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RIBONUCLEASE ACTIVITIES OF THE DEVELOPING CHICK PANCREAS

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SUMMARY

1. Two ribonuclease activities are present in embryonic chick pancreas. Both activities differ in their specificity from bovine pancreatic ribonuclease. One of the activities ('Mg^{2+}-ribonuclease') is due to a soluble magnesium-requiring endonuclease which has been described previously. The other activity ('neutral ribonuclease') which is bound to particles does not require metal ions and has an optimum pH of 7.2. Neutral ribonuclease activity can be partially solubilized by raising the salt concentration. It hydrolyzes RNA core, polyuridylic (poly U) and polycytidylic (poly C) acids but not polyadenylic (poly A) or polyinosinic (poly I) acids.

2. The fact that Mg^{2+}-ribonuclease is actively secreted by the pancreas *in vitro* on stimulation with carbamylcholine shows that it is a digestive enzyme. The properties of neutral ribonuclease, on the other hand, suggest that it is not a digestive enzyme.

3. Differential centrifugation studies on homogenates of pancreas in isotonic sucrose show that Mg^{2+}-ribonuclease, like other digestive enzymes, is located mainly in the zymogen granule and soluble fractions. A large portion of the neutral ribonuclease is associated with heavy particles sedimenting at $250 \times g$, but is not specifically attached to nuclei.

4. Developmental patterns of the activities of the two ribonucleases differ markedly. The specific activity of neutral ribonuclease reaches a maximum between 16 and 18 days of development after which it declines. The specific activity of Mg^{2+}-ribonuclease increases steeply between 19 and 21 days of development and closely parallels the increase in specific activity of amylase during this period. Before 17 days of development, however, the specific activities of Mg^{2+}-ribonuclease and amylase do not increase in a parallel fashion. It is suggested that digestive enzyme activities in chick pancreas increase in a non-parallel manner before 17 days of development and afterwards in a parallel manner.

INTRODUCTION

The pancreas offers particular advantages as a system for the study of molecular mechanisms of differentiation because its differentiation involves the induction

Abbreviations: poly A, polyadenylic acid; poly C, polycytidylic acid; poly I, polyinosinic acid; poly U, polyuridylic acid.

of synthesis of a wide spectrum of characteristic digestive enzymes[1,2]. Among the pancreatic digestive enzymes ribonuclease is of dual interest: firstly as one of a battery of digestive enzymes whose synthesis is accelerated during development and secondly as a potential cause of unwanted RNA degradation in studies of the role of RNA in differentiation.

In a preliminary communication[3] we reported the presence in embryonic chick pancreas of two distinct ribonuclease activities neither of which resembles bovine pancreatic ribonuclease. One of these activities is due to an endonuclease, with a high pH optimum and a requirement for divalent metal ions, which releases oligonucleotides terminating in a 5'-phosphate. This enzyme has been partially purified and studied by ELEY AND ROTH[4,5]. The second nuclease activity has an optimum pH of 7.2 and does not require bivalent metal ions. The latter enzyme is not as well characterized as the former but its ability to hydrolyze RNA core shows that it differs in specificity from bovine pancreatic ribonuclease. In the present paper we compare in more detail the properties of the two ribonuclease activities, their patterns of activity during development and their distribution in subcellular fractions.

MATERIALS AND METHODS

Substrates and enzymes

RNA core and crystalline soybean trypsin inhibitor were obtained from Worthington Biochemical; bovine pancreatic ribonuclease was purchased from Sigma Chemical; polyadenylic (poly A), polyinosinic (poly I) and polycytidylic (poly C) acids were products of Miles Laboratories, while polyuridylic (poly U) acid was obtained from Calbiochem.

Embryos and chicks

These were obtained from White Leghorn X New Hampshire eggs weighing 50–60 g and incubated at 37.5–38°. The developmental ages of embryos and chicks were normalized (according to embryo weights[6] and the criteria of HAMBURGER AND HAMILTON[7]) to correspond with the developmental ages in previous studies[1,8].

