



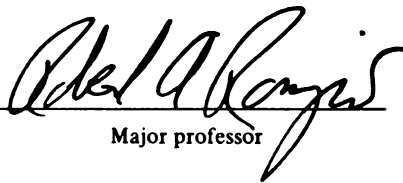
This is to certify that the
thesis entitled
**Glycoprotein Synthesis in the Rat Exocrine Pancreas
During the Secondary Transition Period
of Pancreatic Differentiation**

presented by

Beverly Blum DaGue

has been accepted towards fulfillment
of the requirements for

M.S. degree in Biochemistry


Major professor

Date September 23, 1977

GLYCOPROTEIN SYNTHESIS IN THE RAT EXOCRINE PANCREAS DURING THE
SECONDARY TRANSITION PERIOD OF PANCREATIC DIFFERENTIATION

By

Beverly Blum DaGue

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1977

ABSTRACT

GLYCOPROTEIN SYNTHESIS IN THE RAT EXOCRINE PANCREAS DURING THE
SECONDARY TRANSITION PERIOD OF PANCREATIC DIFFERENTIATION

By

Beverly Blum DaGue

Induction of glycoprotein synthesis in the rat exocrine pancreas during the major secretory protein synthetic period of pancreatic differentiation (the secondary transition) was studied using embryonic pancreatic rudiments cultured in the absence or presence of the differentiation inhibitor 5-bromodeoxyuridine from day 14 of gestation. Rudiments labeled with L[³H]fucose, D[³H]glucosamine, or [³H]N-acetyl-D-mannosamine at various times during the secondary transition period were analyzed by SDS-polyacrylamide slab gel electrophoresis with gel autofluorography and/or Coomassie blue staining. Radioimmunoassays were used to analyze labeled rudiments for "secretory protein-like" and "zymogen granule membrane-like" glycoproteins.

Results suggest that only six or seven of the embryonic pancreas glycoproteins are induced during the secondary transition period. One of these, a high molecular weight zymogen granule membrane-like glycoprotein, appears to be induced only with respect to glycosylation. The major zymogen granule membrane glycoprotein (GP-2) does not appear to be induced during the secondary transition period. Other induced glycoproteins are soluble species, two of which probably correspond to secretory proteins.

to Michael Garrison DaGue

There was a child went forth everyday,
And the first object he look'd upon, that object he became,
And that object became part of him for the day or a
certain part of the day,
Or for many years or stretching cycles of years.

--Walt Whitman--

ACKNOWLEDGMENTS

My sincerest thanks to those who have helped me most along the way, especially to Drs. Boyd O'Dell, Wayne Gonnerman, and Edward Harris, who provided a positive initiation into research and much encouragement; to Dr. Robert Ronzio, for his guidance as my thesis advisor and for his efforts to develop in me a scholarly approach to research; to Dr. Eugene Giroux, of Merrell International, Strasbourg, France, for the opportunity to work in his laboratory; to my parents and the DaGue family for their continuing support; and, most of all, to my husband, Michael, without whom I might have taken this work all too seriously.

TABLE OF CONTENTS

	Page
INTRODUCTION AND HISTORICAL	1
Differentiation of the Rat Exocrine Pancreas	1
Histodifferentiation of the Rat Exocrine	
Pancreas.	2
Cytodifferentiation of the Rat Exocrine	
Pancreas.	3
Biochemical Differentiation of the Rat	
Exocrine Pancreas	4
Changes in Glycoproteins During Cellular	
Differentiation.	8
Glycoproteins of the Adult Rat Pancreas.	11
Glycoprotein Synthesis in the Differentiating	
Rat Pancreas: A Statement of the Problem.	13
MATERIALS AND METHODS	15
Materials.	15
Electrophoresis Reagents.	15
Radiochemicals.	15
Reagents for Liquid Scintillation Counting.	15
Immunochemicals	16
Tissue Culture Media.	16
Miscellaneous Reagents and Materials.	17
Laboratory Animals.	17
Methods.	17
<i>In vitro</i> Culture of Embryonic Rat Pancreas.	17
Procedures for SDS-Polyacrylamide Slab Gel	
Electrophoresis and Gel Autofluorography.	19
<i>In vitro</i> Labeling of Adult Rat Pancreas with	
[³ H]Leucine and [³ H]Fucose.	24
Preparation of Zymogen Granule Membranes and	
Zymogen Granule Content Proteins from Adult	
Rat Pancreas.	24
Preparation of Rabbit Antisera to Bovine Serum	
Albumin and to Rat Pancreas Zymogen Granule	
Membranes and Zymogen Granule Content Proteins.	26
Preparation of Anti Zymogen Granule Membrane	
(IgG)	27
Preparation of Particulate and Soluble Fractions	
from Pancreatic Rudiments for Radioimmunoassay.	28
Radioimmunoassay Procedures	28
Acid Precipitation of Radioactive Protein	31
Immunodiffusion Analysis.	32

	Page
Liquid Scintillation Counting	32
General Assay Procedures.	33
RESULTS	34
5-Bromodeoxyuridine Inhibition of <i>in vitro</i> Pancreatic Differentiation.	34
SDS-Polyacrylamide Slab Gel Electrophoresis Analysis of Protein and Glycoprotein Synthesis in Differen- tiating Pancreatic Rudiments	35
Coomassie Blue Staining Profiles for Cultured Pancreatic Rudiments.	36
Incorporation of [³ H]Leucine into Cultured Pancreatic Rudiments.	41
Incorporation of Radioactive Sugar Precursors into Glycoproteins of Cultured Pancreatic Rudiments	42
Elution of Radioactive Proteins and Glyco- proteins from Gels During Staining and Destaining Procedures	47
Incorporation of [³ H]Leucine and [³ H]Fucose into Zymogen Granule Content and Zymogen Granule Membrane Constituents of Adult Rat Pancreas	48
Fractionation of [³ H]Fucose-Labeled Differentiated Pancreatic Rudiments	55
Incorporation of [³ H]Fucose and [³ H]Glucosamine into Acid-Precipitable Protein During Pancreatic Differentiation.	62
Radioimmunoassay for Zymogen Granule Membrane Glyco- proteins and Secretory Glycoproteins in Differen- tiating Rat Pancreas	65
Characterization of the Anti Zymogen Granule Content (Antiserum)	66
Purification of the Anti Zymogen Granule Membrane (Gamma Globulin)	75
Characterization of the Anti Zymogen Granule Membrane (Gamma Globulin)	79
Accumulation of Antibody-Precipitable Glyco- proteins During the Secondary Transition Period of Pancreatic Differentiation.	82
Identification of Antibody-Complexed Glycopro- teins of Differentiating Rat Pancreas	89
Competition of Adult Rat Pancreas Zymogen Granule Membrane and Zymogen Granule Content Proteins with Glycoproteins of Differentiated Embryonic Pancreatic Rudiments for Antibody Binding	95
Radioimmunoassay of Pancreatic Rudiments Cultured in the Presence of 5-Bromodeoxy- uridine for Zymogen Granule Content and Zymogen Granule Membrane Glycoproteins.	99

	Page
DISCUSSION.	101
Summary of Results	101
Glycosylation of Pancreatic Secretory Glycoproteins. . .	105
Metabolism of Glycoprotein Precursors.	108
Biogenesis of Zymogen Granule Membranes.	109
Glycosyltransferase Induction During Pancreatic Differentiation.	110
Pancreatic Glycoprotein Synthesis and the Model for Pancreatic Differentiation	112
Suggestions for Future Investigations.	114
LIST OF REFERENCES.	116

LIST OF TABLES

Table		Page
1	Relative mobilities of standard proteins on 0.1% SDS-8% acrylamide slab gels.	22
2	Coomassie blue-stained proteins observed on SDS-polyacrylamide slab gels to be induced during the secondary transition period of rat exocrine pancreas differentiation.	39
3	Glycoproteins induced in the rat exocrine pancreas during the secondary transition period of pancreatic differentiation.	46
4	[³ H]Leucine- and [³ H]fucose-labeled components of adult rat pancreas zymogen granule membranes and zymogen granule contents	51
5	Fractionation of [³ H]fucose-labeled differentiated (<i>in vitro</i>) pancreatic rudiments.	56
6	Incorporation of radioactive sugars into acid-precipitable protein by pancreatic rudiments during the secondary transition period of differentiation . . .	63

LIST OF FIGURES

Figure		Page
1	A model for the differentiation of the rat exocrine pancreas as proposed by Rutter and colleagues (Rutter et al., 1968).	6
2	SDS-polyacrylamide gel electrophoresis of [³ H]fucose-, [³ H]glucosamine- and [³ H]leucine-labeled pancreatic rudiments.	38
3	SDS-polyacrylamide gel electrophoresis of pancreatic rudiments labeled with [³ H]N-acetylmannosamine at various stages of differentiation	44
4	SDS-polyacrylamide gel electrophoresis of adult rat pancreas zymogen granule membrane proteins and zymogen granule content proteins labeled <i>in vitro</i> with [³ H]leucine and [³ H]fucose.	50
5	SDS-polyacrylamide gel electrophoresis of subcellular fractions from cultured pancreatic rudiments labeled on the equivalent of 19-20 days gestation with [³ H]fucose	59
6	Electrophoretic comparison of fucoproteins from cultured pancreatic rudiments with the fucoproteins of zymogen granule membranes and the zymogen granule contents from adult rat pancreas	61
7	Titration of anti zymogen granule content (antiserum) with [³ H]leucine-labeled zymogen granule content proteins from adult rat pancreas.	68
8A	Ouchterlony double diffusion test of anti zymogen granule content (antiserum) and anti zymogen granule membrane (IgG) versus zymogen granule content proteins and zymogen granule membranes prepared from adult rat pancreas	71
8B	Microheterogeneity of anti zymogen granule content (antiserum). Double diffusion test of anti zymogen granule content (antiserum) versus various dilutions of a zymogen granule content protein solution.	71

9	Cross-reactivity of anti zymogen granule content (antiserum) with smooth microsomal membranes and NaBr-washed rough microsomal membranes from adult rat pancreas	74
10	Purification of anti zymogen granule membrane (IgG). Removal of gamma globulin fraction cross-reactive with zymogen granule content proteins.	78
11	Cross-reactivity of anti zymogen granule membrane (IgG) with smooth microsomal membranes and NaBr-washed rough microsomal membranes from adult rat pancreas	81
12	Accumulation of "zymogen granule content-like" glycoproteins and "zymogen granule membrane-like" glycoproteins in differentiating rat exocrine pancreas	85
13	Specific radioactivities of the "zymogen granule content-like" glycoproteins and "zymogen granule membrane-like" glycoproteins which accumulate in the rat exocrine pancreas during the secondary transition period of pancreatic differentiation.	88
14	SDS-polyacrylamide gel electrophoresis analysis of radioimmunoprecipitates of soluble and particulate glycoproteins from differentiating pancreas.	91

LIST OF ABBREVIATIONS

5-BrdU	5-bromodeoxyuridine
BSA	bovine serum albumin
Con A	Concanavalin A
EDTA	ethylenediaminetetraacetate
GP-1	glycoprotein-1
GP-2	glycoprotein-2
GP-3	glycoprotein-3
IgG	immunoglobulin G
SDS	sodium dodecylsulfate

INTRODUCTION AND HISTORICAL

Differentiation of the Rat Exocrine Pancreas

Differentiation of the rat exocrine pancreas is considered to be an excellent model for mammalian secretory tissue differentiation. Differentiation of the rat exocrine pancreas may be viewed as the cumulative events of three major processes. These are 1) histogenesis, in which presumptive pancreatic cells divide and orient themselves with respect to each other and surrounding non-pancreatic cells; 2) cytodifferentiation, during which the generation of specific organelles involved in the matured functioning of the cell occurs; and 3) biochemical differentiation, during which the cell begins to synthesize and package those specific secretory proteins which identify it as an exocrine pancreas cell. The histogenesis and cytodifferentiation of the rat exocrine pancreas have been examined thoroughly and documented (Spooner et al., 1970; Pictet et al., 1972). The molecular events underlying these obvious structural changes and via which the exocrine pancreas cell acquires functional maturity still remain largely unknown. Substantial information is available regarding the accumulation of secretory proteins during differentiation. Post-translational modification of these secretory proteins during differentiation has not been investigated, however. Furthermore, the effects of differentiation on protein and glycoprotein constituents of cellular membranes, in particular those of the zymogen granule membrane, have remained

essentially unstudied. Such information is certain to contribute to an understanding of the regulatory mechanisms involved in initiating and coordinating the molecular events of the differentiation process.

Histodifferentiation of the Rat Exocrine Pancreas

The pancreatic tissue arises from the epithelial cells of the embryonic endoderm. These cells are organized in a single layer with a continuous basal lamina and are connected to one another at their apices by junctional complexes. The dorsal pancreatic bud first appears by the twenty-somite stage (ten days gestation) and consists of a single layer of cells arranged in a bulbous protrusion from the dorsal side of the endodermal tube. The apices of the cells form a lumen. Continued cell division and an increase in the height of the cells produce a thickening of the pancreatic bud which by the thirty-five somite stage (day twelve of gestation) consists of approximately three thousand cells. Though the tissue begins to appear stratified, the orientation of the cells remains the same. They continue to be connected to each other by apical junctional complexes, the apical cell surfaces forming a lumen. It is the orientation of cell division which maintains this relationship among the cells. The axis of the mitotic spindle is always parallel to the apical cell surface and apical junctional complexes are formed between daughter cells before cytokinesis is complete.

By day 12 of embryonic development, lobulation of the pancreatic tissue is apparent. The luminal canal branches throughout the tissue; however, it is small and difficult to detect. The pancreatic epithelium remains surrounded by basal lamina which separates it

from the mesenchyme. In the days which follow, the branching pancreatic tissue gradually penetrates into the mesenchyme. Day 15 marks the obvious appearance of the pancreatic lumen. With the development of the lumen, histodifferentiation is complete.

Cytodifferentiation of the Rat Exocrine Pancreas

The development of the mature subcellular structure of the exocrine pancreas parallels the biochemical differentiation of the cell. Between the twenty-four somite stage (middle of day 11 of gestation) and day 14 of embryonic development, the cell resembles other endodermal cells of the gut. This stage is called the "protodifferentiated state." Cells at this stage are characterized by 1) apical junctional complexes and luminal microvilli, 2) both cytoplasmic ribosomal clusters and ribosomes extensively bound to rough endoplasmic reticulum, 3) a rough endoplasmic reticulum consisting of cisternae which are not oriented with respect to the cell nucleus or the cell surface membrane, 4) a golgi apparatus consisting of flattened saccuoles located between the cell nucleus and the apical cell surface, and 5) the presence of mitotic structures as cells undergo division.

On day 15 of embryonic development, when histodifferentiation of the pancreas is essentially complete, the cells begin to acquire the structural characteristics of mature cells. This is considered the beginning of the transition to the "differentiated state." Not all exocrine cells of the pancreas undergo this transition simultaneously; however, the same maturation process occurs for all exocrine pancreas cells.

The transition requires about three days. Several noticeable changes occur in cellular organization and organelle structure. The rough endoplasmic reticulum increases in size and the cisternae become oriented around the nucleus. Condensing vacuoles may be found within the golgi stacks. By day 17 in most exocrine pancreas cells, zymogen granules have begun accumulating between the golgi and the apical surface membrane.

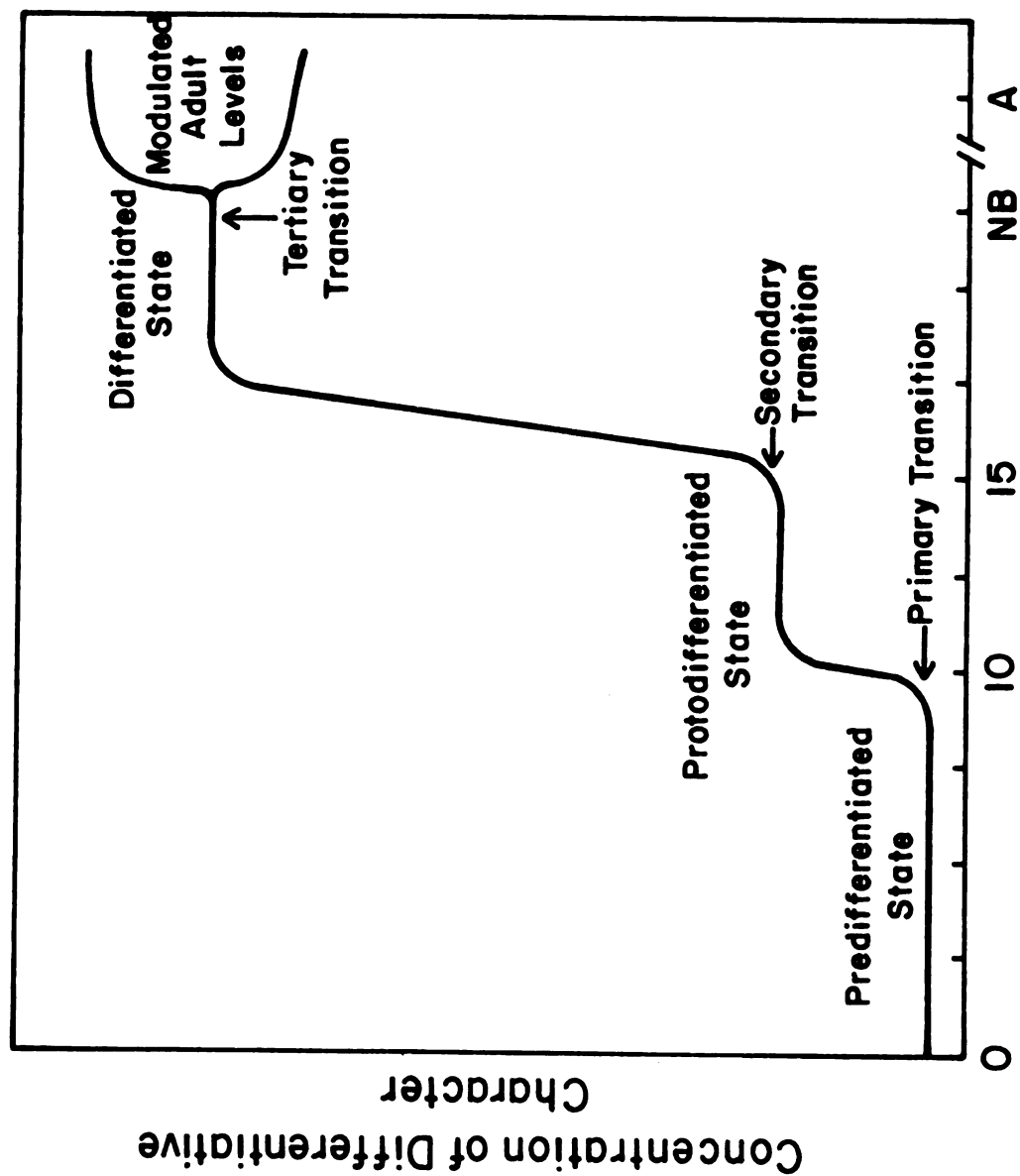
Cytodifferentiation is essentially complete by day 17 of gestation. Only a continued accumulation of zymogen granules is apparent during the remainder of the transition.

Biochemical Differentiation of the Rat Exocrine Pancreas

Concomitant with the histogenesis and cytodifferentiation of the rat exocrine pancreas, occurs the "functional" differentiation of this cell type. Three steady states for the biochemical differentiation of the exocrine pancreas have been proposed by Rutter and his colleagues (Rutter et al., 1968; Kemp et al., 1972). The transitions from one steady state to the next are determined by the levels of secretory proteins present in the acinar cells. Figure 1 illustrates schematically the biochemical differentiation process proposed by Rutter and colleagues.

At about the twenty-four somite stage, presumptive exocrine pancreas cells undergo a transition from the "predifferentiated state" in which none of the specific secretory products can be detected in the cells, to the "protodifferentiated state" in which the cells are characterized by low but detectable levels of the secretory enzymes and proenzymes. Exactly when this transition occurs is not known. The levels of the secretory proteins in the

Figure 1. A model for the differentiation of the rat exocrine pancreas as proposed by Rutter and colleagues (Rutter et al., 1968). "NB" is newborn rat; "A" is adult rat.



Embryonic Age (Days)

Figure 1

protodifferentiated pancreas range from 10^3 (for lipase A) to 10^5 (for amylase and chymotrypsinogen) molecules per cell (Rutter et al., 1968).

The protodifferentiated state lasts until day 14 or 15 of gestation, at which time the second regulatory transition occurs and the rates of synthesis of the secretory proteins increase dramatically. While the rate of total protein synthesis increases negligibly on a per cell basis *in vitro*, the rates of synthesis of amylase and chymotrypsinogen increase 100-fold during the secondary transition period (Kemp et al., 1972). Accumulation curves for the various secretory proteins indicate a synchronous rather than coordinate induction since not all of the secretory proteins are synthesized at an increasing rate at exactly the same time. The differential effect of actinomycin D on the accumulation of the various secretory proteins in cultures of developing pancreatic rudiments also suggests independent regulation of the synthesis of the individual secretory proteins. This effect of actinomycin D, furthermore, suggests that messenger RNAs coding for the secretory proteins are sequentially synthesized during the secondary transition.

