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THE NON-PARALLEL INCREASE OF AMYLASE, CHYMOTRYPSINOGEN AND PROCARBOXYPEPTIDASE IN THE DEVELOPING CHICK PANCREAS

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SUMMARY

Developmental changes in the levels of α-amylase, procarboxypeptidase and chymotrypsinogen were studied in the pancreas of the chick embryo (developmental age 13–20 days) and the newly-hatched chick (developmental age 21–23 days).

1. Levels of zymogens were determined by measuring the activity of the corresponding proteolytic enzyme after activation of the zymogen with trypsin. For this purpose controlled conditions of activation of procarboxypeptidase and chymotrypsinogen were developed which would permit full activation of the zymogens in homogenates of pancreas at different stages of development.

2. When homogenates of pancreas at different stages of development were mixed, enzyme activities were additive. This seems to rule out the possibility that changing concentrations of inhibitors or activators during development influence the activities of the enzymes studied.

3. The specific activities of all three enzymes increase most steeply after 18 days of development reaching a maximum in the 1-day chick (22 days of development). However, in spite of this similarity, the level of each enzyme changes with age in a different and characteristic manner. From 13–22 days of development the specific activities of amylase, carboxypeptidase and chymotrypsin increase 9-fold, 24-fold and 200-fold, respectively.

INTRODUCTION

The differentiation of the exocrine pancreas, both in the chick and in the mouse, is characterized by a steep rise in the level of amylase within the gland[1,2]. In the chick pancreas an early rapid increase in amylase specific activity from 6 to 14 days of development, is followed by a second sharp increase in amylase specific activity at the time of hatching[3]. The second phase of rapid amylase accumulation coincides with a phase of rapid cell division and morphological maturation of the pancreas[4,5].

Abbreviations: ATEE, *N*-acetyl-L-tyrosine ethyl ester; HPLA, hippuryl-DL-phenyllactic acid.

Since the various digestive enzymes perform the same function in the animal, it seemed likely that the biochemical differentiation of the pancreas would involve a rapid accumulation not only of amylase but also of digestive enzymes in general. The purpose of the present work was to determine whether this prediction is fulfilled and if so to ascertain whether different types of digestive enzymes accumulate in a parallel fashion, similar to that of certain bacterial enzymes of related function[6] or in a non-parallel fashion. It is shown that the maturation of the chick pancreas is indeed accompanied by a steep rise in the levels of α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1), procarboxypeptidase (EC class 3.4.2) and chymotrypsinogen (EC 3.4.4.5) but that the pattern of accumulation of each enzyme is distinct from those of the other enzymes studied.

MATERIALS AND METHODS

Embryos and chicks

These were obtained from white Leghorn × New Hampshire eggs weighing 50 to 60 g and incubated at 37.5–38°. The developmental ages of embryos were normalized, according to the criteria of HAMBURGER AND HAMILTON[7], to correspond with the developmental ages in a previous study[1]. Chicks hatched on the 21st day of incubation.

Enzymes and substrates

Bovine trypsin twice-crystallized, salt-free, lyophilized, was obtained from Worthington Biochemical; *N*-acetyl-L-tyrosine ethyl ester (ATEE) was purchased from Yeda, Rehovoth, Israel and hippuryl-DL-phenyllactic acid (HPLA) was the product of Cyclo Chemical.

Preparation of homogenates

Pancreases from 5–40 embryos or chicks were homogenized with a Potter–Elvehjem homogenizer in 0.01 M Tris–HCl buffer (pH 7.6) to give a final protein concentration of 0.7–3.2 mg/ml. Analyses were performed on homogenates which were fresh or had been stored for not more than 48 h at −20°. Amylase and the zymogens of the proteolytic enzymes were stable under these conditions.

Analytical methods

Protein was determined by the method of LOWRY *et al.*[8] using crystalline bovine plasma albumin as standard. Amylase was determined according to BERNFELD[9], 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.

