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SPECIFICITIES OF RAT ALKALINE RIBONUCLEASES AND CYTOCHEMICAL LOCALIZATION OF PANCREATIC-LIKE RIBONUCLEASES

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SUMMARY

Pancreatic-like ribonucleases differ, and may be distinguished, from other alkaline ribonucleases of the rat by their ability to hydrolyse uridine-3′-(α-naphthyl phosphate) (Up-naphthyl). This difference in specificity is not due to endogenous ribonuclease inhibitors; nor is it a steric effect since it is also valid for uridine-3′-phenyl phosphate (Up-phenyl), a new pancreatic ribonuclease substrate, the synthesis of which is described here. The specificity of this substrate is, however, limited by its susceptibility to spleen phosphodiesterase.

Hog spleen alkaline ribonuclease was purified over 100-fold and shown to be inactive vs uridine-3′-(α-naphthyl phosphate) and uridine-3′-phenyl phosphate. However, storage of the purified enzyme, or of enzyme-enriched extracts of spleen and liver, led to the gradual appearance of a low activity against the two substrates. The activity of the purified spleen enzyme vs the α-naphthyl ester was associated with a labile fraction weakly bound by a cation exchanger. The ratio of the activities of this fraction vs RNA and uridine-3′-(α-naphthyl phosphate) was identical with that for pancreatic ribonuclease.

With the aid of uridine-3′-(α-naphthyl phosphate), pancreatic-like ribonuclease activity was found, and cytochemically localized, in the pancreas, the parotid gland and the kidney. It was also found in the duodenal fluid, the serum and the urine. The localization patterns in the pancreas and the parotid gland pointed to its extracellular nature.

The occurrence of pancreatic-like ribonuclease activity in lysosome-like structures in kidney proximal tubule epithelial cells indicated that it is the extracellular enzyme adsorbed from the duodenal fluid into the blood stream and subsequently partially removed by kidney lysosomes.

The abbreviations employed in this text conform in general with the nomenclature recommended by the Commission on Biochemical Nomenclature of I.U.P.A.C. The following non-usual abbreviations are also employed: Up-methyl, uridine-3′-methyl phosphate, Up-naphthyl, uridine-3′-(α-naphthyl phosphate), with similar connotations for Tp-naphthyl and Ip-naphthyl; Up-phenyl, uridine-3′-phenyl phosphate; Up-benzyl, uridine-3′-benzyl phosphate.

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INTRODUCTION

Our present understanding of the structure and function of mammalian ribonucleases is characterized by a marked disproportion between the information accumulated about pancreatic ribonuclease and that available for the alkaline ribonutcleases of other organs. Both the structure and specificity of the former are well established [1], and its function as a digestive enzyme reasonably well documented [2]. By contrast, only several of the alkaline ribonucleases of other organs have been, at best, partially purified, data regarding their specificities are by no means complete, and nothing is known regarding their possible functions [3, 4].

It consequently appeared desirable to obtain some information about the localization of these enzymes, which might conceivably throw some light on their cellular role. For this reason we undertook, several years ago, the synthesis of uridine-3′-(α-naphtyl phosphate) (Up-naphthyl), which was shown to be an in vitro specific substrate for crystalline pancreatic ribonuclease, and simultaneously a satisfactory cytochemical substrate with the use of the diazo dye coupling techniques [5, 6]*. Following initial reports of localization of alkaline ribonucleases in various organs with this substrate, the latter was found to be contaminated with some impurities so that the localization patterns were partially erroneous.

However, even when the substrate was adequately purified, it was found to give results discordant with those obtained by the film-substrate method using RNA as substrate [9], as well as by the labelled-antibody technique [10], in that a variety of tissue sections other than the pancreas, kidney and parotid exhibited a completely negative reaction towards Up-naphthyl. This negative reaction was confirmed with the use of crude tissue extracts free of endogenous ribonuclease inhibitors [11].

The foregoing findings, indicative of some difference in specificities between the ribonucleases of different tissues, led to the present study on the specificity of alkaline ribonucleases and the extent to which pancreatic-type ribonuclease is present in mammalian tissues.

MATERIALS AND METHODS

Phenyl phosphodichloridate was a product of the Aldrich Chemical Co. (U.S.A.). The diazonium salts Fast Red TR and Fast Garnet GBC, as well as *p*-rosaniline chloride, were purchased from G. Gurr (London, Great Britain). Ribonuclease A (EC 2.7.7.16), *Escherichia coli* ribonuclease-free alkaline phosphomonoesterase (EC 3.1.3.1) and snake venom phosphodiesterase (EC 3.1.4.1) were obtained from Worthington (Freehold, N.J., U.S.A.). Highly polymerized yeast RNA was supplied by the Physiological Chemistry Department of the Lodz Medical School. Poly(rU), poly(rC) and poly(rA) were products of Miles (Elkhart, Ill., U.S.A.). The dinucleoside monophosphates were obtained from Sigma (St. Louis, Mo., U.S.A.).

* Actually the specificity of Up-naphthyl is, in some instances, not absolute because of the slight susceptibility to phosphodiesterase II [7]. This difficulty has been overcome by addition of a 5′-*O*-benzyl substituent, and 5′-*O*-benzyluridine-3′-(α-naphthyl phosphate) is a good ribonuclease substrate absolutely resistant to phosphodiesterase II [8].

Thymidine-3′-(*p*-nitrophenyl phosphate) and thymidine-5′-(*p*-nitrophenyl phosphate) were purchased from Raylo Chemicals (Edmonton, Alta., Canada).

The nucleoside-2′,3′-cyclic phosphates were prepared as elsewhere described [12]. Up-naphthyl and inosine-3′-(α-naphthyl phosphate) (Ip-naphthyl) were prepared according to Kole et al. [13] and 5′-*O*-benzyluridine-3′-(α-naphthyl phosphate) as described by Kole et al. [8], with the following additional modification: In order to ensure removal of traces of naphthyl phosphate resulting from slight substrate decomposition during storage, each substrate, following removal of the protecting groups, was treated with an excess of ribonuclease-free *E. coli* phosphomonoesterase for 15 min at 37 °C in 5 mM Tris–HCl buffer, pH 8.0. The sample was then neutralized with dilute HCl and extracted with ether. The purified sample was stored at −20 °C and used within 2–3 days; if storage was prolonged, the phosphatase treatment was repeated.