Secretory proteins fail to accumulate in pancreatic tissue cultured in the presence of the thymidine analogue 5-bromodeoxyuridine (5-BrdU) if it is administered beginning with day 14 or day 15 of embryonic development. 5-BrdU is incorporated into DNA and subsequently alters RNA synthesis (Walther et al., 1974). That 5-BrdU completely blocks differentiation of the exocrine pancreas is further evidence for the dependence of the secondary transition on specific RNA synthesis.

The third steady state of the differentiation profile for exocrine pancreas is attained by day 19 of gestation. At this time most secretory proteins have accumulated to the levels found in the adult rat pancreas. In the mature pancreas, the levels of secretory proteins synthesized remain high but are subject to modulation in response to variation in diet.

Changes in Glycoproteins During Cellular Differentiation

Changes in glycoproteins, in particular cell surface membrane glycoproteins, have been correlated with cellular differentiation. For example, alterations in the types of glycoproteins and their relative exposure to the external environment of the cell have been shown to occur during the development of embryonic chick neural retinal cells using carbohydrate-binding phytoagglutinins of different sugar specificities (Kleinschuster and Moscona, 1972). Cultured murine neuroblastoma cells possess a cell surface membrane glycoprotein and a soluble glycoprotein, each with an apparent molecular weight of 105,000 daltons, only in the differentiated state (Truding et al., 1974).

Red blood cell maturation in the rat appears to involve alterations in the relative concentrations of sialated and nonsialated glycoproteins on the cell surface. Con A binding by surface membrane glycoproteins (nonsialated) of the mature erythrocyte is much less than that observed for the precursor erythroblasts. The opposite variation occurs for sialated surface membrane proteins (detected with positively charged ferric oxide) with the mature erythrocytes bearing a much higher concentration of surface membrane sialoproteins than the erythroblasts (Skutelsky and Farquhar, 1976).

Dictyostelium discoideum, 6-18 hours after the initiation of differentiation (when the amoebae have aggregated and the formation of a fruiting body has begun), exhibits a large increase in the intensity of labeling (by iodination) of a specific plasma membrane glycoprotein. This glycoprotein has a molecular weight of approximately 150,000 daltons and binds Con A. At the same time that the 150,000-dalton species appears in large quantities on the plasma membrane, a plasma membrane glycoprotein of 180,000 daltons becomes virtually undetectable (Geltoskey et al., 1976).

Of greatest significance to a consideration of glycoproteins in the differentiation of the exocrine pancreas are the results of studies of intestinal epithelial cells. Both cell types have their embryonic origin in the gut endoderm.

The lumen of the small intestine is penetrated by a vast number of villi. A single layer of cells covers each villus, and from the tip of each villus to its base there is a gradual decrease in the degree of differentiation of the epithelial cells. Those epithelial cells at the tip of the villus are the most completely differentiated while those at the base of the villus, the crypt cells, are the least differentiated and are mitotically active. Over a period of thirty-six to seventy-two hours, crypt cells move up the villus to its distal end. During this time they undergo differentiation to the mature cell. Methods have been devised for isolating epithelial cells from various levels along the villus (Weiser, 1973) so that it is possible to assay for enzymes and markers of the cells in their various stages of differentiation. A number of significant findings have been reported.

First, the specific activities of the hydrolytic enzymes sucrase and alkaline hydrolase are greatest in differentiated cells at the tips of the villi while they are lowest in the non-differentiated crypt cells. Furthermore, from villus tip cells to crypt cells there is a gradual decrease in the specific activities of these enzymes. *In vivo* incorporation of radioactive sugars into glycoproteins by intestinal epithelial cells also appears to parallel the degree of differentiation of the cells. D-glucosamine, L-fucose, and D-galactosamine are incorporated, *in vivo*, to the greatest extent by differentiated cells at the villus tip. Cells isolated from the tips of villi also incorporate much greater amounts of radioactive D-glucosamine than do isolated crypt cells during *in vitro* incubations with the sugar. Subcellular fractionation of the epithelial cells from the villus tip indicates that most of the glucosamine-labeled glycoproteins appear in the microvillus plasma membrane fraction and that the hydrolytic enzymes are associated with these glycoproteins (Weiser, 1973).

Secondly, when cells are isolated from the various levels of the villi and assayed for cell surface glycosyltransferase: endogenous acceptor activity, completely differentiated cells are found to have the lowest transferase activity for the nucleotide sugars GDP-fucose, GDP-mannose, UDP-N-acetylglucosamine, UDP-galactose, and UDP-glucose. Only the CMP-sialic acid transferase: endogenous acceptor-activity is greater in differentiated villus tip cells than in crypt cells. By definition of the transferase activities, undifferentiated crypt cells must then not only have the highest levels of transferase activities, but in addition must possess on their cell surfaces incomplete glycoprotein acceptors for glycosylation by these various

sugars. When fetal intestinal cells and chemically-induced intestinal tumor cells are assayed for cell surface galactosyltransferase: endogenous acceptor activity, they exhibit activities similar to those of the crypt cells.

The lectin binding ability of intestinal epithelial cells is also a function of cellular differentiation. Crypt cells, fetal cells, and tumor cells are agglutinated by Con A, whereas differentiated villus cells are not (Weiser, 1972 and 1974). Etzler and Branstrator (1974), using fluorescein isothiocyanate-labeled lectins, have observed a decreasing ability of differentiated cells to bind various lectins other than wheat germ agglutinin on villi ten centimeters or more distant from the pylorus. This is contrasted with the ability of less differentiated cells to bind lectins from *Lotus tetragonolobus* (specific for terminal non-reducing α -L-fucose) and *Ricinus communis* (specific for terminal non-reducing α - or β -D-galactose). Completion of cell surface membrane glycoproteins with β -N-acetylglucosamine (specifically bound by wheat germ agglutinin [LeVine et al., 1972]) and N-acetyl-neuraminic acid (bound nonspecifically by wheat germ agglutinin [Greenaway and LeVine, 1975]) appears to be related to the state of differentiation of intestinal epithelial cells.

Glycoproteins of the Adult Rat Pancreas

Previous studies in this laboratory have made it apparent that glycoproteins are important constituents of the adult rat exocrine pancreas. All of the membrane subfractions of the exocrine pancreas cell bear glycoprotein components (Ronzio et al., submitted for publication). Comparative carbohydrate analysis indicates that of all the membrane structures present in the cell, zymogen granule

membranes have the highest ratio of carbohydrate to protein (0.44) on a mass basis. A "plasma membrane-enriched" fraction is also found to be highly glycosylated with a carbohydrate to protein ratio of 0.23. Zymogen granule membranes and the "plasma membrane-enriched" fraction are also the most enriched in protein-bound fucose and galactose.

Each subcellular fraction isolated from rat exocrine pancreas labeled *in vitro* with radioactive glucosamine exhibits specific glucosamine-labeled glycoproteins when analyzed with SDS-polyacrylamide gel electrophoresis. (Plasma membrane, it must be noted, has not been examined by these techniques.) Zymogen granule membranes bear three major glucosamine-labeled proteins with molecular weights of 120,000 (GP-1), 74,000 (GP-2), and 52,000 (GP-3) daltons. GP-2 is by far the most intensely labeled component, containing two-thirds of the radioactivity recovered from polyacrylamide gels. Smooth microsomal membranes derived from the Golgi complex bear a glucosamine-labeled glycoprotein of the same molecular weight as GP-2 in the zymogen granule membrane.

Though the relative carbohydrate contents of the post-microsomal supernate and the zymogen granule content are low by comparison with the membrane fractions from exocrine pancreas, each contains a minimum of three molecular weight classes of glucosamine-labeled glycoproteins. Approximate molecular weights of these glycoproteins are 68,000, 55,000, and 25,000 daltons (Ronzio et al., submitted for publication). Furthermore, *in vitro* stimulation of secretion in pancreatic tissue pre-labeled with radioactive fucose or glucosamine has shown that a linear discharge of soluble glycoproteins occurs

parallel with the secretion of radioactive leucine-labeled proteins (Völkl et al., 1976; Kronquist et al., 1977).

Glycoprotein Synthesis in the Differentiating Rat
Exocrine Pancreas: A Statement of the Problem

It had been observed in this laboratory that embryonic pancreatic rudiments cultured to different stages of development incorporated radioactive L-fucose into a large number of glycoproteins. Some of these glycoproteins appeared to be induced during pancreatic differentiation. Others, notably a glycoprotein(s) with the electrophoretic mobility of the zymogen granule membrane glycoprotein GP-2, were present in both protodifferentiated and differentiated pancreas in relatively the same amounts (Ronzio, unpublished observations). In addition, it has been reported by Walther (1972, 1974) that 1) 5-BrdU, which prevents the secondary transition in developing exocrine pancreas, does not inhibit the synthesis of glycoproteins associated with membrane functions; 2) glycoprotein profiles on SDS-polyacrylamide gels for glucosamine-labeled control and 5-BrdU cultures of embryonic pancreas are similar; and 3) possibly only one secretory protein with the electrophoretic mobility of amylase or procarboxypeptidase A is glycosylated. (A minimum of three glycosylated zymogen granule content proteins were observed by Ronzio and co-workers [submitted for publication].)

Though these observations are not entirely contradictory, it seemed that the answers to some questions about glycoprotein synthesis during pancreatic differentiation needed clarification. How many glycoprotein species are characteristic of exocrine pancreas at different stages of development? Can different species be observed by labeling pancreatic cultures with different glycoprotein sugar

precursors? Are all these glycoproteins constitutive proteins of the exocrine pancreas or is the synthesis of some of them induced during the secondary transition period of differentiation? Are there glycosylated secretory proteins? What are the relative rates of synthesis of the zymogen granule membrane glycoproteins and the secretory proteins at different stages of pancreatic development? Most important of all, how does glycoprotein synthesis during the embryonic development of the pancreas fit into the "Concerted" mechanism for pancreatic differentiation proposed by Rutter and his colleagues (1968)? This mechanism requires only the two regulatory transitions shown in Figure 1 to account for the differentiation of the exocrine pancreas with respect to secretory protein synthesis. Does this mechanism account for glycoprotein synthesis in the differentiating exocrine pancreas as well?

The work reported in this thesis was designed to answer these questions.

MATERIALS AND METHODS

Materials

Electrophoresis Reagents

Sodium dodecylsulfate (sequenal grade) was obtained from Pierce Chemical Co., Rockford, Illinois. Acrylamide (99%), N,N'-methylene-bisacrylamide, and N,N,N',N'-tetramethylethylenediamine were obtained from Ames Co., Elkhart, Illinois. Ammonium persulfate was from Canalco, Rockville, Maryland. Sigma Chemical Co., St. Louis, Missouri, was the source of Coomassie brilliant blue R and β -mercaptoethanol. Bromphenol blue was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Radiochemicals

D-[6-³H(N)]glucosamine hydrochloride (9 Ci/mmole), L-[6-³H]fucose (12 Ci/mmole), [³H(G)]N-acetyl-D-mannosamine (2.2 Ci/mmole), L[4,5-³H(N)]leucine (60 Ci/mmole), and [³H]H₂O (2.03 x 10⁶ dpm/ml, calibrated 9/14/74) were obtained from New England Nuclear, Boston, Massachusetts.

Reagents for Liquid Scintillation Counting

Triton X-100, 2,5-diphenyloxazole, and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene were obtained from Research Products International Corp., Elk Grove Village, Illinois.

Immunochemicals

Goat antirabbit (gamma globulin) was obtained from Calbiochem, La Jolla, California. Rabbit antisera to bovine serum albumin (crystallized, Sigma Chemical Co.) and to subcellular fractions of adult rat pancreas had been prepared in the laboratory (Ronzio and Mohrlök, 1977). Complete Freund's adjuvant was obtained from Difco Laboratories, Detroit, Michigan.

Tissue Culture Media

Powdered Hanks' Balanced Salt Solution (with NaHCO_3), powdered Nutrient Mixture F-12 (with L-glutamine, without NaHCO_3), Minimal Essential Medium Amino Acids Solution (50x, without L-glutamine), Minimal Essential Medium Non Essential Amino Acids Solution (10 mM, 100x), and Antibiotic-Antimycotic Solution (100x, containing 10,000 units Penicillin, 10,000 μg Streptomycin, and 25 μg Fungizone per ml) were obtained from Grand Island Biological Co. (GIBCO, Grand Island, New York) prepared according to their formulations.

5-Bromodeoxyuridine was obtained from Sigma Chemical Co. Bovine Plasma Albumin, Fraction V, was from Miles Laboratories, Inc., Elkhart, Indiana. Dulbecco's Phosphate Buffered Saline was prepared as a 10x solution from reagent grade salts according to the formulation in the GIBCO catalog, except for the substitution of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ for anhydrous CaCl_2 and the addition of 0.1 g phenol red (Sigma Chemical Co.).

All powdered media were prepared according to the package directions and supplemented as described in Methods. Media were filtered through sterile Millipore (0.22 μm) or Gelman Metrical (0.20 μm) filters before use.

Miscellaneous Reagents and Materials

Sucrose (ultrapure) was obtained from Schwarz/Mann, Orangeburg, New York. Soybean trypsin inhibitor and cyanogen bromide-activated Sepharose 4B were obtained from Sigma Chemical Co. Dimethyl sulfoxide was obtained from both Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, and Mallinckrodt, St. Louis, Missouri. ICN Pharmaceuticals, Inc., Plainview, New York, was the source of copper ethylenediamine-tetraacetate. Sephadex G200 was from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey, and DEAE cellulose (Cellex-D) was from Bio-Rad Laboratories, Richmond, California. Soluble starch was obtained from Mallinckrodt. Polyethylene glycol (average molecular weight 6000-7500) was obtained from Matheson Coleman and Bell, Norwood, Ohio. Kodak X-Omat R X-ray film came from Picker X-Ray, Detroit, Michigan, and Kodak RP Royal X-Omat X-ray film came from Grand X-Ray, Grand Rapids, Michigan.

Laboratory Animals

Sprague-Dawley rats, including timed-pregnant female rats, were obtained from Spartan Research Laboratories, Haslett, Michigan.

Methods

In vitro Culture of Embryonic Rat Pancreas

The method used for culturing dorsal pancreatic rudiments from embryonic rats was essentially that described by Ronzio and Rutter (1973), except for the omission of chick embryo extract from the culture medium. Timed-pregnant Sprague-Dawley rats were sacrificed by decapitation on day 14 of gestation and the uteri transferred to sterile plastic petri dishes containing sterile Hanks' Balanced Salt

Solution. Iridectomy knives were used to dissect the pancreases from the embryos. Dissection was carried out in sterile plastic petri dishes containing sterile F-12 medium supplemented with 4% (v/v) Minimal Essential Medium Amino Acids Solution (50x). (This represented a supplement of two times the amounts of the essential amino acids present in Eagle's Medium [Eagle, 1959].) Pancreatic rudiments, with some adhering mesenchyme, were transferred to 2 mm-wide Millipore filter (type THWP) strips glued across the wells of a section of Linbro plastic tray (MVC-96) set into a sterile petri dish. Four to eight rudiments were placed on each filter strip. The culture wells were filled with approximately 0.25 ml of culture medium consisting of F-12 supplemented with 4% (v/v) Minimal Essential Medium Amino Acids Solution (50x), 1% (v/v) Antibiotic-Antimycotic Solution (100x), and L-glutamine to a final concentration of 2.0 mM. Culture dishes were kept under a humidified atmosphere of 95% air-5% CO₂ in a 37°C incubator. The culture medium was changed daily. When rudiments were cultured in the presence of 5-BrdU, 2×10^{-3} M 5-BrdU in water was added to the culture medium to a final concentration of 2×10^{-5} M.

Cultured rudiments were labeled with radioactive sugars on the equivalent of days 14, 17, and 19 of gestation. The appropriate volume of [³H]sugar was transferred to a sterile disposable plastic tube and taken to near dryness with a filtered stream of nitrogen. Sterile culture medium (plus or minus 5-BrdU) was then added to the tube to give a final [³H]sugar concentration of 25 µCi/ml. The culture medium was then replaced by approximately 0.25 ml of this radioactive medium. Rudiments were labeled for 18 hours. Incubation of rudiments with [³H]leucine was carried out in a similar manner.

The final leucine specific radioactivity was 12.4×10^6 dpm/ μ g (Kemp et al., 1972).

At the end of the labeling period, cultured rudiments were harvested. The radioactive medium was aspirated from the culture wells and the wells were rinsed once with Dulbecco's Phosphate Buffered Saline (pH 7.0-7.4). The filter strips with adhering pancreatic rudiments were then removed from the wells and placed into a small amount of Dulbecco's Phosphate Buffered Saline in a petri dish. Using the iridectomy knives, the rudiments were gently scraped or lifted off the filter strips. The rudiments were then transferred to plastic microfuge tubes and centrifuged for 5-15 seconds in a Beckman model 152 microfuge. Usually one to two litters of rudiments were pooled per tube. Residual medium was aspirated from the tubes and the tissue pellets were frozen on dry ice and stored at -80°C .

Procedures for SDS-Polyacrylamide Slab Gel Electrophoresis and Gel Autofluorography

Gel Preparation. The Laemmli (1970) SDS-polyacrylamide gel system was used, with some modifications, for all slab gel electrophoresis analyses. Slab gels, 1.5 mm thick, consisted of a one-centimeter stacking gel above a nine-centimeter separating gel. A 30% acrylamide-0.8% N,N'-methylenebisacrylamide stock solution was used in preparing both the separating gel and the stacking gel. The separating gel of 8% acrylamide contained 0.373 M Tris-HCl (pH 8.8) and 0.1% SDS. Polymerization was with tetramethylethylenediamine and ammonium persulfate at final concentrations of 0.17% (v/v) and 0.08% (w/v), respectively. The stacking gel of 3% acrylamide

contained 0.116 M Tris-HCl (pH 6.8) and 0.1% SDS and was polymerized by the addition of tetramethylethylenediamine and ammonium persulfate to final concentrations of 0.024% (v/v) and 0.09% (w/v), respectively. The concentrations of polymerizing agents in both the stacking and separating gels were modifications of the Laemmli system made by Gahmberg and Hakomori (personal communication).

The day before use, gels were cast between glass plates rinsed with 2% Photo-flo 200 (Kodak).

Sample Preparation. For electrophoresis, usually one to two litters of radioactively-labeled pancreatic rudiments were solubilized in 4% SDS and then mixed with an equal volume of sample buffer containing 0.124 M Tris-HCl (pH 6.8), 20% (w/v) glycerol, 0.002% bromphenol blue, and 10% (w/v) β -mercaptoethanol. All samples contained 2% SDS, 0.062 M Tris-HCl (pH 6.8), 10% (w/v) glycerol, 0.001% bromphenol blue, and 5% (w/v) β -mercaptoethanol when applied to the gels. The prepared samples were boiled 2-5 minutes before application to the gels.

Electrophoresis Conditions. The same buffer was used for both upper and lower reservoirs. This buffer (pH 8.3) consisted of 0.025 M Tris, 0.192 M glycine, and 0.1% SDS.

Gels were electrophoresed at room temperature in a Bio-Rad Laboratories Model 220 slab gel electrophoresis unit. Run time was either 6-8 hours at 25 ma per gel or overnight at a low voltage setting. After overnight runs, the voltage was usually increased on the following morning to 75-100 volts. Gels were electrophoresed until the bromphenol blue tracking dye had migrated 8.5 to 9.0 cm from the top of the separating gel.

Gel Staining Procedure. After completion of electrophoresis, some gels were stained in a solution of 0.1% Coomassie brilliant blue R in 30% (v/v) methanol-10% (v/v) glacial acetic acid (Bonner and Laskey, 1974). Gels were stained 16-24 hours at room temperature. Destaining was accomplished in 30% (v/v) methanol-10% (v/v) glacial acetic acid at room temperature, usually within 48 hours. Stained gels appearing in Figures 2A, 2B, 5A, 5B, and 14A were stained 24 hours, destained for 48 hours, and then stored at 0-4°C in 10% (v/v) glacial acetic acid until photographed.

Procedures for Gel Autofluorography. Gels were prepared for autofluorography according to the procedure outlined by Bonner and Laskey (1974). Gels were first soaked in twenty times their volume of dimethyl sulfoxide for thirty minutes at room temperature. This soaking was repeated for an additional thirty minutes in fresh dimethyl sulfoxide. Gels were then immersed in four times their volume of 20% (w/w) 2,5-diphenyloxazole in dimethyl sulfoxide for three hours. Subsequently, the gels were rinsed in distilled water and then rehydrated for one hour in twenty volumes of distilled water. The gels were then dried on Whatman 3MM filter paper using a Bio-Rad slab gel drier Model 224. Both stained and non-stained gels were prepared for autofluorography by this procedure.

Autofluorographs were obtained by exposure of either Kodak X-Omat R or RP Royal X-Omat X-ray film to the dried gels. In complete darkness a dried gel was sandwiched with a sheet of X-ray film between two glass plates held together by metal clamps. The gel-film sandwich, placed in a small manilla envelope, was wrapped in multiple layers of aluminum foil and stored at -80°C. X-ray films were

exposed for varying lengths of time depending on the radioactivity of samples applied to the gels. Films were not exposed to a hypersensitizing light flash before use (Laskey and Mills, 1975). X-ray films were developed in a Kodak RP X-Omat Rapid Processor.

