SECRETION OF α-AMYLASE BY THE EMBRYONIC CHICK PANCREAS IN VITRO

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ABSTRACT

Pancreases taken from chick embryos secrete amylase in vitro when stimulated by cholinergic drugs. Rates of secretion increase with developmental age. The pancreas isolated together with the duodenal loop from the 8 day embryo is already capable of secretion in vitro. It is therefore concluded that the pancreas acquires the ability to secrete digestive enzymes more than 10 days before the beginning of the prominent biochemical and morphological changes associated with the maturation of the gland.

INTRODUCTION

Histological and biochemical studies indicate that, from about the 19th day of embryonic development until just after hatching (developmental age of 22 days), the chick pancreas undergoes a process of maturation. Among the manifestations of this maturation phase are a burst of cell division, an increase in average cell size as well as changes in the fine structure of the cells such as the expansion of the endoplasmic reticulum, and an increase in the size of the zymogen granules (Zeigel, 1962; Sreebny, unpublished observations; Kulka and Duksin, 1964). Concomitant with these morphological changes is a steep rise in the levels of α-amylase, chymotrypsinogen, and procarboxypeptidase within the pancreas (Kulka and Duksin, 1964; Marchaim and Kulka, 1966).

In the present study, an attempt was made to ascertain whether or not these maturation steps are also manifestations of the functional development of the pancreas, namely, the acquisition of the capacity to secrete digestive enzymes. It is shown that the chick pancreas can secrete amylase in vitro as early as the 8th day of embryonic development. This means that the gland acquires the ability to secrete digestive enzymes long before the beginning of the phase of maturation described above.

MATERIALS AND METHODS

Embryos and Chicks

The embryos and chicks were obtained from eggs of a White Leghorn–New Hampshire cross incubated at 38° to 38.5°C. The degree of development of embryos of various ages corresponded to the appropriate stages established by Hamburger and Hamilton (1951). On the 21st day of incubation, chicks were hatched and given only water.

Analytical Methods

Unless stated otherwise, amylase was determined by the method of Bernfeld (1955), with a unit defined as the amount of amylase that in 3 min at 30°C catalyzes the appearance of reducing groups equivalent to 1 mg of maltose hydrate. In some experiments where greater sensitivity was required, the following micromodification (Ben-Chorin, 1961) of the iodometric method of Smith and Roe (1949) was employed. To 0.8 ml of enzyme in 0.03 M phosphate buffer pH 6.9 containing 7 mM NaCl at 37°C, 0.2 ml of 0.5% soluble starch was added, and the mixture was incubated for 30 min. The reaction was stopped with 1.0 ml of 0.3 N HCl. After adding 10.0 ml of water and 0.1 ml of 0.3% I_2 in 3% KI, the solutions were mixed and allowed to stand for 5 min, and the optical density was read at 620 mμ. The unit for the iodometric method is defined as the amount of amy-

lase that causes the disappearance of 1 mg of starch in 30 min at 37°C. Under our conditions, 1 Bernfeld unit was equivalent to about 25 iodometric units. All of the data presented have been expressed in Bernfeld units.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Incubation Medium

A Krebs medium I basic salt solution (Krebs, 1950) containing 5 mM succinate as sole substrate was used in all experiments. Unless stated otherwise, the medium also contained 100 μg/ml of crystalline soybean trypsin inhibitor (Kunitz, 1947; purchased from the Worthington Corporation, Freehold, New Jersey). In experiments with the pancreas alone or with the pancreas attached to the duodenal loop, neither tryptic nor chymotryptic activity could be detected in the medium when the inhibitor was added.

Incubation Procedure

This was based on techniques developed by other investigators (Hokin, 1951; Bdolah et al., 1964; Schramm et al., 1965). Whole pancreases were taken out with the duodenal loop and dissected from it gently to avoid mechanical damage, and preincubated for 5 to 45 min in 2 to 10 ml of medium gassed continuously with 95% O_2 + 5% CO_2. To start the incubation, a suitable number of pancreases were transferred to 2 or 3 ml of medium containing the appropriate additions in a stoppered Erlenmeyer flask (25 ml or 50 ml) equipped with inlet and outlet tubes for gassing. The flasks were gassed for 5 min with 95% O_2 + 5% CO_2 in aerobic experiments and with 95% N_2 + 5% CO_2 in anaerobic experiments. In kinetic experiments, gassing was repeated after the withdrawal of samples. Both preincubations and incubations were carried out at 37°C in a rotatory shaking water bath (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey) at 100 RPM. Experiments were designed so that the conditions of preincubation of pancreases placed in different flasks were uniform.