Chymotryptic hydrolysis of ATEE was followed at 30° by titration with 0.05 M NaOH in a Radiometer pH-stat type TTT1. The reaction mixture (5.0 ml) contained 2 mM Tris–HCl buffer (pH 7.6), 5 mM ATEE, and 0.6–1.2 units of chymotrypsin. While this work was in progress RYAN, CLARY AND TOMIMATSU[10] reported that purified chicken chymotrypsin hydrolyzed ATEE optimally at pH 8.6. However, for the sake of consistency, determinations were continued at pH 7.6 which gives rates about 25% lower than those at the optimum pH.

Carboxypeptidase activity was determined by following the hydrolysis of HPLA[11] at 30° titrimetrically with 0.05 M NaOH in the pH-stat. The assay system (5.0 ml) contained 4 mM Tris–HCl buffer (pH 7.6), 0.04 M NaCl, 0.01 M HPLA and 0.6–1.2 units of carboxypeptidase.

A unit of chymotrypsin or carboxypeptidase is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmole of substrate per min.

Activation of zymogens

In order to obtain a reliable measure of the amount of a zymogen in a homogenate, precautions must be taken to attain full activation of the zymogen but to avoid destruction of the activation product by proteolysis. REBOUD, BEN ABDEJLIL AND DESNUELLE[12] have shown that steady activities of trypsin and chymotrypsin could be obtained on activating the corresponding zymogens if various conditions such as temperature, concentration of the homogenate and amount of trypsin added were suitably adjusted. In the present investigation conditions were developed which would give full activation of zymogens in homogenates of pancreas from chick embryos at different stages of development.

The activation of chymotrypsinogen with trypsin was sluggish at 0° while at 30° maximum activity was reached rapidly but was unstable. At 20° full activation of chymotrypsinogen was readily obtained in homogenates of pancreas at different stages of development and the product was stable. Although the time taken to attain full activation was variable, maximum chymotryptic activity was consistently reached after 100 min and remained stable for more than 2 h thereafter (Fig. 1). The maximum chymotryptic activity obtained was independent of the amount of trypsin added to the activation system. The chymotryptic activity of unactivated homogenate was usually less than 2% of that obtained after activation.

Procarboxypeptidase was activated rapidly by trypsin at 0°. The time required for complete activation of procarboxypeptidase was variable but never exceeded 100 min, after which time the carboxypeptidase activity remained stable for more

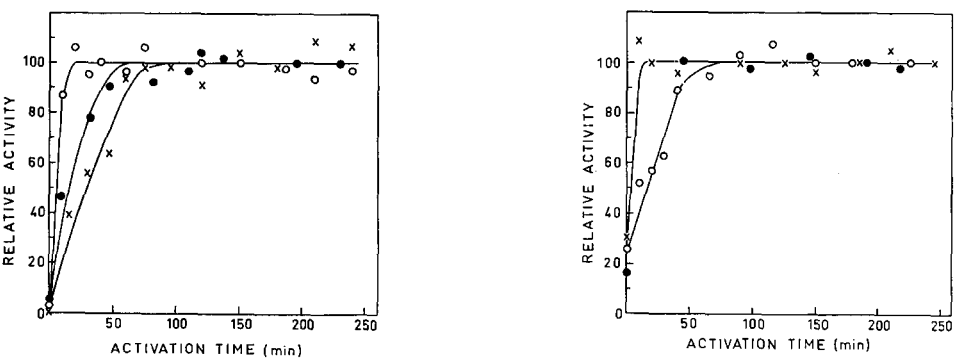

Fig. 1. Kinetics of activation of chymotrypsinogen. Standard conditions of activation were used. Homogenates of pancreas from (×) 15-day embryo, (●) 16-day embryo and (○) 1-day chick (developmental age 22 days). For calculations of relative activities the mean activity of the fully activated homogenate was taken as 100.

Fig. 2. Kinetics of activation of procarboxypeptidase. Standard conditions of activation were used. ×, 15-day embryo; ●, 17-day embryo; ○, 1-day chick (developmental age 22 days). Relative activities were calculated as described under Fig. 1.

than 2 h (Fig. 2). In homogenates of pancreas, at all stages of development tested, activity on HPLA amounting to 10–30% of the final activity after activation was consistently observed before activation.

