°C, 125 mM- H_2SO_4 , then centrifuged at $15 \times 10^6 g$ -min			
P6	S6	100	16
↓			
Neutralization		350	7
↓			
Gel filtration on Sephadex G-25			
↓			
Chromatography on CM-cellulose			
↓			
Purified neutral ribonuclease		32 500 (s.d. $\pm$ 4500)	4

Purification of rat liver neutral ribonucleases

The technique used here is a modification of the method described by Roth (1957) and Gordon (1965a), taking advantage of our observations on the intracellular localization of the enzyme (Bartholeyns *et al.*, 1975b). The purification scheme is summarized in Table 1. All operations were performed at 2°C. Some 20 rats were killed, their livers homogenized and the nuclear fraction was separated from the cytoplasmic extract as described by de Duve *et al.* (1955). Extract corresponding to 0.2 g of liver/ml was submitted to an integrated centrifugal force of 250000g-min (rotor 30, Spinco LHV centrifuge) to sediment a large-granule fraction.

The large-granule fraction was suspended in 10 g of Triton X-100/litre and centrifuged at 15×10^6 g-min (rotor 65, Spinco LHV centrifuge) to separate acid ribonuclease from neutral ribonuclease. The lysosomal membrane is damaged by the detergent; acid ribonuclease is solubilized, whereas neutral ribonuclease is strongly adsorbed on particulate material in non-ionic medium and is thus recovered in the pellet. The pellet was resuspended in a solution of 10 g of Triton X-100/litre in 0.1M-sodium pyrophosphate buffer (pH9)/0.15M-NaCl, and centrifuged again at 15×10^6 g-min; in the presence of salt the neutral ribonuclease was solubilized. The supernatant, adjusted to pH5 in 0.02M-sodium acetate buffer, was incubated for 40 min at 50°C and centri-

fuged at 8500g-min after cooling to 0°C. The supernatant was then submitted to $(\text{NH}_4)_2\text{SO}_4$ fractionation; the fraction precipitated between 27.5 and 55% satn. (212 and 524 g/l respectively) was left for 48 h at 0°C in 125mM- H_2SO_4 and centrifuged at 15×10^6 g-min. The supernatant was used for subsequent steps. All these preliminary purification steps were necessary to eliminate protein not separable from neutral ribonuclease by chromatography.

The supernatant was neutralized and submitted to gel filtration on a column (1.5cm $\times$ 50cm) of Sephadex G-25, with 0.01M-sodium acetate buffer, pH4.5, as the eluent, at a flow rate of 30 ml/h (Fig. 1). Three to five fractions (10 ml each), corresponding to the peak of neutral ribonuclease, were pooled and the concentration of the sodium acetate buffer was increased to 0.5M. The material was further chromatographed on a column (1.5cm $\times$ 50cm) of CM-cellulose. After adsorption in 0.5M-sodium acetate buffer, pH4.5 (75 ml), it was eluted with a gradient obtained by addition of 0.5M-sodium acetate, pH8, to 75 ml of 0.5M-sodium acetate buffer pH4.5 (Fig. 2). Mixing of the two solutions was performed with a linear gradient maker. The total volume of the gradient was 150 ml and the flow rate 30 ml/h. The peak containing ribonuclease was neutralized; the final purification of the enzyme ranged from 25000- to 40000-fold with respect to the initial homogenate, depending on the experiment. The specific activity of a purified preparation is between 1800 and 3000 units/mg of protein, compared with 0.075 unit/mg on average in the liver homogenate.

Fig. 1. Purification of neutral ribonuclease by gel filtration. Gel filtration of the S6 supernatant (see Table 1) was on a column (1.5cm $\times$ 50cm) of Sephadex G-25 in 0.01M-sodium acetate buffer, pH4.5. —, Neutral ribonuclease; ----, protein. Concentrations are expressed with respect to the concentration of the sample layered on the column. To facilitate comparison, they are normalized by multiplying the values obtained by the recovery/100. Recoveries were 99% for protein and 108% for ribonuclease. The fractions pooled for further purification are indicated by the arrow.

Fig. 2. Purification of neutral ribonuclease by chromatography on CM-cellulose.

The sample layered on the column (1.5cm $\times$ 50cm) in 0.5M-sodium acetate buffer was eluted by gradually replacing the sodium acetate buffer, pH4.5, by 0.5M-sodium acetate, pH8.5 (see the Experimental section). —, Neutral ribonuclease; ----, protein. Concentrations are expressed as in Fig. 1. Recoveries were 94% for protein and 83% for ribonuclease. The fractions used for the study of enzyme properties are indicated by the arrow.