At the end of incubation, the pancreases were removed from the medium and homogenized with an appropriate volume (1 to 5 ml) of 0.03 M phosphate buffer pH 6.9 containing 7 mM NaCl. The incubation medium was diluted twofold with the same buffer. If analyses for protein and amylase were not performed on the day of the incubation, samples were stored at −20°C.

At developmental ages when removal of the pancreas only from the embryo proved difficult, the pancreas was isolated and incubated together with the attached duodenal loop. Preincubations and incubations of duodenal loops plus pancreases from 8-day embryos were performed in wide-mouthed vessels in which the tissue was supported on stainless steel grids to facilitate handling.

Calculation of Secretory Rates

In the present work, two types of expression were used: (*a*) *Specific secretion* which is defined as the number of units of amylase secreted per milligram of total initial pancreatic protein; and (*b*) *Per cent secretion* which is calculated as follows:

$$\frac{\text{Units of amylase in medium}}{\text{Units of amylase in medium} + \text{units of amylase remaining in slice}} \times 100$$

(cf. Bdolah et al., 1964).

Specific secretion was used to express results only in cases where we wanted to compare on an absolute scale the capacities of the pancreas at different developmental ages to secrete amylase. In most experiments, results expressed as per cent secretion seemed more pertinent, since this expression emphasizes the relative ability of pancreases of the same age to transfer their complement of amylase from the cells to the medium under different conditions.

RESULTS

Effect of Secretory Stimulants

The isolated pancreas of the 3 day chick was used to compare the relative effectiveness of various drugs to stimulate the secretion of α-amylase (Fig. 1). All the cholinergic agents stimulated secretion to varying degrees, whereas epinephrine was completely inactive. The highest rates of secretion were obtained with carbamylcholine and acetylcholine plus eserine. Carbamylcholine, which was chosen as the stimulant for subsequent experiments, produced maximum rates of secretion at a concentration of 10^{-5} to 10^{-4} M. Since pancreases at different stages of development showed no differences in sensitivity to the level of carbamylcholine in the medium, a concentration of 10^{-4} M was used for routine purposes.

Rate of Secretion of Amylase by Pancreas at Different Stages of Development

The capacity of the pancreas to secrete amylase increases progressively with age (Fig. 2). Specific secretory rates, corrected for the unstimulated control, of the pancreas of the 13 day and 17 day embryo and the 3 day chick were respectively 1.5, 8, and 22 units of amylase/mg protein per hr. At

all three ages, the rate of secretion, when corrected for the unstimulated control, was linear over a period of 3 hr. When the results of similar experiments are expressed as per cent secretion (Tables I and II), it is found that the pancreases of the 13 day embryo and 3 day chick secreted about 40% of their complement of amylase in 3 hr, whereas the pancreas of the 17 day embryo secreted more than 70%. The per cent secretion in the unstimulated control tends to be greater at the earlier stages of development, probably because there is more nonspecific leakage of amylase as a result of greater damage during the dissection.

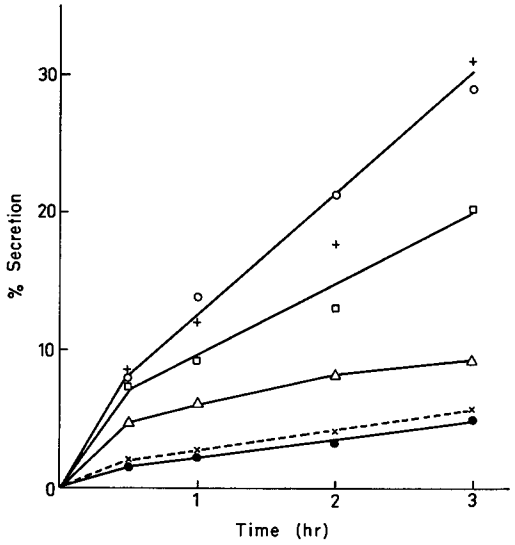

FIGURE 1 Effect of secretory stimulants on the 3 day chick pancreas. Each vessel contained 2 pancreases in 3.0 ml of medium. Stimulants were added to the medium before the introduction of the pancreases to give a final concentration of 10^{-4} M. Control, ●; Epinephrine, ×; Pilocarpine, □; Acetylcholine, △; Acetylcholine + Eserine, +; Carbamylcholine, ○.

