IN VITRO REGENERATION AND DIFFERENTIATION OF THE EMBRYONIC PROTODIFFERENTIATED MOUSE PANCREAS

by

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INTRODUCTION

The embryonic development of the mammalian pancreas has been extensively characterized and temporally defined. As a result, this organ has become an excellent system for investigating organogenesis. Studies dealing with biochemical differentiation of the pancreas in the mouse and rat (Rutter, et al., 1964, 1968; Clark and Rutter, 1972; Ronzio and Rutter, 1973; Walther, et al., 1974; Sanders and Rutter, 1974; Pictet, et al., 1975; de Gasparo, et al., 1975), coupled with studies of morphological differentiation (Hard, 1944; Munger, 1958; Kallman and Grobstein, 1964; Parsa, et al., 1969; Wessells and Cohen, 1967; Wessells and Evans, 1968; Pictet and Rutter, 1972) have been important to the overall understanding of the cellular and molecular mechanisms which are involved in development.

The pancreas anlaga first arise as a result of an interaction between the gut endoderm and the surrounding mesenchyme (Spooner, et al., 1970; Wessells and Cohen, 1967) at 9.5 days of gestation in the mouse and 11 days of gestation in the rat. The dorsal pancreas appears at approximately the 20 somite stage of development immediately behind the rudimentary stomach (Wessells and Cohen, 1967), and the ventral pancreas appears about 12 hours later on the opposite side of the gut, where the biliary duct of the liver and the ventral wall of the gut unite (Spooner, et al., 1970; Pictet, et al., 1972). During this time, the mesodermal cells condense with the pancreatic diverticula. By 15 days in the mouse, acinar structures with central lumena are apparent, and shortly thereafter, zymogen granules and β-granules become visible in the acinar cells and presumptive islet tissue, respectively (Pictet, et al., 1972). By 16-17 days of ges-

tation in both rats and mice, the two pancreatic glands with their separate duct systems merge. The mature pancreas, then, consists of one organ. The exocrine component of this organ is a ramified system of tubules whose cells form a single secretory layer with a basal lamina and an apical lumen. The endocrine component is located as islets between the secretory acinar tissues and is surrounded by its own basal lamina.

The exocrine function of the pancreas is to produce enzymes, fluids, and electrolytes to aid in digestion. The pancreas produces these enzymes in zymogen form so they may be safely secreted, and later, activated in the duodenum. These hydrolytic enzymes include trypsinogen, chymotrypsinogen, procarboxypeptidase A and B, ribonuclease, deoxyribonuclease, lipases A and B, and amylase (see Guyton, 1971). The endocrine function is to produce hormones which regulate metabolism. Both insulin, contained in β-granules, and glucagon, contained in α-granules, are known to be produced in the pancreas (see Unger, 1974). There is also evidence that additional hormones may be located in the pancreas (McGuigan, 1972; Kimmel, et al., 1975; Arimura, et al., 1975).

The study of the accumulation of these enzymes and hormones, particularly by Rutter and his colleagues (Ronzio and Rutter, 1969, 1973; Bradshaw and Rutter, 1972; Clark and Rutter, 1967, 1972; Kemp, et al., 1972; Rutter, et al., 1968; Sanders and Rutter, 1975) have shown that pancreas development can be divided into three states of temporal development. The first state, the predifferentiated state, represents a stage just prior to the time when the pancreas diverticulum arises from the gut endoderm. No specific products can be detected during this period of development. Following this state, insulin and lipase A specific activities rise to a low, but detectable plateau. Initial appearance of these molecules correlates

with initial elevation of the pancreatic diverticulum. It is suspected that the specific activities of other exportable proteins also rise at this time. However, the assays for these proteins have not reached the required sensitivity for detection. These enzyme specific activities, then, remain stable over a definite time period, usually lasting a few days. This plateau, the protodifferentiated state, has been substantiated for other enzymes such as procarboxypeptidase A, chymotrypsinogen, ribonuclease, and amylase (Rutter, et al., 1964, 1968). The protodifferentiated state is followed by another phase, where the specific activities of these enzymes and insulin increase several orders of magnitude until a terminally differentiated state is reached.

The protodifferentiated state is the focal point of the present study. While previous investigations have dealt mainly with the bulbous region of the developing pancreas (Golosow and Grobstein, 1962; Rutter, et al., 1964; Wessells and Cohen, 1967; Ronzio and Rutter, 1973), we now have information implicating the neck region (i.e., the primitive duct) as an important component in its chemical and morphological differentiation. In this paper we report in vitro studies demonstrating that the duct is capable of regenerating a new dorsal pancreas if the original is removed during the protodifferentiated state. This regenerated pancreas differentiates both exocrine and endocrine tissue, and, furthermore, is not retarded in its temporal developmental program when compared with controls. Finally, we have also explored the tissue mass requirements for differentiation in the bulbous and duct regions of protodifferentiated pancreas.

LITERATURE REVIEW

The pancreas has become important in the analysis of the development of normal organ systems. This has come about through extensive and thorough studies into the differentiation of the endocrine and exocrine components of this system. The pancreas, as a whole, has been recognized as a major organ of the body for many centuries. However, the endocrine cells of the pancreas were not discovered until 1869, when Langerhans observed these cells in an islet arrangement amid the exocrine acinar cells. Confusion existed as to whether these islet cells were epithelial or mesenchymal in origin until 1893, when Laguesse (1893) determined through a major study of the developing pancreas, that the islets were epithelial in origin. Since this time, many investigations have been undertaken using increasingly more sophisticated techniques in the analysis of the morphology and cytochemistry of the pancreas.

Many later studies emphasized the endocrine component of this organ. Hard (1944), using light microscopy to examine developing rat pancreas, and Munger (1958), using light and electron microscopy to examine mouse pancreas, were able to determine the approximate gestational age at which endocrine cells begin to differentiate. Hard noted that a few islets were discernable in the 13-day embryo in the rat, but that the majority did not appear until day 16-18. This majority seemed to be derived from the pancreatic tubules, while those which appeared earlier were located in the dorsal lobe of the pancreas only, shortly before the ventral and dorsal lobes of the pancreas fused on day 14 of gestation. Munger, using mouse embryos, also observed differentiated islet tissue at 13-14 days of gestation, and believed these β-granule containing cells to be B-cells.

Both investigators noted differentiated A-cells, containing α-granules, after birth.

There were rapid advances in the knowledge of both endocrine and exocrine pancreas differentiation in the 1960's. Golosow and Grobstein (1962) demonstrated that as in salivary gland, kidney, thymus, and other organs, the normal development of the pancreatic rudiment depended upon an interaction between the epithelium and the surrounding mesenchyme. However, unlike most endodermally derived organs, pancreatic growth was easily supported by kidney, lung, stomach, and salivary mesenchyme, as well as pancreatic mesenchyme. This development resembled that which occurred in vivo, and a direct association of the epithelial and mesenchymal components was not required, as normal in vitro development also occurred when the tissues were placed upon opposite sides of a Millipore filter during the culture period. Kallman and Grobstein (1964) followed the development of mouse pancreas isolated from 11-day embryos and used 13-day salivary mesenchyme transfilter to support differentiation. Their finding concurred with those of previous investigations; early exocrine cell differentiation was first apparent in the changes in the endoplasmic reticulum and its associated particles, followed by an expansion in the cisternae of the endoplasmic reticulum. Rutter, et al. (1964) demonstrated that the amylase specific activity began to rise sharply at the time the cisternae expanded and continued to rise as zymogen granules began to appear.

These data, as well as further data on the accumulation of other exocrine and endocrine products of the pancreas, led to a proposal by Rutter and his colleagues that pancreatic cell differentiation occurred in a bimodal pattern (Clark and Rutter, 1967, 1972; Rutter, et al., 1968; Ronzio and Rutter, 1969, 1973; Kemp, et al., 1972; Bradshaw and Rutter,

1972; Sanders and Rutter, 1975). The primary state of the differentiation process was referred to as the predifferentiated state. This state appeared prior to the budding of the pancreas primordium from the gut epithelium at 9.5 days of gestation in the mouse (Wessells and Cohen, 1967). It was followed by a phase characterized by a sharp increase in the specific activities of insulin (Clark and Rutter, 1967) and lipase (Bradshaw and Rutter, 1972), until a low, constant level was reached. (The assays for other exocrine enzymes have not yet reached such high sensitivity that this increase may be investigated for other exocrine products as well.) The low plateau level, called the protodifferentiated state, remained stable from day 10 through day 13 of gestation until there was a major increase in the specific activities of insulin and the enzymes amylase, carboxypeptidase, trypsin, chymotrypsin, ribonuclease, and lipase. A terminally differentiated state was reached at day 20-21, around the time of parturition (Rutter, et al., 1964). From this time on, these levels were modulated by the requirements made on the pancreas by the individual (see Unger, 1974; and Preshaw, 1974).

It was interesting to note that the glucagon specific activity did not follow the same pattern as that for insulin and exocrine enzymes (Rall, et al., 1973). The specific activity of glucagon was 100 times higher than that of insulin at the onset of pancreas organogenesis, and the level of glucagon expressed as molecules per cell remained fairly stable throughout development. This not only suggested a regulatory role for glucagon in embryonic development, but also agreed with the findings of Pictet et al. (1972) that the differentiated endocrine cells present at the onset of pancreas organogenesis were A-cells, and not B-cells as had been previously proposed.

Wessells and Evans (1968) and Wessells and Cohen (1967) gave complete descriptions of the early stages of pancreas organogenesis. Wessells and Cohen (1967) demonstrated that the ability to form exocrine pancreas under in vitro conditions existed in the gut endoderm by the 8 somite stage, and salivary mesenchyme could support pancreas morphogenesis by approximately 15 somites, even though the dorsal pancreatic rudiment did not appear until about 22 somites. Spooner, et al. (1970) demonstrated in the rat, that the ventral pancreas and dorsal pancreas followed identical patterns of morphologic and biochemical differentiation, although the ventral rudiment did not appear until 12 hours (29-30 somites) after the dorsal rudiment. They also determined that the capacity to form both pancreases was present by 12 somites.

Wessels and Cohen (1967) further determined that fractionation of the growing dorsal epithelium during the protodifferentiated state into smaller pieces, postponed the appearance of zymogen granules or amylase specific activity. However, artificial fusion of pancreatic rudiments giving a 24-fold increase in the mass of protodifferentiated epithelium, did not promote precocious differentiation. It was suggested that the cells would not differentiate because they were insufficiently mature and had been disturbed, stimulating cell mitosis. Differentiation could not occur unless the tissues were left undisturbed after reaching a specific chronological age. Further data of Wessells (1964) showed that mitosis preceded cytodifferentiation in the pancreas, first in the interior of the developing bulbous region, and then in the periphery of the bulbous region. Interestingly, Pictet and Rutter (1972) presented ultrastructural evidence demonstrating the ability of differentiated exocrine and endocrine cells to undergo mitosis.

Another study by Wessells and Cohen (1968) which involved the developing

embryonic pancreas, investigated the effects of collagenase on lung, ureteric bud, and pancreatic epithelia. Although lung morphogenesis terminated and ureteric buds lost their characteristic shape in the presence of collagenase, the pancreatic epithelia were unaffected. This suggested that collagen was necessary for epithelial stabilization in lung and ureteric bud development but was not necessary for pancreatic development.

Hypotheses for the initial formation of the pancreatic diverticula were presented by both Wessell's and Rutter's groups. Wessells and Evans (1968) proposed that the arrangement of filaments in the various elongated cells making up the rudiment at this time, suggested a contractile model for the constriction of the apical ends of pancreatic cells. It was also proposed by Rutter and his colleagues (Pictet, et al., 1972) that an increase in cell number in the gut epithelium at the time the pancreas diverticulum arose from the gut wall, caused lateral pressure, resulting in a slight deformity or out-pocketing in the cell layer. Further mitosis increased the size of the digitation. They also hypothesized that this same process could be repeated for the formation of further digitations in the pancreatic rudiment.

It was demonstrated by Rutter, et al. (1964) that an extract obtained from chick embryos was capable of allowing normal pancreatic development in the absence of suitable mesenchyme. This mesenchyme factor (MF) was partially purified by Ronzio and Rutter (1973), and the ability of the partially purified MF to stimulate the incorporation of ^{3}H-thymidine into DNA of developing pancreas was determined. MF increased the incorporation of ^{3}H-thymidine into DNA 5-8 fold over the initial rates in the absence of MF or mesenchyme. Incorporation of ^{14}C-uridine into RNA and ^{3}H-leucine into protein also increased several fold. Other effects included a 2-3

fold increase in DNA polymerase activity, and a 100-fold increase in amylase activity. Levine, et al. (1973) demonstrated that MF acted at the cell surface, and further investigations by Pictet, et al. (1975) and Filosa, et al. (1975) determined that while cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) neither inhibited nor potentiated the action of MF, dibutyryl cAMP had the ability to reactivate MF which had been deactivated by treatment with sodium periodate and to potentiate the action of active MF. However, dibutyryl cAMP, dibutyryl cGMP, cAMP or cGMP, in combination or used separately, could not replace MF action. This data suggested that the activity of MF was in some way cAMP dependent and that the adenyl cyclase activation site of MF could possibly be altered by periodate oxidation.

Another investigation examined the effects of 5-bromodeoxyuridine on the developing exocrine pancreas (Walther, et al., 1974). This investigation proposed that the inhibition of differentiation in 5-bromodeoxyuridine-treated pancreatic rudiments was caused by an alteration in the ability of regulatory proteins to bind properly to DNA. Recently, other investigations stemming from Rutter's laboratory have dealt mainly with the effects of specific secretagogues such as glucocorticoids, caffeine, cholera toxin, dibutyryl cAMP, glucagon, and glucose on exocrine and endocrine pancreatic development and embryonic secretion (de Gasparo, et al., 1975; Rall, et al., 1975). These studies have demonstrated the ability of the embryonic pancreas to be stimulated by molecules which are either in the embryo during development, or are known to affect differentiated adult tissues.