Molecular Weight Calibration of Gels. Protein standards were included on some of the gels that were stained before preparation for autofluorography. The standard proteins that were used and their molecular weights are listed in Table 1. An average relative mobility

Table 1. Relative mobilities of standard proteins on 0.1% SDS-8% acrylamide slab gels

Standard Protein	Molecular Weight	Reference	Average Relative Mobility
Thyroglobulin (porcine)	165,000	DeCrombrugghe et al., 1966	0.089
Phosphorylase A (rabbit muscle)	94,000	Weber and Osborn, 1969	0.335
Transferrin (human)	74,000	Roberts et al., 1966	0.445
Serum albumin (bovine)	68,000	Weber and Osborn, 1969	0.478
Fumarase (porcine heart)	49,000	Weber and Osborn, 1969	0.670
α -Chymotrypsinogen (bovine pancreas)	25,700	Weber and Osborn, 1969	0.91

for each protein was obtained from measurements made on dried gels and/or photographs taken of the stained gels before the gels were prepared for autofluorography. The relative mobility of a protein

is defined as the ratio of the migration distance of the protein band (measured from the top of the separating gel to the center of the protein band) to the migration distance of the bromphenol blue tracking dye (similarly measured). All protein standards were run on a minimum of two gels.

A plot of log molecular weight as a function of relative mobility for the standard proteins was linear. A least squares analysis supplied the slope (-0.9623) and y-intercept (5.3015) parameters for this plot. The following equation was used to calculate molecular weights of unidentified proteins on experimental slab gels:

$$\text{Molecular Weight} = \text{antilog}[(-0.9623)(\text{Relative Mobility}) + (5.3015)]$$

From this equation, it is estimated that proteins with molecular weights in the range of 22,000 to 200,000 are resolvable in this gel system. If the molecular weights of the standard proteins are in turn calculated using this equation, values within 10% of the known molecular weights are obtained. This is the degree of accuracy normally assigned to protein molecular weight determinations based on migration in SDS-polyacrylamide gels.

It will be noted that ovalbumin appears as a standard in several of the figures presenting stained gel profiles in Results. Ovalbumin was not used, however, in the least squares calculation described above. The electrophoretic behavior of ovalbumin deviated measurably from the linear relationship between log molecular weight and relative mobility observed for the other standard proteins employed.

In vitro Labeling of Adult Rat Pancreas
with [^3H]Leucine and [^3H]Fucose

Pancreatic tissue taken from adult Sprague-Dawley rats was minced into small fragments. The minced tissue was transferred to 50-ml Erlenmeyer flasks (approximately 2 g per flask) in complete Krebs-Ringer bicarbonate buffer (Krebs, 1950) containing 1% bovine plasma albumin, 1% (v/v) Antibiotic-Antimycotic Solution (100x), 2.5 mM Ca^{2+} , 1.2 mM Mg^{2+} , 0.01% soybean trypsin inhibitor, 14 mM glucose, and a complete Minimal Essential Medium (Eagle's) amino acid supplement (Amsterdam and Jamieson, 1974). Tissue was incubated at 37°C for 4 hours in 125 μCi L-[4,5- $^3\text{H}(\text{N})$]leucine or 50 μCi L-[6- ^3H]fucose in a total volume of 5 ml medium per flask. Periodically throughout the incubation period, the flasks were aerated with moistened 95% air-5% CO_2 . At the conclusion of the incubation period, the labeling medium was aspirated from the flasks and the minced tissue was pooled and washed extensively with 0.3 M sucrose containing 0.25 mg/ml soybean trypsin inhibitor.

Preparation of Zymogen Granule Membranes
and Zymogen Granule Content Proteins
from Adult Rat Pancreas

Zymogen granule membranes and zymogen granule content proteins were obtained from unlabeled pancreatic tissue or washed labeled tissue by the fractionation procedure of Ronzio and colleagues (submitted for publication) based on the procedure of Meldolesi et al. (1971). All operations were carried out at 4°C except where noted. The tissue was homogenized in ten volumes of 0.3 M sucrose containing 0.25 mg/ml soybean trypsin inhibitor with four strokes of a glass-teflon Potter-Elvehjem homogenizer driven at 620 revolutions per minute. The homogenate was filtered through several

layers of cheesecloth and centrifuged at 500 x g to sediment nuclei and tissue debris.

To sediment zymogen granules, the 500 x g supernate was centrifuged at 1730 x g for 30 minutes. The tops of the zymogen granule pellets were washed with 0.3 M sucrose containing 0.25 mg/ml soybean trypsin inhibitor to remove co-sedimenting mitochondria, then resuspended in the sucrose-soybean trypsin inhibitor solution and centrifuged for 30 minutes at 1730 x g. (In one preparation, the first 1730 x g supernate was centrifuged at 1730 x g for another 30 minutes. Zymogen granules from this centrifugation were washed, resuspended, and pooled with the first washed zymogen granule pellet. A final centrifugation at 1730 x g for 30 minutes was used to sediment all zymogen granules.)

Final zymogen granule pellets were suspended in a small volume (0.5-1.0 ml) of 0.15 M NaCl. This suspension was added dropwise to 15 times the volume of 0.2 M NaHCO_3 , and the zymogen granules were allowed to lyse at room temperature for approximately ten minutes. The lysed suspension was layered over a discontinuous gradient consisting of 4 ml of 1.0 M sucrose-0.25 mg/ml soybean trypsin inhibitor topped with 4 ml of 0.3 M sucrose-0.25 mg/ml soybean trypsin inhibitor in a nitrocellulose tube. The gradients were centrifuged at 196,000 x g for 1 hour.

The top 4 ml of solution containing zymogen granule content proteins were removed from the gradients, pooled, and stored at -80°C . Two-thirds of the 0.3 M sucrose-0.25 mg/ml soybean trypsin inhibitor layer were pipetted from the tubes and discarded. The zymogen granule membrane felt at the interface of the two layers of sucrose was transferred with a Pasteur pipet to a second nitrocellulose

tube and diluted with an equal volume of 0.25 M NaBr. The NaBr-membrane suspensions were sonicated with four to five pulses (each approximately 5 seconds in duration) of the small probe of a Bronwill sonicator. The tubes were then filled with 0.25 M NaBr and centrifuged at 272,000 x g for 2 hours. The NaBr wash was aspirated from the tubes and the zymogen granule membrane pellets frozen on dry ice and stored at -80°C.

Preparation of Rabbit Antisera to Bovine Serum Albumin and to Rat Pancreas Zymogen Granule Membranes and Zymogen Granule Content Proteins

Antisera to crystalline bovine serum albumin (BSA), rat pancreas zymogen granule membranes, and zymogen granule content proteins were supplied by Ms. S. Mohrlök of this laboratory. They were obtained by immunization of New Zealand white rabbits. Immunization to BSA and zymogen granule content proteins was obtained by injections of these proteins (1-2 mg) dissolved in 0.15 M NaCl and mixed with equal volumes of complete Freund's adjuvant. Zymogen granule membranes (0.4-0.5 mg) were suspended in 0.15 M NaCl plus an equal volume of complete Freund's adjuvant for injections (Ronzio and Mohrlök, 1977).

Rabbits were bled by puncture of the inner marginal vein of the ear. Collected blood was allowed to clot at room temperature or 37°C for 30 minutes and then stored overnight at 0-4°C. The serum was decanted from the clotting tube into clean centrifuge tubes and centrifuged (at 4°C) for thirty minutes at 1000 x g to sediment red blood cells. The serum was stored at -20°C.

Antisera to BSA and zymogen granule content proteins were heated at 56°C for thirty minutes to inactivate complement.

Preparation of Anti Zymogen
Granule Membrane (IgG)

The gamma globulin fraction from antiserum to zymogen granule membrane was prepared according to the procedures of Fraker, Cicurel, and Nisonoff (1974) and Levy and Sober (1960). A known volume of antiserum was precipitated with sufficient 25% Na_2SO_4 (at 25°C) to give a final Na_2SO_4 concentration of 18%. The precipitation mixture was centrifuged at room temperature for 30 minutes at 1265 x g. The supernate was discarded. Water was added to the precipitate and then sufficient 25% Na_2SO_4 was added to make the final Na_2SO_4 concentration 14%. The precipitation mixture was centrifuged as before and the supernate decanted. The final precipitate was dissolved in a small volume of borate-saline buffer (pH 8.0-8.2, containing 0.13 M NaCl, 0.16 M boric acid, and 0.02 N NaOH) and transferred to dialysis tubing. Dialysis was against several changes of this same buffer for 44 hours at 4°C. In preparation for DEAE cellulose chromatography, dialysis was continued against 0.018 M phosphate buffer (pH 6.9) containing 1.05 g Na_2HPO_4 and 1.5 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ per liter. The dialyzed preparation was centrifuged to remove insoluble material.

To remove possible contaminating beta globulins, the dialyzed anti zymogen granule membrane (gamma globulin) preparation was chromatographed on a column (9.5 cm x 1.5 cm, i.d.) of DEAE cellulose equilibrated in 0.018 M phosphate buffer (pH 6.9). Peak fractions (determined by absorbance readings at 280 nm) were pooled and transferred to a dialysis bag. The dialysis bag was then dredged in polyethylene glycol (average MW 6000-7500) to concentrate the gamma

globulin preparation. The concentrated preparation was dialyzed against borate-saline buffer (pH 8.0-8.2), then stored at -20°C.

Preparation of Particulate and Soluble Fractions from Pancreatic Rudiments for Radioimmunoassay

Borate-saline buffer (pH 8.4-8.5, containing 5.0 mM boric acid, 1.25 mM sodium borate, and 0.142 M NaCl) was added to plastic microfuge tubes containing [³H]fucose- or [³H]glucosamine-labeled pancreatic rudiments at a ratio of 75 µl buffer per 10-13 rudiments. The tubes were capped, sealed with parafilm, and taped to the bottom of a shallow plastic dish to which ice water was added. The large probe of a Bronwill sonicator, set at 80% maximum probe intensity, was run up and down the side of the tube, usually for one or two minutes, to completely suspend the tissue. The sonicates were then centrifuged at 111,000 x g for 2 hours at 4°C. The supernate, designated the "soluble fraction", was removed from each tube using a Hamilton syringe. Each pellet was resuspended by sonication in 0.25 M NaBr. The sonicates were centrifuged as before. The resultant supernates were discarded and the pellets, designated the "particulate fraction", were resuspended with sonication in Na₂HPO₄-detergent buffer (pH 7.4, containing 10 mM Na₂HPO₄, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 5 mM disodium EDTA, 12 µM phenylmethylsulfonyl-fluoride, and 0.001% butylated hydroxytoluene [Ronzio and Mohrlök, 1977]).

Radioimmunoassay Procedures

Precipitin Reactions. Double antibody quantitative precipitin reactions were used to determine the levels of glycosylated secretory

proteins (glycosylated zymogen granule content proteins) and glycosylated zymogen granule membrane-like proteins present in pancreatic rudiments at different stages of development. Assays for the soluble glycoproteins were carried out in standard borate-saline buffer (pH 8.4-8.5) containing 5.0 mM boric acid, 1.25 mM sodium borate, and 0.142 M NaCl. A phosphate-detergent buffer (pH 7.4), containing 10 mM Na_2HPO_4 , 1% (v/v) Triton X-100, 1% sodium deoxycholate, 5 mM disodium EDTA, 12 μM phenylmethylsulfonylfluoride, and 0.001% butylated hydroxytoluene, was used for all immunoassays of particulate membrane glycoproteins. Final assay volume in all cases was 300 μl .

Radioimmunoassay reactions for secretory glycoproteins in the soluble fractions prepared from pancreatic rudiments were carried out in the following manner. First buffer was aliquoted into plastic microfuge tubes and then various dilutions of the soluble fractions in borate-saline buffer were added to the tubes. Total volume was 90 μl . Ten microliters of decomplexed rabbit anti zymogen granule content (antiserum) was next added to the tubes followed by vortexing. Reaction mixes were incubated 30 minutes at 37°C. Two hundred microliters goat antirabbit (gamma globulin) (reconstituted in borate-saline buffer) were added to the reactions, and the reaction mixes were then incubated at 25°C for one hour. Subsequently the tubes were stored at 0-4°C for a minimum of 24 hours.

Radioimmunoassays for zymogen granule membrane glycoproteins in particulate fractions prepared from pancreatic rudiments were carried out as follows. Phosphate-detergent buffer was aliquoted into plastic microfuge tubes followed by dilutions of particulate fractions (in phosphate-detergent buffer). Total volume was 90 μl . Ten microliters of anti zymogen granule membrane (IgG) (treated to

remove cross-reactivity with secretory proteins) were added to the tubes and the tubes were vortexed. The reactions were incubated at 37°C for 30 minutes. After vortexing, the tubes were stored at 0-4°C for a minimum of 16 hours. One hundred thirty microliters of phosphate-detergent buffer and 70 µl of goat antirabbit (gamma globulin) (reconstituted in phosphate-detergent buffer) were then added to the reaction mixtures. Following a one-hour incubation at 25°C, the reaction mixtures were stored a minimum of 25 hours at 0-4°C.

Control reactions for measuring nonspecific adsorption of labeled proteins or trapping of free radioactive sugar by the immunoprecipitate were prepared in which 10 µl of decomplexed rabbit anti BSA (antiserum) were substituted for anti zymogen granule content (antiserum) and 40 µl of anti BSA (antiserum) diluted 1 to 10, were substituted for anti zymogen granule membrane (IgG).

Collection of Precipitates. Precipitin reaction mixtures were layered on top of 5 ml of 1 M sucrose (dissolved in the appropriate buffer) in conical centrifuge tubes (Rosen et al., 1975). The reaction tubes were rinsed twice with 200 µl of reaction buffer, and these rinses were pooled with the precipitin reaction mixtures in the centrifuge tubes. The tubes were centrifuged at 2000 x g for 10 minutes (0-4°C). The sucrose solution was removed from the tubes and the precipitates were resuspended in one milliliter of the appropriate buffer. Centrifugation at 2000 x g for 5 minutes was used to sediment the precipitates. The precipitates were washed a second time in the same manner. After removal of the final wash,

the tubes were inverted and stored several hours or overnight at 0-4°C to drain.

Washed and drained precipitates were solubilized in 200 µl of 1% SDS-0.1 N NaOH by heating at 60°C for one hour. Samples of the solubilized precipitates were taken for liquid scintillation counting. The results were expressed as the percent of total acid-precipitable dpm initially added to the precipitin reaction precipitated by the antibody. The percent radioactivity precipitated by the control anti BSA (antiserum) was subtracted from the experimental values.

Acid Precipitation of Radioactive Protein

Protein-bound radioactivity of labeled pancreatic rudiments sonicated in 4% SDS and of rudiment subfractions in borate-saline or phosphate-detergent buffer was determined by acid precipitation. One hundred microliters of carrier BSA solution (500 µg protein) was added to tubes containing known volumes of samples. Cold 20% trichloroacetic acid was then added to the tubes to a final concentration of 10%. Precipitation reactions were left at 0-4°C for at least one hour (usually overnight). Tubes were centrifuged at 2000 rpm for 10 minutes (0-4°C) in a Sorvall GLC-2 centrifuge. The acid soluble fraction was removed from each tube and the precipitate was washed three times by suspension in 200 µl cold 10% trichloroacetic acid followed by centrifugation. The final wash was aspirated off the precipitate.

Washed acid precipitates were solubilized in 200 µl 1% SDS-0.1 N NaOH with heating at 60°C for one hour. Samples of the solubilized precipitates were taken for liquid scintillation counting. Recovery of "acid-soluble" plus "acid-precipitable" radioactivity was usually

approximately 80% of the total radioactivity of the original samples. Contamination of the precipitates with acid-soluble radioactivity was estimated at less than 1% of the total radioactivity based on the results of acid precipitation of known amounts of [^3H]fucose and [^3H]glucosamine in the presence of carrier protein.

"Percent Acid-Precipitable Radioactivity" was calculated as the percent of total original dpm found in 10% trichloroacetic acid precipitates.

Immunodiffusion Analysis

Immunodiffusion analyses of anti zymogen granule content (antiserum) and anti zymogen granule membrane (IgG) were carried out according to the method of Ouchterlony (1958) in 1% agarose gels. Zymogen granule content proteins and zymogen granule membrane used to cross react with the antibodies were prepared as described. Microsomal membrane subfractions (from adult rat pancreas) used in cross-reactivity studies were supplied by Ms. S. Mohrlök of this laboratory. They were prepared according to published procedures (Jamieson and Palade, 1967; Ronzio, 1973a and 1973b). The smooth microsomes had been taken through the NaCl-NaHCO₃ lysis step but had not been washed with 0.25 M NaBr. Rough microsomal membranes were NaBr-washed.

Liquid Scintillation Counting

All radioactive samples were counted in a non-refrigerated Hewlett Packard Model 2405 scintillation counter using the tritium quick set or in a refrigerated Hewlett Packard Tri-Carb Model 3310 with a gain setting of 46%. Counting efficiencies were generally similar for the two instruments. Counting efficiency was determined

by the inclusion of "cold" samples (prepared identically to the experimental samples) to which a [^3H]H₂O standard was added, or by first counting the experimental samples and then adding [^3H]H₂O standard and recounting.

Samples were counted in 10 ml of a scintillation fluid consisting of two parts toluene (containing 0.015% 1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]-benzene and 0.82% 2,5-diphenyloxazole) and one part Triton X-100 (Patterson and Greene, 1965). Usually 0.9 ml 1% SDS-0.1 N NaOH and 0.1 ml 1 N HCl were included in the vials.

General Assay Procedures

Protein analyses were carried out according to a micromodification of the Lowry protein assay (Lowry, 1958; Rutter, 1967). Crystalline BSA was used as a standard.

Samples containing Triton X-100 were analyzed for protein by the method of Wang and Smith (1975) using crystalline BSA as a standard and phosphate-detergent buffer (pH 7.4) for sample dilution. Volumes of all reagents were reduced by a factor of five to accommodate small sample volumes of 40 μl or less with protein values on the order of 1 $\mu\text{g}/\mu\text{l}$.

Amylase was assayed in rudiment sonicates by a micromodification of the Bernfeld (1955) method. Volumes of assay components were reduced by a factor of ten. Maltose hydrate was used to prepare a reference standard curve.

RESULTS

5-Bromodeoxyuridine Inhibition of *in vitro* Pancreatic Differentiation

To test the effectiveness of 5-bromodeoxyuridine (5-BrdU) as an inhibitor of pancreatic differentiation, equal numbers of pancreatic rudiments from 14-day rat embryos were cultured for five days in the absence or presence of 2×10^{-5} M 5-BrdU. Harvested rudiments were sonicated in distilled water and samples were analyzed for amylase activity and protein. The level of amylase found in control rudiments was 20.1 μg eq maltose/ μg protein/minute, whereas the level of amylase in 5-BrdU-treated rudiments was only 1.62 μg eq maltose/ μg protein/minute. Thus, amylase activity was reduced by a factor of twelve in rudiments cultured in the presence of 5-BrdU. The amount of total protein accumulated per 5-BrdU-treated rudiment, however, was essentially the same as that for control rudiments (18 and 19 μg /rudiment, respectively).

These results are not entirely consistent with the findings of Walther and co-workers (1974), who observed that 5-BrdU added to the growth medium at a concentration of 2×10^{-5} M inhibited the accumulation of amylase activity by a factor of twenty-eight and produced half-maximal inhibition of total pancreatic protein accumulation. However, differences in age of the embryos at the time of dissection, embryo dissection technique, number of rudiments sampled, and culture technique all may have contributed to the

apparent differences in effectiveness of 5-BrdU in inhibiting pancreatic differentiation. The results reported here do indicate that 5-BrdU was inhibiting the accumulation of at least one pancreas specific protein well enough that the system could be used to study glycoprotein synthesis during pancreatic differentiation.

Accumulation of zymogen granules in control rudiments during differentiation in culture gave a cobbled appearance to the rudiments when they were examined under the dissecting microscope. 5-BrdU-treated rudiments did not acquire this structural feature. The absence of large numbers of zymogen granules in 5-BrdU-treated rudiments was a further indication that 5-BrdU was effectively inhibiting the differentiation of the rudiments.

The appearance of fluid-filled vacuoles in pancreatic rudiments cultured in the presence of 5-BrdU was also an indicator of the effectiveness of 5-BrdU in inhibiting exocrine cell differentiation. Rutter and colleagues (Githens et al., 1976) have shown that these vacuoles are lined by duct cells, the proportion of which increases dramatically in 5-BrdU-treated pancreatic rudiments.

SDS-Polyacrylamide Slab Gel Electrophoresis Analysis of Protein and Glycoprotein Synthesis in Differentiating Pancreatic Rudiments

Induction of specific protein and glycoprotein synthesis during pancreatic differentiation was first assessed by SDS-polyacrylamide gel electrophoresis of pancreatic rudiments labeled at various times during the secondary transition period of differentiation with radioactive leucine or radioactive sugars. Rudiments were cultured for 18 hours in the presence of the radioactive precursors. Radioactive sugars used to label the rudiments were L[³H]fucose,

[³H]N-acetyl-D-mannosamine, and D[³H]glucosamine. (Fucose and N-acetylmannosamine are normally incorporated at or near the non-reducing ends of glycoprotein carbohydrate chains, N-acetylmannosamine being incorporated as one of the N-acylneuraminic acids. Glucosamine, as a general label for the carbohydrate of glycoproteins, is incorporated throughout the chains as N-acetylglucosamine or N-acetylgalactosamine and terminally as N-acylneuraminic acids.) Control rudiments were labeled at 14 days of gestation or after three or five days in culture (the equivalent of 17-day and 19-day rudiments, respectively). Rudiments cultured in the presence of 5-BrdU were also labeled after three or five days in culture.