Uridine-3′-phenyl phosphate (Up-phenyl). To a solution of 1 mmole 2′,5′-di-*O*-tetrahydropyranyluridine [14] in 5 ml anhydrous pyridine, cooled in an ice bath, was added at 15-min intervals three times 100 μl of phenyl phosphodichloridate (2 mmole). Phosphorylation was allowed to proceed for 4 h at room temperature, using thin-layer chromatography on Al_2O_3 with Solvent D to follow the disappearance of 2′, 5′-di-*O*-tetrahydropyranyluridine ($R_F = 0.71$), and the reaction was terminated by the addition of 3 ml pyridine–water (9:1, v/v). The mixture was concentrated under reduced pressure, with frequent addition of triethylamine to maintain alkalinity, finally brought to dryness, the residue taken up in methanol and subjected to chromatography with Solvent D on Merck Al_2O_3 PF_{254} 16 cm × 20 cm plates (30 g gel per plate and 5000 $A_{260\ nm}$ units product). The band corresponding to 2′,5′-di-*O*-tetrahydropyranyluridine-3′-phenyl phosphate ($R_F = 0.50$) was eluted with three times 10 ml 50% aqueous methanol, the eluate filtered, brought to dryness and the residue stored over P_2O_5 under vacuum. This is necessary because of the fact that, following removal of the tetrahydropyranyl-protecting groups, the substrate exhibits slight instability, particularly at alkaline pH. Hence, prior to use as a substrate, 15 mg product in 0.50 ml water was treated with 40 mg Dowex 50 (H^+) for 30 min to remove the protecting groups. The resin was removed and the solution carefully brought to pH 6 with 0.1 M NaOH and extracted with ethyl ether. The homogeneity of the Up-phenyl (R_F 0.20 and 0.28 with Solvent D on Al_2O_3 plates, both diastereoisomers being susceptible to ribonuclease) was checked by incubation with an excess of ribonuclease, followed by chromatography, showing the formation of only 3′-UMP and free phenol (the latter was revealed by spraying with an alkaline solution of a diazonium salt). Treatment of the substrate solution with *E. coli* alkaline phosphatase and snake venom phosphodiesterase demonstrated the absence of contamination with phenyl phosphate or uridine-5′-phenyl phosphate.

Chromatography. Paper chromatography, ascending, used Whatman 3 MM paper. Thin-layer chromatography utilized Merck (Darmstadt, GFR) TLC Cellulose F plates and Al_2O_3 PF_{254} plates. The following solvent systems were used (v/v): (A) isopropanol–conc. NH_4OH–water (7:1:2, by vol.); (B) ethanol–1 M ammonium acetate, pH 7.5 (5:2, v/v); (C) ethanol–1 M ammonium acetate, pH 7.5 (11:9, v/v); (D) methanol–water (1:1, v/v). Column chromatography of enzymatic hydrolyzates of RNA was as described by Cohn [15] with Dowex 1, formate form.

Phosphate was estimated by the method of Chen et al. [16], protein according to Lowry et al. [17] or Mejbaum-Katzenellenbogen [18], and sugars by the procedure of Dubois et al. [19].

Electrophoresis on polyacrylamide gel at pH 4.5 followed the technique of Ornstein [20] and Davis [21], using amido black for staining the protein. Ribonuclease activity in the gel was localized with the aid of RNA as described by Wolf [22]. Ribonuclease activity against Up-naphthyl was localized in the gel by incubation of the gel column in 3 ml medium containing 2 μmole substrate and 3 mg Fast Red TR in 0.1 M Davis buffer [23] at pH 7.8; incubation was for 3–24 h at room temperature, with frequent adjustment of the pH by means of 0.1 M NaOH and changing the incubation medium at intervals of 2–3 h.

Measurement of enzyme activities

Ribonuclease activity vs RNA was determined by the method of Beard and Razzell [24], but with a 3-fold increase in the volume of the incubation medium, and using 0.05 M Davis buffer, pH 7.2. This permitted the estimation of $5 \cdot 10^{-5}$–$2 \cdot 10^{-4}$ μg of crystalline pancreatic ribonuclease. Activities are expressed as micromoles hydrolyzed per hour at 37 °C [24].

The ribonuclease activities of tissue extracts, and of the purified spleen enzyme, using RNA as substrate are expressed in terms of the equivalent amount of crystalline pancreatic ribonuclease (in μg) which gave the same extent of hydrolysis.

Ribonuclease activity vs Up-naphthyl was determined according to Kole et al. [13]. The incubation medium, total volume 125 μl, included 0.6 μmole substrate, 10 μmole Davis buffer (pH 7.4) and the appropriate amount of tissue extract. Because of the slight alkaline lability of Up-naphthyl, control solutions consisted of buffer and substrate.

Ribonuclease activity vs Up-phenyl utilized 125 μl incubation medium containing 0.6 μmole substrate, 10 μmole Davis buffer (pH 7.4) with incubation at 37 °C for 30–60 min. Liberated phenol was estimated by a modification of the method of Seligman and Nachlas [25], as follows: 1 ml 0.2 M Tris–HCl buffer, pH 9, and 250 μl of an aqueous solution of Fast Red TR (15 mg/ml) were added to the sample. Because of substrate lability in alkaline medium, the coupling reaction was limited to 3–5 min, followed by the addition of 250 μl 40% trichloroacetic acid. The mixture was left for 3 min, the dye formed extracted with 3 ml ethyl acetate and measured spectrally at 360 nm against a control. The resulting calibration curve was linear over the range 0.5–8.0 μg phenol, allowing for the assay of $1 \cdot 10^{-2}$–$5 \cdot 10^{-2}$ μg crystalline pancreatic ribonuclease.

Phosphodiesterase I and phosphodiesterase II activities, with thymidine-5′-(*p*-nitrophenyl phosphate) and thymidine-3′-(*p*-nitrophenyl phosphate) as substrates, were determined according to Razzell [26]. Deoxyribonucleases I and II activities were estimated according to the procedure of Sung and Laskowski [27] for mung bean nuclease, but with the use of 0.1 M Tris–HCl buffer, pH 7, with 5 mM $MgSO_4$ for deoxyribonuclease I, and 0.1 M acetate buffer, pH 5, with 10 mM EDTA for deoxyribonuclease II. Acid ribonuclease was assayed as described by Bernardi and Bernardi [28]; phosphomonoesterase according to Bessey and Love [29]; and 2′,3′-cyclic phosphodiesterase activity against 2′,3′-GMP by thin-layer chromatography of the hydrolysis product.

Preparation of tissue extracts

Pancreatic, parotid gland, kidney, liver and spleen rat extracts, devoid of all nuclease activity with the exception of heat and acid-stable alkaline ribonuclease, were prepared by the procedure of Razzell [11].

Tissue extracts with 100-fold higher activities than the foregoing were prepared from hog liver and spleen by following Steps 1–4 of the liver ribonuclease purification scheme [24].

Purified hog spleen alkaline ribonuclease was prepared by the method of Bardoń et al. [30] for placental ribonuclease, with modifications as described under Results.

Determination of specificity of purified spleen ribonuclease

The products of enzymatic hydrolysis of RNA, poly(rC) and poly(rU) were determined chromatographically according to Uchida and Egami [31], as well as on Merck cellulose plates with Solvents A and C. The mean lengths of the oligonucleotides resulting from exhaustive hydrolysis of RNA were determined by the method of Farkas and Marks [32], using the technique of Chen et al. [16] for phosphate.

The procedure of Uchida and Egami [31] was utilized to measure the percentage hydrolysis of RNA, poly(rC) and poly(rU) from the ratio of the amount of acid-soluble enzymatic hydrolysis products to the amount of mononucleotides formed on alkaline hydrolysis. The percentage enzymatic hydrolysis of dinucleoside monophosphates and nucleoside 2′,3′-phosphates was determined by thin-layer chromatography with Solvents A and B, followed by elution of the hydrolysis products with 0.01 M HCl and spectrophotometry of the eluates. Supplementary controls were utilized in which the spleen enzyme was replaced by an amount of crystalline pancreatic ribonuclease with an equivalent activity vs RNA.