The regulatory mechanisms involved in the differentiation of the pancreas into exocrine and endocrine populations still constitute a basic unsolved problem. How is it that the variety of cells within the endocrine

and exocrine tissues can arise from such a limited cell population? Pictet and his colleagues (Pictet, et al., 1974) have attempted to answer this question by analyzing the effects of MF on the relative proportion of endocrine and exocrine acinar cells in the differentiated pancreas. They have determined that epithelial rudiments in the absence of MF and mesoderm develop unusually high numbers of endocrine cells (greater than 50% of the combined exocrine and endocrine cell population as opposed to approximately 6% in vivo). MF is implicated, once again, as an important determining factor in pancreas morphogenesis and cytodifferentiation. Further analysis of the interaction of MF with cell surface components of embryonic pancreas prior to differentiation will be beneficial in understanding the plasticity and stability of differentiating organ systems. Once this has been determined, a major step will have been made in understanding the regulatory mechanisms involved in embryonic development.

MATERIALS AND METHODS

Animals, Dissections, and Media.

Tissues used in this study were dissected from CD-1 (Charles River Mouse Farms, Wilmington, Mass.) albino mouse embryos. Female mice were mated overnight with males, and the following morning was designated as day 0. After 9, 10 or 12 days of gestation, pregnant females were sacrificed by cervical dislocation, and the uterus with the embryos was quickly removed to sterile Hank's salt solution. Embryos were dissected from the uterus in a plastic petri dish and transferred to a glass petri dish containing sterile Hank's solution and staged according to somite number. The correlation between somite number and hours of development has been previously published by Wessells and Cohen (1967). Primitive guts were excised with iridectomy knives according to the procedure of Wessells and Cohen (1967) and Spooner, et al. (1970). The heads, anterior to the first pharyngeal pouch, were first removed from the embryos, followed by the somites with the aorta, the heart, and the lateral body walls. Excess cardiac tissue, and as much of the primitive liver tissue as possible, was also carefully removed. In 9 and 10 day embryos, the remaining whole gut with its surrounding mesenchyme was cultured on a Millipore filter (Millipore Filter Corporation, Bedford, Mass.) cemented over the top of a hole drilled through a thin Plexiglas slide. Appropriate portions of the whole gut for the experiments employing 12 day embryonic tissues were also cultured on the Millipore filter assemblies. The culture assemblies were first sterilized in 70% ethyl alcohol for 15 minutes, and rinsed in sterile Hank's solution 6 times before they were

placed in sterile Grobstein culture dishes (Grobstein, 1956). The wells of the dishes were filled with medium. Tissues were transferred to the upper surface of the Millipore filter with a sterile micro-tip pasteur pipette, and the culture dish was covered and incubated at 37° C in a water-saturated atmosphere of 5% CO_2 in air. All cultures were fed daily by removing the medium with a suction apparatus and replenishing the filter wells with fresh sterile medium.

The medium used was a modification of Ham's F12 (Cahn et al., 1967) containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), 2x amino acids and pyruvate and supplemented with penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively, Grand Island Biological Co.), and Amphotericin B (5.0 µg/ml, Fungizone, Grand Island Biological Co.). This medium has been used by Spooner and associates to culture chick thyroid epithelium (Spooner, 1970), rat whole guts (Spooner, et al., 1970), and mouse lung epithelium in various recombinations with mesenchymal tissue (Spooner and Wessells, 1970).

When required, the dorsal pancreas of each whole gut was removed with its surrounding mesenchyme after an appropriate number of days in culture. In most cases, the ventral pancreas was undisturbed so that it might be used as a control. It has been substantiated by Spooner et al. (1970) that the dorsal and ventral pancreases exhibit identical patterns of morphological and biochemical differentiation.

Protein and Amylase Assays.

Tissues to be assayed for amylase activity and for protein content were harvested in the following manner. The filter assemblies containing the cultures were placed in a shallow dish of Hank's salt solution. Using

iridectomy knives, the culture was gently peeled from the filter and the control or regenerate pancreas was dissected from the remainder of the culture. The rudiments were then transferred to a microfuge tube, centrifuged for a few seconds in a Beckman 152 microfuge, and the Hank's solution was absorbed from the tube using a twisted Kimwipe. The tissues, in the microfuge tube, were either frozen at -20° C for assay at a later time or were placed in an ice bucket for immediate assay.

For disruption of the tissues and enzyme activation, 50, 100, or 150 µl of deionized glass-distilled water was added to the microfuge tubes and they were taped to the bottom of a large beaker with water and ice added to cover the microfuge tubes. The tip of the sonicator (Sonifier Cell Disruptor W185, 185 watt, Heat Systems-Ultrasonics, Inc., Plainview, L. I., N. Y.) on output control setting 7 was passed along the tube for 30 sec to 1 min. The tubes were placed in ice.

The Rutter (1967) modification of the Lowry protein assay (Lowry, et al., 1951) was used for protein determination. The following solutions were required for the protein assay:

Solution A: 20 gm Sodium carbonate, 4 gm Sodium hydroxide, 0.2 gm Sodium potassium tartrate dissolved in 100 ml water.

Solution B: 0.5 gm $CuSO_4 \cdot 5\ H_2O$ dissolved in 100 ml water.

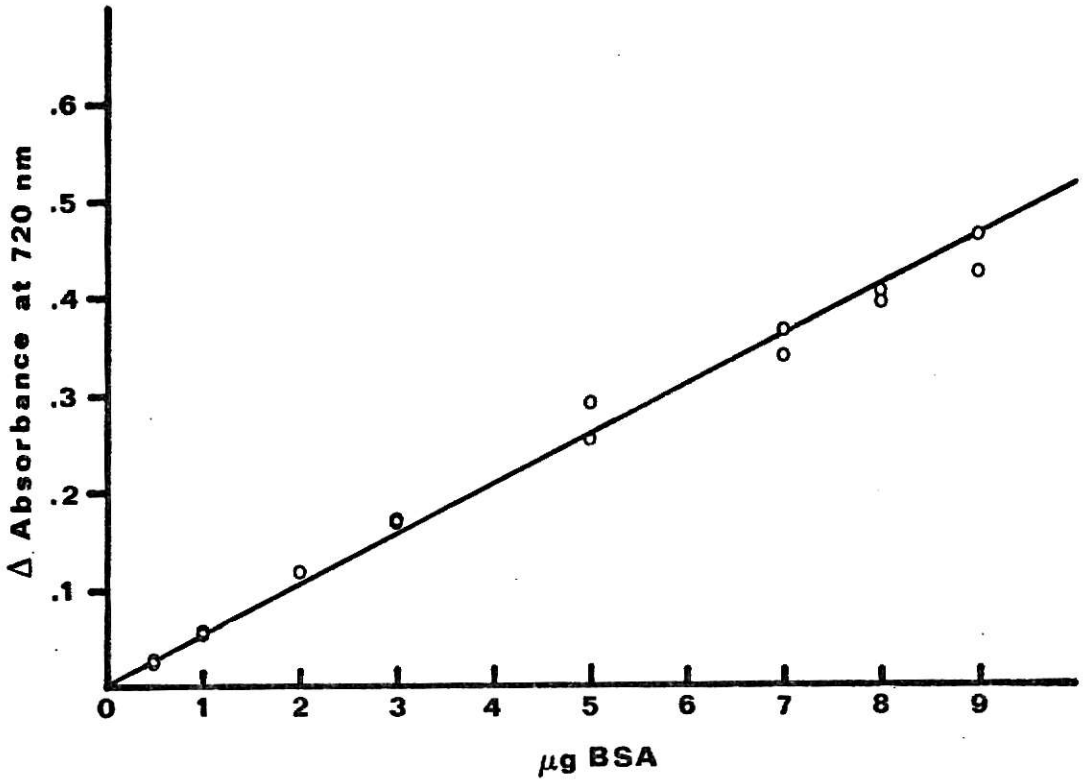
Solution C: 50 parts Solution A mixed with 1 part Solution B immediately before use. This solution should be discarded after 1 day.

Solution D: Dilute the Folin-Cicalteau reagent (Folin and Cicalteau, 1927, Fisher Scientific Company) 1:1 with H_2O immediately before use.

Using crystalline bovine serum albumin (BSA; Sigma) ($E^{1\%}_{1\ cm} = 6.6$ at 280 nm), a series of standards ranging from 0 to 9 µg of protein in 50 µl distilled deionized water proved to give a linear protein curve (Fig. 1)

Fig. 1. A representative protein standard curve. Two sets of standards of increasing concentrations of bovine serum albumin (see text) were determined for each assay. °, optical density recorded at 720 nm.

Fig. 2. A representative maltose standard curve for the amylase assay. Two sets of standards containing increasing quantities of maltose (see text) were determined with each assay. °, optical density recorded at 500 nm.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5
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7
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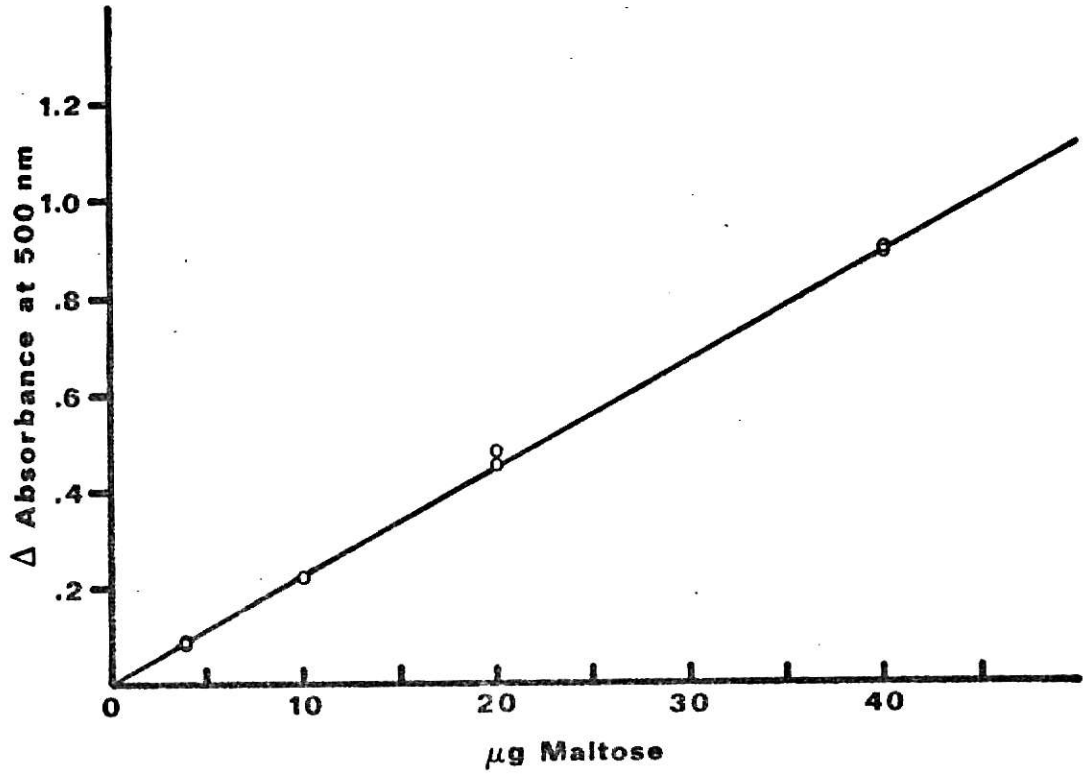
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.6
.4
.2
0
10
20
30
40
μg Maltose

for sample determinations. Samples ranging from 5 to 40 µg (precise amounts were determined from the results of previous assays) were removed from the sonicate and placed in a small test tube. Adequate deionized distilled water was added to bring the total volume to 50 µl. 50 µl of the appropriate bovine serum albumin dilutions were also added to different test tubes. From this point on, both standard and sample tubes were treated alike. To these tubes, 250 µl of Solution C was added. After 30 min, 25 µl of Solution D was added. Following an additional 45 min, absorbance at 720 nm was determined on a Zeiss spectrophotometer (Carl Zeiss, Inc., New York, N. Y.).

Amylase was assayed using a micromodification (Sanders and Rutter, 1974) of the Bernfeld assay (Bernfeld, 1955). The following solutions were required for the amylase assay:

Buffer: 0.05 M Histidine monohydrochloride, pH 6.5.

Maltose: 2 mg/ml in 0.05 M Histidine HCl buffer.

Starch: 1.0% soluble starch in 0.05 M Histidine HCl buffer; add starch to buffer and place it in a boiling water bath until it dissolves. This solution deteriorates and must be closely checked by running a starch "blank" with each assay.

DNA: Dissolve 1 gm 3,5-Dinitrosalicylic acid in 20 ml of 2.0 N NaOH and 50 ml H_2O. Add 30.0 gm Rochelle salt (NaK tartrate) and add sufficient water to 100 ml.

Standards were made by placing increasing amounts of maltose in a small test tube and adding adequate buffer to bring the total volume to 25 µl. This standard curve is linear for 0 to 40 µg of maltose (Fig. 2). A starch blank, containing no maltose, was used with each assay. Sample tubes of 5 to 20 µl were taken from the sonicated tissues and the volume

was brought to 25 µl total with buffer. A volume of 25 µl of starch was added to both standard and sample tubes and they were immediately incubated for 3 to 10 min at 37° C in a circulating water bath (Aquatherm, New Brunswick Scientific Co., Inc., New Brunswick, N. J.). Upon removal from the water bath, 50 µl DNS was added immediately with mixing and the tubes placed in ice. They were then placed in a boiling water bath for 5 min, cooled, and 0.5 ml distilled water was added. Absorbance at 500 nm was determined and a graph was plotted. Specific activity is expressed as µg of maltose hydrate released per min per µg of protein. Controls including either boiled sonicate plus starch or native sonicate minus starch exhibited no amylase specific activity.