Purification of RNA for ribonuclease assay

Yeast RNA purchased from Mann Research Laboratories or Nutritional Biochemicals was purified by the following procedure based on the methods of WOODWARD[9] and SHORTMAN[10]. All steps were performed at 0–4°. To a 6% solution of RNA adjusted to pH 5.5 with NaOH were added 5 vols. of glacial acetic acid. After standing for 15 min the suspension was centrifuged at 2500 × *g* for 15 min. The pellet was redissolved in water with addition of NaOH and the acetic acid precipitation was repeated twice. The precipitate was suspended in 0.5 vol. of water, sedimented and washed twice with 5 vols. of absolute ethanol. The washed precipitate was dried in a desiccator and could be stored in this form indefinitely without affecting its absorbance blank in the ribonuclease assay system.

Batches of the dried RNA were dissolved in water with the aid of NaOH to give a concentration of 6% and pH 6.0. The RNA solution was dialyzed successively against 40 vols. of 0.01 M EDTA (pH 7.5), to remove bivalent metal ions, 40 vols. of 0.15 M NaCl to remove oligonucleotides and finally against 40 vols. of double-distilled

water. The dialyzed solution was diluted to 1% and stored at -20°. RNA prepared by this method gave an absorbance blank of 2 in the ribonuclease assay.

Determination of ribonuclease activities

Both assay procedures were based on the method of KALNITSKY, HUMMEL AND DIERKS[11].

Mg^{2+}-ribonuclease. The assay system contained 40 mM Tris–HCl buffer (pH 8.7), 15 mM magnesium acetate, 14 mg of purified yeast RNA and enzyme in a final volume of 4.0 ml.

Neutral ribonuclease. The assay system contained 40 mM imidazole–HCl buffer (pH 7.2), 5 mM EDTA, 14 mg of purified yeast RNA and enzyme in a final volume of 4.0 ml.

In both assays the reaction was started by adding substrate to the incubation mixture at 37°. Samples of 1.0 ml were transferred at zero time and at suitable intervals thereafter to test tubes containing 0.35 ml of 0.75% uranyl acetate in 25% $HClO_4$. After standing for 15 min at 0° the samples were centrifuged for 15 min at 2000 × *g* at 4°. $A_{260\ m\mu}$ of the clear supernatant, diluted with water, was measured with a Zeiss spectrophotometer. A unit of ribonuclease is defined as the amount of enzyme that gives a ΔA of 1.0 per min in the undiluted supernatant. Absorbance changed linearly with the amount of enzyme or incubation time if the overall change in absorbance, corrected for zero-time blank, did not exceed 21 for Mg^{2+}-ribonuclease or 4.5 for neutral ribonuclease. In some figures and tables activity is expressed in terms of ΔA which is the $A_{260\ m\mu}$ of a 30-fold dilution of the supernatant after precipitation with the uranyl acetate–perchloric acid reagent, corrected for the corresponding zero-time blank.

Other analytical methods

Protein was determined by the method of LOWRY *et al.*[12] using crystalline bovine plasma albumin as standard. Amylase was determined according to BERNFELD[13], a unit being defined as the amount that in 3 min at 30° catalyzes the appearance of reducing groups equivalent to 1 mg of maltose hydrate. DNA was determined by the method of SCHNEIDER[14], modified as described previously[8].

Preparation of homogenates

Pancreases were homogenized in double-distilled water, containing 100 μg/ml of crystalline soybean trypsin inhibitor, to give a final protein concentration of 10–40 mg/ml. Homogenates were stored up to 24 h at 0–4°. The ribonuclease activities were stable under these conditions.

Secretion by pancreas in vitro

Experiments were performed as described previously[15]. At the end of incubation pancreases were homogenized in 0.01 M imidazole–HCl buffer (pH 7.2). This homogenate was suitable for assays of both neutral and Mg^{2+}-ribonuclease. The incubation medium was divided into two portions, one of which was diluted with an equal volume of 0.05 M imidazole–HCl buffer (pH 7.2) (for assay of neutral ribonuclease and amylase) and the other with an equal volume of 0.05 M Tris–HCl buffer (pH 8.4) (for assay of Mg^{2+}-ribonuclease).

