FUNCTIONAL ASPECTS OF THE EXOCRINE PANCREAS IN RELATION TO THE ISLETS OF LANGERHANS

Effects of Islet Hormones on the Synthesis, Storage and Secretion of Amylase

AKADEMISK AVHANDLING

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ÅKE DANIELSSON

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ÅKE DANIELSSON

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The present thesis is based on the following publications, reference to which will be made by citation of the appropriate Roman numerals.

I **Danielsson, Å:** Techniques for Measuring Amylase Secretion from Pieces of Mouse Pancreas. **Anal. Biochem.** in press.

II **Danielsson, Å:** Effects of Glucose, Insulin and Glucagon on Amylase Secretion from Incubated Mouse Pancreas. **Pflügers Arch.** in press.

III **Danielsson, Å:** Effects of Nutritional State and of Administration of Glucose, Glibenclamide or Diazoxide on the Storage of Amylase in Mouse Pancreas. **Digestion** in press.

IV **Carlsöö, B., Danielsson, Å. and Helander, H. F.:** Effects of Starvation on Amylase Storage in Mouse Pancreas and Parotid Gland. A Biochemical and Morphometric Study. **Acta Hepato-Gastroenterol.** 21, 48, 1974.

V **Danielsson, Å. and Sehlin J.:** Transport and Oxidation of Amino Acids and Glucose in the Isolated Exocrine Mouse Pancreas: Effects of Insulin and Pancreozymin. **Acta Physiol.Scand.** in press.

VI **Danielsson, Å., Marklund, S. and Stigbrand, T.:** Effects of Starvation and Islet Hormones on the Synthesis of Amylase in Isolated Exocrine Pancreas of the Mouse. **Acta Hepato-Gastroenterol.** in press.

BACKGROUND

In the pancreas of the rabbit small accumulations of cells, different from the acinar cell, were described by Paul Langerhans in 1869. Later these accumulations were termed the islets of Langerhans and were found to constitute an endocrine gland which produces hormones active in the blood sugar homeostasis. In all species of birds and mammals the islets, which produce insulin, glucagon and probably gastrin, are dispersed throughout the exocrine parenchyma. This arrangement provides a very large area of contact between the two types of tissue, and it is natural to deliberate on whether the intimate relation between the endocrine and exocrine parts of the gland has any physiological implications. In the adrenal, another example of closely associated tissues with distinct secretory functions, the corticosteroids are considered important for the synthesis of adrenaline in the medulla (Wurtman, 1966).

Studies on the cat have shown that the exocrine pancreatic cells situated close to the islets display a pronounced granulation; the granule population is furthermore resistant to discharge by vagal nerve stimulation or cholinergic drugs (Sergeyeva, 1938). In the mouse these juxtainsular cells are larger, as are their nuclei and nucleoli and they also exhibit more nucleoli than those acinar cells which are located farther away from the islets (Hellman, Wallgren and Petersson, 1962). The morphological characteristics suggest that acinar cells in the periinsular region — the "halo zone" — have a higher functional activity than other acinar cells. This has been attributed to a high local concentration of insulin in the "halo zone" (Ferner, 1958). In support of this hypothesis the administration of alloxan — a β-cell cytotoxin — will abolish the "halo zone" phenomenon in rats (Kramer and Tan, 1968). Moreover, the duck pancreas contains two types of islets, only one of which produces insulin and is surrounded by a "halo zone" (Ferner, 1958; Wallgren and Hellman, 1962).

A high local concentration of insulin in the surrounding exocrine tissue could be due either to diffusion of the hormone from the islets or to a specialized arrangement of the vascular system. Reports in the relevant literature are rather contradictory where this matter is concerned. In a number of species, vascular connections have been demonstrated between the islets and the exocrine pancreas (Beck and Berg, 1931; Wharton, 1932; Ferner, 1958; Fujita, 1973; Fujita and Murakami, 1973). It has even been claimed that the blood supply to the exocrine pancreas is exclusively derived from the islet circulation (Wharton, 1932; Fujita, 1973). However, other authors have failed to demonstrate an intimate vascular connection between the two portions of the pancreas (Brunfeldt, Hunhammer and Skouby, 1958; Bunnag, Bunnag and Warner, 1963).