The following conditions of activation were finally adopted: For chymotrypsinogen, trypsin was added to homogenate (diluted to contain 0.4–1.6 mg of protein per ml) to give a final concn. of 0.025 mg per ml. After 100 min at 20° samples were removed at intervals for determination of chymotryptic activity. For procarboxypeptidase, trypsin was added to homogenate (diluted to contain 0.4–1.6 mg of protein per ml) to give a final concn. of 0.15 mg per ml. After 100 min at 0° samples were removed for determination of carboxypeptidase activity.

Enzyme activities were determined for each homogenate at several different times of activation, in order to ensure that full activation of the zymogen had been reached.

RESULTS AND DISCUSSION

Effect of mixing homogenates

A possible objection to the use of enzyme activities as a measure of the levels of enzyme molecules or their precursors in the pancreas at different stages of development is that activities may be affected by changing levels of specific activators or inhibitors. This problem is likely to affect in particular the determination of chymotrypsin which is known to be inhibited by the pancreatic trypsin inhibitor of Kunitz[13]. In order to determine whether changes in the levels of inhibitors or activators during development influence the relative activities of the enzymes studied, we tested the effect of mixing homogenates of pancreas at different stages of development. Table I shows that when homogenates of pancreas from embryos at different stages of development were mixed, enzyme activities were additive. This seems to rule out the possibility that changing concentrations of free activators or inhibitors during development alter the ratios of the activities of the three types of enzymes studied.

TABLE I

EFFECT ON DIGESTIVE ENZYME ACTIVITIES OF MIXING HOMOGENATES OF PANCREAS AT DIFFERENT STAGES OF DEVELOPMENT

The mixtures were prepared by mixing equal volumes of homogenates. Chymotrypsinogen and procarboxypeptidase were activated by standard procedures. In the case of the mixtures the zymogens were activated after mixing the homogenates.

Homogenates of pancreas from	*Amylase (units/ml)*	*Carboxypeptidase (units/ml)*	*Chymotrypsin (units/ml)*
15-day embryo	31	4.1	1.8
18-day embryo	28	7.5	12.5
1-day chick	212	43.5	64.0
Mixture of 15-day embryo + 18-day embryo found	32	5.7	7.5
Calculated	30	5.8	7.2
Mixture of 18-day embryo + 1-day chick found	124	29.5	37.8
Calculated	120	25.5	38.3

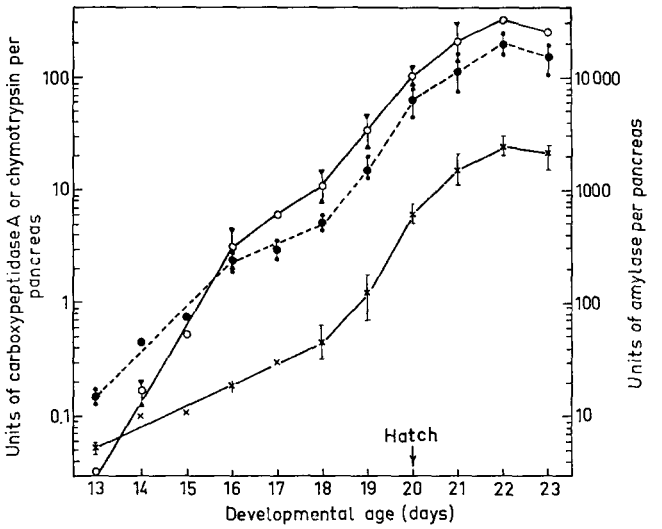

Fig. 3. Changes in the total activities of digestive enzymes during development. Points for developmental ages 13 to 17 days represent the average of determinations on at least 2 homogenates while points from 18 to 23 days represent the average of at least 3 determinations. Vertical bars show the range of individual results which fall outside the points. Amylase, ×—×; carboxypeptidase, ●---●; chymotrypsin, ○—○.

Changes in enzyme activities during development

Total activities of amylase, carboxypeptidase and chymotrypsin increase 500-, 1200- and 10 000-fold, respectively, between 13 and 22 days of development (Fig. 3). The patterns of increase of the three enzyme activities are strikingly different from 13–17 days of development, but are similar from 18–23 days of development. All activities reach a maximum at 22 days.