Preparation of subcellular fractions by differential centrifugation

The homogenizing medium contained 0.25 M sucrose, 3 mM $CaCl_2$, 50 μg/ml of crystalline soybean trypsin inhibitor and 0.01 M Tris–HCl buffer (pH 7.5). 30 pancreases from 20-day embryos or 1-day chicks were homogenized with 6 ml of medium at 400 revs./min in a loose (0.4 mm clearance) Potter–Elvehjem homogenizer with a capacity of 20 ml. After filtering through 0.05 mm stainless steel gauze, the homogenate was centrifuged at 250 × *g* for 5 min. The pellet was resuspended in half the original volume of homogenizing medium and centrifuged again at 250 × *g* for 5 min. The supernatants of the two centrifugations were pooled. Subsequent centrifugations were carried out at 1000 × *g* for 5 min, 2000 × *g* for 15 min and 15 000 × *g* for 20 min. The precipitates were suspended in water for analysis.

Subfractionation of the 250 × g fraction

The following modification of the method of CHAUVEAU, MOULE AND RUILLER[16] was used. The 250 × *g* fraction prepared as described above was suspended in 2.3 M sucrose containing 1.5 mM $CaCl_2$ and 0.01 M Tris–HCl buffer (pH 7.7) (0.5 vol. of the homogenate from which 250 × *g* fraction was prepared). After homogenizing by five up and down strokes of the loose pestle of a Dounce homogenizer the suspension was centrifuged at 44 000 × *g* for 60 min in the SW-39 head of a Spinco preparative ultracentrifuge. Three layers were formed: a bottom pellet, a cloudy sucrose layer and a surface pellet. The surface layer was removed with a spatula, the sucrose layer was decanted, while the bottom pellet containing most of the nuclei was washed twice by resuspending in half the original volume of 0.25 M sucrose in 1.5 mM $CaCl_2$ and 0.01 M Tris–HCl (pH 7.7) and centrifuging for 5 min at 500 × *g*. Nuclear and surface pellets were suspended in water for analysis. Part of the central layer was diluted 3 fold with water and centrifuged for 60 min at 100 000 × *g*. Both the pellet and supernatant from this centrifugation were analyzed for ribonuclease activity.

RESULTS

pH optima

The presence of two ribonuclease activities in homogenates of 16-day embryonic pancreas was initially indicated by a double pH optimum with peaks at pH's 7.2 and 8.5 (Fig. 1A). When bivalent cations were bound by adding EDTA, only the ribonuclease activity with optimum pH at 7.2 was observed. In the late embryo only one pH optimum was evident, a plateau of activity from pH 8.5 upwards (Fig. 1b). When EDTA was added to homogenates from late embryos little residual activity was found at pH 7.2. These observations indicated that at least two separate nucleases are present in embryonic chick pancreas and that their relative amounts change drastically during development. The nuclease activity at pH 8.4–8.7 in presence of bivalent metal ions was therefore called Mg^{2+}-ribonuclease while the residual nuclease activity in presence of EDTA at pH 7.2 was called neutral-ribonuclease.

It is interesting to note that the optimum pH of the Mg^{2+}-ribonuclease observed by us is considerably lower and less sharp than that reported for the partially purified enzyme by ELEY AND ROTH[4]. This difference may be due to the fact that we used

crude homogenates or to the different conditions of assay such as the high concentration of Mg^{2+} used in our studies.

Effect of bivalent metal ions

Little ribonuclease activity was detected at pH 8.4 in homogenates of chick pancreas which had been dialyzed to remove bivalent metal ions. Addition of Mg^{2+}, Mn^{2+} or Co^{2+} markedly stimulated ribonuclease activity, Zn^{2+} stimulated slightly, while Ca^{2+} had no appreciable effect (Fig. 2). Mg^{2+} was the most effective ion for stimulating ribonuclease activity, maximum activity being reached when its concen-