Histochemical localization of ribonuclease

Substrates employed were Up-naphthyl, Ip-naphthyl and 5′-*O*-benzyluridine-3′-(α-naphthyl phosphate). The substrate media, prepared immediately prior to use, included either (a) substrate, (10 mg/ml), 0.1 M Tris–HCl buffer, pH 7.4, and freshly diazotized *p*-rosaniline [33], the volume ratio of the three components being 1:1:2; or (b) substrate (10 mg/ml), 0.2 M Tris–HCl buffer, pH 9, and Fast Red TR (10 mg/ml), with the ratio of components at 2:5:4.

Reactions were carried out with 3–5 μ paraffin, or 10 μ cryostat sections of rat tissues fixed in formol-calcium, and covered with a 50-μl drop of incubation medium as elsewhere described [6]. Incubation times varied from 30 s to 3 h at room temperature, with frequent changes of the incubation medium for long incubation periods. Runs were also made with 3–5-μ paraffin sections of material fixed in Carnoy solution, ethanol or acetone.

In order to exclude the possible inhibition of the enzyme activity by endogenous ribonuclease inhibitors, fresh and/or fixed sections were preincubated with 10^{-3} M *p*-chloromercuribenzoate or 10^{-6} M Pb^{2+}. Acid thermostability of the localized enzyme, and inactivation of the inhibitor, were checked by means of formalin-fixed sections heated for 10 min at 60 °C at pH 3.5, and then subjected to incubation.

RESULTS

Cytochemical

Irrespective of the fixation method employed, only the pancreas, parotid gland and kidney gave positive histochemical reactions with Up-naphthyl. The ribonuclease of these tissues is weakly bound, so that only fixation with formol-calcium gave results free from enzyme diffusion [6]. Figs 1–3 exhibit the cytochemical localization patterns for paraffin sections of the above three tissues.

For the pancreas, 30 s sufficed to give an intense reaction in the cytoplasm of the apical portions of the acinar cells. The remaining portion of the cytoplasm of the acinar cells exhibits a feebler reaction. Appreciable activity can be seen in the lumen of the excretory ducts (Fig. 1). The islets of Langerhans, the blood vessels, the connective tissue and the nuclei of all cells showed no activity, even when the incubation time was prolonged.

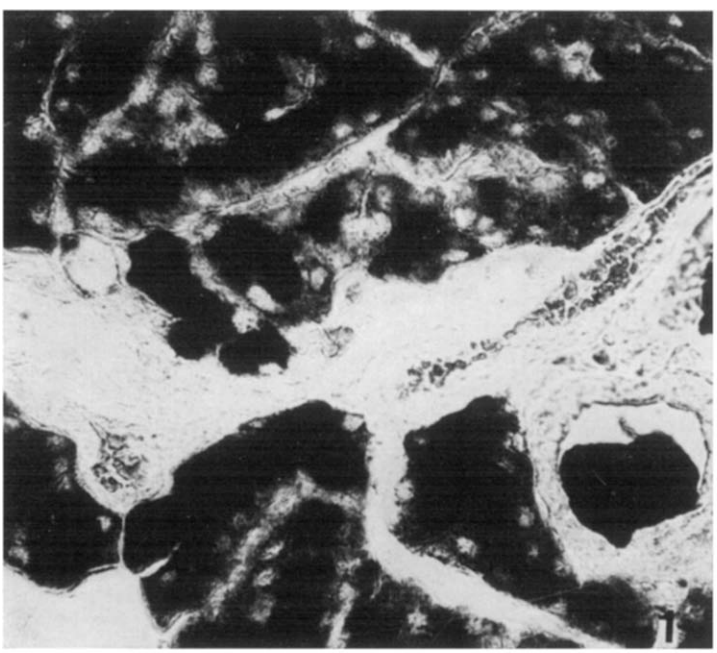

Fig. 1. Pancreas: Ribonuclease activity in apical cytoplasm of acinar cells. Note negative nuclei. Incubated for 3 min (×400).

In the parotid gland (Fig. 2) activity can be seen in the apical portion of the acinar cells. The enzyme also shows up in the lumen of salivary and intercalated ducts. The epithelial cells of the salivary and intercalated ducts, the connective tissue and the cell nuclei are negative.

The kidney exhibits activity uniquely in the epithelial cells of the first segnmet of the proximal convoluted tubule, where the enzyme is localized in lysosome-like structures (Fig. 3).

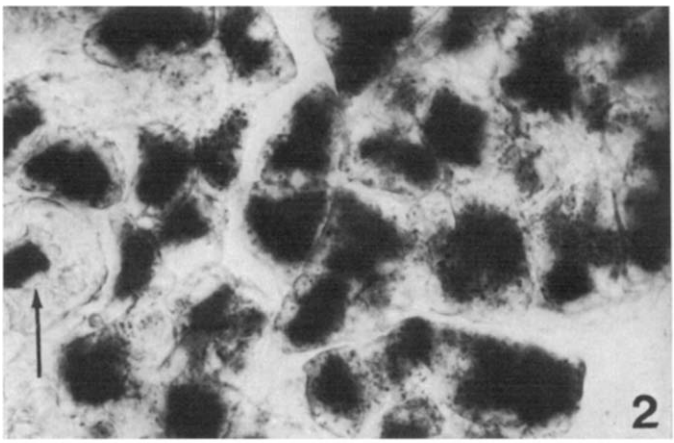

Fig. 2. Parotid gland: Ribonuclease activity in apical cytoplasm of acinar cells. Note activity in the lumen of salivary duct (see arrow). Incubated for 15 min (×560).

Prior heating of the sections in acid medium at 60 °C (see Materials and Methods) was without effect on the localization patterns, showing that the enzyme localized is an alkaline ribonuclease stable to acid and heat.

Since Up-naphthyl is also susceptible to the non-base specific ribonuclease T_2 and to a smaller extent to phosphodiesterase II [13], the three tissues were tested against control substrates, viz. Ip-naphthyl, which is a substrate for ribonuclease T_2 but resistant to pancreatic ribonuclease [13], and 5′-*O*-benzyluridine-3′-(α-naphthyl

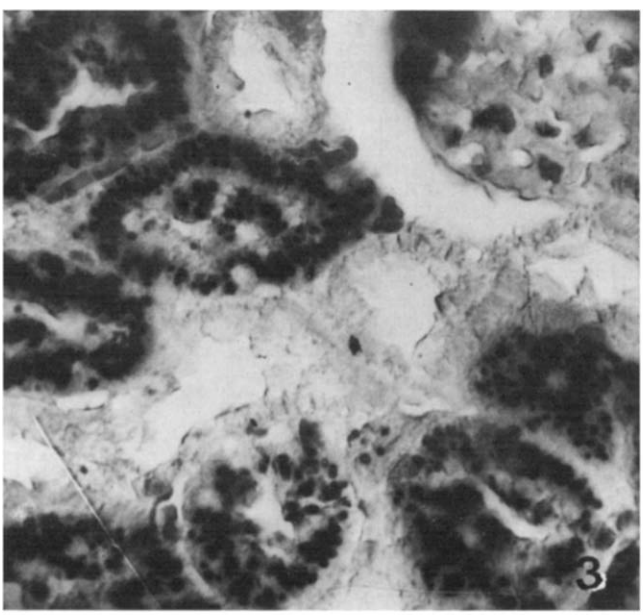

Fig. 3. Kidney: Ribonuclease activity in lysosome-like structures of the epithelium of proximal convoluted tubules. Incubated for 15 min (×860).

phosphate), which is a substrate for pancreatic ribonuclease, but resistant to phosphodiesterase II [8]. With the former control substrate no reaction was observed, whereas with the latter the localization patterns were identical with those obtained with the use of Up-naphthyl. Consequently, the reactions observed with pancreatic, parotid gland and kidney sections are due only to pancreatic ribonuclease.