Fig. 3. Purification of the ribonuclease inhibitor by chromatography on hydroxyapatite

After a first chromatography on DEAE-cellulose, the fractions corresponding to the peak of inhibitor were pooled and adjusted to 0.15M-NaCl/45mM-potassium phosphate buffer, pH 6.4. This preparation was layered on top of a column (1.5cm×60cm) of hydroxyapatite and eluted with a linear gradient obtained from 0.045M- and 0.15M-phosphate buffer, pH 6.4, both in 0.15M-NaCl. Total gradient volume was 400ml. —, Inhibitor; ----, protein. Concentrations are expressed as in Fig. 1; recoveries were 25% for protein and 80% for the inhibitor.

Preparation of highly purified inhibitor

All the steps have to be carried out within 48h at 2°C to prevent inactivation; 0.1 mM-mercaptoethanol was included in all solutions. DEAE-cellulose chromatography of the high-speed supernatant (3500000g-min) obtained from liver homogenate was performed as described by Shortman (1961) and Gribnau *et al.* (1969). The preparation, adjusted to 0.15M-NaCl/45mM-potassium phosphate buffer, pH 6.4, was layered on a column (1.5cm×50cm) of hydroxyapatite (Tiselius *et al.*, 1956). Elution was performed with a linear gradient, obtained by mixing 0.15M-potassium phosphate buffer (pH 6.4)/0.15M-NaCl with the buffer used for layering the sample. The flow rate was 30ml/h and the total volume of the gradient 400ml. Results of an experiment are presented in Fig. 3. In our experimental conditions, most (75%) of the protein remains adsorbed on the DEAE-cellulose column. The inhibitor was purified 8000-fold with a yield of 36% with respect to the liver homogenate (specific activity 80000units/mg of protein). We have verified that the preparation is devoid of neutral ribonuclease activity.

Materials

Sephadex G-25 was from Pharmacia Fine Chemicals AB (Uppsala, Sweden); MN-300 G cellulose,

Table 2. Distribution of neutral ribonuclease and of its natural inhibitor

Values in parentheses are S.D. For inhibitor determination, values were corrected for ribonuclease content; therefore the accuracy is low for tissues with very high ribonuclease activity or very low inhibitor concentration.

Rat tissues	Neutral ribonuclease (4 expts.) (units/g of tissue)	Natural inhibitor (2 expts.) (units/g of tissue)
Heart	1.50 (0.30)	<100
Brain	1.45 (0.06)	170–186
Lung	8.90 (3.34)	743–852
Liver	16.3 (3.25)	1240–2700
Thymus	6.92 (3.84)	300–408
Ovary	3.80 (1.48)	170–203
Stomach	16.0 (6.13)	2160–2270
Spleen	28.8 (10.0)	2300–2400
Kidney	50.4 (13.8)	<100
Adrenal	23.3 (2.23)	1890–2050
Fat	3.53 (2.60)	<100
Intestine (washed)	27.6 (4.77)	3360–4090
Pancreas	413.6 (47.0)	>3000
Blood serum	3.08* (0.16)	<100*

* units/ml.

DEAE-cellulose and CM-cellulose were purchased from Macherey Nagel, Düren, Germany; hydroxyapatite was prepared as described by Tiselius *et al.* (1956).

Poly(A), poly(G), poly(C) and poly(U), yeast RNA type II-S, calf thymus DNA type V, bovine pancreatic ribonuclease A, bovine spleen phosphodiesterase I (EC 3.1.4.1), *Crotalus adamanteus* venom phosphodiesterase II (EC 3.1.4.18), 3'-nucleotidase (EC 3.1.3.6) and 5'-nucleotidase (EC 3.1.3.5) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results and Discussion

Distribution of neutral ribonuclease and its inhibitor in various tissues

A survey of neutral ribonuclease activity and of its inhibitor was made in different tissues (Table 2). As previously, the enzyme is defined as the neutral ribonuclease sensitive to the natural inhibitor. The enzyme was found in appreciable amount in all rat organs tested.

In view of the possible physiological role of the natural inhibitor of neutral ribonuclease, the inhibitor was measured in two of the experiments. It was found in all organs tested; there is, however, no correlation between the amount of inhibitor in different tissues and the activity of neutral ribonuclease. For example, as compared with liver, kidney has a very low

inhibitor concentration, whereas that of neutral ribonuclease is about 3 times as high. Our data on the inhibitor are different from those of Roth (1956, 1967), who has used a crude high-speed supernatant as a source of inhibitor, and the ribonuclease occurring in such preparations was not taken into account. Under our conditions, both the inhibitor and the enzyme are independently measured.