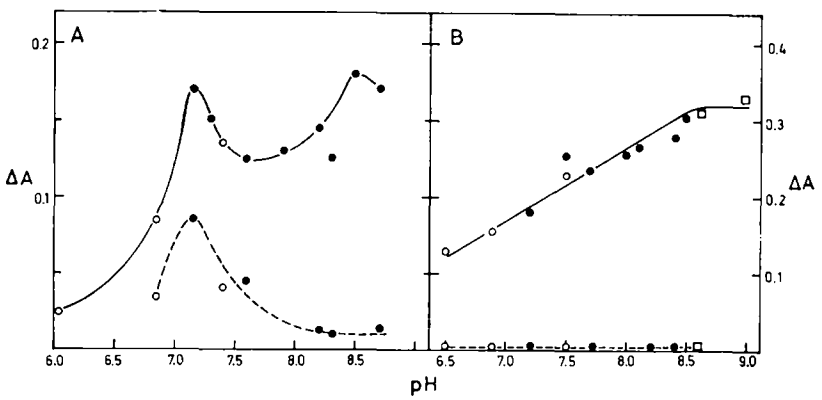
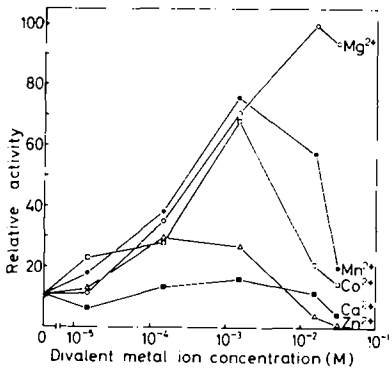


Fig. 1. Effect of pH on ribonuclease activity in presence and absence of Mg^{2+}. A. Homogenate of pancreas from 16-day embryos. Assay systems contained 0.8 mg/ml of protein, 3.3 mg/ml of yeast RNA, 50 mM Tris-HCl buffer (●) or 50 mM imidazole HCl buffer (○), with 15 mM Mg^{2+} (— · —) or 7 mM EDTA (— — —). Activity is expressed as $\Delta A/20$ min per 2.5 mg of protein. B. Homogenate of pancreas from 20-day embryos. Assay systems contained 0.25–1.0 mg/ml of protein, 3.5 mg/ml of yeast RNA, 40 mM Tris HCl buffer (●), 40 mM imidazole-HCl buffer (○) or 40 mM glycine HCl buffer (□) with 15 mM Mg^{2+} (— · —) or 5 mM EDTA (— — —). Activity is expressed as $\Delta A/6$ min per 0.8 mg of protein.

tration was 10 mM. Up to a concentration of 1.5 mM Mn^{2+} and Co^{2+} stimulated ribonuclease activity as effectively as Mg^{2+} but at higher concentrations their effectiveness decreased. The decline in stimulatory ability of Mn^{2+} and Co^{2+} at high concentrations is probably related to the observation that at concentrations of 15 mM or above they precipitate the RNA in the assay system.

Developmental changes of ribonuclease activities

Assays of Mg^{2+}-ribonuclease and neutral ribonuclease activities were based on differences in the pH optimum and bivalent metal ion requirements described above. Fig. 3 shows the developmental changes of the two nuclease activities. The specific activity of neutral ribonuclease reaches a maximum between 16 and 18 days of development after which it declines. In contrast, the specific activity of Mg^{2+}-ribonuclease increases slowly between 14 and 19 days of development after which it rises steeply reaching a maximum at 21 days of development. From 17 to 22 days of development the pattern of increase of the specific activity of Mg^{2+}-ribonuclease closely resembles that of amylase. Before 17 days however, the pattern of activity of Mg^{2+}-ribonuclease markedly differs from that of amylase.



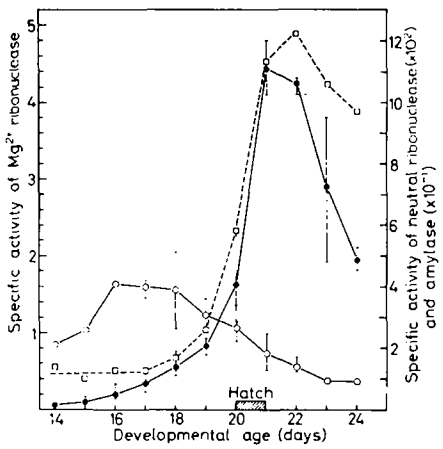


Fig. 2. Effect of bivalent metal ions on ribonuclease activity at pH 8.4. Homogenate of pancreas from 2-day chicks, containing 21 mg of protein/ml ,was dialyzed at 4° first against 5 mM EDTA in 0.05 M Tris-HCl (pH 7.5) and then against 0.05 M Tris-HCl (pH 8.3). Dialyzed homogenate (0.2 ml) was incubated with an assay system containing 40 mM Tris–HCl buffer (pH 8.4), 14 mg of purified yeast RNA, and bivalent metal ions as shown in the figure in a final volume of 4.0 ml. To equalize the conditions of precipitation for each metal ion the uranyl acetate–perchloric acid reagent contained the same ion as in the reaction mixture adjusted to give a final concentration after precipitation of 11 mM.