Light and Electron Microscopy.

Living cultures were viewed and photographed on a Carl Zeiss Photomicroscope II (Carl Zeiss, Inc.).

To prepare specimens for examination by electron microscopy, tissues were gently peeled from the filter assemblies 1 day before they were to be fixed. They were placed in 60 mm plastic tissue culture dishes (Falcon Plastic, Div. of B. D. Laboratories, Los Angeles, Calif.) containing medium and reincubated. This allowed the tissues to adhere to the bottom of the dish before fixation so they could be oriented later on for sectioning. The following day, the medium was removed and the cultures were immediately fixed in 2% glutaraldehyde in 0.07 M Sorenson's phosphate buffer, or in 3% glutaraldehyde in 0.1 M cacodylate buffer for 1 to 2 hours at room temperature. The tissues were washed twice in buffer, for 10 min each, with a 3rd wash lasting for 16 hrs at 4° C, and then post-fixed with 1% OsO_4 for 1 hr in either cacodylate or phosphate buffer, at 4° C. Tissues were washed twice, for 5 min each, in cold distilled water.

Dehydration was accomplished with a graded ethanol series of 50% ethanol, 70% ethanol, 80% ethanol, and 95% ethanol (7 min each at 4° C), and the tissues were allowed to come to room temperature with the first of 2 ten min washes in 100% ethanol.

The alcohol was removed and the embedding resin (Luft's Epon; Luft, 1961; 15 ml Epon 812-DDSA to 35 ml Epon 812-NMA with the addition of approximately 0.75 ml DMP-30) was added to a depth of 5 mm to the petri dishes. The dishes were placed in a vacuum oven for 30 min to remove all remaining alcohol from the tissues before cultures were polymerized 48 hr in a 60° C oven.

After polymerization, the Epon was carefully removed from the plastic petri dish and the embedded tissues were cut from the Epon with a jeweler's saw. Tissues were cemented to flat Epon blocks with Duco Cement (Dupont Corp.). Sectioning was done on a Reichert OM-2 Ultramicrotome (Reichert OmU2, Wien, Austria). Sections were stained with uranyl acetate in 50% ethanol (pH 5.0) and with Reynold's (1963) lead citrate (pH 12.0) and viewed on a Philips 201 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.) operating at 60 kv.

RESULTS

In Vitro Development.

The dorsal pancreas was chosen for these studies because of the ease with which it can be viewed and photographed. Although the ventral pancreas parallels the dorsal pancreas in its temporal development both *in vivo* and *in vitro* (Spooner, et al., 1970), difficulties arose in ventral pancreas manipulations because of the close association of the ventral pancreas with the bile duct, particularly under *in vitro* conditions, illustrated in Fig. 3. Whole guts (gut endoderm plus the surrounding mesenchyme) were isolated at 9 days of gestation, the day the dorsal pancreas first makes it appearance from the gut wall. After 2 days in culture, the dorsal pancreas was clearly visible as a bulbous structure (Fig. 1A). The ventral pancreas, which appeared approximately 12 hours later than the dorsal pancreas, budded from the bile duct on the opposite side of the gut from the dorsal pancreatic bud. A more detailed description of mouse dorsal pancreas organogenesis is given in Wessells and Cohen (1967).

Pancreas Regeneration.

The protodifferentiated state of mouse dorsal pancreas development begins at approximately day 10 and terminates after day 13 of gestation (Rutter, et al., 1964). During this time, the amylase specific activity in the pancreas of the outbred CD-1 mouse remains at a low, constant level of 0.01 µg of maltose hydrate released/min/µg of protein at 37° C (Spooner et al., 1976). This is comparable to the protodifferentiated amylase specific activity obtained by Wessells and Cohen (1967) for the B/ALB C and C3H mouse cross and by Sanders and Rutter (1974) for Sprague-

Fig. 3. Development *in vitro* of the dorsal and ventral pancreas in the mouse. This whole gut was placed in culture at 9 days of gestation. Photograph A was taken after 2 days of culture, during which time the rudimentary organs had begun to form. Photographs B, C, and D were taken at 4, 6 and 8 days of culture, respectively. During the culture period, both dorsal and ventral pancreases increased greatly in size, and by day 8, equivalent to 17 days *in vivo*, they exhibited a slight opacity due to zymogen granule accumulation. The dorsal and ventral pancreases do not fuse *in vitro* as they normally would *in vivo*. DP, dorsal pancreas; VP, ventral pancreas; S, rudimentary stomach; I, rudimentary intestine (67 X).

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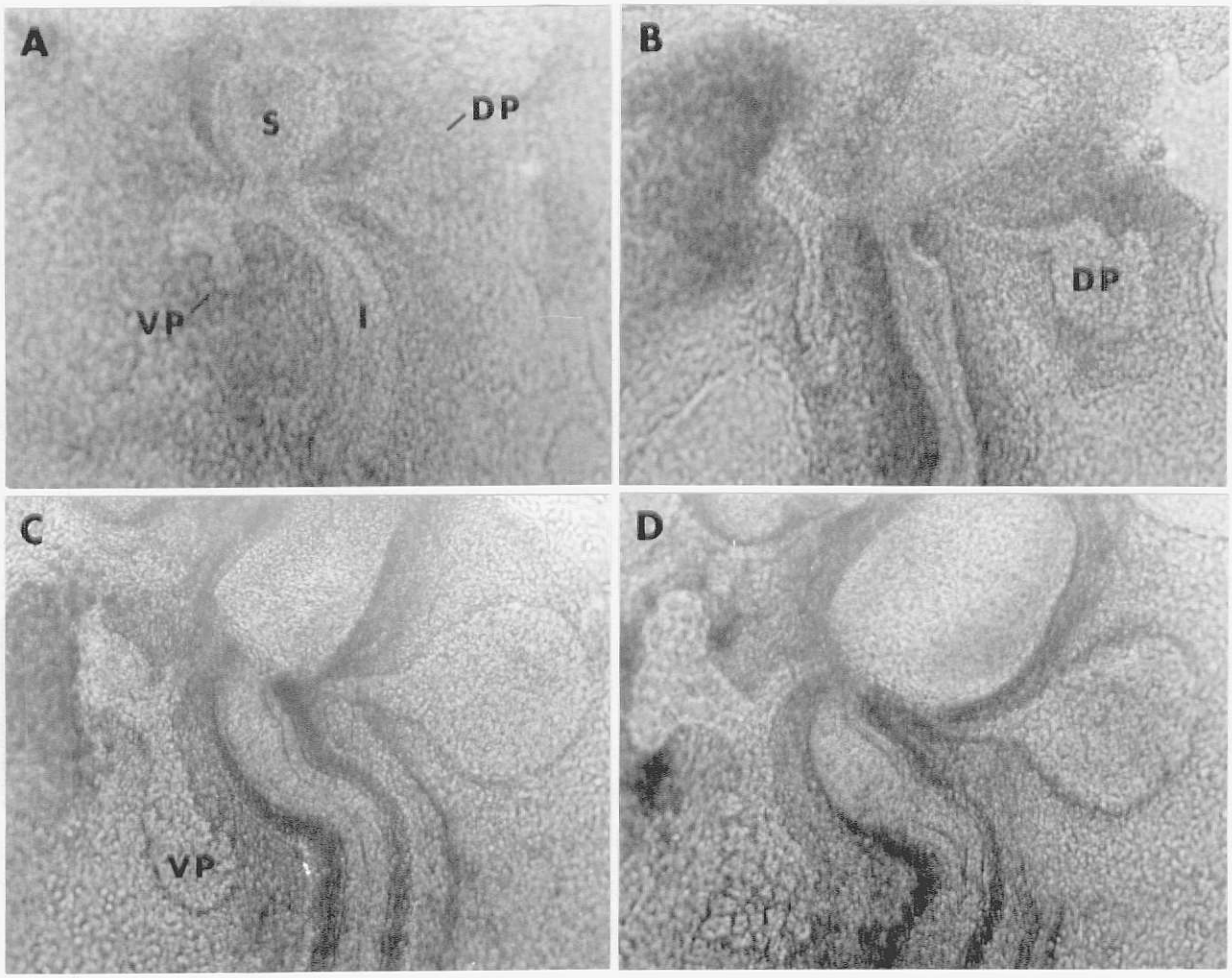
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Dawley rats. In an experiment originally aimed at exploring the influence of the dorsal pancreas on ventral pancreas development, the protodifferentiated dorsal pancreas was surgically removed from whole gut cultures. Unexpectedly, the remaining pancreatic duct regenerated a new pancreas. This observation is illustrated in Fig. 4. Fig. 4A shows a whole gut isolated from the embryo at 9 days of gestation, and grown in culture for 3 days (now equivalent to 12 days of gestation in vivo). The dorsal pancreas was removed on this third day of culture with iridectomy knives and discarded, leaving only the duct (Fig. 4B). Over the next 5 days, the duct regenerated a new pancreas. This new pancreas, and the control ventral pancreas, acquired an opacity due to zymogen granule formation.

Further experimentation has indicated that this phenomenon is probably restricted to the protodifferentiated state in pancreas development. Table 1 illustrates that the whole gut may be removed from the embryo after 9 or 10 days of gestation, and as long as the time of incubation before the cut is made and the pancreas removed is no longer than that which would make it equivalent to 12 or 13 days of normal gestation, the duct will regenerate a new pancreas. The likelihood of this occurring after an equivalency of 14 days in vivo is reached, decreases sharply (Table 1).

Those experiments which began with a day 12 embryo were handled in a slightly different manner from those which began with 9- and 10-day embryos. Only the dorsal pancreas, with a small portion of the adjoining gut endoderm, was isolated from these embryos. Immediately thereafter, the bulbous region of the pancreas was removed and placed on a Millipore filter assembly to serve as a control, while the duct region with the connected gut tissue was placed on a separate Millipore filter assembly. Again, a new pancreas regenerated from the duct. This result showed that

Fig. 4. Regeneration of the dorsal pancreas. This whole gut was placed in culture at 9 days of gestation. Photograph A demonstrates the appearance of the culture after 3 days of incubation. On this third day of culture, the dorsal pancreas was removed, leaving only the duct (B). One day following the removal of the original pancreas (C), the duct appeared rounded, and over the next 5 days (D, E, F, G, H) a pancreas regenerated from the duct. DP, dorsal pancreas; PD, pancreatic duct; RP, regenerating pancreas (67 X).

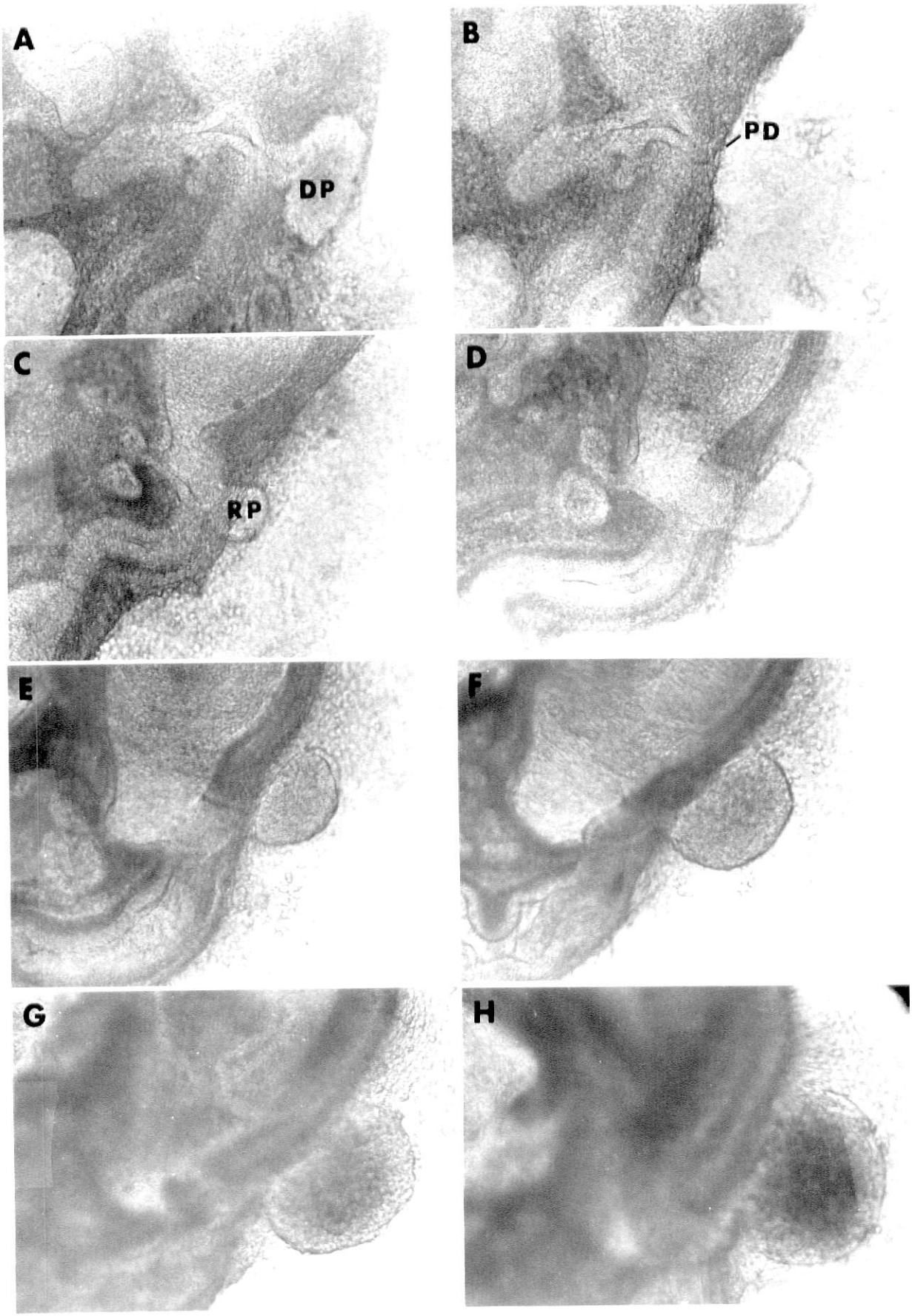

A
DP
B
PD
C
RP
D
E
F
G
H

TABLE I

REGENERATION OF EMBRYONIC DORSAL PANCREAS *IN VITRO*

Embryonic Age in Days of Tissue Explanted to Culture	Day of Culture when Pancreas is Removed (*In Vivo* Equivalent)	Days of Regeneration after Surgery	Total # of Guts	Number of Regenerates
9	3 (12)	5	43	37
10	2 (12)	5	18	15
12[a]	0 (12)	5	31	25
9	4 (13)	4	22	19
10	3 (13)	4	28	17
9	5 (14)	3	16	1

[a]Only the duct with the adjoining gut endoderm was placed in culture. The control tissue was dorsal pancreas removed from the duct and placed on a separate Millipore filter assembly for culture.

a preculture period was not required for the regeneration, and that this process did not require the presence of the stomach, the ventral pancreas, and the major portion of the forming intestine caudal to the dorsal and ventral pancreas (Fig. 5).