The specific activities of all 3 enzymes increase steeply after 18 days of development, reaching a maximum at 22 days (Fig. 4). This further supports the previous suggestion[1] that the biochemical maturation of the pancreas, characterized by rapid accumulation of digestive enzymes, parallels the dramatic morphological maturation of the gland around the time of hatching[4,5]. It should be noted, however, that although there is a rough coincidence in the time of steepest increase of the specific activities of the three enzymes, the specific activity of each changes with age in a different and characteristic manner. The lack of parallelism of the patterns of accumulation of the enzymes in the pancreas is brought out by the fact that from 13–22 days the overall increase of specific activity is 9-fold for amylase, 24-fold for carboxypeptidase and 200-fold for chymotrypsin.

A number of surveys have been made of developmental changes in the activities of enzymes of related function. Notable amongst these are studies of changes in activities of the urea cycle enzymes during the metamorphosis of the tadpole[16] and investigations of patterns of activity of various enzymes of hexose phosphate metabolism in the liver during mammalian development[17–20]. The specific activities of all urea cycle enzymes increase steeply during metamorphosis. Among the enzymes of carbohydrate metabolism there are also pairs or groups of enzymes whose levels change during development according to similar temporal patterns. However, it is

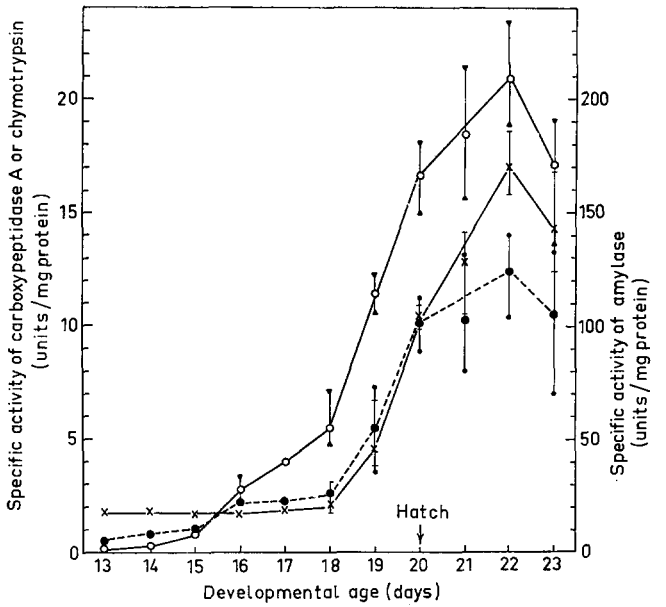

Fig. 4. Changes in the specific activities of digestive enzymes during development. For details see Fig. 3.

impossible on the basis of the data available to decide whether changes in activities of such related enzymes are 'parallel'* in the sense that constant proportions between the activities are maintained throughout development. The developmental changes in the activities of the three types of pancreatic enzymes studied in the present investigation are clearly 'non-parallel', in spite of the fact that the time of steepest increase in activity is similar for all of them. Further work is required to determine whether the changes in the specific activities of the enzymes studied result from changes in the pattern of the synthesis of the enzyme molecules *de novo*. It also remains to be ascertained whether the hydrolysis of ATEE and HPLA are each catalyzed by a single molecular species or by a group of enzymes of similar specificity. If the observed changes of enzyme activities during development indeed reflect accurately changes in the rate of synthesis of enzyme molecules *de novo*, our results indicate that the biosynthesis of each of the three types of enzyme is controlled separately. REBOUD *et al.*[14,15] have shown that in adult rat pancreas the rates of biosynthesis of various digestive enzymes vary independently in response to changes in diet. However, the mechanisms controlling the synthesis of digestive enzymes in adult and embryonic pancreas are not necessarily identical.

* The term 'parallel' seems to be more appropriate to denote constant ratios of the activities of several enzymes during induction or repression than the term 'coordinate' used for micro-organisms (*cf.* ref. 6). A close coordination of the temporal patterns of synthesis of various enzymes need not necessarily result in a constant ratio of their activities.

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