Fig. 3. Developmental changes of specific activities of Mg^{2+}- ribonuclease, neutral ribonuclease and amylase. Homogenates contained 10–60 pancreases. Each point from 14 to 22 days represents the mean of determinations on at least 3 homogenates, while points for 23 and 24 days are the mean of 2 determinations. Vertical bars show the range of individual values. Specific activities are expressed as units per mg protein. Data for all enzymes are from the same series of homogenates. ●, Mg^{2+}-ribonuclease; ○, neutral ribonuclease; □, amylase.

Secretion of Mg^{2+}-ribonuclease in vitro

The pattern of accumulation of Mg^{2+}-ribonuclease during development suggested that it is a digestive enzyme. In order to test whether this is indeed the case we examined the ability of the pancreas from 1-day chicks to secrete Mg^{2+}-ribonuclease *in vitro*. Table I shows that on treatment *in vitro* with carbamylcholine chick pancreas secreted Mg^{2+}-ribonuclease as effectively as it secreted amylase. Since the specific activities of both enzymes in the medium after secretion were about 3 times as high as specific activities of the corresponding enzyme in the pancreas, the secretion must have been highly selective. Pancreas from 17-day embryos could also secrete Mg^{2+}-ribonuclease under similar conditions. These experiments clearly show that Mg^{2+}-ribonuclease is a digestive enzyme.

The assay methods used were not sensitive enough to permit accurate determinations of the amount of neutral ribonuclease secreted, however, analyses indicated little, if any, secretion of neutral ribonuclease. In the experiment summarized in Table I less than 10% of the neutral ribonuclease present in the pancreas was secreted.

Separation of ribonuclease activities on the basis of solubility

When embryonic pancreas is homogenized in water, almost all of the neutral ribonuclease activity is bound to particles while most of the Mg^{2+}-ribonuclease

TABLE I

SECRETION OF Mg^{2+}-RIBONUCLEASE AND α-AMYLASE BY ISOLATED PANCREAS

Each vessel contained 4 pancreases from 1-day chicks (developmental age 22 days) in 3.0 ml of Krebs-Ringer bicarbonate medium. Vessels were shaken for 4 h at 37°.

$$\% \text{ secreted} = \frac{\text{units of enzyme in medium}}{\text{units of enzyme in medium} + \text{units of enzyme in pancreas}}$$

Additions	*Mg^{2+}-ribonuclease*			*Amylase*		
	% secreted	*Specific activity (units/mg protein) in*		*% secreted*	*Specific activity (units/mg protein) in*	
		medium	*pancreas*		*medium*	*pancreas*
None	8	2.7	4.7	12	87	109
10^{-4} M carbamyl choline	58	9.2	2.8	56	286	96

activity is soluble (Table II). Addition of detergents such as Triton X-100 or sodium deoxycholate to the homogenate decreases the amount of Mg^{2+}-ribonuclease bound to the particles but does little to increase the solubility of the neutral ribonuclease activity. Extraction of the particles containing neutral ribonuclease with salt so-

TABLE II

SEPARATION OF RIBONUCLEASE ACTIVITIES ON THE BASIS OF SOLUBILITY

Homogenate containing 45 pancreases from 19-day embryos in 17 ml of water was divided into equal portions to which detergents were added as shown below. Samples of homogenate were centrifuged for 60 min at 100 000 × *g* and the supernatants were collected. Pellets were then suspended in medium having the same volume and containing the same additions as the corresponding supernatant.

Additions	*% of neutral ribonuclease activity in*		*% of Mg^{2+}-ribonuclease activity in*	
	Pellet	*Supernatant*	*Pellet*	*Supernatant*
None	100	0	21	79
1.0% Triton X-100	100	0	14	86
0.5% Sodium deoxycholate	89	11	6	94

lutions, however, results in the solubilization of more than 50% of the activity (Table III).

Distribution of ribonuclease activities in subcellular fractions

Most of the Mg^{2+}-ribonuclease was found in the 1000 × *g* (zymogen granule) fraction and in the supernatant (Table IV). This distribution is similar to that of other digestive enzymes.