Regeneration would also occur if there was a 3-day preincubation period before removal of the stomach, ventral pancreas with the bile duct, and the gut endoderm. In this experiment, whole guts were isolated from 9 day embryos. Following the 3 day preincubation, the above mentioned tissues plus the dorsal pancreas were removed, leaving only a bit of gut and the attached duct (Fig. 6). Portions of salivary mesenchyme taken from 13 day embryos were added to the Millipore filters of 10 cultures to insure an adequate amount of mesenchyme conducive to differentiation. This method has been used to support pancreas differentiation in the past (Wessells and Cohen, 1967). No salivary mesoderm was added to the remaining 2 cultures. In 5 of the 10 cultures to which salivary mesenchyme was added, a new pancreas appeared and grew from the remaining pancreatic duct tissue. There was no obviously visible regenerate apparent in the remaining 5 cultures, although the endodermal tissue remaining on the filter frequently took on an opacity and became bubbly in appearance. In those tissues in which no salivary mesenchyme was added, 1 of 2 cultures definitely regenerated a new pancreas.

The importance of the presence of the duct was examined by totally removing all pancreas tissue including the duct from cultures isolated at 9 days of gestation and incubated for 3 days. In 11 cultures, no pancreas replaced the original over the succeeding 5 days of culture. This leads to the conclusion that the duct is required for regeneration.

Amylase Specific Activity Comparison.

A major question which now arises deals with the differentiated state of these regenerated dorsal pancreases. Do these regenerates contain the exocrine and endocrine products which appear in a normal pancreas? To investigate this question, the amylase specific activities of both control (pancreas tissue left undisturbed for the entire culture period) and regenerated pancreas were measured.

Guts were first isolated at 9 days of gestation, allowed to grow 3 days before the dorsal pancreas was removed (equivalent to 12 days of gestation in vivo), and then continued another 5 days in culture before the pancreases were harvested and assayed (Table II). Remarkably, regenerates and controls exhibited the same amylase specific activity. This was a 240-fold increase over protodifferentiated levels of enzyme activity. The level in the regenerated pancreas had been expected to be 10- to 100-fold less than the control pancreas, as the control had almost 3 days "head-start" on the regenerate. These data demonstrate, however, that the regenerate is not retarded in its development when compared with undisturbed control pancreas, as monitored by amylase specific activity.

These data were further supported by analyzing the amylase specific activity of control and regenerated pancreas first cultured at 12 days of gestation. Here, there was a 420-fold increase over protodifferentiated levels of amylase specific activity, and both regenerate and control cultures exhibited similar specific activities, as before (Table II). This also revealed that the stomach, ventral pancreas, and other tissues removed at the time of first culture were not a requirement in the normal chemical differentiation of the regenerate pancreas.

In most cases, the regenerate appeared smaller than the control dorsal

Fig. 5. Pancreas regeneration in the absence of the majority of whole gut tissues. The whole gut was isolated at 12 days of gestation, and all gut tissue anterior and posterior to the gut endoderm adjoining the pancreatic duct was removed along with the dorsal and ventral pancreases (A). Over the next 5 days in culture, a new pancreas regenerated from the remaining duct (B, C, D, E and F). It is difficult to ascertain whether this remaining duct is the dorsal or ventral duct. It has been demonstrated in the rat, that the ventral pancreas is also capable of regeneration (B. S. Spooner, unpublished observations). PD, pancreatic duct; RP, regenerating pancreas (67 X).

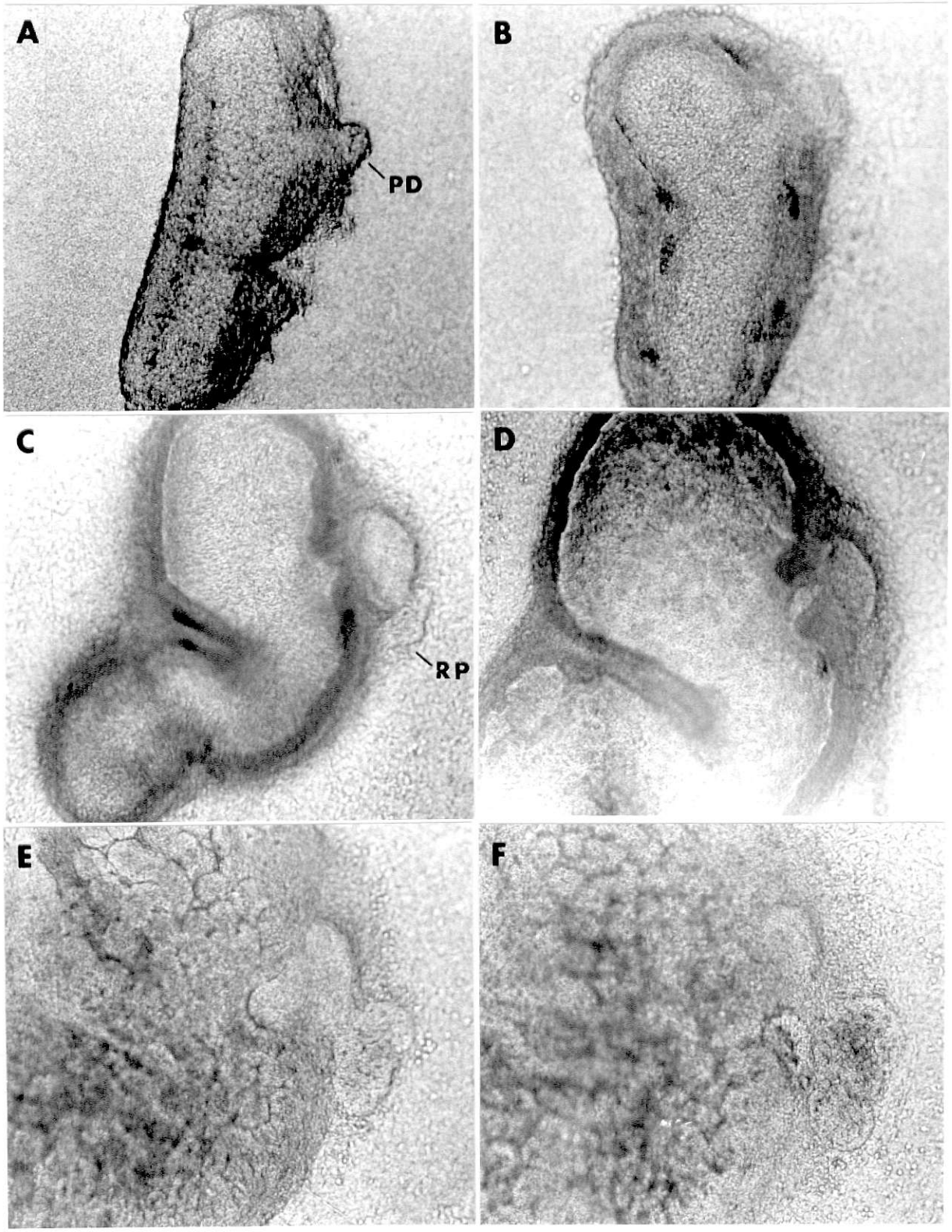
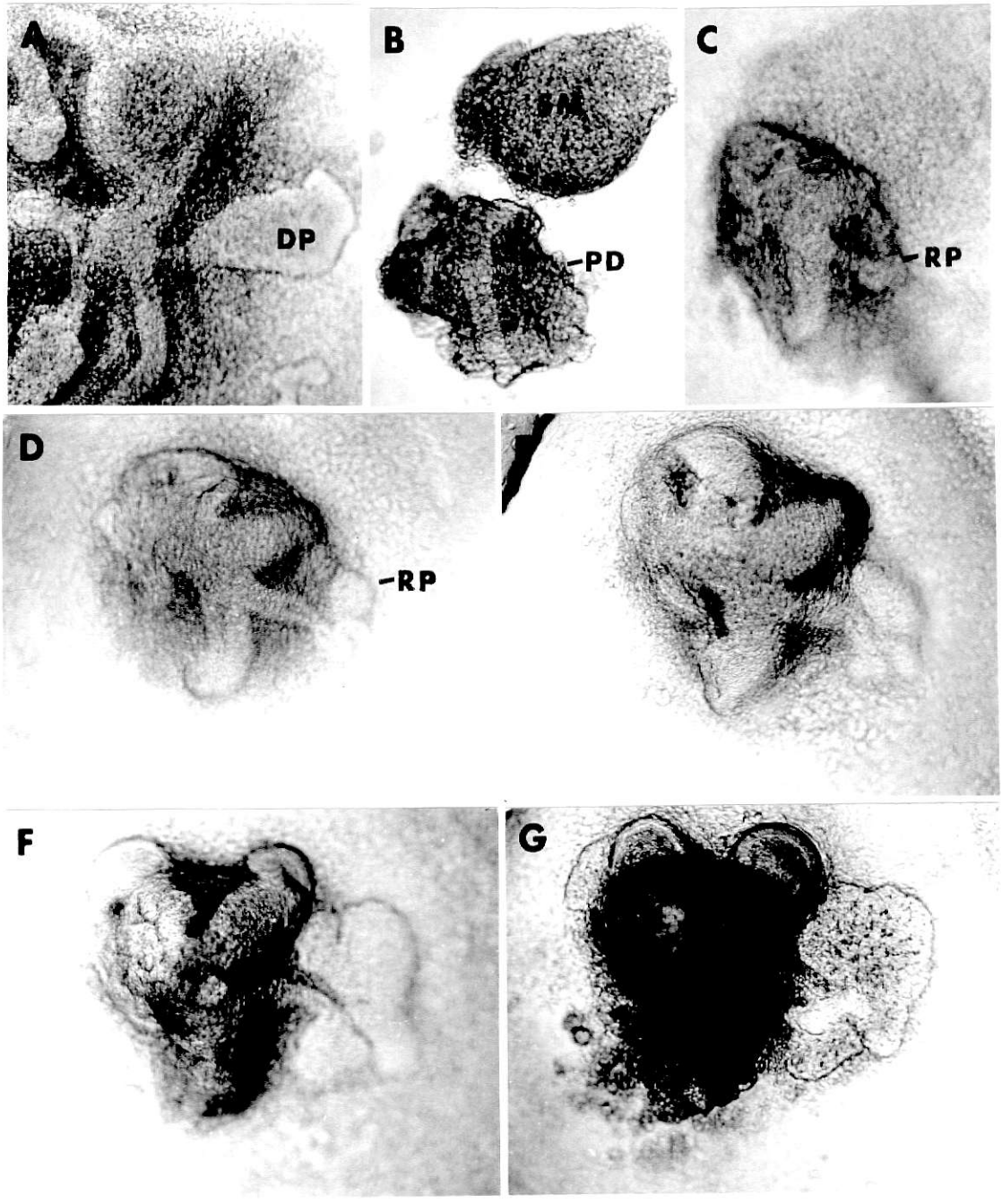

A
PD
B
C
RP
D
E
F

Fig. 6. Pancreas regeneration in the absence of the majority of whole gut tissues and in the presence of salivary mesenchyme. Three days (A) after culturing a 9 day whole gut, the dorsal pancreas, ventral pancreas, stomach, and all material caudal to the gut endoderm adjoining the pancreatic duct, was removed. A portion of 13 day salivary mesoderm was added to the cultures (B) to ensure adequate mesenchyme for the regeneration process. Again, the dorsal pancreatic duct regenerated a new pancreas during the remaining 5 days of culture (C, D, E, F, G). Note that the pancreas maintains a trilobular appearance in the beginning stages of regeneration similar to early salivary gland morphogenesis (see Spooner, 1973, for a discussion of salivary morphogenesis). DP, dorsal pancreas; PD, pancreatic duct; RP, regenerating pancreas; SM, salivary mesenchyme (67 X).


A
DP
B
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D
RP
F
G

TABLE II

AMYLASE SPECIFIC ACTIVITY IN EMBRYONIC CONTROL AND REGENERATED PANCREAS

	Embryonic Age in Days Tissue Explanted to Culture	Days of Culture Pancreas Removed (In Vivo Equivalent)	Days of Regeneration Past Surgery	Total Days of Culture (In Vivo equivalent)	Amylase Specific Activity[a] (± S.E.M.)	Amylase Treatment over Protodiff.[b] Levels
Experimental	9	3 (12)	5	8 (17)	2.4 (± .69)[c]	240X
^{I}Control	9	-	-	8 (17)	2.4 (± .46)[d]	240X
Experimental	12	0 (12)	5	5 (17)	4.2 (± 1.6)[e]	420X
^{II}Control	12	-	-	5 (17)	4.8 (± 1.2)[f]	480X

[a]Expressed as µg of maltose hydrate released/min/µg of protein at 37° C.