The absence of any positive reaction with the other rat tissues tested was not due to inhibition by an endogenous ribonuclease inhibitor [34], since neither preincubation of the preparations with *p*-chloromercuribenzoate or Pb^{2+}, nor treatment with warm acid, altered the negative reactions.

A similar type of activity against Up-naphthyl was observed also in rat serum, urine and duodenal fluid. The serum activity, however, differed from that of the other body fluids and tissues by its thermolability.

Hydrolysis of Up-naphthyl by tissue extracts

The negative reaction exhibited towards Up-naphthyl by tissues other than the pancreas, parotid gland and kidney prompted us to examine the activities, towards this "substrate" and RNA, of tissue extracts which were first treated to inactivate all nuclease activities except those due to heat- and acid-stable ribonuclease [11]. Serum was not subjected to this treatment, which led to an appreciable loss of its ribonuclease activity; the absence of phosphodiesterase II in serum was confirmed by demonstrating the absence of activity towards thymidine-3'-(*p*-nitrophenyl phosphate).

The results shown in Table I demonstrate that the ribonucleases of rat pancreas and parotid gland exhibit activity towards both RNA and Up-naphthyl, the ratio of activities vs the two substrates being 100:1, i.e. as for crystalline pancreatic ribonuclease. For kidney extracts this ratio was 50–75. By contrast, liver and spleen extracts are completely inactive vs Up-naphthyl.

TABLE I

ACTIVITY OF HEAT- AND ACID-STABLE ALKALINE RIBONUCLEASES OF VARIOUS RAT TISSUE EXTRACTS, MEASURED AGAINST RNA AND Up-NAPHTHYL

Tissue	Substrate hydrolyzed (μmole/h per mg tissue at 37 °C)	
	RNA	Up-naphthyl
Pancreas	480	3.8
Parotid	4.3	0.04
Spleen	0.84	—*
Kidney	0.55	0.01
Liver	0.42	—*

* No detectable activity.

Confirmation of the absence of activity of liver and spleen ribonuclease vs Up-naphthyl required the use of higher levels of these enzymes, necessitating their partial purification from hog tissues (see Materials and Methods). Such purified preparations made it feasible to examine the effect of a 30-fold higher level of ribonuclease activity (measured vs RNA, see Table II) than the amount of pancreatic ribonuclease

TABLE II

ACTIVITIES OF VARIOUS TISSUE RIBONUCLEASES VS RNA AND Up-NAPHTHYL

Ribonuclease preparation	Activity in μmole substrate hydrolyzed by a given quantity of enzyme at 37 °C		Ratio of activity vs RNA to activity vs Up-naphthyl
	RNA	Up-naphthyl	
Bovine pancreas (crystalline)	2	0.02	100
Rat pancreas (tissue extract)	2	0.02	100
Rat parotid gland (tissue extract)	2	0.02	100
Rat kidney (tissue extract)	2	0.027	74
Rat liver (tissue extract)	2	—*	—
Hog liver (100 times concentrated tissue extract)	60	—*	—
Hog liver (100 times concentrated tissue extract stored at −25 °C)	40	0.04	1000
Rat spleen (tissue extract)	28	—*	—
Hog spleen (100 times concentrated tissue extract)	50	—*	—
Hog spleen (100 times concentrated tissue extract stored at −25 °C)	50	0.03	1700

* No detectable activity.

necessary for hydrolysis of 0.02 μmole Up-naphthyl. From Table II it will be seen that, even with these high levels of liver and spleen ribonuclease, no detectable hydrolysis of the substrate could be observed.

Influence of storage on ribonuclease activities of liver and spleen

Storage of partially purified preparations of liver and spleen ribonuclease at 0 or–25 °C led to the appearance of traces of activity vs Up-naphthyl, of the order of 10^{-3} of the activity of these preparations vs RNA (Table II), hence quite different from the ratio of activities towards the two substrates of the ribonucleases of pancreas, kidney and parotid. The appearance of trace activity vs Up-naphthyl in these stored preparations was further examined with the aid of a purified preparation of spleen ribonuclease.

Purification of hog spleen alkaline ribonuclease

The procedure applied was that of Bardon et al. [30] for placental ribonuclease, with modifications of Steps I–V. Activities were measured against RNA unless otherwise stated.

Step I (1000 × g supernatant). Fresh spleen was homogenized with four vol. of 0.25 M saccharose for 1 min. The homogenate was centrifuged for 10 min at 1000 × g and the precipitate discarded.

Step II (sulphonosalicylic acid supernatant). The supernatant was treated with sulphonosalicylic acid to extract protein, the quantity of which was 4-fold greater than in the case of placenta [30].

Step III (tannin–caffeine). Treatment of the partially purified supernatant with tannin precipitated a protein fraction which included most of the activity. This fraction was in turn freed from tannin by treatment with caffeine [30], but with the use of a 4-fold excess by weight of the latter relative to the amount of protein.

Step IV (DEAE-cellulose). The material liberated from the tannin complex, now devoid of phosphodiesterase I and II activities, was deposited on a DEAE-cellulose column, and elution carried out as described in the legend to Fig. 4. The ribonuclease activity was recovered in Fractions 20–35 (Peak I). Fractions 25–32 were pooled, dialyzed against 0.005 M acetate buffer, pH 5.6, and concentrated 10-fold by evaporation under reduced pressure at 30 °C [24]*.

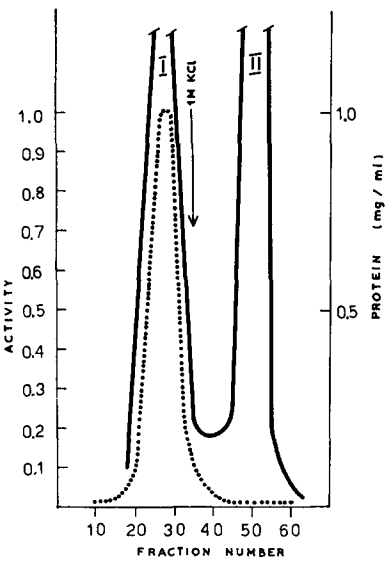

Fig. 4. DEAE-cellulose chromatography of hog spleen protein fraction isolated by the tannin–caffeine procedure. 500–1000 mg protein was deposited on a 35 cm × 3.5 cm column equilibrated with 0.02 M acetate buffer, pH 5.6. Elution was started with the same buffer, and collecting 4-ml fractions, giving Peak I. Elution was then continued with the same buffer containing 1 M KCl to give Peak II. ———, protein content in mg/ml [18]; · · · · · ·, ribonuclease activity vs RNA, expressed as $A_{260\ nm}$ due to 1 μl effluent after a 15-min incubation.