Neutral ribonuclease, on the other hand, was located mainly in the fractions sedimenting at 250 × *g* and 1000 × *g* (Table IV). Possible explanations of this

TABLE III

EXTRACTION OF NEUTRAL RIBONUCLEASE FROM PARTICLES BY SALT SOLUTIONS

Pancreases from 20-day embryos were homogenized in water (0.4 ml per pancreas). Samples containing 5.0 ml of the homogenate were centrifuged at 100 000 × *g* for 60 min and the supernatant was discarded. The pellets were suspended in 4.0 ml of extraction medium and the centrifugation was repeated. After collecting the supernatant the pellet was suspended in 3.5 ml of extraction medium. Activities of neutral ribonuclease in the extraction medium and the pellet were determined.

Extraction medium	*% of activity extracted*
Water	5
0.5 M KCl	50
1.0 M KCl	56

distribution are that neutral ribonuclease is located in the nuclei or present in red blood cells contaminating the homogenate. Measurements of the specific activity of ribonuclease at pH 7.2 in red blood cells showed that it is too low to contribute significantly to the neutral ribonuclease activity of the pancreas through contamination. In order to determine whether neutral ribonuclease is associated with the cell nuclei a subfractionation was performed on the 250 × *g* fraction to separate

TABLE IV

DISTRIBUTION OF RIBONUCLEASE ACTIVITIES IN SUBCELLULAR FRACTIONS

Homogenate containing 100 pancreases from 1-day chicks in 20 ml of medium was prepared and fractionated as described under MATERIALS AND METHODS.

Fraction No.	*Centrifugal force (× g)*	*Centrifugation time (min)*	*Neutral ribonuclease*		*Mg^{2+}- ribonuclease*	
			Units per fraction	*% of activity in homogenate*	*Units per fraction*	*% of activity in homogenate*
Homogenate	—	-	10.2	100	553	100
1	250	5	5.9	57	36	6
2	1 000	10	2.1	21	163	29
3	2 000	15	1.6	16	42	8
4	15 000	20	0.7	7	33	6
Supernatant	—	—	2.3	22	243	46
Total	-	—	12.6	124	517	94

nuclei from unbroken cells and other components of the fraction. Table V shows that after centrifugation in 2.3 M sucrose[16] most of the neutral ribonuclease activity was found in the sucrose and surface layers whereas most of the nuclei (as indicated by DNA determination and microscopic examination) were in the bottom pellet. The neutral ribonuclease in the sucrose layer was attached to particles which could be sedimented after diluting the sucrose. The widely varying ratio of ribonuclease

TABLE V

SUBFRACTIONATION OF 250 × *g* FRACTION BY CENTRIFUGATION IN 2.3 M SUCROSE

The 250 × *g* fraction was prepared from homogenate of 97 pancreases from 20-day embryos in 19 ml of medium and the subfractionation was performed as described under MATERIALS AND METHODS.

Fraction	*Neutral ribonuclease*		*DNA per fraction (mg)*	*Protein per fraction (mg)*	*Units of neutral ribonuclease per mg DNA*	*Units of neutral ribonuclease per mg protein*
	Units per fraction	*% of units in 250 × g fraction*				
Crude 250 × *g* fraction	3.2	100	4.2	22.5	0.73	0.013
Nuclear pellet	0.9	28	2.0	5.8	0.45	0.016
Sucrose phase	1.2	37	0.1	6.6	12	0.018
Surface layer	1.1	35	0.7	8.9	1.5	0.017
Total	3.2	100	2.8	21.3	-	

activity/DNA in different fractions serves further to emphasize the lack of correlation between the distribution of neutral ribonuclease and the nuclei. Thus neutral ribonuclease activity does not appear to be associated with the majority of the pancreatic nuclei. However, the possibility still exists that neutral ribonuclease is associated with a class of pancreas nuclei sedimenting more slowly than the bulk. Otherwise it has to be assumed that neutral ribonuclease is attached or adsorbed to rapidly sedimenting particles of unknown nature.