[b]Protodifferentiated.

[c]This is the mean of 13 assays averaging 5 pancreatic rudiments per assay.

[d]This is the mean of 7 assays averaging 4 pancreatic rudiments per assay.

[e]This is the mean of 3 assays averaging 11 pancreatic rudiments per assay.

[f]This is the mean of 3 assays averaging 8 pancreatic rudiments per assay.

(and ventral) pancreas. Analysis of protein content substantiates that observation. The regenerates in Exp. I of Table II had an average protein content of 1.2 µg per pancreatic rudiment, while controls contained 2.0 µg per rudiment. In Exp. II of Table II, regenerates contained 2.2 µg of protein and controls 14.4 µg of protein per rudiment.

Electron Microscopy

Electron microscopic analysis were made to further compare regenerate differentiation to controls. Initially, we focused on the cellular composition of the duct from which the regenerate formed, and on the entire pancreas of a normally developing pancreatic rudiment. For these studies, whole guts were isolated from 9-day embryos and were grown in culture. On the third day, the dorsal pancreas of each whole gut was removed with its surrounding mesenchyme. The whole guts were then gently peeled from the filter assemblies and placed in plastic tissue culture dishes filled with medium. The guts adhered to the bottom of the dish overnight and were fixed the next morning. This procedure allowed the mesenchyme to adhere well enough to withstand the fixation and embedding procedure without lifting from the plastic. Trimming was carefully done so that the dorsal pancreatic duct and the ventral pancreas were visible on the block face and sections could be made containing both.

The normal pancreas at this stage of development (approximately 13 days in vivo) contains cells which exhibit wide differences in electron densities (Fig. 7). The more electron-dense cells are believed to be the precursors of the endocrine cell population (Wessells and Evans, 1968). Cells of varying electron density also characterize the duct area of the pancreas (Fig. 8). This variance is true for the dorsal pancreatic duct, as well as

for the control ventral pancreatic duct. The usual large ratio of nucleus to cytoplasm in these cells is illustrated in Fig. 9. It is important to note that no zymogen granules are visible in these or any other cells that have been examined at this stage in pancreas development. The only secretory granules which have been found are α-granules in differentiated A-cells (Fig. 10). These A-cells, which synthesize glucagon, are quite common in the bulbous region of the pancreas, but have not been located in either of the pancreatic ducts. No cells containing β-granules have been observed anywhere in the pancreas at this stage. These observations are consistent with those of Pictet, et al. (1972). In addition, there is some indication of initial acinar formation, but there is no evidence that the cells are forming zymogen granules (Fig. 7).

In order to assess the ultrastructural differentiation of the regenerating pancreas, the dorsal pancreas was removed on the third day of culture, and the gut was allowed to continue in culture on the filter assembly for 4 days before being transferred to the petri dishes. The day following the transfer, it was discovered that contractions of differentiated smooth muscle in the cultures had caused the whole guts to "ball up", and they were unable to adhere to the bottom of the culture dish. It was therefore necessary to dissect the regenerated and control pancreases from the whole guts, place them in separate culture dishes, and incubate them one more day. They adhered to the dish sufficiently well for routine fixation and embedding procedures.

The differentiating regenerate pancreas was comparable to control pancreas. The acinar cells were arranged normally around a central lumen, and zymogen granules were quite abundant in the apical cytoplasm (Fig. 11). Other structures in these acinar cells also appeared to be normal. Ribo-

Fig. 7. Pancreatic cells during the protodifferentiated state (cultured at 9 days of gestation and incubated 4 days). This electron micrograph demonstrates the large differences in electron densities at this stage. Endocrine secretory granules would be difficult to discern at this magnification, however, no exocrine zymogen granules are visible, and there is very little rough endoplasmic reticulum. Several large vacuoles (V) are present in many cells, and an acinus may be forming in the lower left portion of the micrograph (arrows) (8,460 X).

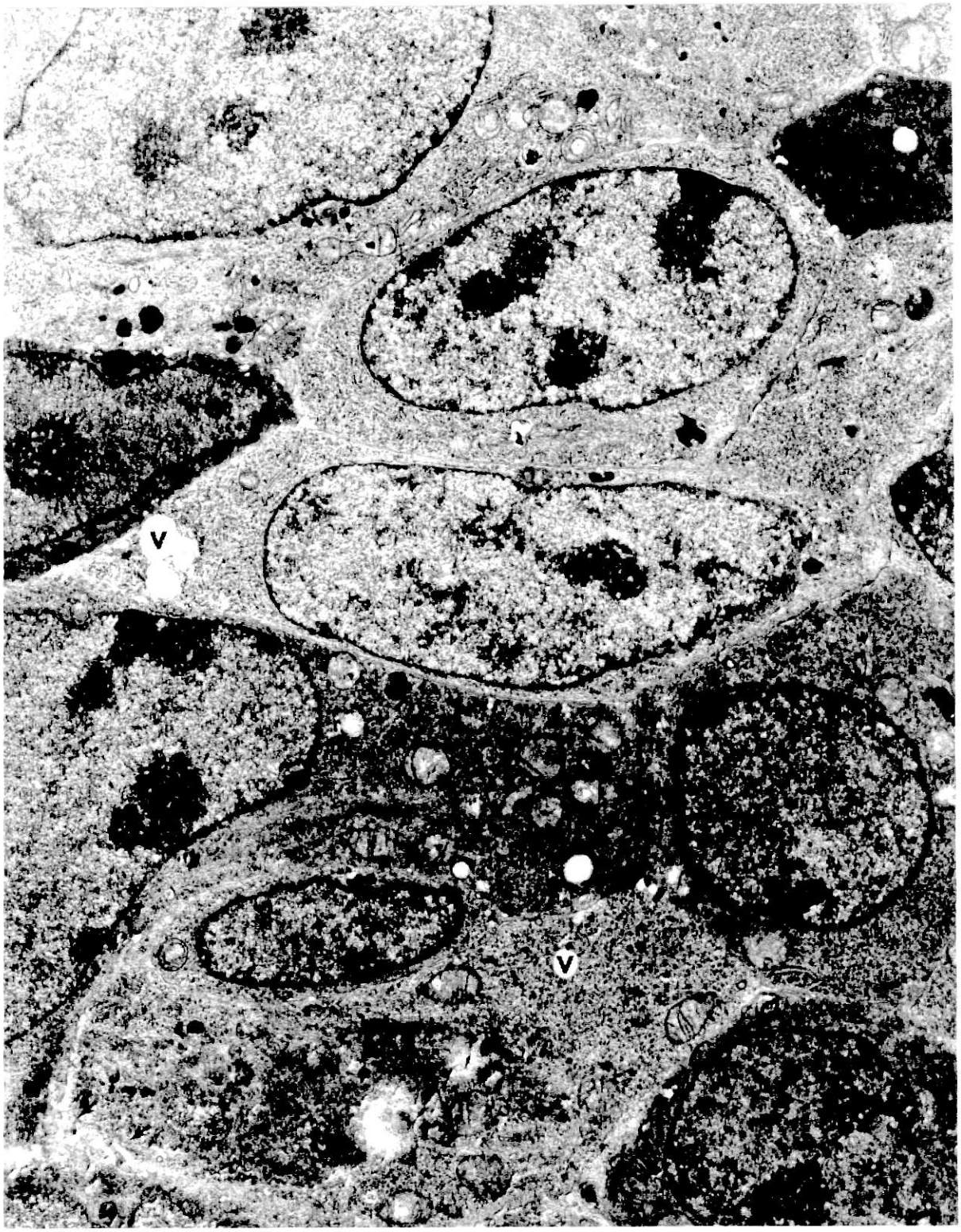

V
V

Fig. 8. Regenerate pancreas duct cells one day after removal of the original pancreas. These cells are similar to those found in the bulbous and duct regions of control pancreas, demonstrating that the differences in electron density extend into the pancreatic duct region. The electron-dense cell is positioned at the periphery of the duct, and a basement membrane (BM) is present. Microvilli (MV) surround this cell on all sides except for the basal surface (20,800 X).

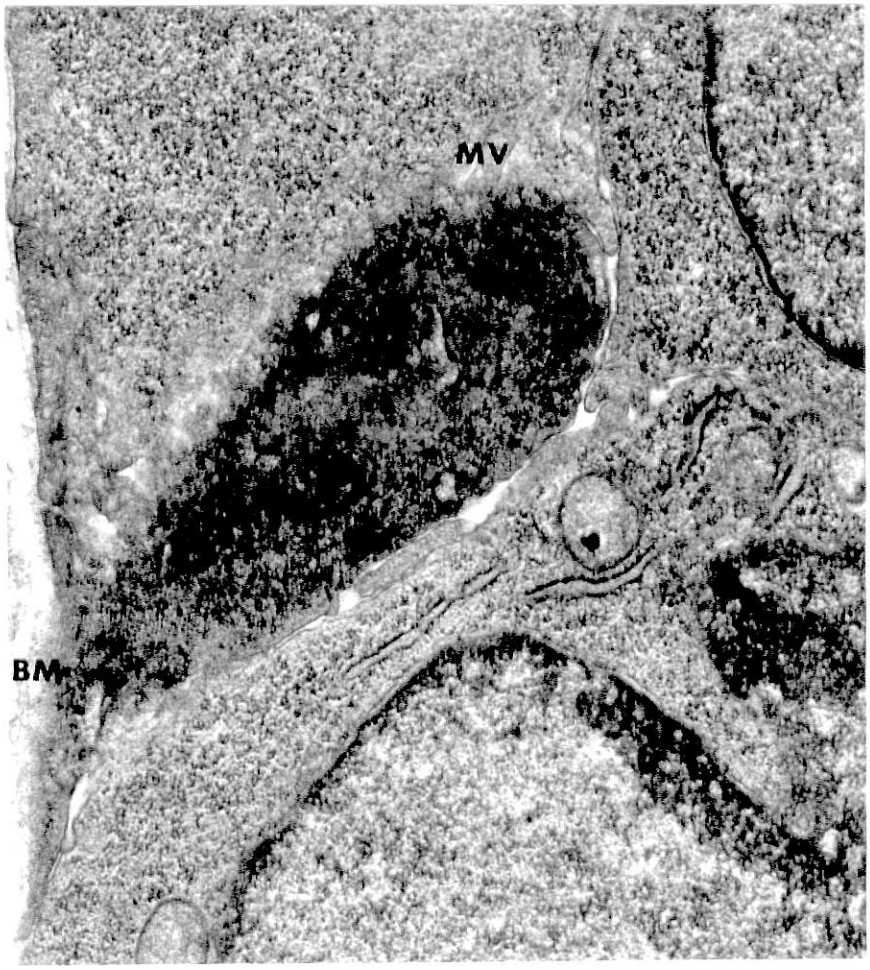

MV
BM

Fig. 9. Cells from the protodifferentiated pancreas demonstrating a high nuclear to cytoplasmic ratio. The high ratio of nucleus (N) to cytoplasm (C) is a basic characteristic of protodifferentiated pancreas cells. The electron dense cell in the upper portion of the micrograph is believed to be an "undifferentiated" endocrine cell (Wessells and Evans, 1968). The less dense cell will presumably become an exocrine cell. No secretory granules are visible in this micrograph (27,500 X).