Properties of purified rat liver particulate neutral ribonuclease

Purification of liver neutral ribonuclease has been attempted before by Roth (1957) and Gordon (1965a). The specific-activity increase reported by Roth (1957) was only 85-fold, and Gordon (1965a) was unable to measure the protein concentration in the most purified preparations but evaluated the overall purification as 6700-fold. Our purification varies between 25000- and 40000-fold, depending on the experiment. As mentioned above, this improvement can be attributed to the preliminary purification steps used here, on the basis of the lysosomal localization of the enzyme. It has been verified that the characteristics of the purified ribonuclease correspond to those described for the inhibitor-sensitive enzyme present in liver homogenate. Purified ribonuclease has an activity between 1800 and 3000 units/mg of protein, depending on the preparation, which can be compared with 2500 units/mg of protein for commercial bovine ribonuclease A (Sigma type I-A). The purified preparation has been submitted to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis [10% polyacrylamide in 0.01 M-sodium phosphate buffer (pH 7.2)/2 M-urea/0.2% sodium dodecyl sulphate; a single band of protein was observed after staining of the gels in Coomassie Blue. Migrations of purified ribonuclease and of bovine pancreatic ribonuclease A were similar.

The purified preparation of particulate neutral ribonuclease had no detectable deoxyribonuclease or phosphodiesterase activity. Taking into account the sensitivity of our enzyme assays, it can be estimated that the activity towards DNA or *p*-nitrophenyl-thymidylate 5'-phosphate is less than 1% of that for RNA. Using artificial homopolynucleotides at a concentration of 2 mg/ml, we found, for 1 unit of ribonuclease activity at pH 7.5 towards yeast RNA measured under standard conditions, an activity smaller than 0.01 unit for poly(A) or poly(G) and of 0.4 and 1.3 units for poly(U) or poly(C) respectively. Liver particulate neutral ribonuclease thus shows specificity towards pyrimidine bases; this conclusion agrees with the results of Reid & Nodes (1959).

Techniques for separation of mono-, di-, tri- and tetra-nucleotides (Thirion & Kaesberg, 1968; Fiers *et al.*, 1965) have been applied to establish whether liver particulate neutral ribonuclease is an endo- or an

Fig. 4. *Chromatography of reaction products* RNA was incubated at pH 7.5 for 24 h at 37°C in the presence of 20 units of purified rat liver neutral ribonuclease (—) or in the absence of enzyme (---). Reaction products were chromatographed on a column (50 cm × 1.5 cm) of DEAE-cellulose, developed at 0°C with a linear gradient of sodium acetate in 7 M-urea/10 mM-Tris/HCl buffer (pH 7.5)/1 mM-EDTA; the flow rate was 30 ml/h. In these conditions, oligonucleotides were separated according to the number of phosphate groups present (Fiers *et al.*, 1965), mononucleotides being eluted first. Concentrations are expressed as in Fig. 1.

exo-nuclease (Fig. 4). Only a single peak was observed for the reaction products of RNA incubated in the absence of enzyme; after incubation with rat liver purified ribonuclease, peaks of material corresponding to oligonucleotides of different size were observed. Hence, like pancreatic ribonuclease, the liver enzyme is an endonuclease.

We have also analysed the oligonucleotides liberated after exhaustive reaction of the purified enzyme. After incubation of the acid-soluble reaction products in the presence of phosphodiesterase I or II at pH 6.5 and 8.8 respectively, chromatography was performed on Whatman 3MM paper, with 1 M-ammonium acetate/95% (v/v) ethanol (2:5, v/v) as eluent. Identification of spots under u.v. light showed that only phosphodiesterase II, requiring a free 5'-hydroxyl end, gave new nucleotides from products of reaction with pancreatic or liver ribonucleases. This observation has also been confirmed by the fact that 3'-nucleotidase was active on the products resulting from exhaustive digestion of RNA by purified neutral ribonuclease, whereas 5'-nucleotidase was not effective. Rat liver particulate neutral ribonuclease is thus similar to bovine pancreatic ribonuclease A, i.e. an endonuclease yielding oligonucleotide 3'-phosphates as end of product of reaction, and specific for pyrimidine bases.