Specific Activity of Amylase in the Pancreas and in the Incubation Medium after Secretion

A criterion distinguishing active secretion from the nonspecific leakage of cell contents is the ability of a gland to transfer digestive enzymes selectively to the medium. To test whether or not this condition is fulfilled, the specific activities of amylase in the medium and in the pancreas were compared after secretion (Table I). After stimulation with carbamylcholine, the specific activity of amylase in the medium was invariably greater than that in the pancreas, whereas, in the unstimulated controls, the specific activity of the amylase in the medium more closely resembled that in the pancreas. The selective export of amylase was particularly marked in the case of the embryonic pancreas which secreted amylase with a specific activity up to 20 times higher than that of the amylase remaining in the gland. Moreover, the specific activity of the amylase secreted into the medium was uniformly high at all ages tested, varying only by a factor of 2, in spite of the wide

TABLE I

Specific Activities of Amylase in the Medium and in the Pancreas After Secretion

Pancreas from:	Stimulant added	Per cent secretion	Specific activity of amylase		SA* amylase in medium / SA amylase in pancreas
			in medium	in pancreas	
			units/mg protein		
13 day embryo	None	7	92	21	4.4
	Carbamylcholine	31	220	11	20.0
17 day embryo	None	22	71	23	3.1
	Carbamylcholine	74	176	13	13.6
3 day chick	None	6	170	177	1.0
	Carbamylcholine	41	304	126	2.4

In the experiment on the 13 day embryo, each vessel contained 10 pancreases in 3.0 ml of medium from which soybean trypsin inhibitor was omitted. Experiments on the 17 day embryo and 3 day chick were performed with 2 pancreases per vessel in 2.0 and 3.0 ml of medium, respectively. The incubation time was 3 hr, and the carbamylcholine concentration was 10^{-4} M.

* SA, specific activity.

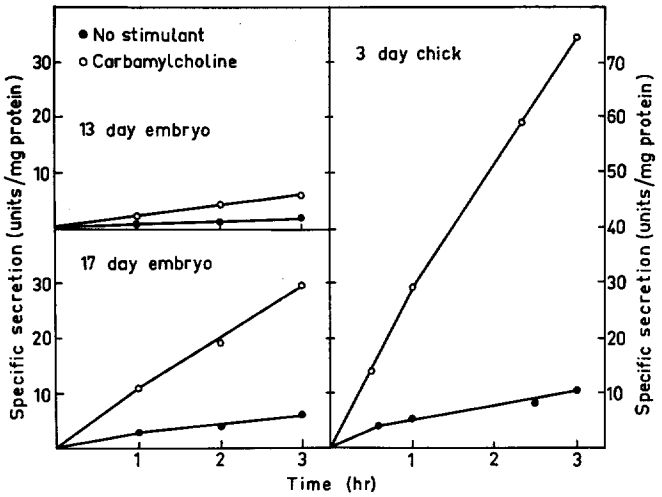

FIGURE 2 Comparison of the specific secretory rates of pancreas at various stages of development. Each vessel contained 2 pancreases in 3.0 ml of medium for the 3 day chick and in 2.0 ml for the 17 day embryo. Ten pancreases from 13-day embryos were incubated per vessel with 3.0 ml of medium from which soybean trypsin inhibitor was omitted. In the experiment on the 13-day pancreas, amylase was determined by the iodometric method but the results presented above have been converted to Bernfeld units. The carbamylcholine concentration was 10^{-4} M in all experiments.

variation (about eightfold) of the initial specific activity of amylase within the glands (cf. Kulka and Duksin, 1964). This finding may indicate a high specific activity of amylase within the zymogen granules throughout development.

Factors Inhibiting Secretion

Two kinds of inhibitory effect were studied: (*a*) interference with the supply of energy for secretion by anaerobiosis or 2,4-dinitrophenol, DNP, (Hokin, 1951; Bdolah, et al., 1964); and (*b*) blocking of the stimulatory action of carbamylcholine by atropine (Schucher and Hokin, 1954). At all ages tested, each of these two types of inhibition decreased the rate of secretion in the presence of carbamylcholine to about that of the unstimulated control (Table II).