Specificities

The specificity of Mg^{2+}-ribonuclease towards synthetic polynucleotides has been

TABLE VI

HYDROLYSIS OF SYNTHETIC POLYNUCLEOTIDES BY SOLUBLE AND PARTICLE-BOUND NEUTRAL RIBONUCLEASE

50 pancreases from 18-day embryos were homogenized in 9.0 ml of water and the homogenate was centrifuged at 100 000 × *g* for 60 min. The supernatant was rejected and the precipitate was suspended in 7.0 ml of 1 M KCl after which the suspension was again centrifuged at 100 000 × *g*. The supernatant which contained 3.2 mg of protein per ml was tested for activity on the substrates listed below. The pellet was suspended in 6.0 ml of water to give a protein concentration of 3.4 mg/ml and the activity of the suspension on various substrates was tested. Assay systems contained 1.65 mg of polynucleotide 3.5 μmoles of EDTA and 20 μmoles of imidazole HCl (pH 7.2) in a volume of 0.5 ml. The reaction was started by adding 0.2 mg of enzyme. Vessels were incubated for 2 h at 37°. Samples of 0.16 ml were removed and placed in microcentrifuge tubes containing 0.06 ml of uranyl acetate perchloric acid reagent. Other details of the procedure were as described under MATERIALS AND METHODS.

Substrate	*Neutral ribonuclease activity*	
	Supernatant (ΔA)	*Particles (ΔA)*
Yeast RNA	0.66	0.34
Poly U	0.11	0.08
Poly C	0.07	0.09
Poly A	0	0
Poly I	0	0

described elsewhere[3-5]. It is an endonuclease which hydrolyzes both poly A and poly U to short-chain oligonucleotides terminating in a 5'-phosphate. Neutral ribonuclease hydrolyzes poly U and poly C but not poly A or poly I (Table VI). In this respect its specificity resembles that of bovine pancreatic ribonuclease. Both Mg^{2+}-ribonuclease and neutral ribonuclease hydrolyze RNA core (the non-dialyzable residue remaining after the action of bovine pancreatic ribonuclease on yeast RNA), the former slightly more slowly and the latter slightly more rapidly than yeast RNA (Table VII). Thus both enzymes differ in their specificity from bovine pancreatic

TABLE VII

ACTIVITY OF Mg^{2+}- AND NEUTRAL RIBONUCLEASE ON RNA CORE

Homogenate of 47 pancreases from 19-day embryos in 7.5 ml of water was dialyzed at 4° first against 5 mM EDTA in 0.05 M Tris-HCl buffer (pH 7.5) and then against 0.05 M Tris-HCl buffer (pH 8.3). Assay systems for Mg^{2+}-ribonuclease and neutral ribonuclease were as described under MATERIALS AND METHODS except that RNA core replaced yeast RNA where indicated. Incubation times and amounts of homogenate or bovine pancreatic ribonuclease were such as to give ΔA = 0.33 in the Mg^{2+}-ribonuclease assay system and ΔA = 0.15 in the neutral ribonuclease assay system with yeast RNA as substrate. Activities are expressed as ΔA/min per ml of enzyme.

Enzyme preparation	*Mg^{2+}-ribonuclease assay system*			*Neutral ribonuclease assay system*		
	Activity on		*Ratio of activities*	*Activity on*		*Ratio of activities*
	Yeast RNA (ΔA)	*RNA core (ΔA)*	*RNA core/ yeast RNA*	*Yeast RNA (ΔA)*	*RNA core (ΔA)*	*RNA core/ yeast RNA*
Homogenate of 19-day pancreas	0.175	0.108	0.62	0.015	0.018	1.20
Bovine pancreatic ribonuclease (1 μg/ml)	0.330	0	0	0.340	0.012	0.03

ribonuclease. Both soluble and particulate preparations of neutral ribonuclease showed the same specificity towards synthetic polyribonucleotides. In view of the low activity of the preparations, it is doubtful whether the slightly differing ratios of activity on various substrates in the supernatant and the particle fraction are significant.

DISCUSSION

The separation of Mg^{2+}-ribonuclease and neutral ribonuclease on the basis of their different solubilities (Table II) shows unequivocally that there are at least two different ribonucleases in embryonic chick pancreas. The Mg^{2+}-ribonuclease activity is probably due to a single enzyme which has been partly purified and characterized by ELEY AND ROTH[4,5]. It is not clear, however, whether the neutral ribonuclease activity is due to one or more nucleases. Both Mg^{2+}- and neutral ribonuclease activities are obviously different from bovine pancreatic ribonuclease.