Step V (SE-Sephadex C-25). The pooled concentrated Fractions 25–32 were deposited on an SE-Sephadex C-25 column equilibrated with 0.05 M acetate buffer, pH 5.6. The column was eluted with a gradient as described in the legend to Fig. 5. The first peak contained 95% of the protein with negligible ribonuclease activity. Active protein was then eluted as four unresolved protein peaks (Fig. 5). No improvement in resolution could be obtained with the use of a milder gradient or change of buffer.

Step VI (Sephadex G-100). Fractions 78–92 from Step V (Fig. 5) were subjected to gel filtration on Sephadex G-100 as described in the legend to Fig. 6, to give a symmetrical peak (Fractions 45–58) of active protein. This was preceded by several peaks with only traces of ribonuclease activity.

Step VII (rechromatography on SE-Sephadex C-25). Fractions 45–58 from the

* This dialysis and concentration procedure was also applied to Steps V–IX.

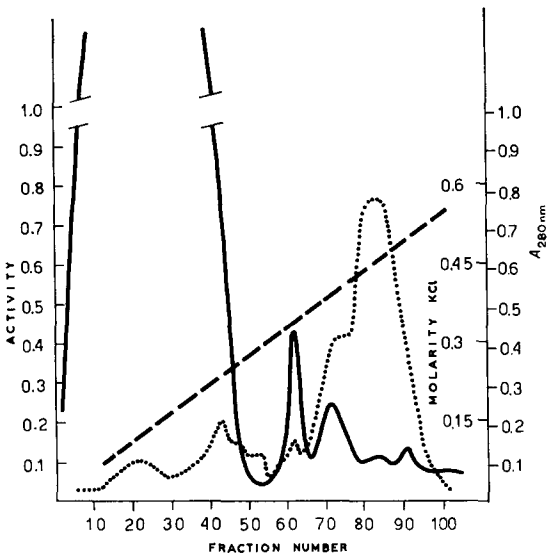

Fig. 5. SE-Sephadex C-25 chromatography of alkaline ribonuclease-rich fractions (25–32) from DEAE-cellulose chromatography in Fig. 4. 10 ml (100 mg protein) was deposited on a 25 cm × 2 cm column and eluted with a gradient formed between equal volumes of 0.05 M acetate buffer, pH 5.6, and 0.05 M acetate buffer, pH 4.4, containing 0.6 M KCl, collecting 4-ml fractions. Note that the peak of active protein is centred at 0.45 M KCl. ———, absorption at 280 nm; ·····, ribonuclease activity ($A_{260\,nm}$/1 μl per 15 min).

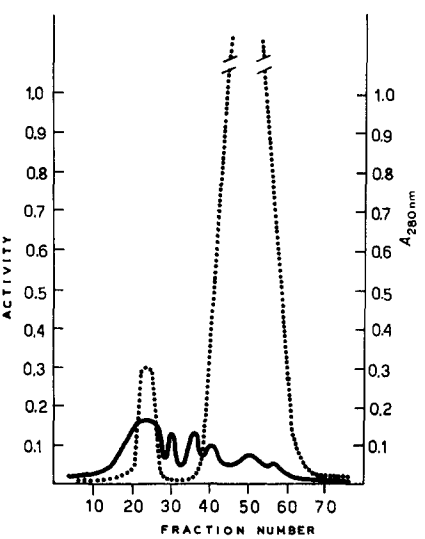

Fig. 6. Sephadex G-100 gel filtration of alkaline ribonuclease-rich fractions (78–92) from SE-Sephadex C-25 chromatography in Fig. 5. 5 ml ($A_{280\,nm}$ = 5) was loaded on a 55 cm × 2.5 cm column equilibrated with 0.05 M acetate buffer, pH 5.6. Elution was with same buffer containing 0.1 M KCl, collecting 3-ml fractions. ———, absorption at 280 nm; ·····, ribonuclease activity ($A_{260\,nm}$/1 μl per 15 min).

G-100 column were rechromatographed on SE-Sephadex C-25 to give two protein peaks (Fig. 7). The first of these (Fractions 13–22), weakly bound to the resin, exhibited low ribonuclease activity. The second (Fractions 54–110), eluted at higher KCl concentrations (0.35–0.5 M), was highly active.

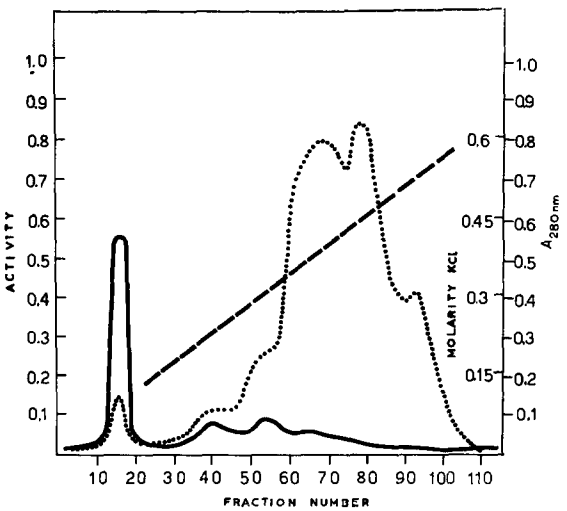

Fig. 7. SE-Sephadex C-25 rechromatography of ribonuclease-rich fractions (45–58) from Sephadex G-100 chromatography described in Fig. 6. Elution was carried out as in Fig. 5, with the collection of 2-ml fractions. ———, absorption at 280 nm; ·····, ribonuclease activity ($A_{260\,nm}$/1 μl per 15 min).

Step VIII (heating). The pooled fractions of the second peak from the SE-Sephadex column were reduced in volume to give a protein concentration of 3 mg/ml, and an acetate buffer concentration of 0.05 M, pH 3.5. The mixture was heated for 5 min at 100 °C. This treatment had no effect on the total or specific ribonuclease activity; nor did it lead to any turbidity, pointing to the absence of thermolabile protein in the preparation.

Step IX (CM-Sephadex C-25). The heated preparation from the previous step was rechromatographed on CM-Sephadex C-25, with elution as in Fig. 5, giving an elution pattern identical to that for SE-Sephadex C-25 in Step VII (Fig. 7).

Rechromatography on SE-Sephadex of the major highly active peak, after it had been stored for some time, repeatedly gave the same elution pattern as in Steps VII and IX (i.e. as in Fig. 7), in which the small peak, weakly bound to the cation, exhibited relatively low specific activity towards RNA and Up-naphthyl (Fig. 8). We shall refer to this as Peak A, and its properties will be discussed below.

The results of the overall purification scheme are presented in Table III. It should be emphasized that the extraction of active protein with sulphonosalicylic acid (Step II, see Table III) led to the initial removal of more than 98% of the non-active protein, including the endogenous ribonuclease inhibitor [34]. For this reason, the calculated degree of purification, over 100 (Table III), is based on Step II as the starting material.