TABLE II

Inhibition of Secretion by 2,4-Dinitrophenol, Atropine, and Anaerobiosis

Additions	Gas phase	Per cent secretion by pancreas from: 13 day embryo	17 day embryo	3 day chick
None	O_2/CO_2	18	22	6
Carbamylcholine	O_2/CO_2	45	74	41
Carbamylcholine + DNP	O_2/CO_2	16	14	6
Carbamylcholine + atropine	O_2/CO_2	28	19	11
Carbamylcholine	N_2/CO_2	—	32	12

Each vessel contained 2 pancreases in 2.0 ml of medium for the 13 day and 17 day embryo and in 3.0 ml for the 3 day chick. Stimulants and inhibitors were added to the medium before the introduction of the pancreases to give the following final concentrations: carbamylcholine, 10^{-4} M; atropine, 10^{-4} M, and 2,4-dinitrophenol (DNP), 10^{-3} M. The incubation time was 3 hr. In the experiment with the 13 day pancreas, amylase was determined by the iodometric method.

Secretion by Pancreas Attached to the Duodenal Loop

Since the separation of the pancreas from the duodenal loop proved difficult in embryos aged 12 days or less, experiments were performed on the pancreas isolated with the duodenal loop. This preparation, like the pancreas alone, secreted amylase in vitro when stimulated by carbamylcholine (Table III). Secretion could not be ascribed to the duodenal loop since it contained no amylase. In most experiments, the per cent secretion in the presence of carbamylcholine plus DNP was less than that of the unstimulated control, a fact which indicates that there is some endogenous

TABLE III

Secretion by Pancreas Attached to the Duodenal Loop

Age of embryo	Stage (Hamburger and Hamilton, 1951)	Per cent secretion: no stimulant	+ carbamylcholine	+ carbamylcholine + DNP
days				
8	34	18	40	10
10	36	18	39	13
14	40	21	68	12

In experiments on the 8, 10, and 14 day embryo, 4, 3, and 2 glands, respectively, were incubated per vessel in 2.0 ml of medium, containing 10^{-4} M carbamylcholine with or without 10^{-3} M DNP where indicated. In the case of the 8- and 10-day embryos, amylase was determined by the iodometric method. The incubation time was 3 hr.

stimulation of secretion. These experiments show that the pancreas can secrete amylase as early as the 8th day of embryonic development. The secretory ability of the pancreas from embryos younger than 8 days was not examined because of difficulties in taking out the gland.

DISCUSSION

Our observations on the relative effectiveness of various cholinergic drugs in stimulating secretion and on the inhibitory effects of DNP, anaerobiosis, and atropine agree closely with the data of Hokin and his collaborators on the adult pigeon pancreas (Hokin, 1951; Hokin and Hokin, 1953; Schucher and Hokin, 1954). The inhibition by DNP or anaerobiosis shows that the secretion observed in vitro is an active, energy-requiring process, whereas the inhibitory action of atropine emphasizes the specific physiological action of carbamylcholine as a stimulant. The high specific activity of amylase released into the medium, relative to that of the gland after secretion, confirms that the export of amylase in vitro is a selective process analogous to secretion in the intact animal. These observations eliminate the possibility that a nonspecific effect of carbamylcholine on the leakage of enzymes from the gland has been mistaken for secretion in this study. It should be remembered, however, that although our experiments are a valid test of the ability of the pancreas to secrete in vitro, no conclusions can be drawn from them concerning its state of activity in vivo.

The present experiments clearly demonstrate that the pancreas is capable of secreting amylase as early as the 8th day of embryonic development. It is concluded, therefore, that the gland acquires the ability to secrete digestive enzymes long before the initiation, on the 19th day of development, of the dramatic morphological and biochemical changes which characterize the maturation phase.