Labeled rudiments were sonicated in 4% SDS and samples were prepared for electrophoresis on 0.1% SDS-polyacrylamide slab gels. Approximately the same amount of radioactivity for each sample was applied to the gels. Gels were stained with Coomassie brilliant blue and/or prepared for autofluorography.

Coomassie Blue Staining Profiles for Cultured Pancreatic Rudiments

The Coomassie blue staining profiles for fucose- and glucosamine-labeled pancreatic rudiments are shown in Figure 2A. Arrows indicate either distinct proteins or heterogeneous unresolvable bands of protein induced during differentiation. In this system, "induced" proteins are those proteins which appear in the culture equivalent of 19- to 20-day control rudiments at significantly greater levels than in 14- to 15-day control (protodifferentiated) rudiments and rudiments cultured in the presence of 5-BrdU to the equivalent of 19-20 days gestation.

Figure 2. SDS-polyacrylamide gel electrophoresis of [^3H]fucose-, [^3H]glucosamine-, and [^3H]leucine-labeled pancreatic rudiments.

Samples of cultured pancreatic rudiments, labeled at different stages of differentiation, were electrophoresed on 0.1% SDS-8% acrylamide slab gels as described in Methods. Gels were stained and/or prepared for autoradiography. Coomassie blue staining profiles for labeled rudiments are shown in Figure A. Figure B is the fluorograph prepared from the stained gel in Figure A. Figure C is the fluorograph of an identically prepared gel that was not stained before preparation for autoradiography.

Fluorographs of [^3H]fucose- and [^3H]glucosamine-labeled samples were obtained from films (RP Royal X-Omat) exposed to the prepared gels for 22 days. Fluorographs of [^3H]leucine-labeled samples were from films (RP Royal X-Omat) exposed to prepared gels for three days.

Gels were prepared so that on the basis of initial radioactivity measurements, 20,000 cpm were applied for samples B-G and 200,000 cpm were applied for samples H and I. The actual amounts of acid-precipitable radioactivity for each sample applied to the gels (dpm values in parentheses) were determined after electrophoresis.

Lane A shows the protein standards, 1) porcine thyroglobulin (165,000), 2) human transferrin (74,000), 3) bovine serum albumin (66,000), 4) ovalbumin (43,000), and 5) bovine pancreatic ribonuclease A (13,700) at the bromophenol blue tracking dye.

Lanes B-D show the profiles for cultured pancreatic rudiments labeled with [^3H]fucose on the equivalent of B) 14-15 days gestation (28 μg protein; 52,900 dpm), C) 19-20 days gestation (40 μg protein; 47,800 dpm), and D) 19-20 days gestation, 5-BrdU culture (33 μg protein; 42,100 dpm).

Lanes E-G show the profiles for cultured pancreatic rudiments labeled with [^3H]glucosamine on the equivalents of E) 14-15 days gestation (39 μg protein; 36,600 dpm), F) 19-20 days gestation (10 μg protein; 40,800 dpm), and G) 19-20 days gestation, 5-BrdU culture (14 μg protein; 32,800 dpm).

Lanes H and I show the profiles for cultured pancreatic rudiments labeled with [^3H]leucine on the equivalent of H) 19-20 days gestation (2.9 μg protein; 450,000 dpm) and I) 19-20 days gestation, 5-BrdU culture (2.5 μg protein; 540,000 dpm).

Arrows indicate differentiation-induced protein and glycoprotein species.

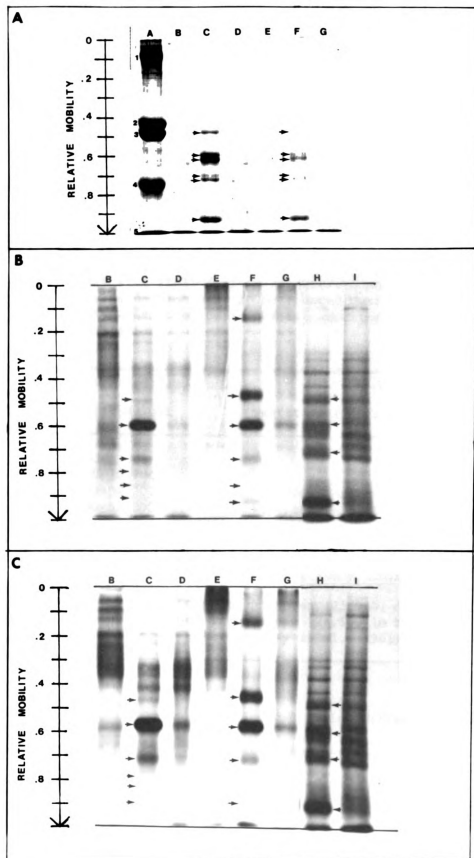


Figure 2

A minimum of 29 protein species can be identified in this gel system as early as day 15 of pancreatic development. Stained protein profiles, as expected, are similar for corresponding samples of fucose- and glucosamine-labeled rudiments. The majority of the proteins that are detected appear to be constitutive. Six induced Coomassie blue-stained proteins are detected. The relative mobilities and calculated molecular weights of these proteins are summarized in Table 2.

Table 2. Coomassie blue-stained proteins observed on SDS-polyacrylamide slab gels to be induced during the secondary transition period of rat exocrine pancreas differentiation

Relative Mobility	Molecular Weight	Tentative Identification
0.47	70,000	
0.47-0.49	67,600-70,700	
0.59-0.61	51,800-54,200	Amylase (56,000; Sanders, 1970)
0.61-0.63	49,600-51,800	Procarboxypeptidase B (50,000; Sanders, 1970)
0.70-0.72	40,600-42,400	Lipase (37,000-43,000; Vandermeers and Christophe, 1968)
0.72-0.73	39,700-40,600	Procarboxypeptidase A (36,000; Walther et al., 1974)
0.93-0.94	24,900-25,500	Chymotrypsinogen (23,000; Sanders, 1970) or Trypsinogen (24,500 in bovine pancreas; Kay et al., 1961)

Among the proteins known to be induced during differentiation of the rat exocrine pancreas are eight of the secretory proteins. (More than a dozen proteins have been resolved for pancreatic secretions from various animal species.) These proteins are amylase,

procarboxypeptidase B, lipases, procarboxypeptidase A, chymotrypsinogen, trypsinogen, and ribonuclease.

With the exception of the proteins with relative mobility 0.47 and 0.47-0.49, the induced proteins in Figure 2A may be tentatively identified as secretory proteins on the basis of their estimated molecular weights and relative abundance. In the order of increasing mobility, these induced proteins are probably amylase, procarboxypeptidase B, lipase(s), procarboxypeptidase A, and chymotrypsinogen and/or trypsinogen. The induced proteins with relative mobilities of 0.47 and 0.47-0.49 correspond in molecular weight ($\sim 69,000$) to a rat pancreas zymogen granule content glycoprotein(s) previously observed by Ronzio and co-workers (submitted for publication) and to an unidentified differentiation-induced rat pancreas protein observed by Walther and co-workers (1974). Ribonuclease is expected to have migrated with the tracking dye in this gel system as did bovine pancreatic ribonuclease A which has a molecular weight similar to that of the rat pancreas enzyme (13,000 daltons; Walther et al., 1974). Thus, the differentiation-dependent induction of ribonuclease could not be determined in this gel system since a number of low molecular weight proteins are expected to migrate with the tracking dye.

The molecular weights of the induced proteins observed in Figure 2A correspond with the "known" molecular weights of the assigned secretory proteins within the 10% error range imposed upon molecular weight determinations based on protein migration in SDS-polyacrylamide gels (Weber and Osborn, 1969). Protein glycosylation can result in electrophoretic mobilities which indicate

molecular weights which differ from those determined by other techniques (Segrest and Jackson, 1972).

Incorporation of [^3H]Leucine into Cultured Pancreatic Rudiments

The incorporation of [^3H]leucine into the equivalent of 19- to 20-day embryonic pancreatic rudiments cultured with and without the differentiation inhibitor 5-BrdU was also used to study general protein induction during pancreatic differentiation. As is shown by the fluorograph profiles in Figure 2B, Lanes H (control) and I (5-BrdU-treated), a large number of proteins can be detected in embryonic pancreatic rudiments labeled with [^3H]leucine. The complexity of the profiles makes it somewhat difficult to assess which labeled proteins are "induced" proteins. Only four protein bands can be identified with any certainty as induced. The relative mobilities of these proteins are 0.47-0.50 (66,100-70,700 daltons; this band was seen as a doublet in one gel with components having relative mobilities 0.47-0.48 and 0.48-0.50), 0.58-0.62 (50,700-55,400 daltons), 0.69-0.72 (40,600-43,400 daltons) and 0.89-0.93 (25,500-27,900 daltons). Each of these induced [^3H]leucine-labeled proteins appears to correspond to an induced protein in the Coomassie blue-stained gel of Figure 2A, as expected on the basis of work from Rutter's group (Kemp et al., 1972). The latter three proteins are tentatively identified as amylase, lipase, and chymotrypsinogen/trypsinogen. Even though there are slight variations in the measured ranges of mobilities from those of the induced proteins of Figure 2A, the relative positions of the induced [^3H]leucine-labeled proteins in the fluorograph are the same as those for the Coomassie blue-stained induced proteins.

Incorporation of Radioactive Sugar
Precursors into Glycoproteins of
Cultured Pancreatic Rudiments

Gel autofluorographs showing [^3H]fucose- and [^3H]glucosamine-labeled glycoproteins from differentiating pancreatic rudiments are presented in Figure 2B. Similarly, in Figure 3 is shown the gel autofluorograph of [^3H]N-acetylmannosamine-labeled glycoproteins from cultured pancreatic rudiments at several stages of differentiation. (These fluorograph profiles for each of the radioactive sugar precursors of pancreatic glycoprotein synthesis are representative of the results obtained from analyses of a minimum of two separate pools of cultured rudiments labeled with the respective glycoprotein precursors.) In all cases, equally complex glycoprotein profiles are characteristic of pancreatic rudiments as early as 14-15 days of gestation. In addition, the fluorographic profiles for [^3H]fucose- and [^3H]glucosamine-labeled glycoproteins of the protodifferentiated pancreas are very similar, exhibiting as many as fifteen glycoprotein species. Fewer [^3H]N-acetylmannosamine-labeled species are detected in the protodifferentiated pancreas; however, no novel glycoprotein species are detectable using this precursor. The majority of glycoproteins present in the protodifferentiated pancreas appear to have molecular weights greater than 80,000 daltons. Most of these glycoproteins are found in the differentiated pancreas at somewhat reduced concentrations relative to the total glycoprotein content of the differentiated pancreas.

The number of glycoproteins induced during pancreatic differentiation varies from five to seven species depending on the radioactive sugar used to label the glycoproteins and the resolution of glycoprotein bands on specific gel autofluorographs. The

Figure 3. SDS-polyacrylamide gel electrophoresis of pancreatic rudiments labeled with [^3H]N-acetylmannosamine at various stages of differentiation.

Pancreatic rudiments were cultured plus or minus 5-BrdU and labeled with [^3H]N-acetylmannosamine at various times during the secondary transition period of differentiation. Samples of the labeled rudiments were electrophoresed on a 0.1% SDS-8% acrylamide slab gel, and the gel was prepared (without staining) for autofluorography as described in Methods. Exposure of X-Omat R X-ray film to the prepared gel was for six weeks. Total radioactivity (free plus protein-bound) values for the samples applied to the gel ranged from 3700 to 4000 cpm. Shown are the profiles for cultured rudiments labeled on the *in vitro* equivalent of A) 14-15 days gestation, B) 17-18 days gestation, C) 17-18 days gestation (cultured in the presence of 5-BrdU), D) 19-20 days gestation, and E) 19-20 days gestation (cultured in the presence of 5-BrdU). Arrows indicate differentiation-induced glycoproteins.

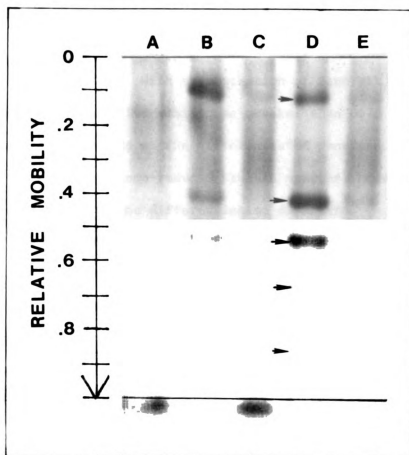


Figure 3

relative mobilities and corresponding molecular weights of the induced glycoproteins are summarized in Table 3. Bands are designated I through VII in order of increasing mobility.

In general, the same induced glycoprotein species can be detected regardless of the radioactive precursor used to label pancreatic rudiments. However, a given glycoprotein may incorporate different precursors in different amounts. For example, Band II is only labeled very slightly with [^3H]fucose, whereas it is intensely labeled when the precursors are [^3H]glucosamine and [^3H]N-acetylmannosamine. With the exception of Band VII, induced glycoproteins with relative mobilities greater than 0.75 are relatively minor constituents of the differentiated pancreas. They appear to correspond to non-induced protein species, however, so their glycosylation may be differentiation dependent.

Band I, observed in [^3H]glucosamine- and [^3H]N-acetylmannosamine-labeled pancreatic rudiments, is somewhat unique among the major induced glycoproteins. Band I is apparent in Coomassie blue-stained profiles for [^3H]fucose- and [^3H]glucosamine-labeled rudiments, fluorographs for [^3H]fucose-labeled rudiments, and fluorographs of [^3H]leucine-labeled rudiments. Yet in all of these cases, Band I does not appear to be induced. Furthermore, while this high molecular weight glycoprotein is not detected in the protodifferentiated pancreas labeled with [^3H]glucosamine or [^3H]N-acetylmannosamine, it is labeled to maximum intensity with these glycoprotein precursors by the middle of the secondary transition period during differentiation.

Comparison of relative mobilities of protein species and glycoprotein species that appear to be induced during pancreatic differentiation suggests that at least three induced glycoproteins

Table 3. Glycoproteins induced in the rat exocrine pancreas during the secondary transition period of pancreatic differentiation

Relative mobility values represent the range of average relative mobilities for a given band determined from three fluorographs in the cases of [³H]fucose- and [³H]glucosamine-labeled rudiments and two fluorographs in the case of [³H]N-acetylmannosamine-labeled rudiments.

^aNot induced; ^bNot detected; ^cAppears as a doublet in one fluorograph and as a broad band in other fluorographs; ^dDetected in only two of three fluorographs examined.

Band Number	[³ H]Fucose-Labeled Glycoproteins		[³ H]Glucosamine-Labeled Glycoproteins		[³ H]N-Acetylmannosamine-Labeled Glycoproteins	
	Relative Mobility	Molecular Weight	Relative Mobility	Molecular Weight	Relative Mobility	Molecular Weight
I	N.I. ^a		0.14-0.15	144,000-147,000	0.12-0.14	147,000-153,000
II	0.46-0.49	67,600- 72,200	0.42-0.48	69,100- 78,900	0.42-0.45	73,900- 78,900
III	0.55-0.60	53,000- 59,200	0.54-0.60	53,000- 60,500	0.54-0.56	57,900- 60,500
IV	0.68-0.75 ^c	38,000- 44,400	0.68-0.74 ^c	38,800- 44,400	0.66-0.68	44,400- 46,400
V	0.74-0.80	34,000- 38,800	N.D. ^b		N.D.	
VI	0.79-0.86	29,800- 34,800	0.79-0.86 ^d	29,800- 34,800	N.D.	
VII	0.87-0.91	26,600- 29,100	0.87-0.92	26,100- 29,100	0.86	29,800

correspond to induced protein species tentatively identified as secretory proteins. Band III corresponds with the induced protein tentatively identified as amylase. Band IV may correspond to lipase and/or procarboxypeptidase A. (Some fluorographs indicated the presence of two induced glycoproteins in this region of the profile.) Band II appears to correspond to the induced protein species with an approximate molecular weight of 69,000 daltons. As was stated earlier, this is probably the zymogen granule content glycoprotein previously observed by Ronzio and colleagues (submitted for publication). It too is apparently a differentiation-induced secretory glycoprotein.

A fourth glycoprotein species, the most rapidly migrating induced glycoprotein, Band VII, may correspond to the induced protein tentatively identified as chymotrypsinogen and/or trypsinogen. However, due to variability in relative mobilities from one gel to the next, it is not possible to determine whether or not the two species co-migrate. In some cases, Band VII appears to migrate just behind the induced protein species (Figure 2). Ronzio and co-workers have observed a glycoprotein species of similar molecular weight (25,000 daltons) in zymogen granule content preparations from adult rat pancreas labeled *in vitro* with radioactive glucosamine (submitted for publication).

Elution of Radioactive Proteins and Glycoproteins from Gels During Staining and Destaining Procedures

Figure 2C shows the fluorograph obtained for a non-stained gel prepared identically to the stained gel from which the photograph and fluorograph were obtained in Figures 2A and 2B, respectively.

The only difference between the two gels was staining with Coomassie blue dissolved in 30% (v/v) methanol-10% (v/v) glacial acetic acid. X-ray film exposure to the gels was for the same period of time at -80°C. A general elution of radioactivity from the gel appears to occur during staining and destaining. No selective elution of glycoproteins could be detected, though the process does seem to reduce the fuzziness (due to microheterogeneity?) associated with glycoprotein bands of relative mobility 0.2-0.4 and to have effectively eluted some of the high molecular weight [^3H]leucine-labeled proteins. In addition, elution of the broad fuzzy bands migrating in front of the tracking dye in Figure 3 occurred when an identical gel was stained. Possibly these bands contain glycolipids which would be highly susceptible to solubilization in the gel staining and destaining solutions.

Incorporation of [^3H]Leucine and [^3H]Fucose
into Zymogen Granule Content and Zymogen
Granule Membrane Constituents of Adult
Rat Pancreas

For the purpose of facilitating the identification of proteins and glycoproteins found in the embryonic pancreas, adult rat pancreatic tissue was labeled *in vitro* with [^3H]leucine and [^3H]fucose. Zymogen granule membranes and zymogen granule content proteins were subsequently obtained from the labeled tissue and samples were electrophoresed on 0.1% SDS-polyacrylamide slab gels. Fluorographs obtained from the 2,5-diphenyloxazole-impregnated dried gels are shown in Figure 4. Table 4 summarizes the relative mobilities and molecular weights of the labeled species.

The most intensely [^3H]leucine- and [^3H]fucose-labeled component of zymogen granule membranes is a protein with a molecular weight

Figure 4. SDS-polyacrylamide gel electrophoresis of adult rat pancreas zymogen granule membrane proteins and zymogen granule content proteins labeled *in vitro* with [^3H]leucine and [^3H]fucose.

Adult rat pancreas was labeled *in vitro* with [^3H]leucine or [^3H]fucose, and zymogen granule membrane and zymogen granule content subfractions of the tissue were obtained as described in Methods. Samples were electrophoresed on 0.1% SDS-8% acrylamide slab gels. Gels were prepared for autofluorography without first being stained. X-Omat R X-ray films were exposed to the dried gels for eight weeks. Fluorograph profiles are shown for A) [^3H]leucine-labeled zymogen granule membrane (4,450 dpm), B) [^3H]leucine-labeled zymogen granule content proteins (8,500 dpm), C) [^3H]fucose-labeled zymogen granule membrane (7,100 dpm), and D) [^3H]fucose-labeled zymogen granule content proteins (3,400 dpm). Arrows indicate labeled species present in the profiles.

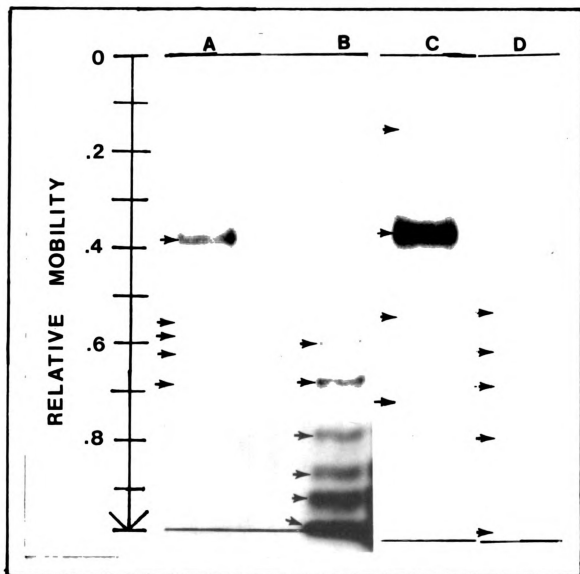


Figure 4

Table 4. [³H]Leucine- and [³H]fucose-labeled components of adult rat pancreas zymogen granule membranes and zymogen granule contents

Protein relative mobilities were obtained from fluorographs of polyacrylamide slab gels shown in Figure 4. ^aNot detected.