Comparison of neutral ribonucleases isolated from rat liver, blood and pancreas

We have also compared the properties of neutral ribonucleases from rat liver, serum and pancreas. For

Fig. 5. Ribonuclease activity as a function of pH. Inhibitor-sensitive ribonuclease activity from rat liver (●), pancreas (▲) and blood (■) was measured as a function of pH. For all the determinations 0.05 M-acetate/Tris/glycine buffer mixture adjusted to the required pH was used. Incubation was performed during 10 min at 37°C under standard assay conditions on partially purified preparations (see the text).

serum and pancreas, the enzymes were only roughly purified (50- and 60-fold respectively) by following the scheme described in Table 1 from step S3 to step S6.

The pH curves observed for the three ribonucleases are the same, with an optimum at pH 8 (Fig. 5). Liver, blood and pancreas ribonucleases have the same sensitivity toward various agents: they are stable to heating for 50 min at 60°C, pH 5; they are not affected by very acid medium (24 h in 125 mM- H_2SO_4); they do not require Mg^{2+} and are not influenced by EDTA. Using synthetic homopolyribonucleotides, we have also verified that rat liver and rat pancreas neutral ribonucleases have the same specificity towards polyribonucleotides and pyrimidine bases; they are all endonucleases and give 3'-phosphate-terminated oligonucleotides. Determination of the apparent K_m for RNA by Lineweaver-Burk plot for rat liver neutral ribonuclease, rat pancreas ribonuclease and ribonuclease A gave the same value, 1.32 g/litre. (The molecular weight of the yeast RNA used, presumably heterogeneous, was not determined. Assuming an average molecular weight of 327 for a mononucleotide, the value of 1.32 corresponds to a 4 mM concentration for mononucleotides.)

Properties of the purified inhibitor and kinetics of the inhibition

The purification scheme derived from Shortman (1961) gave in our hands a purification and yield slightly higher (8000-fold and 36%; s.d. 500 and 3%

Fig. 6. Substrate saturation kinetics of ribonuclease A in the presence of different inhibitor concentrations. Bovine pancreatic ribonuclease (5 ng) was incubated for 10 min at 37°C in the presence of 4 units (■) or 1.3 units (▲) of purified inhibitor, and in the absence of the inhibitor (●) (specific activity 80000 units/mg). RNA concentration was adjusted to 4 g/litre in all cases just before the end of the reaction to avoid co-precipitation artifacts. The data are presented by a Lineweaver-Burk (1934) plot.

respectively) than those reported by Shortman (1961) (6000-fold and 20%), and by Gribnau *et al.* (1969) (4000-fold and 10%) who used the same method. As initially shown by Gribnau *et al.* (1970), we have observed an inactivation of the inhibitor above pH 8.5. Stored at 0°C, the inhibitor is slowly inactivated; a partial protection can be obtained by addition of thiol reagents (0.1 mM-dithiothreitol or -mercaptoethanol) or Ca^{2+} (10 mM- CaCl_2).

We have found that the inhibition of ribonuclease is apparently non-competitive by measuring the influence of increasing concentrations of the substrate on the activity of bovine pancreatic ribonuclease A, in the presence of different concentrations of inhibitor. Fig. 6 shows that the maximum velocity of the reaction is lowered by the presence of the inhibitor, whereas the apparent K_m (1.32 g/litre) is not modified. These observations agree with those reported by Bishay & Nicholls (1973) with bovine pancreatic ribonuclease A and inhibitor obtained from rat kidney and liver.

Provided that highly purified (8000-fold) inhibitor is used the percentage inhibition of ribonuclease is proportional to inhibitor concentration (Fig. 7). Crude preparations did not show the same linear relation between enzyme activity and inhibitor concentration (Gribnau *et al.*, 1970). The same inhibition kinetics was observed for neutral liver ribonuclease and pancreatic ribonuclease A (Fig. 8); when the

Fig. 7. Sensitivity of hepatic ribonucleases to partially (a) or highly (b) purified natural inhibitor. Acid (▲), neutral (●) and alkaline (■) ribonucleases were determined in the presence of different inhibitor concentrations. A rat liver large-granule fraction (2mg of tissue/ml) was used as source of the enzymes. The reaction was performed at 37°C for 10min under standard assay conditions (Bartholeyns *et al.*, 1975a). The specific activities of the inhibitor preparation were respectively 2000 (a) and 80000 (b) units/mg of protein. Alkaline ribonuclease was measured at pH8.5 rather than at pH9, to avoid inactivation of the inhibitor.