Diabetes mellitus is often accompanied by morphological changes in the exocrine pancreas (Warren, LeCompte and Legg, 1966). Furthermore, the total weight of the gland is reduced (Vartiainen, 1944) and the exocrine secretion is often impaired (Chey, Shay and Shuman, 1963; Vacca, Henke and Knight, 1964). Alloxan-diabetic rats display a decreased content of pancreatic amylase (Christophe et al., 1971). Ben Abdeljlil, Palla and Desnuelle (1965) and Palla, Ben Abdeljlil and Desnuelle (1968) reported that alloxan reduces the amylase content but increases the level of chymotrypsinogen. Daily injections of insulin restored the amylase content, but did not reverse the effect on the content of chymotrypsinogen. Administration of insulin to alloxan-diabetic rats has been reported to enhance the incorporation of labelled amino acids into rat pancreatic amylase (Söling and Unger, 1972) and total protein (Palla, Ben Abdeljlil and Desnuelle, 1968).

The main physiological stimuli of secretion from the exocrine pancreas are the gastrointestinal hormones. It is of interest that both cholecystokinin-pancreozymin (CCK-PZ) and secretin have also been found to stimulate insulin secretion (Dupré et al., 1969). Hinz et al. (1971) and Goberna et al. (1971) found that this effect on insulin release is dependent on an adequate function of the exocrine parenchyma.

Against this background of evidence suggestive of functional interrelationships between the endocrine and exocrine pancreas, it was decided to try and establish whether the islet hormones affect the synthesis and release of pancreatic enzymes in the mouse pancreas. Amylase is a major protein component of the mouse pancreas (Danielsson, Marklund and Stigbrand, 1974) and most authors have found that the different pancreatic enzymes are released concomitantly (Wormsley and Goldberg, 1972). For these reasons amylase was used as an index of the exocrine pancreatic function. During the course of the work, the scope of the study had to be widened to include various method developments as well as certain complementary morphological and biochemical studies on the pancreas. The main purposes of the investigation may be summarized as follows:

1. The development of methods for a) the microdetermination of amylase activity, b) the purification of pancreatic amylase, and c) the **in vitro** incubation of pancreas pieces.

2. The study of the effects of starvation as well as modifiers of insulin release on the synthesis and storage of amylase.

3. The study of the **in vitro** effects of insulin, glucagon and glucose on the synthesis and release of amylase.

4. The study of the **in vitro** effects of insulin, glucose and CCK-PZ on the oxidation of glucose and on the uptake and oxidation of alanine and leucine.

METHODS

ANIMALS AND EXPERIMENTAL CONDITIONS

All experiments were performed on pancreatic glands of mice obtained from a colony bred in our laboratory (Hellman, 1965). This stock carries a gene, **ob**, which in the homozygous state gives rise to a syndrome of obesity and hyperglycemia. However, in the present investigations only female mice of normal phenotype were used.

For the **in vivo** investigations (III, IV, VI) mice were given intraperitoneal injections of test substances, and the control animals received the same amounts of solvent. After starvation or treatment the animal was briefly etherized and decapitated. Blood was collected for serum analyses of glucose and insulin (III) and the pancreas was quickly removed. Tissue specimens for light and electron microscopy (IV) as well as for amylase determinations were collected. For the **in vitro** studies the glands were prepared in different ways according to the type of experimental design employed.

ASSAY PROCEDURES

Serum glucose was determined according to Lowry et al. (1964) by recording the fluorescence of $NADPH_2$ in a hexokinase/glucose-6-phosphate dehydrogenase system. Insulin was assayed radioimmunologically using separation of free and antibody-bound insulin with ethanol precipitation (Heding, 1966). A modification of the Kissane and Robins (1958) procedure was used to measure DNA fluorometrically. Quantitation of ^{14}C- and ^{3}H-labelled compounds was performed in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Amylase was determined using a micromodification of the dinitrosalicylate (DNS) method (I). Samples (10 μl each) were incubated at 25° C for 10 min with 10 μl 2 % starch solution. The reaction was interrupted by adding 20 μl DNS-reagent. After boiling for 10 min, the samples were diluted with 200 μl distilled water and were measured spectrophotometrically The results were calculated from a standard curve of maltose. One unit of amylase was defined as the enzyme activity liberating reducing groups corresponding to 1 μmol of maltose per min.

LIGHT AND ELECTRON MICROSCOPIC TECHNIQUES (IV).