Zymogen Granule Membranes				Zymogen Granule Contents			
<u>[³H]Leucine-Labeled</u>		<u>[³H]Fucose-Labeled</u>		<u>[³H]Leucine-Labeled</u>		<u>[³H]Fucose-Labeled</u>	
Relative Mobility	Molecular Weight	Relative Mobility	Molecular Weight	Relative Mobility	Molecular Weight	Relative Mobility	Molecular Weight
N.D. ^a		0.16 (GP-1)	140,000	N.D.		0.54	60,500
0.38 (GP-2)	86,300	0.37 (GP-2)	88,200	0.60	53,000	0.61	51,800
0.56 (GP-3)	57,900	0.54 (GP-3)	60,500	0.69	43,400	0.68	44,400
0.59	54,200	N.D.		0.80	34,000	0.78	35,600
0.63	49,600	N.D.		0.88	28,500	N.D.	
0.69	43,400	0.72	40,600	0.93	25,500	N.D.	
				0.99	22,300	0.98	22,800

of 86,000-88,000 daltons (relative mobility 0.37-0.38). This protein has been designated GP-2 by Ronzio and co-workers (Ronzio, 1973b; MacDonald and Ronzio, 1972; Lewis et al., 1977). It is known to be highly glycosylated and has been shown to have an apparent molecular weight of 70,000-83,000 daltons in 1% SDS-9% acrylamide gels.

Two other major fucoproteins are found in the zymogen granule membrane. They correspond with GP-1 (120,000-130,000 daltons) and GP-3 (52,000 daltons) observed by Ronzio and colleagues. Here GP-1 and GP-3 have apparent molecular weights of approximately 140,000 and 58,000-60,000 daltons, respectively. GP-1 and GP-3 did not label well with [³H]leucine which is in agreement with previous findings of Ronzio and co-workers (Kronquist et al., 1977).

It will be noted that in the gel system employed in these experiments, GP-1, GP-2, and GP-3 all have molecular weights estimated to be somewhat greater than the molecular weight values obtained by Ronzio and co-workers. Several factors may be operating to produce this apparent inconsistency. First, other investigators have found that proteins of high and low molecular weights migrate non-linearly in the Laemmli gel system (Wray and Perdue, 1974). Secondly, glycoproteins, especially those containing 10% or more carbohydrate, bind less SDS on a weight basis than do nonglycosylated proteins. Glycoproteins, therefore, exhibit a decreased charge to mass ratio which results in decreased mobility on SDS gels (Segrest and Jackson, 1972). Finally, it will be noted that the molecular weights of these zymogen granule membrane glycoproteins were previously determined in 1% SDS gels of both 9% and 12% acrylamide. The gels utilized in these experiments were of 8% acrylamide and contained only 0.1% SDS. Although gel samples contained 2% SDS, it is not unlikely that some

SDS bound to glycoproteins was lost as the glycoproteins migrated into the 0.1% SDS-polyacrylamide gels. Furthermore, it may be assumed that the anomalous behavior observed here for zymogen granule membrane glycoproteins extends also to soluble glycoproteins.

Tentative identification of most of the [^3H]leucine-labeled zymogen granule content proteins can be made on the basis of estimated molecular weights. The proteins in order of decreasing molecular weight are probably amylase, lipase, procarboxypeptidase A (or carboxypeptidases A and B in the event of activation of the zymogen granule content solution), unknown, chymotrypsinogen and/or trypsinogen (or chymotrypsin and/or trypsin), and ribonuclease.

Comparison of the [^3H]leucine-labeled proteins from adult rat pancreas zymogen granule content with the differentiation-induced pancreatic proteins listed in Table 2 indicates some differences in the two sets of proteins. Procarboxypeptidase B, for example, is either absent or co-migrates with amylase in the [^3H]leucine-labeled zymogen granule content protein fluorographic profile. The [^3H]leucine-labeled protein of molecular weight 28,500 (relative mobility 0.88) of the zymogen granule content does not appear to be an induced protein in differentiating pancreas. One major disparity is the position of procarboxypeptidase A in the profiles. The protein band with relative mobility 0.80 in the [^3H]leucine-labeled zymogen granule content corresponds in calculated molecular weight most nearly with the known molecular weight of procarboxypeptidase A. However, the relative abundance of procarboxypeptidase A in embryonic pancreas observed by Walther and co-workers (1974) strongly suggests that the protein with relative mobility 0.72-0.73 in differentiating rat pancreas is procarboxypeptidase A.

The possibility of proenzyme activation in the [^3H]leucine-labeled zymogen granule content proteins preparation cannot be eliminated and would provide one explanation for the variations in the mobilities of secretory proteins; that is, proenzymes of the differentiating pancreas may be present in their activated forms (with lower molecular weights) in the preparation of secretory proteins from adult pancreas. Had activation occurred, however, the presence of a large amount of low molecular weight peptides should be evident in the gel profile. It is not.

A number of zymogen granule content proteins appear to label with [^3H]fucose. Only two of these proteins are significantly labeled. The approximate molecular weights of these zymogen granule content fucoproteins are 36,000 and 23,000 daltons. Rat pancreas procarboxypeptidase A, carboxypeptidases A and B, and lipase (delipidated) all have molecular weights of approximately 36,000. Procarboxypeptidase A was suspected to migrate with a somewhat slower mobility (0.72-0.73) in differentiating rat pancreas, however. The 23,000 dalton fucoprotein may be ribonuclease. Bovine and porcine pancreatic ribonucleases are known to be glycosylated (Plummer and Hirs, 1963; Reinhold et al., 1968). This low molecular weight fucoprotein migrates very near the bromphenol blue tracking dye on the gel and may, therefore, consist of a number of unresolved glycoproteins of low molecular weight. Faintly labeled fucoproteins with relative mobilities 0.61 and 0.68 may correspond with amylase and lipase, respectively.

When comparing the fucosylated zymogen granule membrane glycoproteins and zymogen granule content glycoproteins of the adult rat pancreas with fucoproteins of embryonic rat pancreas, several

observations may be made. First, a heterogeneous fucoprotein band of molecular weight similar to that of GP-2 appears to be present in the pancreas from as early as 14-15 days of gestation. It does not appear to be one of the glycoproteins which undergoes induction during the secondary transition period of pancreatic differentiation. Glycoprotein Band I of embryonic pancreas appears to correspond to zymogen granule membrane GP-1. Band I, though not induced in terms of fucosylation, seems to be induced in rudiments labeled with [^3H]glucosamine and [^3H]N-acetylmannosamine.

Uncertainty in the identification of the zymogen granule content proteins confounds rather than simplifies the identification of differentiation-induced fucoproteins. It is probable that the intensely labeled zymogen granule content fucoprotein with relative mobility 0.78 corresponds with one of the differentiation-induced fucoproteins designated Band IV, Band V, or Band VI.

Fractionation of [^3H]Fucose-Labeled Differentiated Pancreatic Rudiments

In order to determine whether or not the differentiation-induced glycoproteins of the pancreas are primarily soluble or membrane-bound glycoproteins, [^3H]fucose-labeled 19- to 20-day pancreatic rudiments (cultured from day 16 of gestation instead of day 14) were fractionated into "soluble" and "particulate" components. Fractionation was carried out essentially as described in Methods. Rudiments (a total of sixteen) were sonicated in 0.3 M sucrose containing 0.25 mg/ml soybean trypsin inhibitor and 12 μM phenylmethylsulfonylfluoride, and the sonicates were centrifuged at 111,000 x g for two hours. The supernate was designated the "soluble" fraction. The pellet was resuspended with sonication

in 0.25 M NaBr and centrifugation was repeated. The supernate from this centrifugation was designated the "first NaBr wash." A second NaBr wash was carried out, and the final pellet was designated the "particulate" fraction. Table 5 shows the protein content and specific radioactivity of each of the fractions obtained.

Table 5. Fractionation of [^3H]fucose-labeled differentiated (*in vitro*) pancreatic rudiments

Fraction	Total Protein (μg)	Specific Radioactivity (dpm/ $\mu\text{g} \times 10^{-6}$)	% of Recovered Radioactivity
Homogenate	894	2.42	
"Soluble" fraction	513	1.19	58.9
First NaBr wash	116	0.19	9.4
Second NaBr wash	28	0.06	3.0
"Particulate" fraction	180	0.58	28.7

Eighty-three percent of the initial radioactivity was recovered during the fractionation. The results suggest that a single NaBr wash is sufficient to remove a large portion of the adsorbed soluble proteins and trapped non-protein bound radioactivity from the particulate fraction.

Acid precipitation of the homogenate indicated that 49% of the [^3H]fucose incorporated by the rudiments was protein-bound after 18 hours of incubation with this precursor.

Figure 5A shows the Coomassie blue staining profiles for the soluble and particulate fractions obtained from the [^3H]fucose-labeled rudiments. Figure 5B shows the corresponding fluorograph from the stained gel. The salient feature of these electrophoretic profiles is that virtually all of the fucoproteins shown earlier to be induced during differentiation (Figure 2B) are found in the soluble fraction and the first NaBr wash of the particulate fraction. The one glycoprotein with relative mobility 0.12-0.15 (Band I) which appears to be differentiation-induced in [^3H]glucosamine- and [^3H]N-acetylmannosamine-labeled rudiments (though not in [^3H]fucose-labeled rudiments) is primarily a particulate glycoprotein.

The soluble fucoproteins tend to have molecular weights less than 74,000 daltons. One notable exception is the fucoprotein with molecular weight 157,000-160,000 daltons (relative mobility 0.10-0.11). The bulk of the fucoproteins with mobilities of 0.2 to 0.5 are particulate (membrane-bound) glycoproteins.

In an effort to identify the fucoproteins of the soluble and particulate fractions from the (*in vitro*) differentiated pancreatic rudiments as zymogen granule membrane or secretory glycoproteins, the fluorographic profiles for these fractions were compared with the profiles for [^3H]fucose-labeled zymogen granule membranes and zymogen granule content proteins from adult rat pancreas. This comparison is presented in Figure 6. The zymogen granule content fucoprotein with relative mobility 0.78 probably corresponds with a soluble (induced) embryonic fucoprotein with relative mobility 0.66-0.75 (Band IV, Table 3) which is suggested to be procarboxypeptidase A and/or lipase. It may, however, correspond to the induced embryonic fucoprotein, designated Band V, the identity of

Figure 5. SDS-polyacrylamide gel electrophoresis of subcellular fractions from cultured pancreatic rudiments labeled on the equivalent of 19-20 days gestation with [^3H]fucose.

Soluble and particulate fractions were obtained from pancreatic rudiments cultured from day 16 of gestation to the equivalent of day 19 and then labeled 18 hours with [^3H]fucose. The fractions were analyzed on 0.1% SDS-8% acrylamide slab gels. Gels were stained and/or prepared for autoradiography. RP Royal X-Omat X-ray films were exposed to the prepared gels for 22 days.

Figure A shows the Coomassie blue staining profiles for these subcellular fractions, while Figure B is the fluorograph obtained from the same stained gel.

Protein standards appearing in lane A are 1) porcine thyroglobulin (165,000), 2) human transferrin (74,000), 3) bovine serum albumin (66,000), 4) ovalbumin (43,000), and 5) bovine pancreatic ribonuclease A (13,700) migrating with the bromphenol blue tracking dye.

The Coomassie blue staining profiles and fluorographic profiles are shown for the B) homogenate (68 μg protein; 185,000 dpm total; 90,000 dpm acid-precipitable), C) soluble fraction (80 μg protein; 186,000 dpm total; estimated 94,000 dpm acid-precipitable), D) first NaBr wash of the particulate fraction (44 μg protein; 71,000 dpm total), and E) particulate fraction after a second NaBr wash (22 μg protein; 72,000 dpm total; estimated 62,000 dpm acid-precipitable).

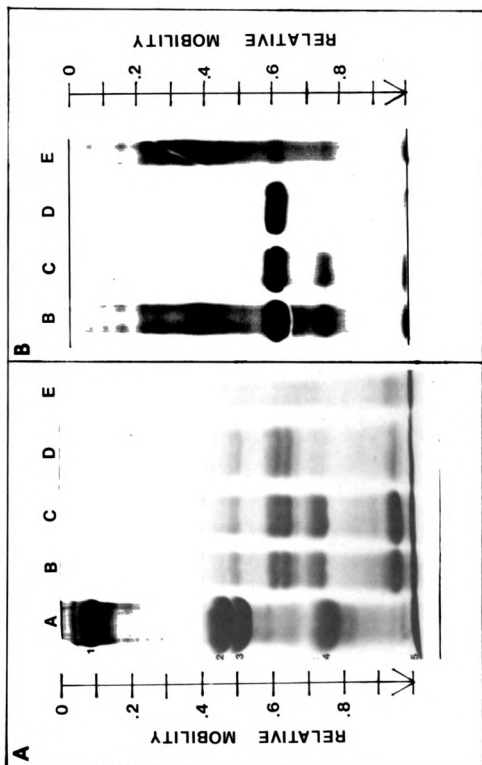


Figure 5

Figure 6. Electrophoretic comparison of fucoproteins from cultured pancreatic rudiments with the fucoproteins of zymogen granule membranes and the zymogen granule contents from adult rat pancreas.

Samples were prepared and electrophoresed as described in Figure 4 and Figure 5. Fluorographic profiles for labeled subfractions from pancreatic rudiments were obtained from an unstained gel. Shown are the A) homogenate from [^3H]fucose-labeled rudiments (185,000 dpm total; 90,000 dpm acid-precipitable), B) soluble fraction from [^3H]fucose-labeled rudiments (186,000 dpm total; estimated 94,000 dpm acid-precipitable), C) [^3H]fucose-labeled zymogen granule content proteins from adult rat pancreas (3,400 dpm), D) [^3H]fucose-labeled zymogen granule membrane proteins from adult rat pancreas (7,100 dpm), and E) particulate fraction (after a second NaBr wash) from [^3H]fucose-labeled rudiments (116,000 dpm total; estimated 98,500 dpm acid-precipitable).

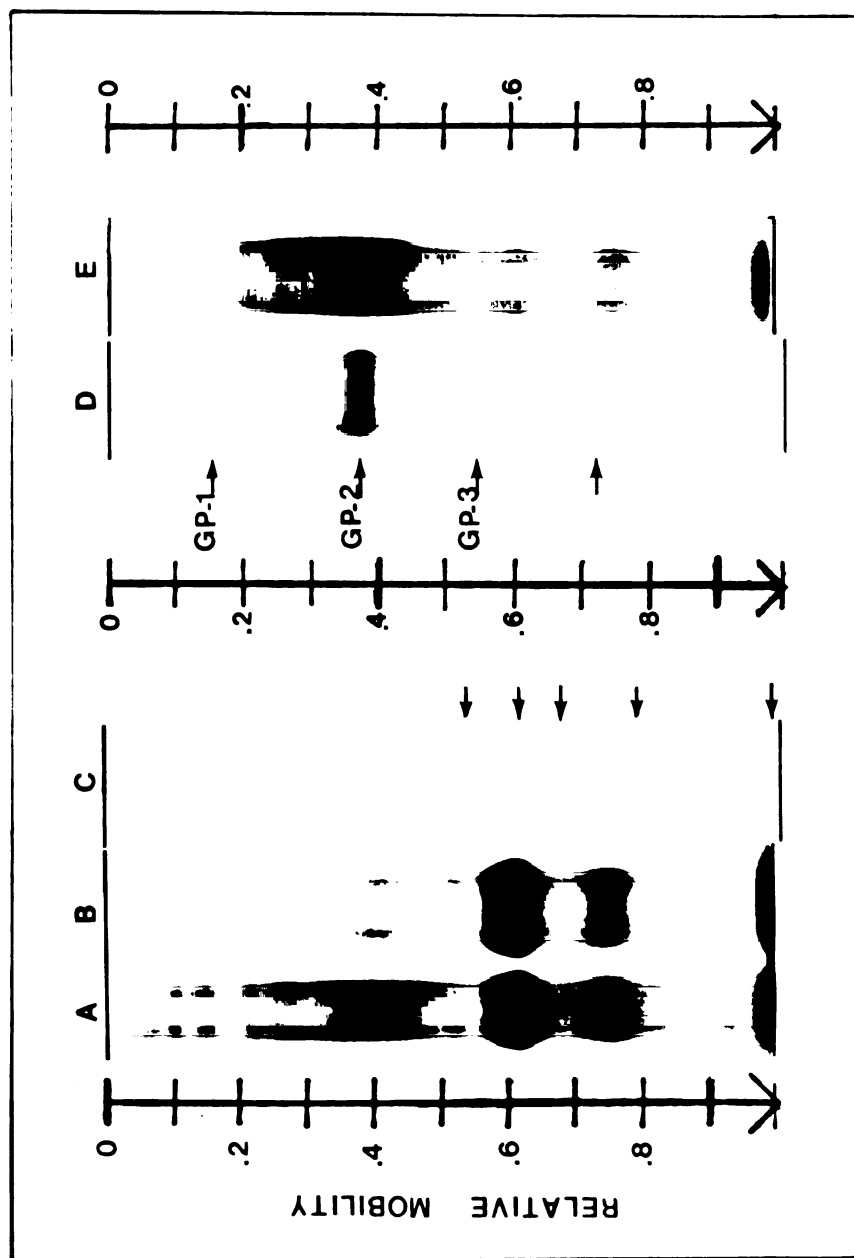


Figure 6

which is not known. The relative mobility of GP-1 from zymogen granule membrane correlates well with the relative mobility of a fucosylated protein (Band I) in the particulate fraction from differentiated pancreatic rudiments. GP-2 migrates similarly to a broad heterogeneous band of particulate fucoproteins from differentiated rudiments.

Incorporation of [3 H]Fucose and [3 H]Glucosamine into Acid-Precipitable Protein During Pancreatic Differentiation

It was not possible, because of the quantity of embryonic tissue needed, to determine the pool sizes and kinetics of incorporation for the radioactive sugars used to label glycoproteins synthesized during *in vitro* pancreatic differentiation. The second best alternative was to measure the amount of radioactive glycoprotein precursor incorporated into 10% trichloroacetic acid-precipitable protein at various times during the secondary transition period of pancreatic differentiation. From the acid precipitation data, it is possible to estimate the sizes of the free sugar pools (acid soluble radioactivity). The rates of glycoprotein synthesis may also be estimated if the precursor pool size is assumed not to change during the labeling period and no conversion of the precursor into other potential protein or glycoprotein precursors occurs. Table 6 summarizes the results of 10% trichloroacetic acid precipitation of samples of [3 H]fucose-labeled and [3 H]glucosamine-labeled rudiments. Due to the low level of radioactivity incorporated during labeling of pancreatic rudiments with [3 H]N-acetylmannosamine (one-tenth that incorporated during [3 H]fucose labeling and one-twentieth that incorporated during [3 H]glucosamine labeling, on the basis of total radioactivity per μ g protein) rudiments labeled with

Table 6. Incorporation of radioactive sugars into acid-precipitable protein by pancreatic rudiments during the secondary transition period of differentiation

Soluble and particulate fractions from [³H]fucose- and [³H]glucosamine-labeled pancreatic rudiments were prepared as described in Methods. Samples of the fractions in the presence of carrier protein were precipitated with 10% trichloroacetic acid. Two experiments were conducted with cultured rudiments labeled on the equivalents of 14-15 or 17-18 days gestation. Four experiments were carried out with cultured rudiments labeled on the equivalent of 19-20 days gestation. Values are $\pm$ mean deviation. ^aNot determined.

Equivalent Embryonic Age of Rudiments when Labeled (days)	Fraction	Acid-Precipitable Radioactivity (dpm/ μ g protein/18 hours)	% of Total Radioactivity Precipitated by 10% Trichloroacetic Acid		
			[³ H]fucose	[³ H]glucosamine	[³ H]glucosamine
14-15	Soluble	1906 $\pm$ 560		1816 $\pm$ 441	28.3 $\pm$ 1.2
	Particulate	1850 $\pm$ 212		1756 $\pm$ 252	84.0 $\pm$ 0.7
17-18	Soluble	1121 $\pm$ 114		1730 $\pm$ 615	35.6 $\pm$ 3.6
	Particulate	1447 $\pm$ 43		1954 $\pm$ 248	81.8 $\pm$ 1.7
19-20	Soluble	570 $\pm$ 122		1095 $\pm$ 369	40.3 $\pm$ 2.4
	Particulate	1426 $\pm$ 396		1909 $\pm$ 485	79.2 $\pm$ 2.4
19-20 (cultured in the presence of 5-BrdU)	Soluble	N.D. ^a		1728	25.6
	Particulate	N.D.		2816	80.6

[³H]N-acetylmannosamine were not examined in this and subsequent experiments.

The results in Table 6 suggest, first of all, that over a constant labeling period of 18 hours, the rate of particulate glycoprotein synthesis relative to total particulate protein synthesis remains essentially unchanged during the secondary transition period. This seems to be in keeping with the observation that possibly only one particulate glycoprotein is induced during the secondary transition period of differentiation. Total particulate protein per cultured embryonic pancreatic rudiment appears to increase by no more than a factor of two over this period.

Soluble glycoprotein synthesis *declines* relative to total soluble protein synthesis during the secondary transition period. Soluble fucoprotein synthesis in the differentiated pancreas is roughly one-third that of the protodifferentiated pancreas. At the same time, the amount of soluble "glucosamine"-glycoprotein synthesis in the differentiated pancreas is sixty percent that of the protodifferentiated pancreas. It appears that synthesis of the differentiation-induced soluble glycoproteins is not commensurate with total soluble protein synthesis, which increases six- to tenfold (on a per rudiment basis) during the secondary transition period.