Fig. 8. Kinetics of the inhibition of bovine pancreatic ribonuclease A

Highly purified inhibitor (specific activity 80000 units/mg) was incubated at 37°C for 10min in the presence of 2 (▲) and 4 (●) ng of pancreatic ribonuclease A, and the ribonuclease activity was measured under standard conditions at pH7.6.

ribonuclease A content was doubled for the same quantity of inhibitor, the inhibition was decreased by half. The process of the inhibition is therefore a titration of ribonuclease by the inhibitor.

It appears likely that the high purification obtained here allowed the removal of an impurity interfering with the kinetics of the enzyme-inhibitor complex-formation. The mechanism of the inhibition of ribo-

nuclease has not been elucidated; a thiol group of the inhibitor is probably involved in the enzyme-inhibitor complex, since the inhibitor is protected by mercaptoethanol, and it can be inactivated by *N*-ethylmaleimide or *p*-chloromercuribenzoate. Up to now it has not been possible to study in detail the structure of the inhibitor, since it is inactivated during the numerous purification steps necessary for preparation of a purified protein for amino acid analysis.

It should be noted that this finding raises some doubts on the validity, on an absolute basis, of the method proposed by Shortman (1961) for the estimation of the amount of inhibitor. Like this author, we have found good reproducibility of the assay with the preparations of roughly the same purity. Care should, however, be taken when preparations of different degrees of purification are compared. Provided that an inhibition of less than 50% is used for the assay, as recommended by Shortman (1961), the error due to a modification in kinetics will only introduce a small underestimation of the amount of inhibitor in the preparation.

Conclusion

On the whole, the characteristics of highly purified liver particulate ribonuclease are strikingly similar to the properties of both serum and pancreatic ribonucleases. The hypothesis of a pancreatic origin of

neutral ribonuclease is thus substantiated by the observations reported here.

The yield of the purification procedure used in this study (100 µg/100 g liver wt.) did not allow us to compare the immunological properties of pancreatic, serum and liver ribonucleases. Purification on a large scale (1100 rats) of neutral ribonuclease from different organs has been performed by Gordon (1965a,b), who concluded that liver and kidney ribonucleases are immunologically identical, but different from spleen or pancreas ribonucleases, the latter being also immunologically identical. He came to no conclusion about the serum enzyme. The observations by Gordon (1965a,b) apparently disagree with the hypothesis of uptake by liver of serum ribonuclease of pancreatic origin. However, it is difficult to assess the effect of the natural inhibitor on the assays for immunological enzyme inhibition in Gordon's (1965a,b) work, since the inhibitor was not measured. Further, it is not known if any antibodies against the inhibitor were present in the antisera used. Further investigations on these points are required for the interpretation of these immunological observations. It is also worth while mentioning here that a recent re-evaluation by Karn *et al.* (1976) of data on inhibition of amylase by antibodies in tissues has led to the conclusion that rat liver α -amylase was probably of pancreatic origin and contributed by blood plasma.

Zan-Kowalczevska *et al.* (1974) have also compared the properties of ribonuclease from various organs. A synthetic substrate (uridine 3'-naphthyl phosphate) was used. Although the activity was clearly detected in pancreas, serum and lysosome-like structures of kidney, no reaction was observed in liver and spleen. However, the activity towards the synthetic substrate is only 1 % of the activity towards RNA. The reaction could thus be specific for a minor component of ribonuclease activity. Zan-Kowalczevska *et al.* (1976) did not take into account the possible interference of the natural ribonuclease inhibitor in their assay; in serum and kidney, where pancreatic-type ribonuclease was detected, there is a much lower inhibitor/ribonuclease ratio than in liver and spleen, where the ratio is high and the enzyme is not detected (see Table 2).

Our hypothesis of an uptake of plasma neutral ribonuclease of pancreatic origin by the liver agrees with the observation by Reddi (1975), who found that human serum ribonuclease was similar to human pancreatic ribonuclease, which was probably its primary source; Reddi (1976) has also shown that patients with pancreatic tumours have a marked increase of serum ribonuclease. Alpers & Isselbacher (1967), Zan-Kowalczevska *et al.* (1974) and Liebow & Rothman (1975) have described transfer of pancreatic ribonuclease and other digestive enzymes, passing from the intestinal tract and the duodenal fluid into the blood stream. Moreover, Zan-

Kowalczevska *et al.* (1974) and Davidson (1973) have shown that ribonuclease was then partially removed from the blood stream by kidney lysosomes. Our work goes one step further and shows that the rat liver lysosomal system also removes blood serum ribonuclease.

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