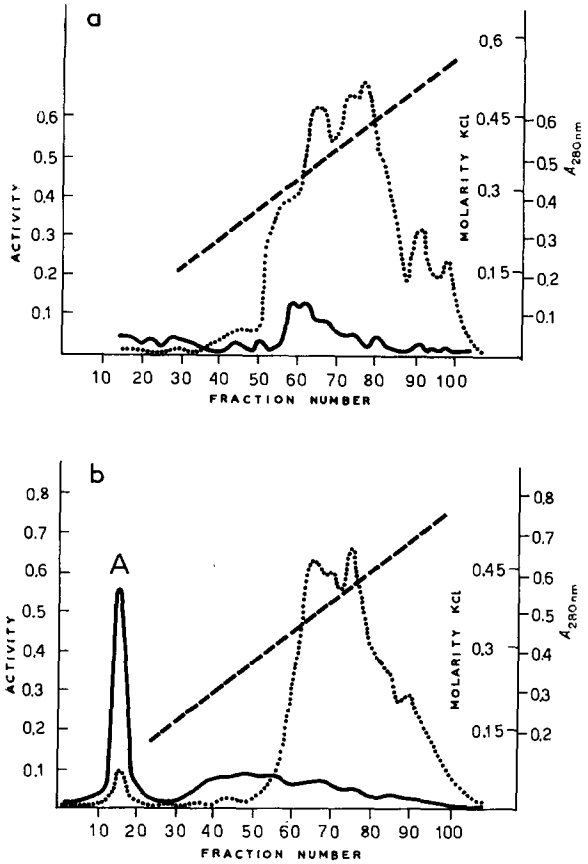

Fig. 8. Effect of storage of purified spleen ribonuclease on its elution pattern from SE-Sephadex C-25: the pooled, concentrated (by evaporation) and dialyzed preparation obtained after Step IX was rechromatographed on SE-Sephadex C-25 and eluted as described in the legend to Fig. 5. (a) rechromatographed immediately following dialysis; (b) rechromatographed after storage for 48 h at 4 or −25 °C. ———, absorption at 280 nm; ·····, ribonuclease activity ($A_{260\,nm}$/1 μl per 15 min).

Properties of spleen alkaline ribonuclease

The purified enzyme was found to contain about 7% by weight of sugar, suggesting it is a glycoprotein. Electrophoresis on polyacrylamide gel at pH 4.5 demonstrated the presence of five bands, all with activity vs RNA (Fig. 9).

The enzyme was quite labile at pH values above 8. By contrast, it was remarkably stable at pH values below 5.6, even at 100 °C for 5 min. It lost activity slowly on prolonged storage below 5 °C.

The preparation was completely devoid of the following enzyme activities: phosphodiesterases I and II, acid ribonuclease, deoxyribonucleases I and II, phosphomonoesterase, 2′,3′-cyclic phosphodiesterase.

Specificity. The results of specificity tests are shown in Table IV. The enzyme hydrolyzed RNA to give fragments with a mean length of about six residues terminated by a pyrimidine nudeoside 2′,3′-cyclic phosphate. Exhaustive hydrolysis of

TABLE III

PURIFICATION SCHEME FOR HOG SPLEEN ALKALINE RIBONUCLEASE

Purification step	Volume (ml)	Protein (mg)	Activity	
			Total (μmole RNA/h)	Specific (μmole RNA/h per mg protein)
I. Supernatant (1000 × *g*)	10.000	385 000	42 550	0.1
II. Supernatant sulphonosalicylic acid	11.000	7 000	302 400	43
III. Tannin–caffeine*	70	—	237 600	—
IV. DEAE	100	4 500	144 000	32
V. SE-Sephadex C-25	120	240	63 620	265
VI. G-100 Sephadex	80	75	49 800	660
VII. SE-Sephadex C-25	37	18.7	34 800	1860
VIII. Heating for 10 min at pH 3.5 and 100 °C	5	17	34 800	2040
IX. CM-Sephadex C-25	110	8	32 700	4100

* The use of tannin led to an increase in ultraviolet absorption at 280 nm, and to turbidity in the Lowry et al. [17] method for protein. Hence in Steps IV–VI protein was estimated by the method of Mejbaum-Katzenellenbogen [18].

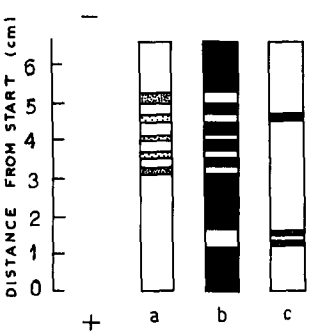

Fig. 9. Electrophoresis on polyacrylamide gel of purified spleen alkaline ribonuclease: a, protein stained with amido black; b, activity vs RNA, localized according to Wolf [22]; c, activity of Peak A vs Up-naphthyl, localized as described in Materials and Methods.

RNA showed that about 50% of the products consisted of fragments with an average of more than ten residues.

Poly(rC), poly(rU) and pyrimidine nudeoside 2′,3′-cyclic phosphates were hydrolyzed at rates similar to those for pancreatic ribonuclease [35]. Poly(rA), purine dinucleoside monophosphates and purine nucleoside 2′,3′-cyclic phosphates were fully resistant.

Freshly prepared preparations showed no activity against either Up-naphthyl or Up-phenyl.

It remained to establish whether the inactivity of spleen alkaline ribonuclease vs Up-naphthyl is a phenomenon limited only to this particular ester of 3′-UMP. For example, thymidine-3′-(*p*-nitrophenyl phosphate) is a good substrate for phosphodiesterase II, but thymidine-3′-(α-naphthyl phosphate) (Tp-naphthyl) is not

TABLE IV

SUBSTRATE SPECIFICITY OF HOG SPLEEN ALKALINE RIBONUCLEASE

Substrate (20 μmole/ml)	Incubation time (h) at 37 °C	Amount of ribonuclease* (μg)	Hydrolysis products	Acid-soluble products (%)	Liberated mononucleotides (%)
RNA	1–22	0.1	Fragments with average of six residues terminated by pyrimidine nucleoside 2′,3′-cyclic phosphate	40–50	2
RNA	1	0.004	Acid-soluble oligonucleotides	37	—
Poly (C)	1	0.1	Oligonucleotides; 2′,3′-CMP; 3′-CMP	—	40
Poly (C)	1	0.004	Acid-soluble oligonucleotides	32	—
Poly (U)	5	0.1	Oligonucleotides with terminal cyclic phosphate, and 2′,3′-UMP	—	5
Poly (U)	1	0.05	Acid-soluble products	8.3	—
Poly (A)	22	0.1	No detectable hydrolysis	0	0
CpA	0.25	0.004	2′,3′-CMP and Ado	—	50
ApA	3	0.04	No detectable hydrolysis	—	0
2′,3′-UMP	24	0.4	3′-UMP	—	23
2′,3′-GMP	24	0.4	No detectable hydrolysis	—	0
Up-naphthyl	3	0.4	No detectable hydrolysis	—	0
Up-phenyl	1	0.4	No detectable hydrolysis	—	0

* The amount of spleen enzyme is expressed in terms of the equivalent amount of pancreatic crystalline ribonuclease giving the same degree of hydrolysis of RNA to acid-soluble products.