The findings reported here further emphasize a point implicit in previous work (Zeigel, 1962; Kulka and Duksin, 1964), i.e., that the differentiation of the chick pancreas involves at least two phases, an early phase of partial differentiation and a later maturing phase. That the pancreas is already partially differentiated prior to the onset of the maturation phase on the 19th day of development is indicated by the facts that digestive enzymes are synthesized on a small scale, a few zymogen granules are present in the cells (Zeigel, 1962), and a limited capacity for secretion exists. During the maturing phase, the gland grows rapidly, enzyme production is accelerated, zymogen granules become larger and more numerous, and secretion becomes more efficient. The process may be likened to the scaling up of a pilot plant to factory production. The two-step differentiation of the chick pancreas seems to differ markedly from the differentiation of the mouse pancreas in which the formation of acini, the appearance of zymogen granules, and the accelerated synthesis of digestive enzymes coincide in a single phase (Grobstein, 1964; Rutter et al., 1964; Kallman and Grobstein, 1964).

Evidence has been presented to show that the zymogen granules represent a vital and final stage in the transport of digestive enzymes from their site of synthesis to the acinar lumen (Caro and Palade, 1964). It seems likely, therefore, that Zeigel's observation (1962) that zymogen granules are already found in the chick pancreas on the 6th day of embryonic development, may be directly related to the observation of its early ability to secrete amylase reported here. These observations suggest that the zymogen granules may be an indispensable component of the secretory apparatus and that their presence in a pancreatic cell is a sign of its ability to secrete digestive enzymes. Although the present studies do not prove this hypothesis, they point out the potential value of studies with embryonic pancreases to de-

termine the role of zymogen granules and other cell organelles in secretion. The embryonic mouse or rat pancreas in which zymogen granules appear at a relatively late stage of development (Munger, 1958; Sjöstrand, 1962) may be particularly suitable for such investigations, since they would facilitate a comparison of the secretory ability of a gland before and after the appearance of zymogen granules.

We wish to thank Mrs. Rachel Ampel for her excellent technical assistance. The participation of Mr. D. Duksin in preliminary experiments is also gratefully acknowledged.

Received for publication 28 December 1965.

REFERENCES

BDOLAH, A., BEN-ZVI, R., and SCHRAMM, M., 1964, *Arch. Biochem. and Biophysics*, 104, 58.

BEN-CHORIN, H., 1961, M. Sc. thesis, The Hebrew University of Jerusalem, Israel.

BERNFELD, P., 1955, *in* Methods in Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Academic Press Inc., 1955, **1**, 149.

CARO, L. G., and PALADE, G. E., 1964, *J. Cell Biol.*, **20**, 473.

GROBSTEIN, C., 1964, *Science*, **143**, 643.

HAMBURGER, V., and HAMILTON, H. L., 1951, *J. Morphol.*, **88**, 49.

HOKIN, L. E., 1951, *Biochem. J.*, **48**, 320.

HOKIN, M. R., and HOKIN, L. E., 1953, *J. Biol. Chem.*, **203**, 967.

KALLMANN, F., and GROBSTEIN, C., 1964, *J. Cell Biol.*, **20**, 399.

KREBS, H. A., 1950, *Biochim. et Biophysica Acta*, **4**, 249.

KULKA, R. G., and DUKSIN, D., 1964, *Biochim. et Biophysica Acta*, **91**, 506.

KUNITZ, M., 1947, *J. Gen. Physiol.*, **30**, 291.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., 1951, *J. Biol. Chem.*, **193**, 265.

MARCHAIM, U., and KULKA, R. G., 1966, in preparation.

MUNGER, B., 1958, *Am. J. Anat.*, **103**, 1.

RUTTER, W. J., WESSELS, N. K., and GROBSTEIN, C., 1964, *Nat. Cancer Inst. Monograph*, **13**, 51.

SCHRAMM, M., BEN-ZVI, R., and BDOLAH, A., 1965, *Biochem. and Biophysic. Research Commun.*, **18**, 446.

SCHUCHER, R., and HOKIN, L. E., 1954, *J. Biol. Chem.*, **210**, 551.

SJÖSTRAND, F. S., 1962, *in* Ciba Foundation Symposium on The Exocrine Pancreas, (A. V. S. de Reuck and M. P. Cameron, editors), London, J. & A. Churchill Ltd., 1962, 1.

SMITH, B. W., and ROE, J. H., 1949, *J. Biol. Chem.*, **179**, 53.

ZEIGEL, R. F., 1962, *J. Nat. Cancer Inst.*, **28**, 269.