The tissue samples were fixed in buffered 4 % glutaraldehyde, postfixed in OsO_4 and embedded in Epon. Pancreatic acini and cells were selected

from areas distant from the islets to avoid an influence on the measurements from the "halo zones". For quantitative light microscopy roughly 1 μ thick sections were lightly stained with toluidine blue and examined by phase contrast microscopy. In micrographs of randomly selected regions, the surface area and the number of granules within this defined area were estimated and expressed as granules per μ^2. For quantitative electron microscopy about 800 Å thick sections were contrasted and examined in the electron microscope. Exocrine cells which displayed a portion of the apical and basal cell surfaces as well as the nucleus were photographed. The volume densities of the secretory granules and of the nuclei were calculated using the point-counting method (Weibel and Elias, 1967). In addition, the secretory granule profiles were measured in a particle size analyser and the results used to calculate the mean diameter of the granules according to the method of Bach (1967).

BIOSYNTHESIS OF AMYLASE AND TOTAL PROTEIN (VI).

Pieces of microdissected exocrine pancreas were incubated in a supplemented Krebs-Ringer bicarbonate buffer also containing 20 amino acids according to Campagne and Gruber (1962), as well as L-(U-^{14}C)leucine (7.5 mCi/mmol). After incubation the tissue was homogenized by sonication in 50 mM phosphate buffer (pH 6.9) and amylase was isolated from other pancreatic proteins and from free L-(U-^{14}C)leucine in one step by isoelectric focusing in a thin layer of Ampholine^{R}-containing polyacrylamide gel. The amylase containing gel strip was cut out and dissolved in H_2O_2. Total pancreatic proteins were obtained by precipitation and repeated washing with TCA. Incorporation of L-(U-^{14}C)leucine into amylase and total protein was expressed in relation to the tissue content of DNA.

AMYLASE SECRETION

A Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with pyruvate, glutamate and fumarate as well as glucose and albumin was used for batch--incubations and perifusions. In the batch-incubation experiments the pancreas pieces were incubated in vessels specially designed for continuous equilibration of the medium with O_2-CO_2 (95:5) (I). Amylase activities were determined in incubation media and supernatants of pancreas homogenates, and related to the wet weight of incubated tissue. The dynamics of the release process were investigated in a specially designed perifusion system (I).

METABOLISM OF GLUCOSE AND AMINO ACIDS (V).

Pieces of exocrine pancreas, practically devoid of islets, were incubated in a Krebs-Ringer bicarbonate buffer. Oxidation studies were performed with different concentrations of ^{14}C-labelled D-glucose, L-alanine or L-leucine, as well as unlabelled test substances. After being trapped in hyamine the

produced $^{14}CO_2$ was measured. The uptake and transport of L-alanine and L-leucine were studied with ^{3}H-labelled amino acids using a double-label technique with ^{14}C-labelled L-glucose as an extracellular space marker. This made it possible to correct for extracellular and contaminating ^{3}H-amino acids without washing the tissue. The data were expressed as mmol substrate oxidized or taken up per kg dry weight of pancreas.

STATISTICS

In the **in vitro** experiments (I, II, III, V, VI) statistical significances were determined by computing **t**-values from the mean ± S.E.M. of the differences between paired test and control incubations in a series of repeated but separate experiments. In the **in vivo** studies (III, IV, VI) differences between group means were tested by the ordinary Student's **t**-test.

RESULTS AND DISCUSSION

CHARACTERISTICS OF THE **IN VITRO** INCUBATED EXOCRINE PANCREAS

Because the exocrine cells synthesize and secrete proteins at a high rate, the importance of oxygen and exogenous substrates for the adequate function of pancreas pieces **in vitro** was explored (I). Dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, or anoxia reduced the basal secretion and abolished carbamylcholine-stimulated release of amylase. Continuous equilibration of the medium with O_2-CO_2 (95:5) during the incubation was necessary to achieve optimum secretory responses to carbamylcholine. The secretory responses were larger and more reproducible in media supplemented with pyruvate, glutamate, fumarate and glucose than in media containing glucose alone. These results are consistent with the findings that aerobic glycolysis accounts for less than 10 % of the ATP production in rat exocrine cells (Bauduin, Colin and Dumont, 1969), and that the oxidation of D-glucose is fairly slow in the isolated exocrine pancreas of the mouse (V).