Since Mg^{2+}-ribonuclease, like amylase, is selectively secreted by pancreas *in vitro*, it is obviously a digestive enzyme. All the other digestive enzymes of chick

TABLE VIII

RATIOS OF ACTIVITIES AMYLASE /Mg^{2+}-RIBONUCLEASE, AMYLASE/CARBOXYPEPTIDASE AND AMYLASE/CHYMOTRYPSIN AT DIFFERENT STAGES OF DEVELOPMENT

Ratios of amylase/Mg^{2+}-ribonuclease were calculated from the data in Fig. 3. Ratios amylase/carboxypeptidase and amylase/chymotrypsin were calculated from the data of MARCHAIM AND KULKA[1].

Age (days)	*Ratio of activities*		
	Amylase/ Mg^{2+}-ribonuclease	*Amylase/ Carboxy-peptidase*	*Amylase/ Chymotrypsin*
13	—	35.0	150
14	250	22.0	60
15	83	15.0	21
16	62	7.7	6.1
17	35	10.0	5.3
18	29	8.5	4.0
19	31	8.5	4.3
20	35	10.3	6.3
21	25	12.5	7.0
22	28	13.7	8.1
23	37	13.6	8.4

pancreas studied in this and in other laboratories[1,8,17,18] resemble their mammalian counterparts. It is interesting, therefore, that the role of digesting RNA should be performed by an enzyme of a completely different type from mammalian pancreatic ribonuclease. Hydrolysis of RNA by homogenates of turkey, duck and pigeon pancreas also depends on the presence of Mg^{2+} suggesting that the Mg^{2+}-ribonuclease is characteristic of avian pancreas in general (H. HELLER and R. G. KULKA, unpublished experiments). As no exhaustive comparative studies of pancreatic ribonucleases have been performed, the evolutionary significance of the presence of Mg^{2+}-ribonuclease in avian pancreas is not clear. It should be of interest to determine whether the avian or the mammalian type of ribonuclease is present in the pancreas of more primitive vertebrates. Although Mg^{2+}-ribonuclease has no counterpart among mammalian pancreatic digestive enzymes, it does closely resemble magnesium-requiring endonucleases present in pig-and guinea pig-liver nuclei[19,20] and in rat-liver mitochondria[21,22].

The role of the neutral ribonuclease of chick pancreas remains a mystery. Its insolubility as well as the decrease in its specific activity during development seem to rule out the possibility that it is a digestive enzyme. It may be significant that the specific activity of neutral ribonuclease reaches a peak between 16 and 18 days of development, just before the maturation phase of the pancreas. It would be premature, however, on the basis of the data presented in this paper, to speculate about a possible function of neutral ribonuclease during development. Perhaps neutral ribonuclease is not a component of exocrine pancreas cells but is associated with some other type of cell whose relative number falls during development. This hypothesis would account for the decrease in the specific activity of neutral ribonuclease during the later stages of development.

Mg^{2+}-ribonuclease provides an example of yet another digestive enzyme whose

specific activity increases steeply from 18 to 22 days of development during the maturing phase of the chick pancreas. In a previous paper from this laboratory[1] we emphasised the non-parallel nature of the accumulation of digestive enzymes in the developing chick pancreas. Although there is no doubt that up to 16 days of development the activities of amylase, Mg^{2+}-ribonuclease, chymotrypsin and carboxypeptidase do not all increase in parallel, the data for 16–22 days of development need to be reevaluated. In Table VIII activities of various enzymes relative to that of amylase have been calculated for each developmental age. Ratios of activities amylase/chymotrypsin and amylase/carboxypeptidase change markedly from 13 to 16 days of development, while the ratio of activities amylase/Mg^{2+}-ribonuclease changes rapidly from 14 to 17 days of development. From 16 or 17 days onwards, however, the ratios of the activities of the various enzymes remain surprisingly constant. The data strongly suggest that around 17 days of development there is a shift from non-parallel to parallel accumulation of enzymes. The possibility that there are groups of two or more enzymes whose activities change in parallel before 17 days of development (*cf.* ref. 2) is not, however, excluded. It is tempting to suggest that at 17 days there is a switching from control of synthesis of enzymes individually or in groups to an overall control mechanism common to all digestive enzymes.

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