The ratio of protein-bound radioactive sugar to total radioactive sugar for the soluble and particulate fractions remains relatively constant during *in vitro* differentiation of the pancreas. Approximately 50% of the labeled fucose found in the soluble fraction is protein-bound, while 85-95% of the labeled fucose in

the particulate fraction is acid-precipitable. The amount of protein-bound radioactivity in the soluble fraction from [^3H]glucosamine-labeled rudiments remains at about 30-40% throughout the secondary transition, while the particulate fraction contains 80-85% acid-precipitable radioactivity from [^3H]glucosamine labeling. (It must be noted that the acid precipitates of the particulate fractions were never extracted with organic solvents to remove glycolipid. A portion of the acid-precipitable radioactivity of the particulate fractions may, therefore, be glycolipid-bound rather than protein-bound.)

Estimates of the free sugar pool sizes were made on the basis of the amount of acid-soluble radioactivity found in the soluble fractions from rudiment sonicates. The amount of free [^3H]fucose per pancreatic rudiment appears to increase by no more than a factor of two during the secondary transition. The apparent size of the free radioactive sugar pool from [^3H]glucosamine labeling increases by a factor of four during the secondary transition. These changes in free sugar pools could reflect an increase in cell volume with cytodifferentiation and/or an increased rate of sugar transport. Whether or not these small changes in the free radioactive sugar pools are significant with respect to glycoprotein synthesis of the differentiating pancreas is not known.

Radioimmunoassay for Zymogen Granule Membrane
Glycoproteins and Secretory Glycoproteins in
Differentiating Rat Pancreas

The most apparent structural change that occurs in the exocrine pancreas during cytodifferentiation is the accumulation of zymogen granules. This occurs in conjunction with the accumulation of the

cell specific secretory proteins. Of considerable interest is whether the zymogen granule membrane glycoprotein constituents undergo differentiation-dependent induction in synchrony with the induction of secretory proteins. The best way to answer this question seemed to be to make use of an indirect radioimmunoassay technique employing rabbit antibodies developed in the laboratory against zymogen granule membranes from adult rat pancreas. Similarly, the use of an anti zymogen granule content (antiserum) was expected to provide more certain evidence for the glycosylation of pancreatic secretory proteins and the accumulation of secretory glycoproteins *in vitro* during the secondary transition period of pancreatic differentiation.

A description of the antibodies used in the radioimmunoassays follows. Subsequently, the results of assays for zymogen granule membrane glycoproteins and secretory glycoproteins in cultured pancreases at various stages of the secondary transition are reported.

Characterization of the Anti Zymogen Granule Content (Antiserum)

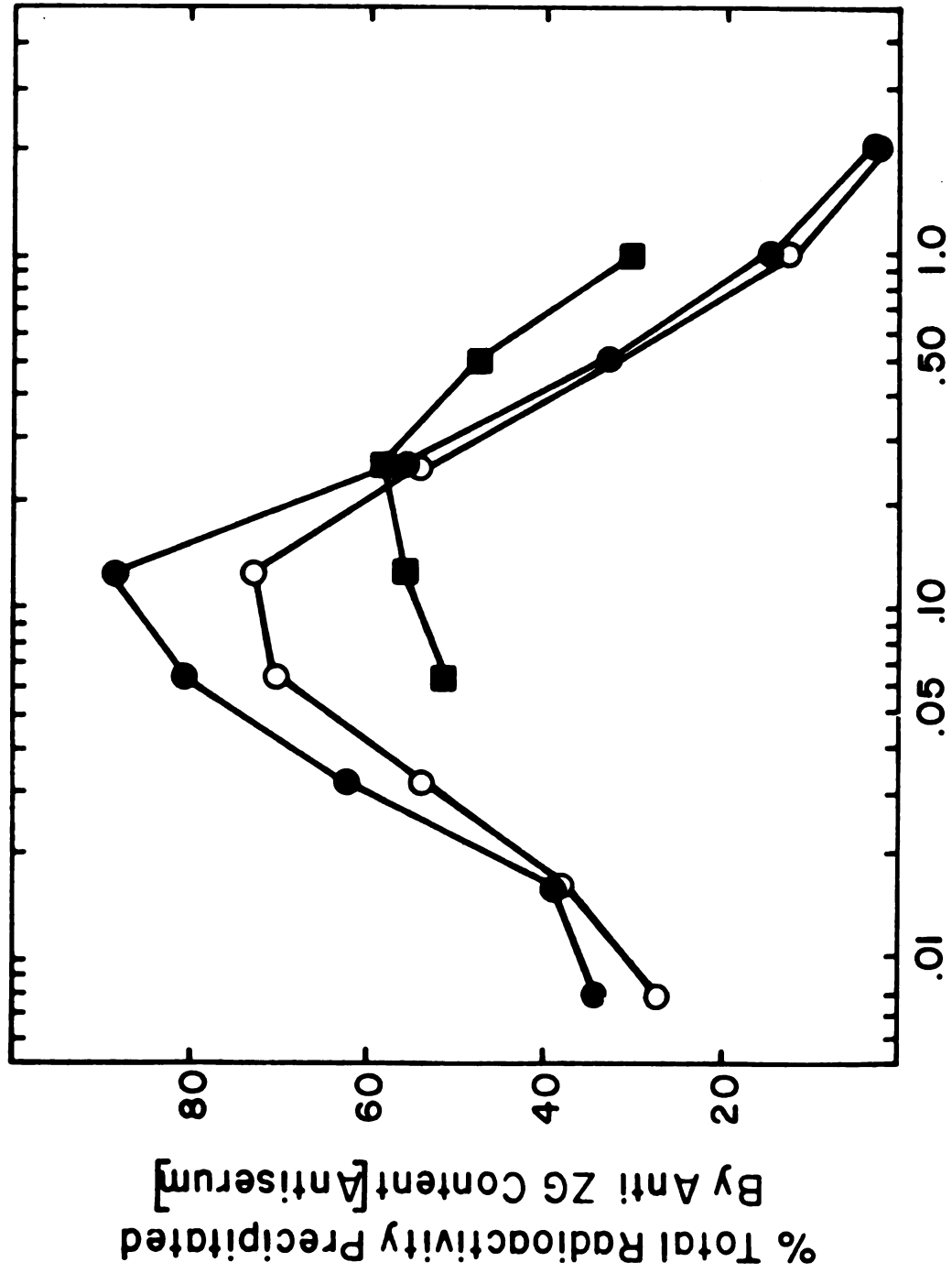
Figure 7 shows the results of both direct and indirect titration of the anti zymogen granule content (antiserum) with a preparation of [³H]leucine-labeled zymogen granule content proteins from adult rat pancreas. In the direct titration, 50 μ l of antiserum were incubated with 50 μ l of zymogen granule content proteins (concentrated and dialyzed against borate-saline buffer, pH 8.4-8.5) at various dilutions in borate-saline buffer for one hour at 25°C. The reactions were then stored at 0-4°C for 24 or 72 hours. Precipitates were collected by centrifugation and washed three times by resuspension in borate-saline buffer followed by centrifugation. The washed

Figure 7. Titration of anti zymogen granule content (antiserum) with [^3H]leucine-labeled zymogen granule content proteins from adult rat pancreas.

Fifty microliters of de complemented anti zymogen granule content (antiserum) were reacted with 50 μl of various dilutions of a [^3H]leucine-labeled preparation of zymogen granule content proteins (245 cpm/ μg) as described in the text for direct titration of the antiserum. The percent of total added radioactivity precipitated by the antiserum was determined for precipitates collected and washed after 24 hours (-O-) and 72 hours (-●-) at 0-4°C.

Indirect titration of the antiserum (10 μl) with 10 μl of various dilutions of [^3H]leucine-labeled zymogen granule content proteins was also carried out using the double antibody precipitation technique described in Methods for the radioimmunoassay of soluble glycoproteins from pancreatic rudiments. Precipitates were collected and the percent of total added radioactivity precipitated by the antiserum was determined for the precipitates (-■-).

ZG = zymogen granule.



μg/μl ZG Content Proteins

Figure 7

precipitates were solubilized and samples taken for liquid scintillation counting. As seen in Figure 7, the equivalence point for the antiserum occurred when zymogen granule content proteins were added to the assay mixture at a concentration of 0.126 $\mu\text{g}/\mu\text{l}$. The total amount of protein precipitated by 50 μl of the antiserum at the equivalence point in the direct titration was 0.46 μg and 0.55 μg for the 24-hour and 72-hour 0-4°C incubation periods, respectively.

A titration curve was also obtained for the anti zymogen granule content (antiserum) with [^3H]leucine-labeled zymogen granule content proteins in a double antibody (indirect) precipitation reaction using a commercial preparation of goat antirabbit (gamma globulin) to precipitate the first antigen-antibody complex. The assay system was the same as that described in Methods for radioimmunoassay of zymogen granule content glycoproteins in the soluble fractions from labeled pancreatic rudiments. A titer of 1.5 μg zymogen granule content proteins per 10 μl antiserum was obtained in the double antibody assay. Use of the second antibody appears to increase the titer efficiency of the antiserum.

To further characterize the anti zymogen granule content (antiserum), the cross-reactivity of the antiserum with various subcellular fractions prepared from adult rat pancreas was examined using double immunodiffusion in 1% agarose gels (Ouchterlony, 1958). The antiserum was tested for cross-reactivity with NaBr-washed zymogen granule membranes, smooth microsomal membranes, and NaBr-washed rough microsomal membranes. In Figure 8A, it can be seen that the anti zymogen granule content (antiserum) (wells 1 and 4), in addition to forming precipitin lines with zymogen granule content proteins (wells 5 and 6), also reacted with NaBr-washed

Figure 8A. Ouchterlony double diffusion test of anti zymogen granule content (antiserum) and anti zymogen granule membrane (IgG) versus zymogen granule content proteins and zymogen granule membranes prepared from adult rat pancreas.

Immunodiffusion was carried out in a 90-mm glass dish filled with 10 ml 1% agarose dissolved in 10 mM Na_2HPO_4 buffer (pH 7.4) containing 1% (v/v) Triton X-100, 1% sodium deoxycholate, 5.0 mM disodium EDTA, 12 μM phenylmethylsulfonylfluoride, 0.001% butylated hydroxytoluene, and 0.01% Trypan blue. The center well contained 20 μl rabbit anti zymogen granule membrane (IgG) (4.9 $\mu\text{g}/\mu\text{l}$). Wells 1 and 4 contained 20 μl rabbit anti zymogen granule content (antiserum). Wells 2 and 3 each contained 20 μl of solubilized zymogen granule membrane (0.43 $\mu\text{g}/\mu\text{l}$ and 0.21 $\mu\text{g}/\mu\text{l}$, respectively). wells 5 and 6 each contained 20 μl zymogen granule content proteins in borate-saline buffer (pH 8.4) (0.6 $\mu\text{g}/\mu\text{l}$ and 0.3 $\mu\text{g}/\mu\text{l}$, respectively). This plate was photographed after 65 hours at room temperature in a moist chamber.

Figure 8B. Microheterogeneity of anti zymogen granule content (antiserum). Double diffusion test of anti zymogen granule content (antiserum) versus various dilutions of a zymogen granule content protein solution.

Immunodiffusion was carried out in a 100-mm plastic dish filled with 8 ml of 1% agarose dissolved in borate-saline buffer, pH 8.4-8.5. The center well contained 20 μl of undiluted anti zymogen granule content (antiserum). Wells 1-6 contained 20 μl of various dilutions of zymogen granule content protein solution containing 2.02 $\mu\text{g}/\mu\text{l}$. Well 1, 1 to 2 dilution; Well 2, 1 to 4; Well 3, 1 to 8; Well 4, 1 to 16; Well 5, 1 to 32; Well 6, undiluted. The plate was photographed after 45 hours at room temperature in a moist chamber.

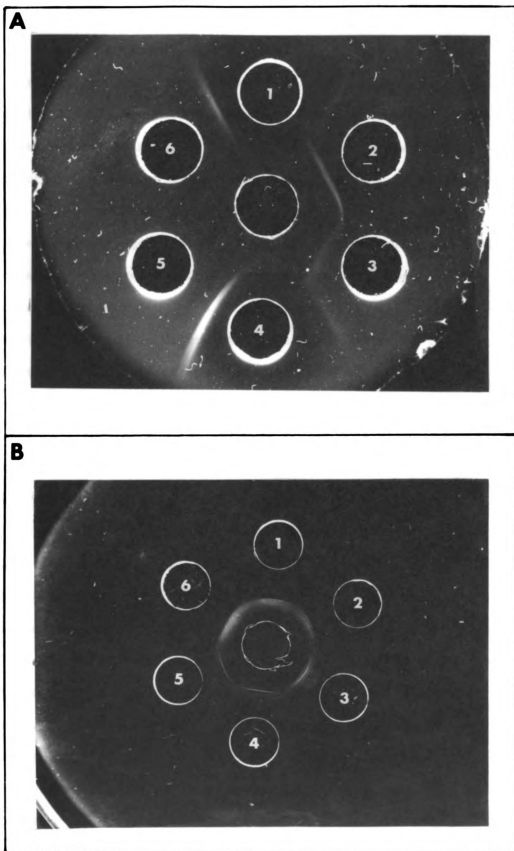


Figure 8

zymogen granule membrane proteins. Several explanations could account for the cross-reactivity. It is possible that the zymogen granule membrane preparation used in the experiment, even after NaBr washing, may still have contained adsorbed secretory proteins (approximately 6%; Ronzio, submitted for publication). Of more interest is the possibility that similar antigenic determinants exist for one or more of the zymogen granule content proteins and zymogen granule membrane proteins.

The microheterogeneity of the anti zymogen content (antiserum) can be seen in Figure 8B. This 1% agarose gel was prepared in borate-saline buffer (pH 8.4-8.5) rather than the phosphate-detergent buffer (pH 7.4) used in Figure 8A. Better resolution of the multiple precipitin lines occurs in this gel. At least five separate precipitin bands can be observed with close examination of the pattern. Such microheterogeneity of the antiserum is expected in view of the large number of secretory proteins present in the zymogen granule content preparations used to induce the formation of the antibodies.

Figures 9A and 9B show the cross-reactivity of the anti zymogen granule content (antiserum) with smooth microsomal membranes and NaBr-washed rough microsomal membranes, respectively. The smooth microsomal membrane preparation had not been washed with NaBr and was, therefore, expected to exhibit reactivity with the antiserum due to adsorbed secretory proteins. In addition to reactions of identity, however, there appear to be at least two reactions of partial identity (spurs pointing toward well 1) which suggest the presence of antibodies in the antiserum which react only with a component of smooth microsomal membranes or a non-secretory protein contaminant of the membrane preparation.

Figure 9. Cross-reactivity of anti zymogen granule content (antiserum) with smooth microsomal membranes and NaBr-washed rough microsomal membranes from adult rat pancreas.

A. Immunodiffusion was carried out as described in Figure 8A. The center well contained 20 μ l of anti zymogen granule content (antiserum). Wells 1 and 4 contained 20 μ l of 0.6 μ g/ μ l and 0.3 μ g/ μ l zymogen granule content proteins, respectively. Wells 2, 3, 5, and 6 contained 20 μ l of solubilized smooth microsomal membranes. Well 2, 12 μ g/ μ l; Well 3, 3 μ g/ μ l; Well 5, 0.8 μ g/ μ l; Well 6, 0.2 μ g/ μ l. This preparation of smooth microsomal membranes had not been washed with 0.25 M NaBr and, therefore, probably contained a minimum of 10% (by mass) adsorbed soluble proteins including zymogen granule content proteins.

B. Immunodiffusion was carried out as described in Figure 8A. The center well contained 20 μ l of anti zymogen granule content (antiserum). Wells 1 and 4 contained 20 μ l of 0.6 μ g/ μ l and 0.3 μ g/ μ l zymogen granule content proteins, respectively. Wells 2, 3, 5, and 6 contained 20 μ l of solubilized NaBr-washed rough microsomal membranes. Well 2, 18 μ g/ μ l; Well 3, 4.5 μ g/ μ l; Well 5, 1.1 μ g/ μ l; Well 6, 0.3 μ g/ μ l.

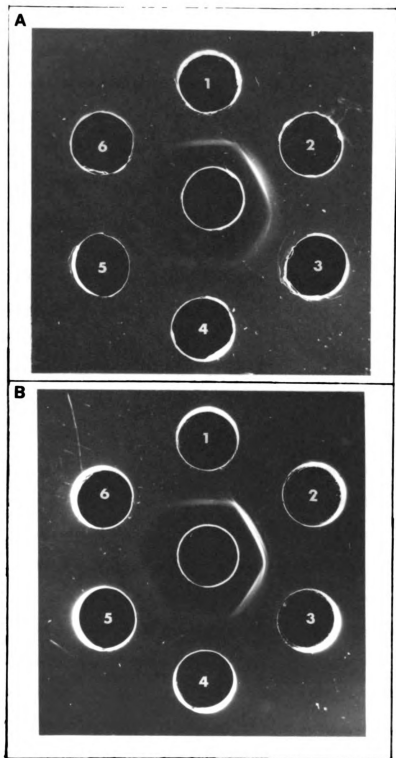


Figure 9

Cross-reactivity of the anti zymogen granule content (antiserum) with NaBr-washed rough microsomal membranes (Figure 9B) was similar to that seen for smooth microsomal membranes. In addition to reactions of identity and partial identity, at least one precipitin line is present that indicates there are antibodies in the antiserum that react only with a component of rough microsomal membranes or a possible contaminant of the membranes (reaction of nonidentity between center well and well 2 of plate).

Purification of the Anti Zymogen
Granule Membrane (Gamma Globulin)

Ouchterlony double diffusion plates showed that the anti zymogen granule membrane (gamma globulin) preparation cross reacted with zymogen granule content proteins (not shown). Passage of the gamma globulin through a column of Sepharose 4B to which zymogen granule content proteins had been bound was the method used to remove this cross-reactivity.

The procedure used to couple zymogen granule content proteins to cyanogen bromide-activated Sepharose 4B was taken from the Pharmacia Fine Chemicals publication, *CNBr-activated Sepharose 4B Immobilization of Biopolymers, Con A-Sepharose for Affinity Chromatography of Polysaccharides and Glycoproteins*. A lyophilized preparation of adult rat pancreas zymogen granule content proteins was dissolved in and dialyzed against high ionic strength borate-saline buffer (pH 8.4-8.5) containing 5.0 mM boric acid, 1.25 mM sodium borate, and 0.46 M NaCl (calculated ionic strength was approximately 0.5). Ten milliliters of this solution of zymogen granule content proteins (55 mg protein) was added to 2 g of CNBr-activated Sepharose 4B which had been swollen in 10^{-3} N HCl and

washed with high ionic strength borate-saline buffer. After two hours at room temperature, the absorbance at 280 nm of the solution above the beads had reached a relatively constant value. The gel suspension was then washed on a scintered glass filter with high ionic strength borate-saline buffer. This was followed by washing and soaking in 1 M ethanolamine-HCl (pH 8.0) for two hours to block any unreacted binding sites on the gel. The final step in the preparation of the affinity gel consisted of alternately washing the gel with 0.1 M acetate buffer (pH 4) and 0.1 M borate buffer (pH 8.5), each containing 1 M NaCl. The gel with coupled zymogen granule content proteins was then washed with standard borate-saline buffer (pH 8.4-8.5) containing 5.0 mM boric acid, 1.25 mM sodium borate, and 0.142 M NaCl.

Approximately 2.6 ml of anti zymogen granule membrane (gamma globulin) were applied to a column packed with the affinity gel (6.5 ml bed volume in a 10-ml glass pipet). Elution of unbound gamma globulin occurred with a single bed volume of standard borate-saline buffer (pH 8.4-8.5) (Figure 10). Approximately 58% of the applied gamma globulin was recovered in the borate-saline eluted peak. Elution of the column with 0.2 M glycine (pH 2.8) in 0.5 M NaCl to remove the bound cross-reacting gamma globulin component did not yield a sufficient quantity of proteins, even after concentration, to react with zymogen granule content proteins on double diffusion plates.

The borate-saline eluted anti zymogen granule membrane (gamma globulin) was found to not react with zymogen granule content proteins on Ouchterlony double diffusion plates. In Figure 8A, the ratio of gamma globulin to zymogen granule content proteins, on a

Figure 10. Purification of anti zymogen granule membrane (IgG). Removal of gamma globulin fraction cross-reactive with zymogen granule content proteins.

Anti zymogen granule membrane (IgG) was prepared as described in Methods from the rabbit antiserum. This gamma globulin preparation (2.6 ml; approximately 7.4 mg/ml) was then chromatographed on a 6.5 ml column of Sepharose 4B to which zymogen granule content proteins (7.8 mg/ml gel, maximally) had been covalently coupled (see text). Fractions were pooled as indicated. The borate-saline eluted IgG fraction was concentrated and used in all radioimmunoassays for zymogen granule membrane proteins.