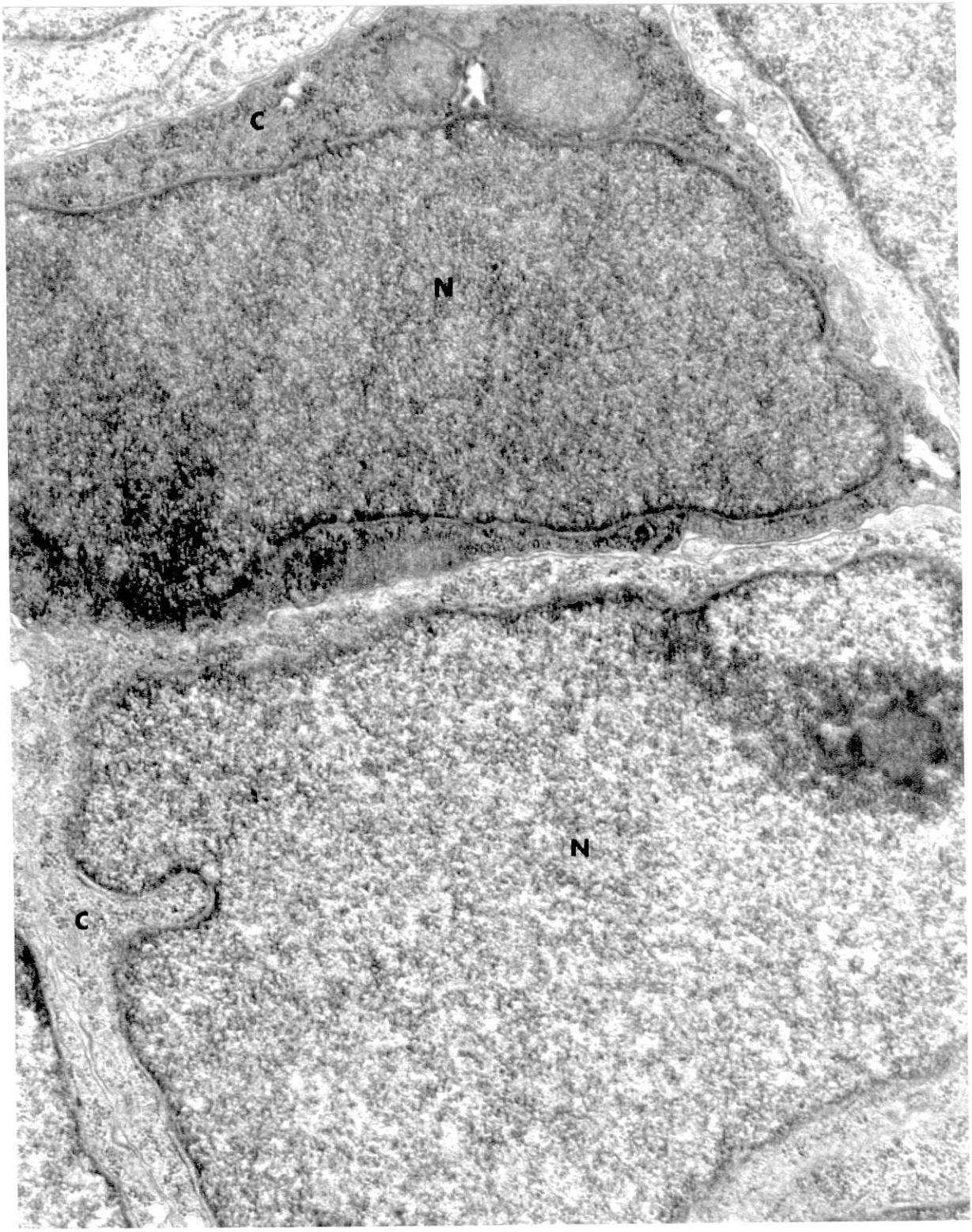
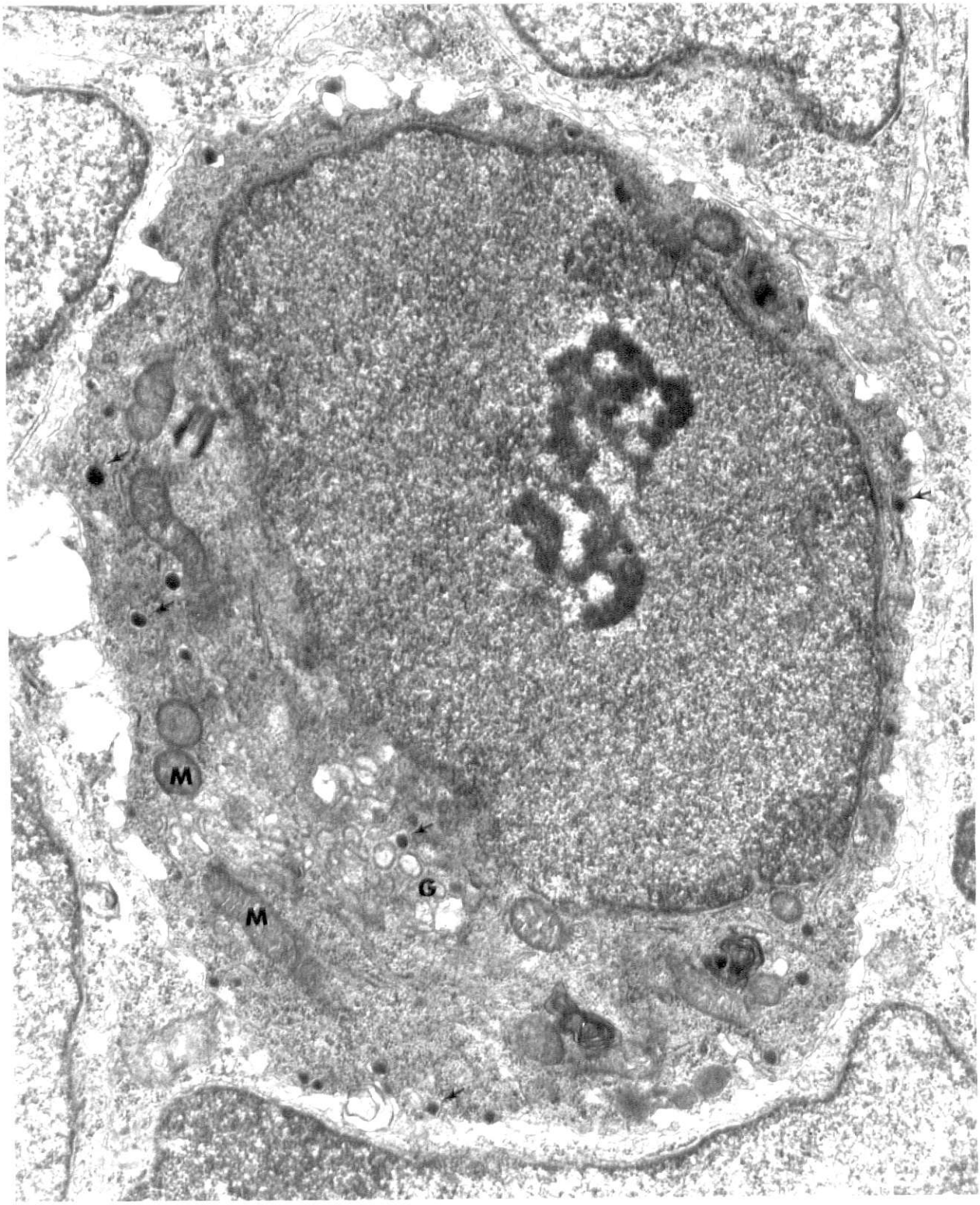

C
N
N
C

Fig. 10. The differentiated A-cell in the protodifferentiated pancreas. A-cells are found from the onset of pancreas budding, into the terminally differentiated state. This interphase cell has several α-granules (arrows) which are characterized by a dark core within a halo surrounded by a membrane. Numerous mitochondria (M) and Golgi complexes (G) are also present. In the differentiated pancreas, the endocrine islets are separated from exocrine acinar cells by two basement membranes, one belonging to the endocrine cells and one belonging to the exocrine cells. This single endocrine cell, completely surrounded by potential exocrine cells, has no basement membrane, but shows areas of vesiculation along zones of contact with the surrounding cells (20,800 X).


M
G
M

somes were found both free and associated with the abundant endoplasmic reticulum (Fig. 11, Fig. 12A). An organized Golgi complex, as in Fig. 11 and Fig. 12B, was also common, and characteristic junctional complexes were present between cells at the lumenal surface (Fig. 13). The endocrine tissue of regenerated pancreas contained at least two types, and possibly three types, of cells (Fig. 14). All of these endocrine cell types contained large Golgi vesicles (Fig. 15). Secretory granules were frequently found in the area of the Golgi complex. In agreement with observations made by Pictet and Rutter (1972), endocrine cells were many times found adjacent to zymogen-containing exocrine cells (Fig. 16).

A random count of 370 cells in various sections taken from 2 different specimens of regenerated pancreas revealed that 21% of the cells were differentiated endocrine cells, while 68% were differentiated exocrine cells. Pictet, et al. (1974) determined that rat pancreas epithelial rudiments cultured in the presence of mesenchymal factor (MF) showed the same percentage of endocrine cells as pancreatic rudiments grown in vivo, approximately 6%. They also demonstrated that pancreatic epithelia grown in the absence of MF contained greater than 50% endocrine tissue. In the present study, the pancreatic mesenchyme was removed with the epithelium at the time of surgical removal of the dorsal pancreas. No embryo extract, from which MF is derived, was present in the culture medium. This might explain the larger amount (21%) of endocrine tissue in the regenerated pancreas.

In summary, the regenerated pancreas appears to undergo normal temporal differentiation and produces both exocrine and endocrine cells.

Duct Isolation

The experiments described above were all conducted with the duct connected to another portion of the gut. In order to determine if the duct

Fig. 11. The acinar arrangement of the regenerated pancreas. These zymogen granule (Z) containing cells are arranged around a central lumen (L) exactly as in control pancreas maintained undisturbed in culture for the full 9 days. There is a large quantity of Golgi material (G) and rough endoplasmic reticulum (RER). Microvilli (MV) line the apical surface of these cells (4, 990 X).

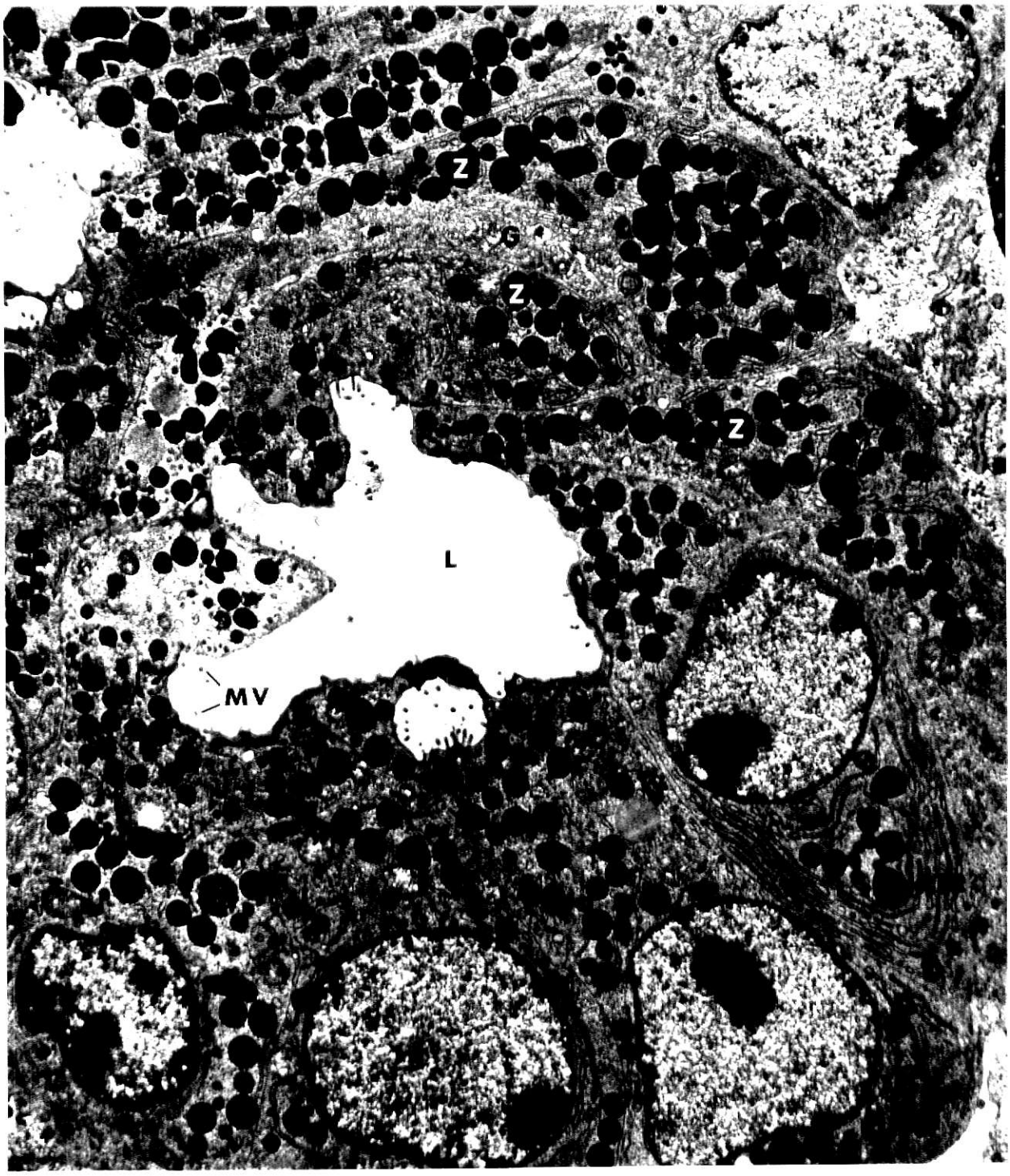

Z
G
Z
Z
L
MV

Fig. 12. Differentiated exocrine tissue in the regenerated pancreas. As in differentiated control pancreas, the acinar exocrine cells in micrograph A have a prominent rough endoplasmic reticulum (RER). Micrograph B shows a high magnification of the type of Golgi apparatus (G) common to these cells. Z, zymogen granules (A, 22,000 X; B, 41,200 X).