[7]. The activity of spleen ribonuclease was, therefore, tested against uridine-3′-methyl phosphate (Up-methyl) and uridine-3′-benzyl phosphate (Up-benzyl), both of which are substrates of pancreatic ribonuclease. The results were negative, but could conceivablybe interpreted as due to the low activity of the spleen enzyme, since both these substrates are hydrolyzed at a rate at least 100-fold less than Up-naphthyl (Witzel, H., personal communication). This prompted the synthesis of Up-phenyl, which was found to be hydrolyzed by pancreatic ribonuclease in 0.1 M Davis buffer, pH 7.4, at 37 °C at a rate of about 4 mmole/h per mg protein, hence about the same as for Up-naphthyl and over 100-fold faster than the methyl and benzyl esters of 3′-UMP [36]. It follows that the lack of activity of spleen ribonuclease against the α-naphthyl ester of 3′-UMP is not due to specific steric hindrance by the bulky α-

naphthyl substituent, but is a general phenomenon for esters of 3′-UMP (excluding, of course, nucleoside esters, e.g. uridilyl-3′→5′-nucleoside).

It should, however, be pointed out that Up-phenyl, although a convenient substrate for pancreatic ribonuclease, is less specific than Up-naphthyl since it was found to be 50-fold more susceptible to phosphodiesterase II than Up-naphthyl.

Fraction A of spleen ribonuclease

As mentioned above, hog spleen ribonuclease on storage exhibits slight activity vs Up-naphthyl (Table II), such that the ratio of the rate of hydrolysis of RNA to that of Up-naphthyl is of the order of 10^3. The activity against Up-naphthyl is completely absent in the freshly prepared enzyme. The activity vs Up-naphthyl in a stored enzyme preparation is eluted from an SE-Sephadex C-25 column as a minor peak A (Fig. 8) which contains all the activity vs Up-naphthyl and about 10% of the activity against RNA.

Fraction A is relatively labile as compared to the main fraction and, following storage for 24 h at 4 °C, retains only about 30% of its activity both against RNA and Up-naphthyl.

The specific activity of Fraction A vs RNA is about 5-fold lower than that of the main fraction, while the ratio of its rate of hydrolysis of RNA to the rate of hydrolysis of Up-naphthyl is approximately the same as for pancreatic ribonuclease, i.e. 100:1. Apart from this, its specificity is similar to that for the main fraction of the purified spleen enzyme.

Electrophoresis of Fraction A on polyacrylamide gel at pH 4.5 gave the five protein band patterns exhibited by the main fraction (Fig. 9a), all exhibiting activity vs RNA, but only one with activity against Up-naphthyl (Fig. 9c). In addition two closely-spaced bands were located with activities against both Up-naphthyl (Fig. 9c) and RNA. Both of these were in the neighbourhood of the anode, and gave a negative-staining reaction with the protein-staining reagent, probably because of their low protein contents.

DISCUSSION

The localization of alkaline ribonuclease active against Up-naphthyl in the secretory region of the acinar cells of the pancreas and parotid gland (Figs 1 and 2), together with its weak binding to the tissues, point to the extracellular digesitve role of this enzyme. The alkaline ribonuclease in serum and the kidney is probably the same enzyme which, after passage into the blood stream, is removed by the kidney. This is supported by its localization in the cytoplasmic structures of the epithelial cells of the proximal convoluted tubules known to be involved in the removal of protein from blood plasma [37]. The presence of pancreatic ribonuclease in blood serum and kidney is in accordance with the observed passage of labelled pancreatic ribonuclease from the intestinal tract to the blood stream [38], and the removal of serum alkaline ribonuclease from the blood stream, at least partially via the proximal convoluted tubules. The extracellular origin of the kidney lysosomal enzyme derives support from the recent findings of Rosso et al. [39], who reported an unusually high level of inhibitor-free ribonuclease in the kidney cytoplasmic fraction of the unilaterally nephrectomized rat and attributed this to an "extrarenal" ribonuclease.

The foregoing points to the necessity of examining the activity of other tissue ribonucleases vs Up-naphthyl. We have begun such studies and find, e.g. that duodenal fluid exhibits activity against Up-naphthyl, the ratio of activities vs RNA and Up-naphthyl varying from 10 to 20, hence different from the corresponding ratio for pancreatic ribonuclease. The relatively high level of activity of duodenal ribonuclease vs Up-naphthyl is rather unusual in the light of the postulate of Zendzian and Barnard [2] as to the pancreatic origin of duodenal ribonuclease.

The alkaline ribonucleases of other tissues, the specificities of which in many respects resemble that of pancreatic ribonuclease [4], nonetheless differ from the latter by their inertness towards Up-naphthyl. If any doubts previously existed about this difference on the basis of the cytochemical results (which could conceivably be interpreted as due to some unusual binding of the enzyme in these tissues), they are resolved unequivocally by the properties of the purified spleen enzyme (Table IV), which is inactive not only against Up-naphthyl, but likewise against Up-phenyl.

This difference in specificities between the alkaline ribonucleases of the pancreas, parotid gland and kidney as compared to other tissues forcibly recalls the heterogeneity of alkaline ribonucleases earlier reported by Beard and Razzell [24] and Gordon [40].

The present findings explain the negative cytochemical reaction for alkaline ribonuclease in spleen, liver and other tissues of the rat, and demonstrate that the applicability of Up-naphthyl as a cytochemical substrate for rat ribonucleases is, for the moment, limited to the pancreas, parotid gland and kidney. It may still be employed as a colorimetric substrate [13] for distinguishing pancreatic-type ribonucleases, in body fluids (serum, urine) and tissue extracts, from other types of alkaline ribonucleases.

The cytochemical results obtained with Up-naphthyl are, of course, not comparable with those of other authors based on the film-substrate technique [9], which uses an RNA substrate hydrolyzed not only by various ribonucleases, but also by phosphodiesterases. However, the specificity of the film-substrate technique may be improved with the use of additional oligo- and polynucleotide substrates [41, 42].

Our cytochemical results (Figs 1–3), are not in agreement with some reported by Wolf et al. [43] based on the use of the indigogenic principle with the synthetic substrate uridine-2′(3′)-(5-bromo-4-chloro-3-indolyl)phosphate. It is, however, not possible to account for the disagreement since the foregoing authors did not describe the synthesis of their substrate, nor did they present any criteria as to its purity.

The appearance, in crude liver and spleen extracts stored at low temperature, of activity vs Up-naphthyl is probably due to the formation of some new form of the enzyme corresponding to Fraction A (Fig. 8) of the purified spleen enzyme, which contains all the activity towards Up-naphthyl. This spontaneous formation of Fraction A accounts for the variable ratio of activities vs RNA and Up-naphthyl noted during storage of crude tissue extracts or preparations of purified enzyme, since the overall activity towards RNA is relatively unaffected.

It is also of interest that Fraction A, although resembling pancreatic ribonuclease by its relative rates of hydrolysis of RNA and Up-naphthyl, nonetheless differs from it in that it exhibits a lower affinity for the SE-Sephadex exchanger. Actual trial showed that it eluted with 0.05 M acetate buffer, as compared to 0.05 M acetate buffer plus 0.1 M KCl for pancreatic ribonuclease.