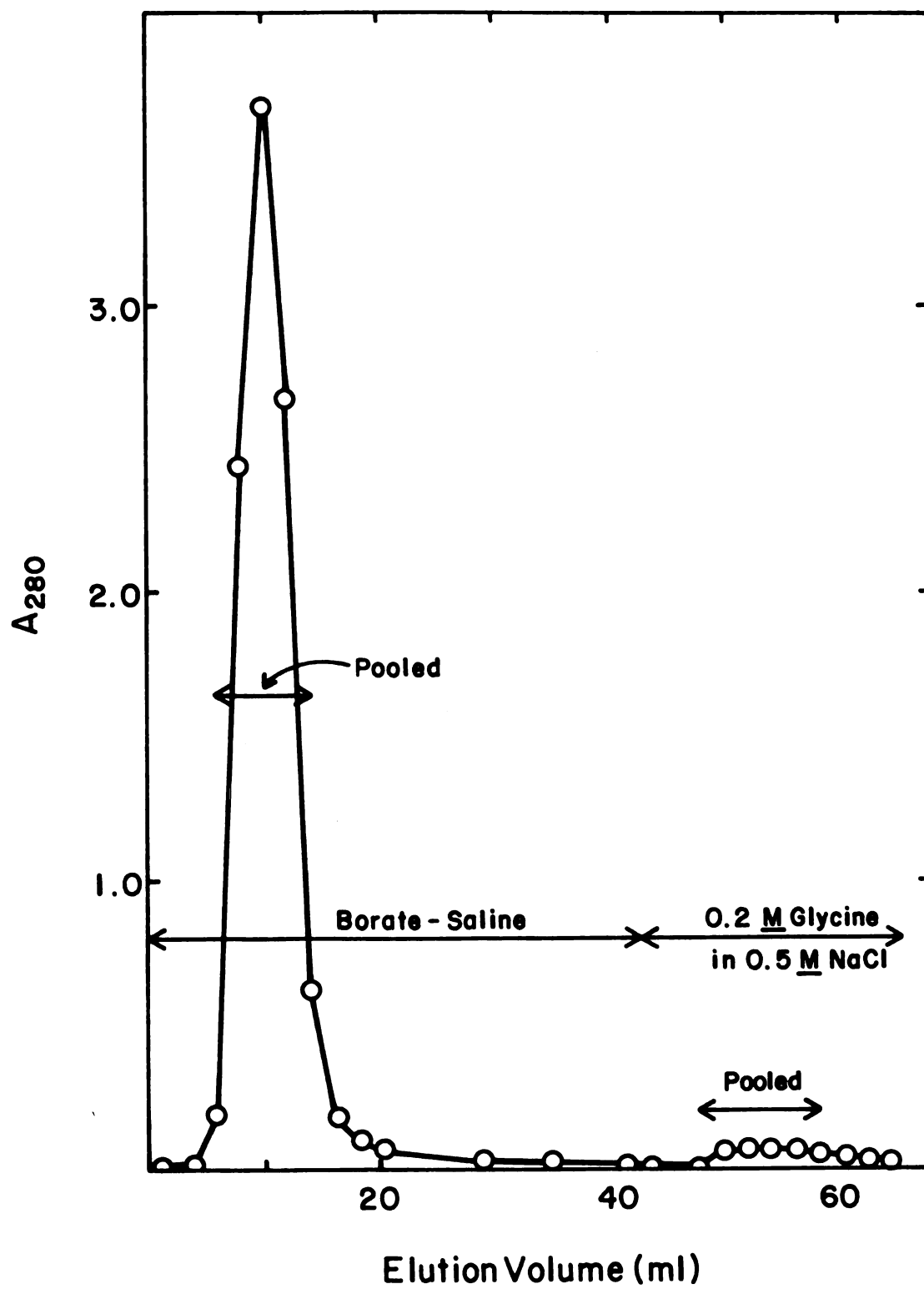


Figure 10

protein basis, was equivalent to that previously used to test for cross-reactivity of the untreated gamma globulin with zymogen granule content proteins. In the latter case, a positive reaction was observed.

Characterization of the Anti Zymogen
Granule Membrane (Gamma Globulin)

Attempts were made to determine the titer of the treated anti zymogen granule membrane (IgG) for zymogen granule membrane proteins using the double antibody precipitation system described in Methods for assay of particulate proteins from labeled pancreatic rudiments. Using this procedure, 10 μ l of the IgG were found to precipitate as much as 8.6 μ g of [3 H]leucine-labeled adult rat zymogen granule membrane protein. Two preparations of [3 H]leucine-labeled zymogen granule membrane were used to titrate the anti zymogen granule membrane (IgG). An equivalence point was never obtained in the titration curves suggesting that the value stated above was still in the antibody excess region of the titration. Between 20 and 45% of the total [3 H]leucine-labeled protein present in the zymogen granule membrane preparations was precipitated in the double antibody precipitation system.

Cross-reactivity of the treated anti zymogen granule membrane (IgG) with smooth microsomal membranes and with NaBr-washed rough microsomal membranes from adult rat pancreas was tested in Ouchterlony immunodiffusion gels. Figures 11A and 11B indicate cross-reactivity of the treated anti zymogen granule membrane (IgG) with both of these membrane subfractions. The precipitin bands suggest the presence of at least two reactions of identity for the IgG with zymogen granule membranes and smooth microsomal membranes as well as two reactions

Figure 11. Cross-reactivity of anti zymogen granule membrane (IgG) with smooth microsomal membranes and NaBr-washed rough microsomal membranes from adult rat pancreas.

A. Immunodiffusion was carried out as described in Figure 8A. The center well contained 20 μ l of anti zymogen granule membrane (IgG). Wells 1 and 4 contained 20 μ l of 0.4 μ g/ μ l and 0.2 μ g/ μ l solubilized zymogen granule membrane proteins, respectively. Wells 2, 3, 5, and 6 contained 20 μ l of solubilized smooth microsomal membranes. Well 2, 12 μ g/ μ l; Well 3, 3 μ g/ μ l; Well 5, 0.8 μ g/ μ l; Well 6, 0.2 μ g/ μ l. This preparation of smooth microsomal membranes had not been washed with 0.25 M NaBr to remove adsorbed soluble proteins.

B. Immunodiffusion was carried out as described in Figure 8A. The center well contained 20 μ l of anti zymogen granule membrane (IgG). Wells 1 and 4 contained 20 μ l of 0.4 μ g/ μ l and 0.2 μ g/ μ l solubilized zymogen granule membrane proteins, respectively. Wells 2, 3, 5, and 6 contained 20 μ l of solubilized NaBr-washed rough microsomal membranes. Well 2, 18 μ g/ μ l; Well 3, 4.5 μ g/ μ l; Well 5, 1.1 μ g/ μ l; Well 6, 0.3 μ g/ μ l.

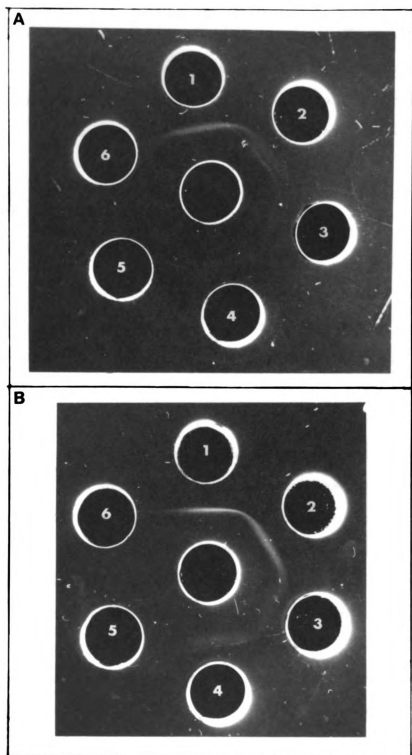


Figure 11

of identity with zymogen granule membranes and rough microsomal membranes. These results suggest that common antigenic determinants may be present on these three membrane subfractions of the exocrine pancreas. In addition, they correlate well with recent evidence obtained in this laboratory for the existence of golgi membrane glycoproteins which appear similar to zymogen granule membrane glycoproteins (Ronizio and Mohrlök, 1977).

Accumulation of Antibody-Precipitable
Glycoproteins During the Secondary
Transition Period of Pancreatic
Differentiation

The anti zymogen granule content (antiserum) and the treated anti zymogen granule membrane (IgG) described were used to assay protodifferentiated and differentiating pancreatic rudiments for secretory glycoproteins and zymogen granule membrane glycoproteins. Soluble and NaBr-washed particulate fractions were prepared as described in Methods from rudiments labeled with [^3H]fucose and [^3H]glucosamine. The soluble fractions were analyzed for secretory glycoproteins using the anti zymogen granule content (antiserum) in the double antibody precipitation procedure outlined in Methods. NaBr-washed particulate fractions were assayed for zymogen granule membrane glycoproteins using the treated anti zymogen granule membrane (IgG) in a similar double antibody system. Samples were titrated to equivalence with the rabbit antibodies. Final assay results were calculated as the percent of total acid-precipitable radioactivity added to the immunoassay reaction precipitated by the antibodies, minus the percent of acid-precipitable radioactivity precipitated by control antiserum (anti bovine serum albumin [antiserum]).

Figure 12 shows the accumulation of antibody-precipitable [^3H]fucose- and [^3H]glucosamine-labeled glycoproteins during the secondary transition period of pancreatic differentiation. Between the *in vitro* equivalent of 14-15 and 19-20 days gestation, a marked increase occurs in the amounts of [^3H]fucose- and [^3H]glucosamine-labeled soluble glycoproteins of the pancreas which are precipitable by the anti zymogen granule content (antiserum). Antibody-precipitable fucoproteins increase from 2.4 to 40.1% of the total soluble fucoproteins during this time. A similar rise from 2.0 to 46.7% is observed for soluble [^3H]glucosamine-labeled glycoproteins. (Mean deviations for these values ranged from 1.2 to 4.6%.) It is presumed that few membrane-bound proteins were present in the soluble fractions prepared from labeled rudiments so that the antibody-precipitated proteins do, in fact, represent zymogen granule content glycoproteins. The results of rudiment fractionation shown in Figure 5 suggest that this is probably the case, since only a relatively small proportion of the radioactive particulate proteins appears in the profiles of the soluble fraction.

The accumulation of anti zymogen granule content (antiserum)-precipitable glycoproteins in differentiating pancreatic rudiments parallels the accumulation of pancreatic secretory proteins known to occur during differentiation. Furthermore, it supports earlier evidence that one or more of the secretory proteins is glycosylated.

In contrast to the observed accumulation of soluble glycoproteins during differentiation of the rat pancreas is the apparent steady state of glycoproteins precipitated by the anti zymogen granule membrane (IgG). *In vitro*, during the secondary transition period of differentiation, the percent of total acid-precipitable particulate

Figure 12. Accumulation of "zymogen granule content-like" glycoproteins and "zymogen granule membrane-like" glycoproteins in differentiating rat exocrine pancreas.

Soluble and NaBr-washed particulate fractions were obtained from cultured pancreatic rudiments labeled with [^3H]fucose or [^3H]glucosamine on the *in vitro* equivalents of 14-15, 17-18, and 19-20 days gestation. Soluble fractions were assayed for "zymogen granule content-like" glycoproteins using the anti zymogen granule content (antiserum) in the double antibody precipitin reaction described in Methods. NaBr-washed particulate fractions were assayed for "zymogen granule membrane-like" glycoproteins using the treated anti zymogen granule membrane (IgG) in a similar double antibody precipitin reaction. Results are expressed as the percent of the total acid-precipitable radioactivity precipitated from the sample by the antiserum or IgG at equivalence. The percent of acid-precipitable radioactivity precipitated by the anti BSA (antiserum) was subtracted from all values to correct for trapping of free [^3H]sugar and non-specific precipitation of labeled glycoproteins in the immunoprecipitates. Results are shown for soluble fractions from [^3H]fucose-labeled rudiments (-O-) and [^3H]glucosamine-labeled rudiments (-●-), and for NaBr-washed particulate fractions from [^3H]fucose-labeled rudiments (-Δ-) and [^3H]glucosamine-labeled rudiments (-▲-).

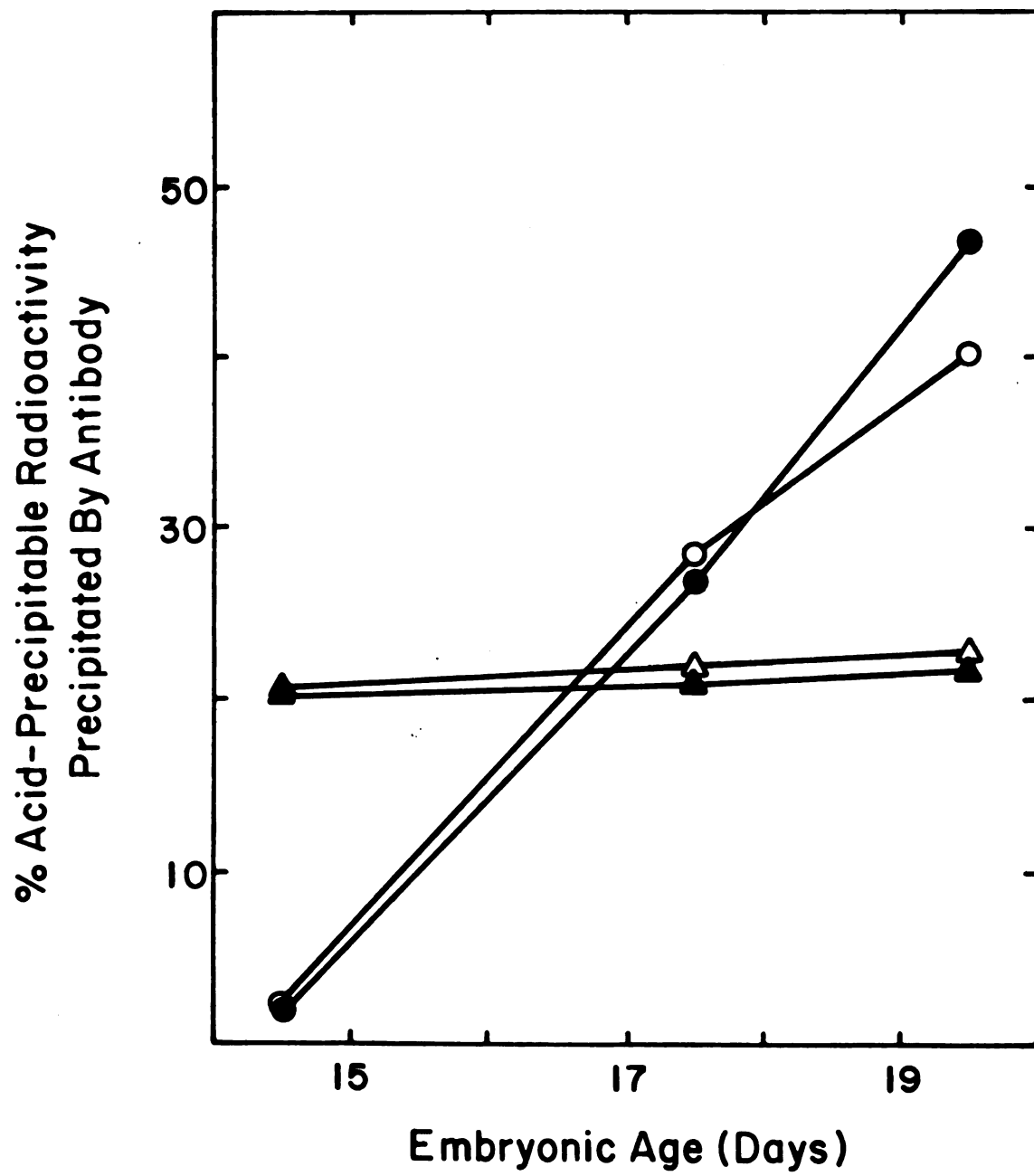


Figure 12

glycoproteins precipitated by this antibody ranges from 20.4 to 22.8% (mean deviation, 0.8-2.9%) for [^3H]fucose-labeled glycoproteins and 20.6 to 21.9% (mean deviation, 3.2-4.0%) for [^3H]glucosamine-labeled glycoproteins. The slight increase in the antibody-precipitable glycoproteins observed during this period is not statistically significant. These results suggest that the particulate glycoproteins, especially those of the zymogen granule membrane, are present in the protodifferentiated pancreas and are not induced parallel to the induction of glycosylated and non-glycosylated secretory proteins.

Figure 13 shows the levels of incorporation of [^3H]fucose and [^3H]glucosamine into antibody-precipitable glycoproteins relative to the total "soluble" plus "particulate" protein present at a given stage of pancreatic differentiation. The specific radioactivity of [^3H]fucose-labeled zymogen granule content-like glycoproteins increases nearly 100-fold between the *in vitro* equivalent of 14-15 and 17-18 days of gestation. The specific radioactivity of [^3H]glucosamine-labeled zymogen granule content-like glycoproteins increases by a factor of two hundred during this same period. After 17-18 days, no significant change occurs in the specific radioactivities of the glycoproteins. (Mean deviations for 17-18 and 19-20 day [^3H]glucosamine-labeled embryonic rudiments were 111 and 145 dpm/ μg , respectively. Thus, the apparent increase is not statistically significant.)

Relative to total particulate plus soluble protein present in the cultured pancreatic rudiment, [^3H]fucose and [^3H]glucosamine incorporation into anti zymogen granule membrane (IgG)-precipitable particulate glycoproteins actually declines during differentiation.

Figure 13. Specific radioactivities of the "zymogen granule content-like" glycoproteins and "zymogen granule membrane-like" glycoproteins which accumulate in the rat exocrine pancreas during the secondary transition period of pancreatic differentiation.

Results shown in Figure 12 for the accumulation of anti zymogen granule content (antiserum)-precipitable glycoproteins and anti zymogen granule membrane (IgG)-precipitable glycoproteins during pancreatic differentiation were used to calculate the specific radioactivities of "zymogen granule content-like" and "zymogen granule membrane-like" glycoproteins present in differentiating pancreas. Specific radioactivities are expressed as the amount of acid-precipitable radioactivity precipitated by the antibody relative to total soluble plus particulate protein (dpm/ μ g protein). Results are shown for [3 H]fucose-labeled (-O-) and [3 H]glucosamine-labeled (-●-) "zymogen granule content-like" glycoproteins and for [3 H]fucose-labeled (-Δ-) and [3 H]glucosamine-labeled (-▲-) "zymogen granule membrane-like" glycoproteins.

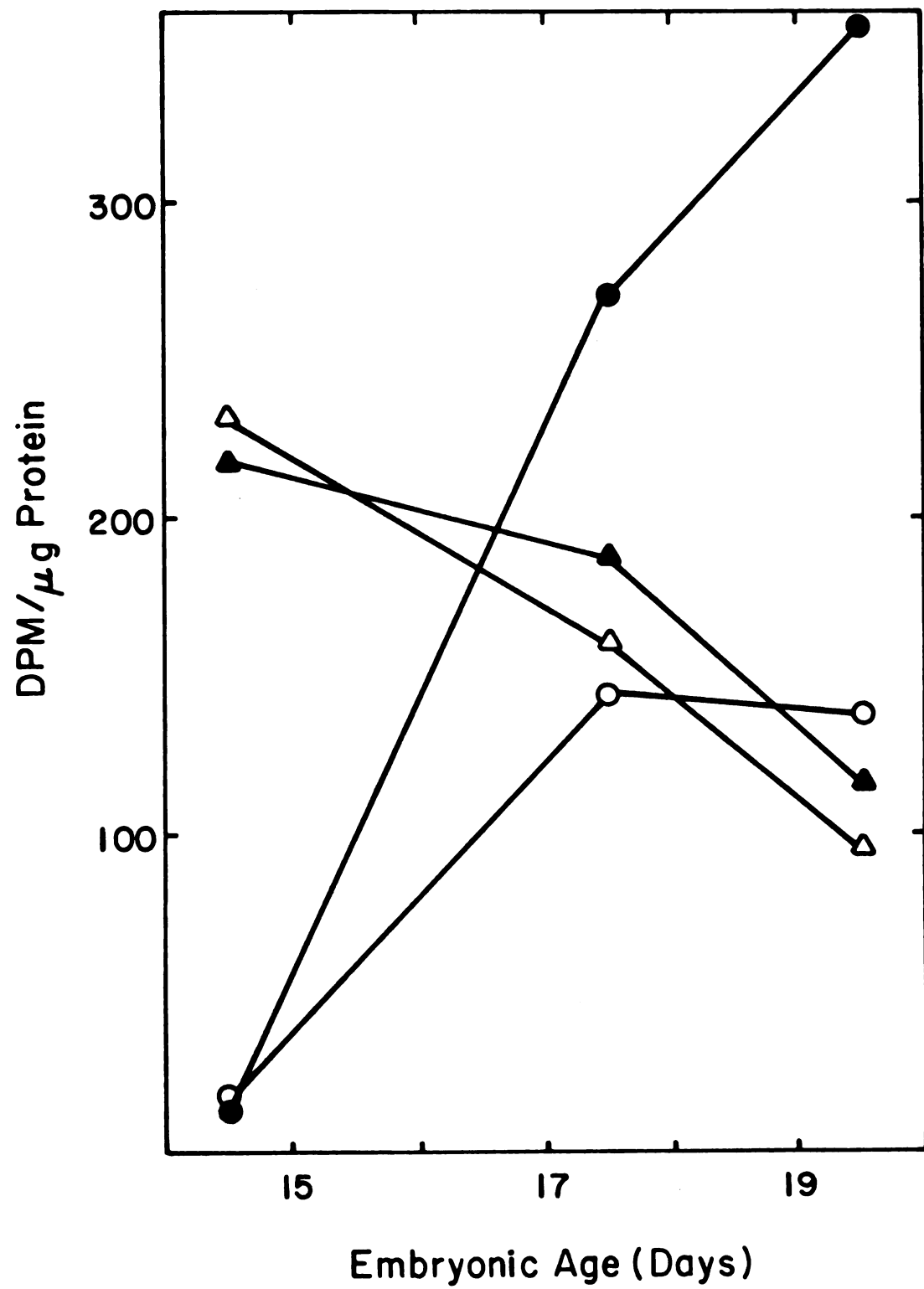


Figure 13

There is approximately a two-fold decrease in the specific radioactivities of both [^3H]fucose- and [^3H]glucosamine-labeled zymogen granule membrane-like glycoproteins during the transition from the protodifferentiated to the differentiated pancreas. Since the proportion of total particulate glycoproteins that is antibody-precipitable remains essentially constant during the secondary transition period of differentiation, this decline in the specific radioactivities of zymogen granule membrane-like glycoproteins is probably partially accounted for by the eight-fold increase per rudiment in the average soluble plus particulate protein content over this same time period.