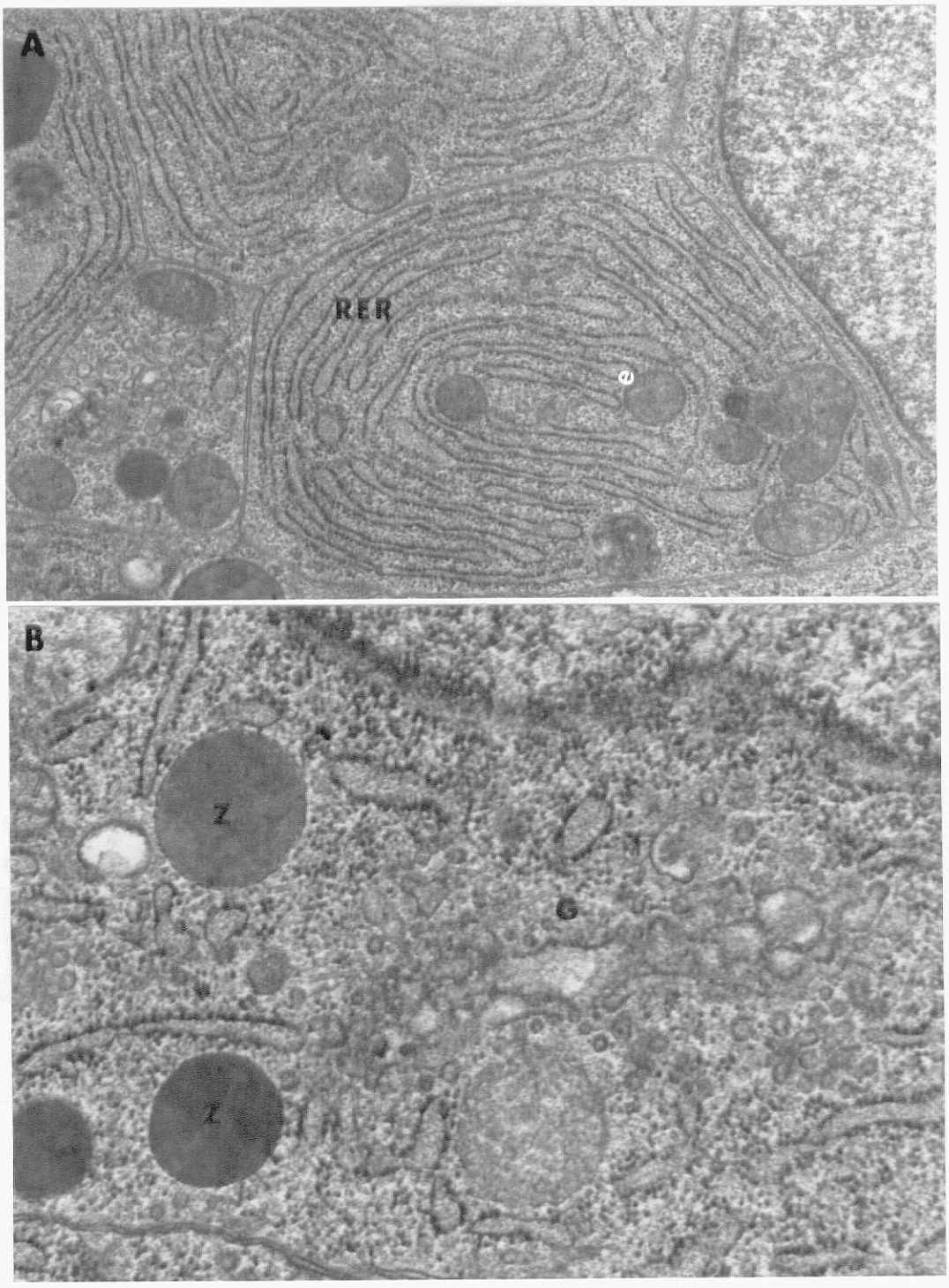

A
RER
a
B
Z
G
Z

Fig. 13. The apical surface of exocrine cells of the regenerated pancreas. The microvilli (MV) take on a fuzzy appearance (glycocalyx) as differentiation continues. Junctional complexes (arrows) are made up of a tight junction, intermediary junction, and a desmosome (Pictet, et al., 1972). The lumen (L) contains a large amount of filamentous material which seems to show a continuity with the glycocalyx of the microville. Z, zymogen granule (17,600 X).

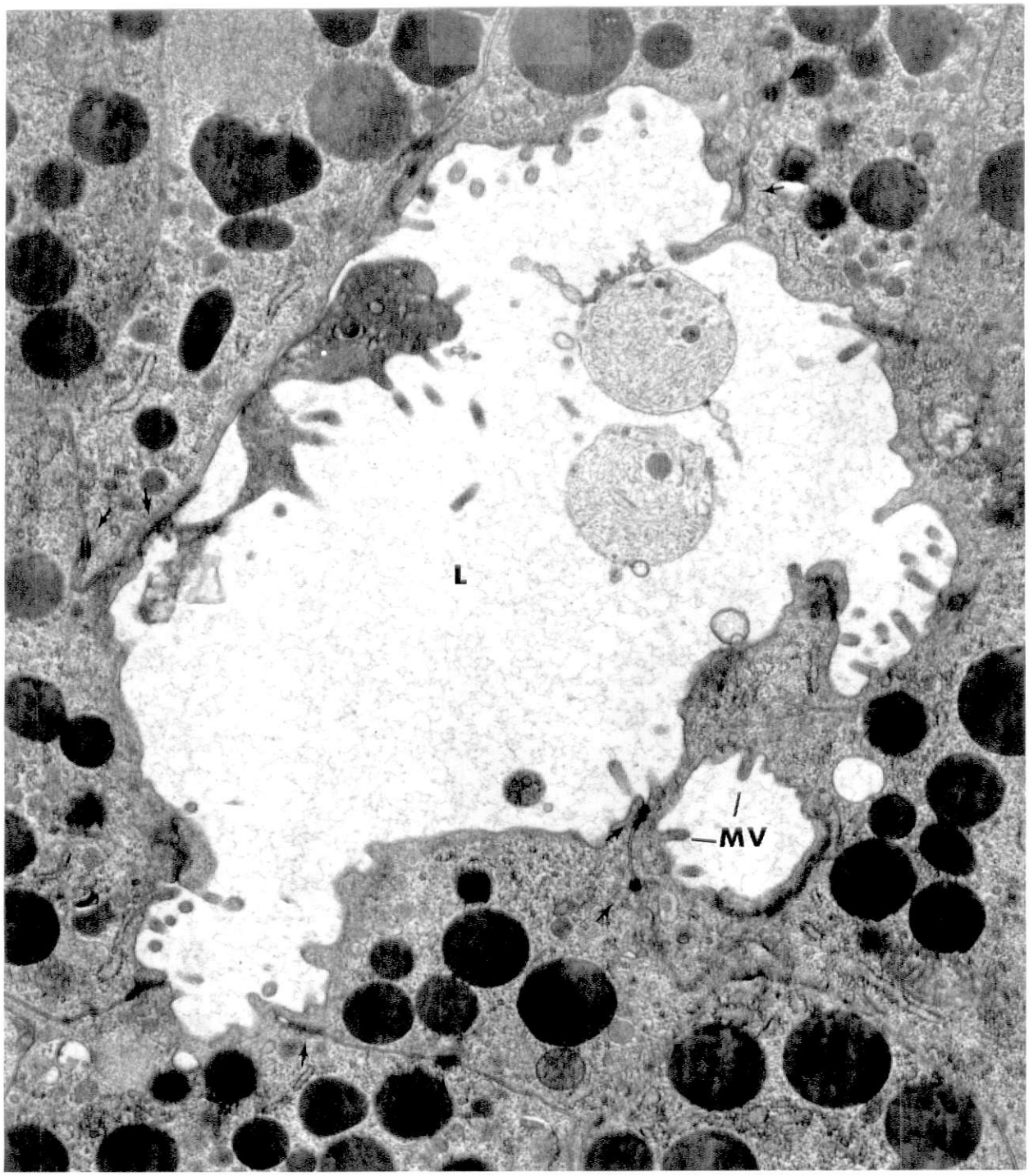

L
MV

Fig. 14. Endocrine tissue in the regenerated pancreas. Pictet and Rutter (1972) demonstrated 3 types of endocrine cells in the rat; A-cells, B-cells, and D-cells. D-cells were infrequent in appearance. In these endocrine cells from the regenerated mouse pancreas, cells 1 and 3 appear to have smaller secretory granules than cell 2 and, particularly, cell 4. Cell 4 definitely has much larger granules than cell 2. On the basis of size, there appears to be at least two, and possibly three, types of endocrine cells (41,200 X).

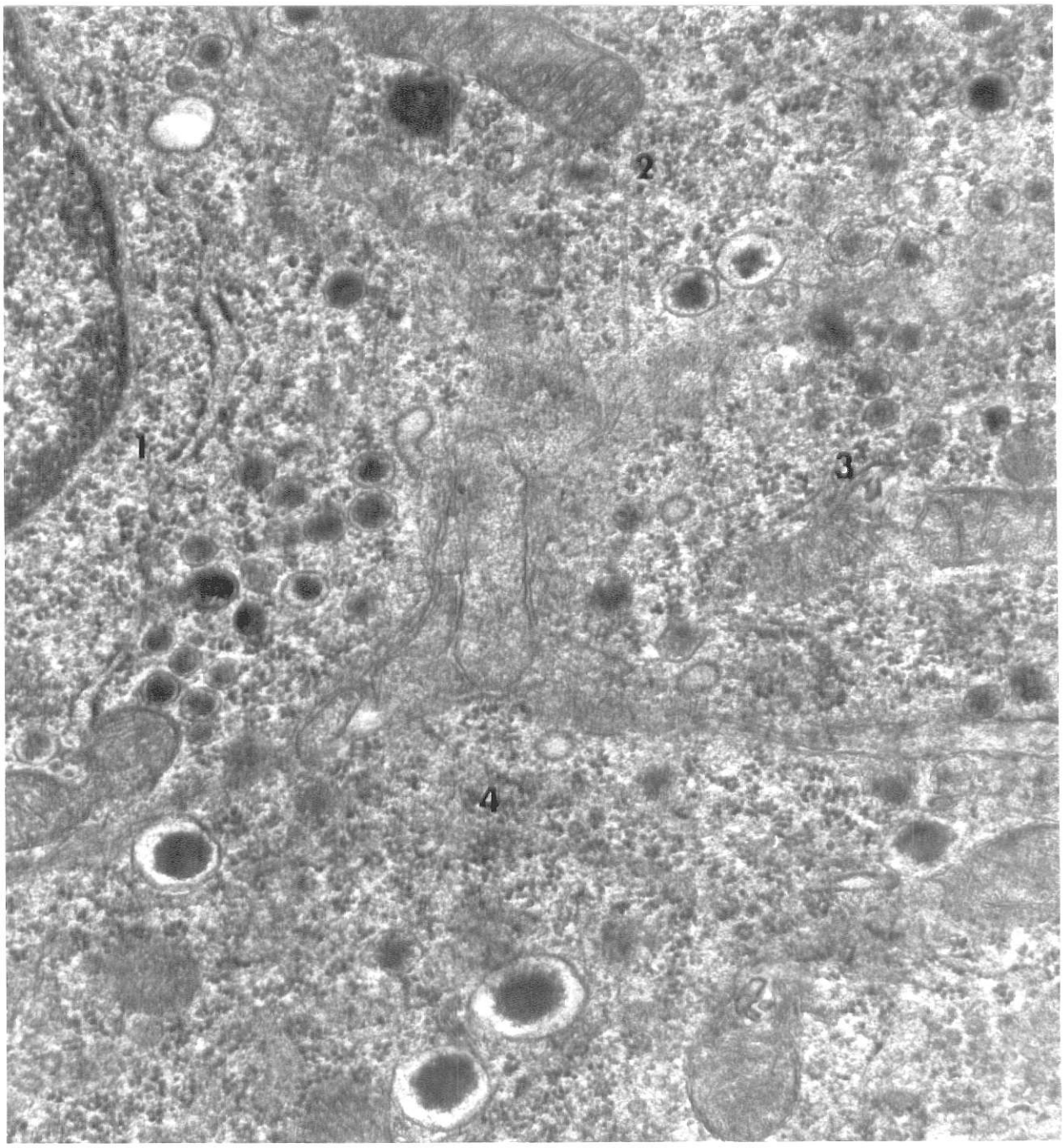

1
2
3
4
2

Fig. 15. The prominent Golgi apparatus in regenerate pancreas endocrine tissue. Secretory granules (S) are frequent inhabitants of the Golgi complex. Also, there is often dense material located within the Golgi vesicles themselves (arrow). Multivesiculate bodies (MVB) may be found throughout many areas of pancreatic endocrine tissue (41,200 X).