As regards the polymorphism of the purified enzyme on SE- or CM-Sephadex C-25, as well as on polyacrylamide gel where the separation of the peaks is better defined, a similar polymorphism has been reported by Reinholt et al. [44] for pancreatic juice ribonuclease and attributed to differences in the composition of the polysaccharide component of the enzyme.

It should be noted that earlier purification schemes for spleen alkaline ribonuclease [45, 46] were more recently refined by Lee et al. [47]. The procedure of the latter authors involved several steps analogous to ours and yielded a ribonuclease preparation of similar specific activity towards RNA. The enzyme was reported to be electrophoretically homogeneous, since it gave one band on cellulose acetate. Since our preparation gave five bands on polyacrylamide gel (Fig. 9), we ran it on cellulose acetate as described by Lee et al. [47]. Under these conditions it also migrated as a single sharp band.

It will undoubtedly be of interest to extend the above studies to other mammalian tissues. The availability of a relatively specific substrate such as uridine-3′-(α-naphthyl phosphate) which distinguishes between pancreatic-like and other alkaline ribonucleases should be a useful tool in studies both on the localization and function of these enzymes.

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Following submissian of the above manuscript, a paper appeared [48] in which it was shown that, following intravenous injection into mice of labelled bovine pancreatic ribonuclease, the latter is rapidly transferred to the kidney and taken up by the lysosomes (cf. [49] where it undergoes partial degradation. This is in accord with the lysosomal locolization of pancreatic-like ribonuclease in the present study by cytochemical methods.

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REFERENCES

1 Richards, F. M. and Wyckoff, H. W. (1971) in The Enzymes (Boyer, P. D., ed.), Vol. 4, pp. 647–806, Academic Press, New York
2 Zendzian, E. N. and Barnard, E. A. (1967) Arch. Biochem. Biophys. 122, 699–713
3 Roth, J. S. (1967) in Methods in Cancer Research (Bush, H., ed.), Vol. 3, pp. 153–242, Academic Press, New York
4 Shugar, D. and Sierakowska, H. (1967) Prog. Nucleic Acid Res. Mol. Biol. 7, 369–429
5 Sierakowska, H., Zan-Kowalczewska, M. and Shugar, D. (1965) Biochem. Biophys. Res. Commun. 19, 138–143
6 Zan-Kowalczewska, M., Sierakowska, H. and Shugar, D. (1966) Acta Biochim. Pol. 13, 237–250
7 Sierakowska, H. and Shugar, D. (1971) Acta Biochim. Pol. 18, 143–152
8 Kole, R., Sierakowska, H. and Shugar, D. (1971) Biochem. Biophys. Res. Commun. 44, 1482–1487
9 Daoust, R. (1965) in International Review of Cytology (Bourne, C. H. and Danielli, J. F., eds), Vol. 18, pp. 191–221, Academic Press, New York

10 Gordon, J. and Myers, J. (1966) Biochim. Biophys. Acta 113, 187–189
11 Razzell, W. E. (1967) Experientia 23, 321–325
12 Szer, W. and Shugar, D. (1963) Biochem. Prep. 10, 139–144
13 Kole, R., Sierakowska, H. and Shugar, D. (1971) Acta Biochim. Pol. 18, 187–197
14 Fromageot, H. P. M., Griffin, B. E., Reese, C. B. and Sulston, J. E. (1967) Tetrahedron 23, 2315–2331
15 Cohn, W. E. (1955) in The Nucleic Acids (Chargaff, E. and Davidson, J. N., eds), Vol. 1, pp. 211–241, Academic Press, New York
16 Chen, P. S., Toribara, T. Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
18 Mejbaum-Katzenellenbogen, W. (1955) Acta Biochim. Pol. 2, 279–294
19 Dubois, R. J., Thompson, K. A., Hamilton, J. K., Reberes, P. A. and Smith, F. (1956) Anal. Chem. 28, 350–356
20 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321–349
21 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
22 Wolf, G. (1968) Experientia 24, 890–891
23 Davis, M. T. (1959) The Analyst 84, 248–251
24 Beard, J. R. and Razzell, W. E. (1964) J. Biol. Chem. 239, 4186–4193
25 Seligman, A. M. and Nachlas, M. M. (1949) Am. J. Physiol. 159, 337–342
26 Razzell, W. E. (1963) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. 6, pp. 236–258, Academic Press, New York
27 Sung, S. C. and Laskowski, Sr, M. J. (1962) J. Biol. Chem. 237, 506–511
28 Bernardi, A. and Bernardi, G. (1966) Biochim. Biophys. Acta 129, 23–31
29 Bessey, O. A. and Love, R. H. (1952) J. Biol. Chem. 196, 175–178
30 Bardoń, A., Pamuła, Z. and Hillar, M. (1969) Acta Biochim. Pol. 16, 119–126
31 Uchida, T. and Egami, F. (1967) J. Biochem. Tokyo 61, 44–53
32 Farkas, W. R. and Marks, P. A. (1968) J. Biol. Chem. 243, 6464–6473
33 Barka, T. and Anderson, P. J. (1962) J. Histochem. Cytochem. 10, 741–753
34 Roth, J. S. (1958) J. Biol. Chem. 231, 1085–1095
35 Heppel, L. A., Ortiz, P. J. and Ochoa, S. (1957) J. Biol. Chem. 229, 679–710
36 Witzel, H. and Barnard, E. A. (1962) Biochem. Biophys. Res. Commun. 7, 294–299
37 Longley, J. B. (1969) in The Kidney (Rouiller, C. and Muller, A. F., eds), Vol. 1, pp. 157–259, Academic Press, New York
38 Alpers, D. H. and Isselbacher, K. (1967) J. Biol. Chem. 242, 5617–5622
39 Rosso, P., Diggs, J. and Winick, M. (1973) Proc. Natl. Acad. Sci. U.S. 70, 169–172
40 Gordon, J. (1965) Arch. Biochem. Biophys. 112, 429–435
41 Daoust, R. and Morais, R. (1972) J. Histochem. Cytochem. 20, 350–357
42 Sierakowska, H. and Shugar, D. (1962) Acta Biochim. Pol. 8, 427–436
43 Wolf, P. L., Horwitz, J. P., Freisler, J., Vazquez, J. and Von der Muehll, E. (1968) Experientia 24, 1290–1291
44 Reinhold, V. N., Dunne, F. T., Wriston, J. C., Schwartz, M., Sarda, L. and Hirs, C. H. W. (1968) J. Biol. Chem. 243, 6482–6494
45 Kaplan, H. S. and Heppel, L. A. (1956) J. Biol. Chem. 222, 917–922
46 Dalaney, R. (1963) Biochemistry 2, 438–444
47 Lee, W. Y., Atallah, N. A., Gyenes, L. and Sehon, A. H. (1971) Arch. Biochem. Biophys. 147 270–283
48 Davidson, S. J. (1973) J. Cell. Biol. 59, 213–222
49 Davidson, S. J. Hughes, W. L. and Barnwell, A. (1971) Exp. Cell Res. 67, 171–187