Using a system with continuous gassing of a supplemented buffer, both batch-incubated and perifused pieces of mouse pancreas responded to known cholinergic and gastrointestinal secretagogues. However, continuous stimulation of secretion from perifused mouse pancreas with CCK-PZ or carbamylcholine produced an initial rise of amylase release, followed by a decline in the release curve. Similar results were obtained by Matthews, Petersen and Williams (1973) after stimulation of the perifused rat pancreas with acetylcholine. Repeated pulse-stimulations seemed to be more effective in provoking amylase secretion (I). The reasons for the transient nature of the secretory response to physiological stimuli are not clear.

Stimulation of secretion by CCK-PZ was accompanied by an increased rate of oxidation of D-glucose, L-alanine and L-leucine (V). An enhanced oxidation of palmitate has previously been observed in CCK-PZ stimulated pigeon pancreas (Webster, Gunn and Tyor, 1966). The increased rate of oxidation of amino acids was not caused by an enhanced uptake, since this process was unaffected by CCK-PZ in the mouse pancreas (V). The oxidation of D-glucose in the exocrine cells was low compared to that of L-alanine and L-leucine (V).

EFFECTS OF STARVATION ON THE EXOCRINE PANCREAS

There has been a certain amount of controversy concerning the effects of nutritional state on protein synthesis in the pancreas. On the one hand, starvation was found to reduce the amylase content (Webster et al., 1972) and to inhibit the protein synthesis in the rat (Morisset and Webster, 1972) and the pigeon pancreas (Webster and Tyor, 1966); in the pigeon pancreas, starvation also induced a reduction of the polysome content and a reduced capacity of the polysomes to synthesize protein (Black Jr and Webster, 1973). On the other hand, Poort and Kramer (1969) claimed that the rate of protein synthesis in the mammalian pancreas is fairly unaffected by variation of the nutritional state including starvation. In the present study, starvation for 24 hrs clearly reduced the rate of incorporation of L-(U-^{14}C)leucine into pancreatic amylase and total protein (VI). This inhibition of the synthesis may explain why starvation also induced a considerable fall in the total amylase content (III) and decreased the number of granules per unit cell area (IV). Refeeding restored the amylase content within 12 hrs (III).

EFFECTS OF GLUCOSE AND ISLET HORMONES ON THE SYNTHESIS AND STORAGE OF AMYLASE

Starvation decreased the rate of amylase and total protein synthesis (VI) as well as the content of amylase (III) and zymogen granules (IV) in the pancreas. These effects may be due to the lowering of blood sugar, since the administration of glucose to starved mice increased the amylase content (III) and enhanced the synthesis of both amylase and total protein (VI). The capacity of injected glucose to increase the amylase synthesis and content was abolished by concomitant administration of diazoxide, a potent inhibitor of insulin secretion. It is therefore conceivable that the effects of glucose on amylase synthesis and content were mediated by stimulation of insulin release. **In vivo** administration of insulin has previously been shown to restore the reduced amylase content and the decreased rates of amylase and

protein synthesis in alloxan-diabetic rats (Palla, Ben Abdeljlil and Desnuelle, 1968; Söling and Unger, 1972). In non-diabetic rats, injection of insulin enhances both the secretion and the synthesis of rat pancreatic proteins. Couture, Dunnigan and Morisset (1972) proposed that these effects are not due to insulin acting directly on the pancreas but to hypoglycemia causing stimulation of the vagal nerve and the release of gastrointestinal factors. This hypothesis was not supported by the present studies, since stimulation of amylase synthesis and content was observed in hyperglycemic animals with increased serum levels of endogenous insulin (III, VI). On the other hand, studies on the **in vitro** effects of glucose, insulin and glucagon did not reveal any changes in amylase and total protein synthesis (VI), suggestig that the **in vivo** effects of glucose may not be mediated by a direct action of the sugar itself or of insulin on the pancreas. Reservations must, however, be made for the possibilities that the responsiveness of the pancreas may be altered by the incubation procedure and that the incubation time may be too short in relation to the conceivable latency of direct insulin effects.