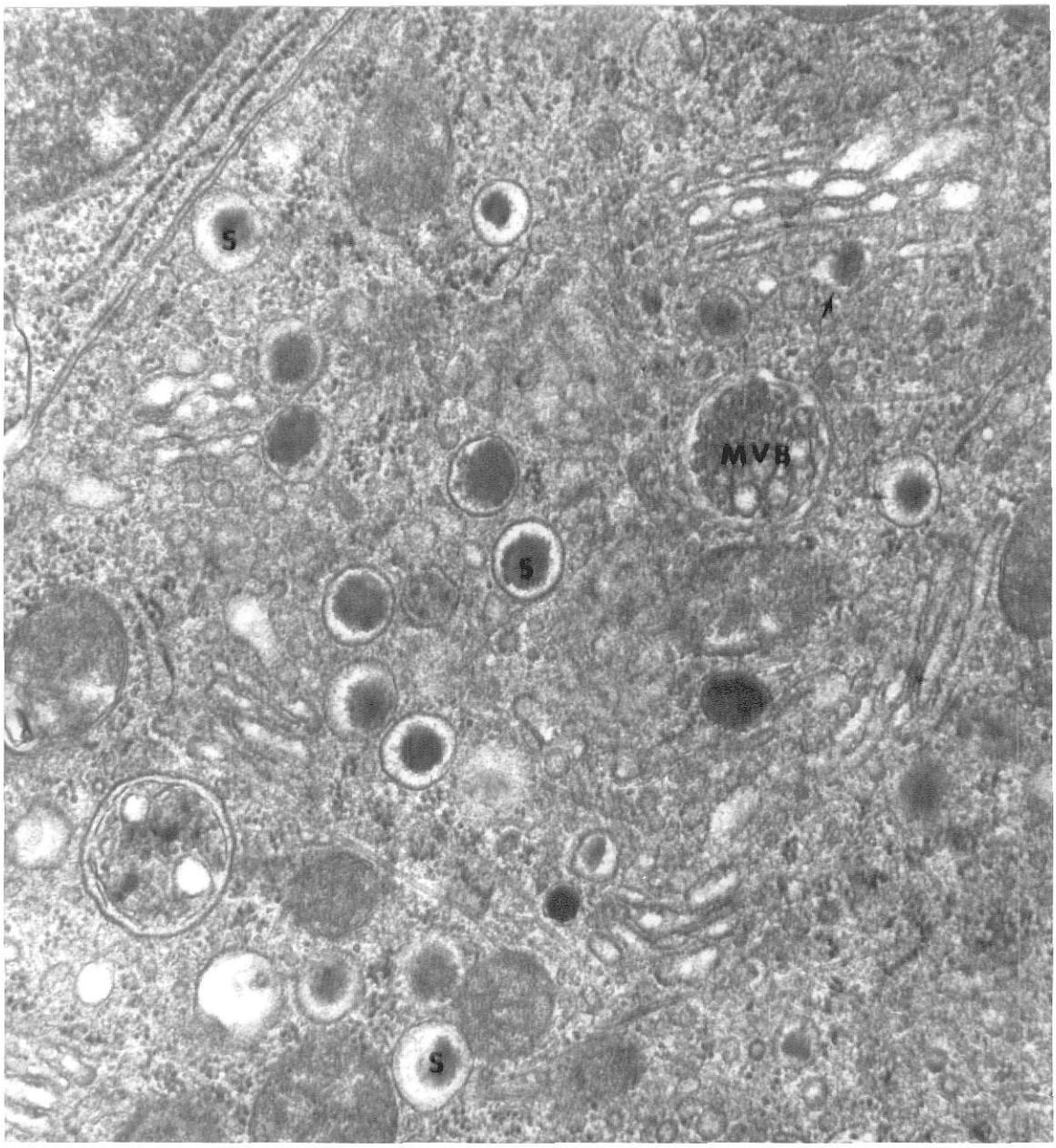
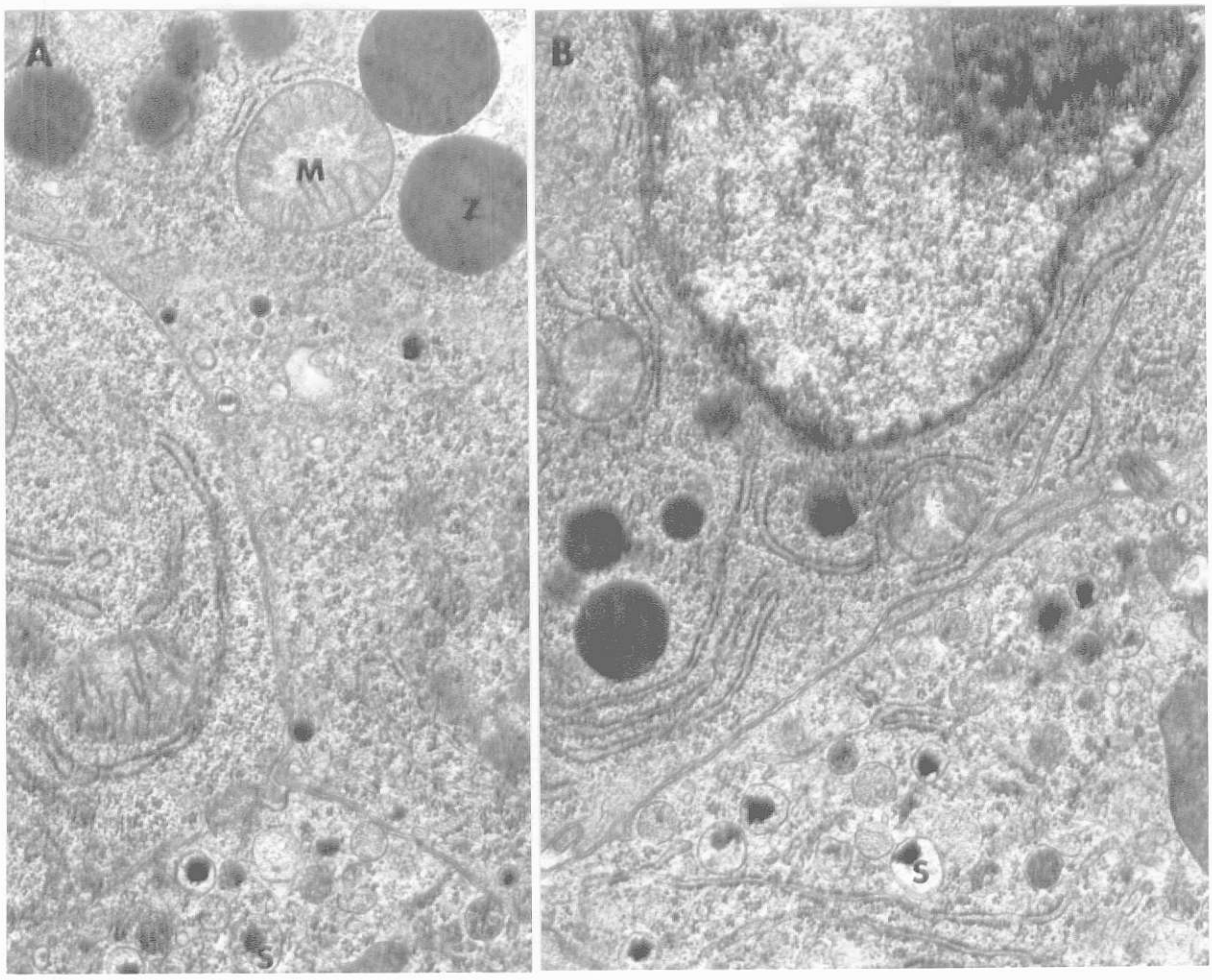

S
MVB
S
S

Fig. 16. The close proximity of developing exocrine and endocrine cells. Both micrograph A and B illustrate the ability of exocrine and endocrine cells to occupy adjoining positions. There is no indication of basement membrane being formed by either of the cell types. This observation is many times also true in tissue from control pancreas. S, endocrine secretory granules; Z, zymogen granules; M, mitochondria (A, 20,800 X; B, 20,800 X).


A
M
Z
S
B
S

could regenerate entirely on its own, the following experiment was performed.

The pancreas with its duct was removed from a 12-day embryo. At that time, the duct was removed from the pancreas and cultured, with adherent mesenchyme, on a Millipore filter assembly. At the same time, a piece of the pancreatic bulb, nearly equivalent in size to the duct (approximately 0.18 mm in diam.), was cultured on a separate Millipore filter assembly. After 5 days in culture, the tissues were harvested and assayed for amylase specific activity. Amylase specific activity did not rise to differentiated levels in the tissue taken from the bulbous portion of the pancreas. The duct tissue, however, exhibited a specific activity well within the range of that found in differentiating pancreas (1.7 ± 0.77). Analysis of the protein content at the beginning of culture and at the end of culture, showed that the bulb tissue increased in size from 0.14 µg to 0.28 µg, a 2 fold difference. The duct tissue increased its protein content by 3.8 fold, from 0.13 µg to 0.49 µg.

These results demonstrate that the duct tissue had the ability to maintain a developmental program, while tissue of the same size from the pancreatic bulb did not retain this ability.

DISCUSSION

The observation that the embryonic dorsal pancreas can regenerate offers the opportunity to investigate the temporal stability of a well characterized developing organ. The results have demonstrated that the regenerated organ contains the same differentiated features that comprise the normal organ. Most impressively, it was the duct tissue that regenerated a complete pancreas, which within a short period of time, at least by 5 days after the pancreas was removed, was equal to control pancreas in amylase specific activity. These data suggest that although the regenerate had not reached the size of the control, the ability of the tissue to differentiate according to the normal time schedule was not altered.

The results should be considered with respect to the observations made by Wessells and Cohen (1967). Their experiments addressed the question of critical tissue mass in development. In other words, was there a specific tissue mass which had to be reached before an organ could complete its development? They approached this problem by repeatedly reducing the size of isolated pancreas in the protodifferentiated state. By maintaining this pancreatic tissue in a size comparable to that of protodifferentiated pancreas or smaller, they were able to prevent attainment of differentiated levels of amylase specific activity. Fusion of pancreas tissue to create a larger mass, however, did not "speed up" the differentiation process.

The data of Wessells and Cohen (1967) suggested that a critical tissue size had to be reached before the pancreas could continue with its development and become "terminally differentiated." Our results are, at first appearance, in contrast to this idea. The electron microscopy and enzyme specific activity data reveal no obvious retardation in the developmental

program.

Comparison of the differentiative abilities of isolated duct versus similar sized pieces from the bulb of the pancreas, allowed insight into this apparent discrepancy. The data demonstrated that while the bulb of the pancreas was subject to a critical tissue mass requirement, the duct was not subject to that same requirement. The experiments of Wessells and Cohen (1967) did not include the pancreatic duct, but involved only the bulb region. Thus, the duct appears to be a specialized region of the developing pancreas. This observation implies a regional difference in the protodifferentiated pancreas that is not visible from a morphological standpoint.

The large amount of endocrine tissue (21%) in the regenerates may be explained by the findings of Pictet, et al. (1974). They noted that pancreatic epithelia grown in the presence of mesenchymal factor (MF) gave rise to the same percentage of endocrine cells as pancreas grown in vivo, about 6%. Those grown in the absence of MF, had greater than 50% endocrine tissue. During the surgical removal of the dorsal pancreas in the experiments of this study, the pancreatic mesenchyme was removed with the epithelium, leaving only a small portion of pancreatic mesenchyme surrounding the duct. In addition, no embryo extract, from which MF is derived (Ronzio and Rutter, 1973), was added to the culture medium. The greater ratio of endocrine to exocrine cells could be explained by inadequate amounts of MF. This alteration might, then, affect the amylase specific activity of the regenerate, as a smaller amount of exocrine tissue would be expected to produce a lower level of enzyme activity. Such a decrease is not observable, and may suggest a possible "compensation" mechanism at work in the exocrine tissue.

If the above is true, perhaps the amount of mesenchyme left surrounding

the small piece of tissue removed from the pancreatic bulb in the duct isolation studies, was not sufficient to allow normal exocrine tissue differentiation and give detectable levels of amylase specific activity, while the amount of mesenchyme left surrounding the duct was capable of doing so. The possibility exists, therefore, that an abnormally large amount of endocrine tissue was present in those pieces taken from the bulb. In addition, the difference in the amount of mesenchyme in the bulb and duct pieces would alter the protein levels of these tissues. These possibilities are worthy of experimental investigation.

Electron microscopy has demonstrated that the duct contains no differentiated endocrine tissue during the protodifferentiated state, when the dorsal pancreas is removed, or even later on, as the embryo steadily matures (Wessells and Evans, 1968; Pictet and Rutter, 1972). The A-cells, the only overtly differentiated cell type present during the early protodifferentiated state, are located only in the bulb portion of the pancreas. The presence of endocrine tissue, containing both A- and B-cells, in the regenerated pancreas, indicates that the duct is capable of giving rise to the endocrine, as well as the exocrine tissue, and that the A-cells, present in the protodifferentiated pancreas, are probably not the sole precursor cells of the entire endocrine population. The suggestion that the more electron dense, "undifferentiated" cells are precursors of the endocrine population (Wessells and Evans, 1968), and our observation that these electron-dense cells are present in the duct region, also lend support to this idea.

Several other questions remain unanswered in this system. At present it is not known whether the regenerate pancreas simply continues with the developmental program as though another part of it had never been lost, or whether the regenerate must "catch up" in order to obtain a comparable

level of amylase specific activity to that found in the control. This could be determined by measuring the specific activity of amylase at earlier time points in the regeneration process. Further, analyses of other enzymes, such as lipase A, lipase B, carboxypeptidase A, carboxypeptidase B, trypsin, chymotrypsin, and ribonuclease, as well as the hormones insulin and glucagon, would give a more complete picture of the total process.

Compatible with this question is another which inquires into a possible local increase in mitotic activity at the point where the pancreas was removed. In vertebrate limb regeneration, cell mitosis is restricted to a region just beneath the cap of the amputated limb (for review, see Hay, 1974). The cells which form the mesenchymatous blastema are derived from cells which were once differentiated cell types, but have lost their differentiated characteristics. In contrast, the cells of the protodifferentiated pancreatic duct have not yet reached a terminally differentiated state. The mitosis which occurs in the duct after the removal of the dorsal pancreas, may be merely a continuation of the morphogenetic process which was set in motion at an earlier stage.

In summation, this study has determined that the protodifferentiated duct is capable of regenerating a new pancreas containing both exocrine and endocrine tissue. This knowledge has enabled the regenerating pancreas to be used as a system to investigate the temporal stability of the developmental program of the pancreas. This program was found to be unexpectedly stable, since the regenerate pancreas was not retarded with regard to amylase specific activity levels, when compared to undisturbed control pancreas. Finally, in experiments testing the ability of the duct to regenerate only in the presence of its own mesenchyme, it was determined, that the duct was not subject to the same critical mass requirement as is the bulbous

region of the pancreas. A piece of bulb tissue, nearly equivalent in size to the duct, was unable to show (after 5 days in culture) differentiated levels of amylase specific activity, while the duct tissue was capable of doing so.

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IN VITRO REGENERATION AND DIFFERENTIATION OF THE EMBRYONIC PROTODIFFERENTIATED MOUSE PANCREAS

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ABSTRACT

Studies of the temporal sequence of mammalian pancreas development have demonstrated the existence of a protodifferentiated state that is characterized by low, constant levels of amylase specific activity. In order to investigate this state further, guts from 9 day mouse embryos were cultured for 3 days, during which a protodifferentiated dorsal pancreas formed, consisting of a neck region or duct that extended from the intestinal wall and terminated in a large bulb of tissue. The pancreas bulb was then removed, leaving only the duct. This day corresponded to day 12 of gestation. During 5 days of additional culture, 37 of 43 ducts regenerated a pancreas with an amylase specific activity equal to that of control pancreas (2.4 μg of maltose hydrate released/min/μg of protein at 37° C). Guts placed in culture at 12 days, with removal of the pancreas the same day, gave similar results. Other combinations of starting embryonic age plus culture time prior to pancreas removal totaling 12-13 days also underwent regeneration. Only 1 of 18 pancreases regenerated from ducts equivalent to 14 days of gestation. Removal of the stomach, the intestine, and the ventral pancreas did not affect the ability of the dorsal pancreas to regenerate. However, removal of the duct at the presumptive intestinal wall did not result in regeneration. Regenerated pancreas differentiation was further confirmed by electron microscopic demonstration of zymogen granules in exocrine cells and of at least 2 types of secretory granules in endocrine cells. Although isolated protodifferentiated duct was shown to differentiate in culture, equivalently small fragments taken from the bulb did not differentiate. The results demonstrate that the protodifferentiated duct can regenerate a new pancreas including both exocrine and endocrine tissue, that the regenerated pancreas is not retarded in its development when compared with control pancreas, and

that the protodifferentiated duct is not subject to the same critical tissue mass requirement as is the bulb of the pancreas.