EFFECTS OF GLUCOSE AND ISLET HORMONES ON THE SECRETION OF AMYLASE

In perifusions as well as in batch-incubation experiments, glucose rapidly inhibited both the basal and CCK-PZ-stimulated amylase secretion from the mouse pancreas (II).Insulin too, at very high concentrations, exerted a similar action. However, the glucose-induced reduction of secretion did not seem to be mediated by the release of insulin, as diazoxide did not counteract the effect of glucose. Further support for the independence of the glucose and insulin effects was obtained by studying secretion from microdissected pieces of pancreas (VI). The inhibition of secretion seems to be confined to the secretory process, since, as described above, glucose and insulin **in vitro** had no effect on amylase and total protein synthesis. Cyclic AMP has been shown to be an intracellular effector of pancreatic amylase secretion (Kulka and Sternlicht, 1968; Bauduin et al., 1971) and insulin decreases the content of cAMP of the liver (Exton et al., 1966). It is not known, however, whether insulin affects cAMP in the exocrine pancreas.

Glucagon did not affect the basal or CCK-PZ-stimulated amylase secretion from incubated pancreas (II). This is in contrast to results obtained **in vivo.** The administration of small doses of glucagon to man and dog induced a marked reduction of protein output from the pancreas during continuosly stimulated secretion (Dyck et al., 1969, 1970). The latter observation may perhaps be explained by the glycogenolytic action of glucagon, leading to hyperglycemia. The increased serum glucose level might reduce the discharge of pancreatic enzymes, either alone or in conjunction with the accompanying stimulation of insulin secretion.

EFFECTS OF GLUCOSE AND INSULIN ON THE METABOLISM OF THE EXOCRINE PANCREAS

Glucose inhibited the oxidation of L-alanine. This effect may have been due to the inhibition of uptake, which was also noted in the experiments (V). The rate of oxidation of glucose itself was relatively low compared to that of L-alanine and L-leucine (V). Insulin stimulated the oxidation of D-glucose but did not affect the oxidation of L-alanine or L-leucine. Insulin also failed to enhance the uptake of L-alanine and L-leucine **in vitro,** which is of interest in relation to the suggestion that insulin stimulates the synthesis of pancreas proteins **in vivo** by enhancing amino acid uptake (Söling and Unger, 1972).

SUMMARY AND CONCLUSIONS

1. a) A micromodification of the dinitrosalicylate method for measuring reducing groups made it possible to assay an amylase activity liberating less than 10 μg of maltose equivalents.

   b) By means of isoelectric focusing in Ampholine^{R}-containing polyacrylamide gels, amylase was highly purified from extracts of tissue samples with less than 1 mg dry weight.

   c) Routines were worked out for the incubation of pancreas pieces in closed vials and in a perifusion system for the study of secretory dynamics. Continuous equilibration with O_2-CO_2 (95:5) and the supplementation of incubation buffers with glucose, pyruvate, glutamate and fumarate were beneficiary for the magnitude and reproducibility of amylase secretory responses to established secretagogues.

2. Starvation decreased the synthesis and the pancreas content of amylase and diminished the population of zymogen granules. The decreases in amylase synthesis and content were counteracted by injections of glucose to starved mice. This effect of glucose was in turn abolished by injecting diazoxide, an inhibitor of glucose-induced insulin release, indicating that the glucose effect was mediated via the discharge of insulin.

3. Insulin, glucagon and glucose, when added to the incubation medium, failed to affect the synthesis of amylase. However, glucose reduced both the basal and the CCK-PZ-induced amylase secretion in a dose-dependent fashion. Insulin too, at a very high concentration, inhibited the secretion of amylase. The effects of glucose and insulin seemed to be mutually independent.

4. Insulin, at this high concentration, stimulated glucose oxidation without affecting the oxidation or uptake of alanine or leucine. CCK-PZ stimulated the oxidation of glucose, alanine and leucine in the pancreas pieces. Glucose inhibited the uptake and oxidation of alanine.

With regard to the overriding question that initiated the present study, it is concluded that the **in vivo** experiments, notably those demonstrating the effects of glucose and diazoxide on amylase synthesis and content, support the idea that insulin influences the specific functions of the acinar cells.

The **in vitro** experiments revealed that insulin and glucose reduced amylase secretion. If the latter finding holds true for the **in vivo** situation, glucose and/or insulin may well contribute to the maintenance of a high content of amylase in the exocrine pancreas. It is conceivable that the periinsular acinar cells are exposed to high concentrations of insulin. The observed effects of insulin on the biosynthesis and secretion of amylase might explain the appearance of "halo zones" around the islets. The failure of insulin and glucose to affect the **in vitro** biosynthesis of amylase may indicate that the **in vivo** effect of glucose is not a direct one or mediated by release of insulin.

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