Identification of Antibody-Complexed
Glycoproteins of Differentiating
Rat Pancreas

[^3H]Fucose- and [^3H]glucosamine-labeled glycoproteins from soluble and particulate fractions of pancreatic rudiments at various stages of differentiation were precipitated with anti zymogen granule content (antiserum) and anti zymogen granule membrane (IgG), respectively, in the double antibody systems described in Methods. The washed precipitates were solubilized in sample buffer and applied to SDS-polyacrylamide slab gels. After electrophoresis was complete, gels were prepared for autofluorography and, subsequently, X-ray films were exposed to the dried gels. The resultant fluorographs are shown in Figure 14B (from [^3H]fucose-labeled rudiments) and Figure 14C (from [^3H]glucosamine-labeled rudiments).

Interpretations of the fluorographs must take into consideration two factors. First, the large quantities of goat gamma globulin present in the immunoprecipitates have distorted the electrophoretic

Figure 14. SDS-polyacrylamide gel electrophoresis analysis of radioimmunoprecipitates of soluble and particulate glycoproteins from differentiating pancreas.

Soluble and particulate fractions were prepared from [^3H]fucose- and [^3H]glucosamine-labeled rudiments (cultured from day 14 of gestation) and analyzed for zymogen granule content glycoproteins and zymogen granule membrane glycoproteins using the described double antibody precipitation techniques. Soluble and particulate fractions and radioimmunoprecipitates of both fractions were solubilized in sample buffer and electrophoresed on 0.1% SDS-8% acrylamide slab gels.

Figure A shows the Coomassie blue staining profiles for A) the soluble fraction from [^3H]glucosamine-labeled 19-20 day rudiments, B) the immunoprecipitate of the soluble fraction from [^3H]glucosamine-labeled 19-20 day rudiments, C) the particulate fraction from [^3H]glucosamine-labeled 19-20 day rudiments, D) the immunoprecipitate of the particulate fraction from [^3H]glucosamine-labeled 19-20 day rudiments, and E) protein standards, 1) porcine thyroglobulin, 2) human transferrin, 3) bovine serum albumin, 4) ovalbumin, and 5) bovine pancreatic ribonuclease A at the bromphenol blue tracking dye. Arrows indicate the 50,000 dalton subunit of gamma globulin.

Figures B and C are fluorographs of unstained gels exposed to RP Royal X-Omat X-ray film for four weeks.

Figure B shows the particulate and soluble fractions and the immunoprecipitates of those fractions from [^3H]fucose-labeled pancreatic rudiments. A) Particulate fraction from 14-15 day rudiments (11,300 dpm); B) Immunoprecipitate of particulate fraction from 14-15 day rudiments (10,400 dpm); C) Particulate fraction from 17-18 day rudiments (29,200 dpm); D) Immunoprecipitate of particulate fraction from 17-18 day rudiments (25,700 dpm); E) Particulate fraction from 19-20 day rudiments (22,900 dpm); F) Immunoprecipitate of particulate fraction from 19-20 day rudiments (20,000 dpm); G) Soluble fraction from 17-18 day rudiments (16,000 dpm); H) Immunoprecipitate of soluble fraction from 17-18 day rudiments (13,800 dpm); I) Soluble fraction from 19-20 day rudiments (13,800 dpm); J) Immunoprecipitate of soluble fraction from 19-20 day rudiments (11,200 dpm).

Figure C shows the particulate and soluble fractions and the immunoprecipitates of those fractions from [^3H]glucosamine-labeled pancreatic rudiments. Array of samples is as in Figure B.

A) 15,000 dpm; B) 12,800 dpm; C) 45,700 dpm; D) 48,300 dpm; E) 20,200 dpm; F) 16,900 dpm; G) 43,000 dpm; H) 36,800 dpm; I) 24,000 dpm; J) 21,400 dpm.

The "dpm" values applied to gels for soluble and particulate fractions are acid-precipitable radioactivity values.

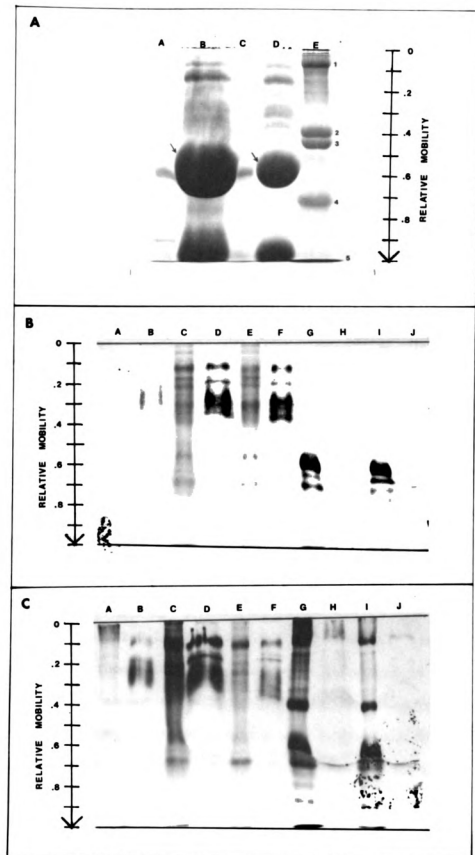


Figure 14

profiles for the immunoprecipitates. Thus, the relative mobilities of proteins migrating in the region of the 50,000 dalton subunit of the gamma globulin have been altered by the presence of the gamma globulin subunit. (See, in particular, Figure 14A. Arrows indicate the 50,000 dalton subunit of gamma globulin in the immunoprecipitate samples.) Furthermore, some of the radioactive species migrating in this region may have been masked by the large amount of gamma globulin present. Secondly, comparisons of the immunoprecipitates from fractions of pancreatic rudiments at different stages of differentiation are not valid because of the wide variations in the amounts of radioactivity in the samples applied to the gels.

Most of the glycoproteins previously observed to be particulate glycoproteins (Figure 5) were present in immunoprecipitates of particulate fractions of [^3H]fucose- and [^3H]glucosamine-labeled rudiments at all three stages of differentiation examined (protodifferentiated, mid-secondary transition, differentiated). Noticeably absent from the immunoprecipitate of the particulate fucoproteins of the *in vitro* equivalent of 14- to 15-day embryonic rudiments, however, is the high molecular weight species with relative mobility 0.12-0.15 (Band I) seen in Figure 2B. Since this is not an induced fucoprotein, its absence is speculated to be due to an insufficient amount of radioactivity in the gel sample to permit its detection by autofluorography over a four-week film exposure period. This high molecular weight particulate protein does appear in the fluorograph of the immunoprecipitate of the particulate fraction from protodifferentiated pancreas labeled with [^3H]glucosamine, though certainly not to the extent that it is

present in the 19- to 20-day cultured embryonic rudiment sample containing approximately the same amount of radioactivity. This high molecular weight particulate glycoprotein is thought to be GP-1 of zymogen granule membranes (see Figure 6).

Zymogen granule membrane component GP-2 appears (on the basis of its relative position in the electrophoretic profile) to migrate with a relative mobility of 0.28-0.35 in the anti zymogen granule membrane (IgG) immunoprecipitates of particulate fractions from differentiating pancreas. It is present at a somewhat higher position on the gels than usual due to the obstruction of its normal migration by the 50,000 dalton subunit of gamma globulin in the samples.

Not all of the particulate glycoproteins complexed by the anti zymogen granule membrane (IgG) correspond with known zymogen granule membrane glycoproteins. In view of the cross-reactivity of the IgG with smooth and rough microsomal membranes of the adult rat pancreas, these other species are thought probably to be glycoproteins of the corresponding membrane structures in differentiating pancreas.

The number of glycoproteins precipitated by anti zymogen granule content (antiserum) in reactions with soluble fractions from [³H]fucose-labeled and [³H]glucosamine-labeled rudiments is few. Only one [³H]fucose-labeled species and only three [³H]glucosamine-labeled species are observed in the *in vitro* equivalents of 17- to 18-day and 19- to 20-day embryonic pancreatic rudiments.

The electrophoretic migration of the one fucoprotein present in immunoprecipitates of soluble fractions from labeled rudiments is distorted by the gamma globulin subunit from the precipitin reactions. It is not clear whether this fucoprotein corresponds to one of the

induced fucoproteins that migrate on this gel with relative mobilities of 0.54-0.62 and 0.69-0.71 or to the non-induced fucoprotein with a relative mobility of 0.64-0.66. The results of titration of the soluble fractions of fucose-labeled rudiments with the anti zymogen granule content (antiserum) showed the complexed glycoprotein(s) to be induced during differentiation (Figure 12). This result and the curvature of the fucoprotein band in the gel strongly suggest that the anti zymogen granule content (antiserum) complexes with the induced fucoprotein (previously designated as Band III) seen to have a relative mobility of 0.54-0.62 on this gel. Earlier, this fucoprotein was tentatively identified as amylase.

The three major glycoproteins of soluble fractions from [^3H]glucosamine-labeled pancreatic rudiments which are complexed by the anti zymogen granule content (antiserum) have relative mobilities of 0.08-0.11, 0.31-0.38, and 0.68-0.69 (in the immunoprecipitates of the soluble fraction from the *in vitro* equivalent of 19- to 20-day embryonic pancreases). The soluble glycoprotein of unusually high molecular weight was observed previously in gels of differentiating pancreatic rudiments labeled with [^3H]fucose and [^3H]glucosamine. It did not appear as an induced protein or glycoprotein species and is not thought to be a secretory protein because of its high molecular weight. The migrations of the other two species have been distorted by the goat gamma globulin subunit. On the basis of the curvature of the bands and the relative abundance of glycoproteins in the soluble fractions from which the precipitates were obtained, it is suggested that these two precipitated species correspond to the two differentiation-induced [^3H]glucosamine-labeled glycoproteins previously designated as Band II and Band III. Both glycoproteins

have been tentatively identified as secretory proteins, the faster migrating species as amylase.

The absence of the intensely labeled fucoprotein of adult rat pancreas zymogen granule contents with relative mobility 0.78 from antibody precipitates of soluble proteins from pancreatic rudiments is not understood. It may be that it simply was not detected by the X-ray film over the four-week exposure period, or that it was a zymogen granule content protein not recognized by the antiserum.

Competition of Adult Rat Pancreas Zymogen
Granule Membrane and Zymogen Granule
Content Proteins with Glycoproteins of
Differentiated Embryonic Pancreatic
Rudiments for Antibody Binding

Further identification of the antibody-precipitated glycoproteins from differentiated pancreas (14-day embryonic rudiments cultured to the equivalent of day 19 and then labeled 18 hours with precursor) was obtained by the use of adult rat pancreas zymogen granule content proteins and zymogen granule membrane proteins as competitive antigens in the double antibody precipitation reactions. To determine the proportion of antibody-precipitable soluble glycoproteins that were actually zymogen granule content proteins, 10 μ l of anti zymogen granule content (antiserum) were first incubated at 37°C for thirty minutes with 24 μ g of zymogen granule content proteins (10 μ l of 2.4 μ g protein/ μ l) in a total reaction volume of 90 μ l. Figure 7 indicates that this is well into the antigen excess region of the titration curve for the antiserum in double antibody precipitation reactions. Ten microliters of soluble fractions from [3 H]fucose- or [3 H]glucosamine-labeled rudiments (diluted for maximum precipitation) were then added to the reaction

mixtures and the tubes were incubated at 37°C for thirty minutes more. Goat antirabbit (gamma globulin) was subsequently added to the reactions which were then incubated at 25°C for one hour and finally stored at 0-4°C for 24 hours. Precipitates were collected and washed and radioactivity levels determined as described in Methods.

"Percent Competition" by the added zymogen granule content proteins was calculated as follows:

$$\frac{(A-B) - (C-B)}{(A-B)} \times 100\%$$

where

$$A = \left[\frac{(\text{dpm precipitated by anti zymogen granule content [antiserum]})}{(\text{acid-precipitable dpm added to reaction})} \times 100\% \right]$$

$$B = \left[\frac{(\text{dpm precipitated by anti BSA [antiserum]})}{(\text{acid-precipitable dpm added to control reaction})} \times 100\% \right]$$

$$C = \left[\frac{(\text{dpm precipitated by anti zymogen granule content (antiserum) in the presence of competitor zymogen granule content proteins})}{(\text{acid-precipitable dpm added to reaction})} \times 100\% \right]$$

Results for two separate cultures of [³H]fucose-labeled rudiments and two separate cultures of [³H]glucosamine-labeled rudiments showed a range of competition for both types of soluble glycoproteins. Zymogen granule content proteins competed with soluble [³H]fucose-labeled glycoproteins from 10.6% to 32.6%, while competition with soluble [³H]glucosamine-labeled glycoproteins for binding by the antiserum ranged from 10.9% to 37.2%. These results suggest that possibly not more than a third of the soluble embryonic pancreatic glycoproteins complexed by the anti zymogen granule content (antiserum) were actually secretory glycoproteins. The possibility that the

zymogen granule content preparation was an inadequate competitor to embryonic pancreatic secretory proteins or that an insufficient amount of competitive zymogen granule content proteins was added to the assay reaction for complete inhibition of binding by embryonic pancreatic secretory glycoproteins cannot be excluded, however. Alternatively, the relative proportions of adult rat pancreas zymogen granule content proteins and labeled embryonic pancreas zymogen granule content proteins may have been such that an equal opportunity for combining with the antibody binding sites did not exist for the two antigens. If this was the case, the experiments are not an accurate measure of competition of the two antigens for binding.

Similar experiments were carried out using zymogen granule membrane proteins to compete with particulate [^3H]fucose- or [^3H]glucosamine-labeled glycoproteins from the cultured equivalent of 19- to 20-day embryonic rudiments for binding by the anti zymogen granule membrane (IgG). Thirteen micrograms of zymogen granule membrane protein were mixed with appropriately diluted samples of radioactive particulate fractions in phosphate-detergent buffer (pH 7.4), and then anti zymogen granule membrane (IgG) (10 μl) was added to the mixture. The procedure for double antibody precipitation of particulate glycoproteins was then carried out as described in Methods. Precipitates were collected, washed, solubilized, and samples were then taken for liquid scintillation counting. Results were calculated as in the competition experiments for soluble glycoproteins. The results of experiments involving two cultures of [^3H]fucose-labeled rudiments showed that 31.6% ($\pm 1.2\%$, mean deviation) of the particulate glycoproteins precipitated by the

gamma globulin represented zymogen granule membrane-like glycoproteins. Similarly, 32.0% ($\pm 8.0\%$, mean deviation) of the [^3H]glucosamine-labeled particulate glycoproteins complexed by this antibody were zymogen granule membrane-like glycoproteins. The results of these competition experiments are consistent with the cross-reactivity of the anti zymogen granule membrane (IgG) with smooth and rough microsomal membranes from adult rat pancreas and the results of the gel electrophoresis-fluorographic analysis of immunoprecipitates of particulate fractions from embryonic pancreas. They suggest that not all of the particulate glycoproteins complexed by the antibody are zymogen granule membrane glycoproteins. It is likely, however, that the percent competition observed in these experiments does not represent the maximum amount of zymogen granule membrane or zymogen granule membrane-like species in the *in vitro* 19- to 20-day embryonic pancreatic rudiment. Since the equivalence point was never reached in titrations of the zymogen granule membrane (IgG) with zymogen granule membranes from adult rat pancreas, the problem was to establish the amount of competitor zymogen granule membrane proteins which would be in the antigen excess region of the precipitation curve for the double antibody assay reaction. Thus, it is proposed that a minimum of 30% of the particulate glycoproteins from embryonic pancreas that are complexed by the anti zymogen granule membrane (IgG) are zymogen granule membrane or zymogen granule membrane-like glycoproteins.

(It will be noted that the radioimmunoassay and competition assay procedures used in these studies were not the standard procedures normally used. Certain technical difficulties prevented

the use of the standard radioimmunoassay methods. Subsequently, interpretation of the results is somewhat limited.)

Radioimmunoassay of Pancreatic Rudiments
Cultured in the Presence of 5-Bromodeoxy-
uridine for Zymogen Granule Content and
Zymogen Granule Membrane Glycoproteins

A further check for differentiation-dependent induction of zymogen granule membrane-like glycoproteins and secretory glycoproteins was made on pancreatic rudiments cultured in the presence of 5-BrdU to inhibit differentiation. The particulate and soluble fractions from [³H]glucosamine-labeled pancreatic rudiments that had been cultured in the presence of 5-BrdU from day 14 to the equivalent of day 20 of gestation were assayed for zymogen granule membrane glycoproteins and zymogen granule content glycoproteins using the double antibody radioimmunoassays described in Methods. Of the acid-precipitable particulate-bound radioactivity, 22.9% was precipitated by the anti zymogen granule membrane (IgG). Only 8.7% of the acid-precipitable soluble glycoproteins from these pancreatic rudiments was precipitated by the anti zymogen granule content (antiserum). The specific radioactivities of the particulate and soluble glycoproteins were 336 and 72 dpm/μg (particulate plus soluble) protein, respectively. Comparison of these values with those for cultured 14- to 15-day and 19- to 20-day control [³H]glucosamine-labeled embryonic rudiments indicates that, while 5-BrdU inhibits the synthesis of soluble (secretory) glycoproteins, it has no effect on particulate zymogen granule membrane or zymogen granule membrane-like glycoprotein synthesis. Precipitation of the particulate fraction from the [³H]glucosamine-labeled 5-BrdU-treated rudiments in the presence of competitor zymogen granule

membrane proteins showed that a minimum of 39% of the antibody-precipitable radioactivity was associated with zymogen granule membrane or zymogen granule membrane-like glycoproteins. This result agrees well with the value obtained for control [^3H]glucosamine-labeled rudiments (32%) and lends additional support to the proposal that zymogen granule membrane glycoproteins follow a unique differentiation program.

DISCUSSION

Summary of Results

The results of these studies confirm the earlier finding in this laboratory that a large number of constitutive glycoprotein species characterize the rat exocrine pancreas throughout differentiation. In addition, the results of these studies have shown that a minimum of six glycoprotein species undergo differentiation-dependent induction with respect to *de novo* protein synthesis and/or glycosylation (in contrast to a previous report by Walther and co-workers, 1974). Essentially the same glycoprotein species appear to be induced regardless of the sugar precursor used to study induction; however, specific induced glycoproteins preferentially incorporate certain sugars. The largest number of induced glycoproteins is detected by [³H]fucose labeling; some of these glycoproteins, however, appear to be minor constituents of the pancreas. Fewer induced species are detected with [³H]N-acetylmannosamine labeling, this sugar being incorporated in much smaller quantities than fucose and glucosamine.

Of all the induced glycoproteins observed, only one is found primarily in the particulate fraction of the tissue. The others are all soluble glycoproteins.

The differentiation-induced particulate glycoprotein was estimated to have a molecular weight of 144,000-154,000 daltons. Induction of this glycoprotein is essentially complete by day 17-18

of gestation (the time when zymogen granules first appear in the acinar cell) and is apparent only in fluorograph profiles of embryonic rudiments labeled with [^3H]glucosamine and [^3H]N-acetylmannosamine. Induction, thus, seems to occur only in terms of extension and completion of the carbohydrate moiety since 1) synthesis of the protein portion of this glycoprotein is not induced during the secondary transition, 2) incorporation of glucosamine is most likely to be throughout the carbohydrate chains, and 3) incorporation of N-acetylmannosamine is expected to be as carbohydrate chain-terminating sialic acid. This particulate glycoprotein does label with [^3H]fucose; however, fucose incorporation does not increase during the secondary transition period of differentiation, suggesting that completion of certain carbohydrate chains of the glycoprotein with fucose is enzymatically possible in the protodifferentiated pancreas. It is suggested that, rather than being a differentiation-induced protein, this particulate glycoprotein is the substrate for one or more differentiation-induced glycosyl transferases.

Comparison of radioactive profiles for [^3H]fucose-labeled zymogen granule membrane from adult rat pancreas with the [^3H]fucose profile for the particulate fraction from pancreatic rudiments cultured from day 14 to the equivalent of day 20 of gestation suggests that this unique high molecular weight glycoprotein corresponds to the zymogen granule membrane component GP-1. Precipitation of the glycoprotein by anti zymogen granule membrane (IgG) is further evidence that it is a zymogen granule membrane-like glycoprotein.

A minimum of two of the soluble induced glycoproteins are thought to be secretory proteins. The molecular weights of these

glycoproteins (calculated from their mobilities in SDS-polyacrylamide gels), their correspondence with similarly migrating induced protein species most certain to be secretory proteins, their relative abundance, and their recognition by the anti zymogen granule content (antiserum) are all evidence supporting the tentative identification of these induced glycoproteins as secretory proteins. These two induced glycoproteins have molecular weights of approximately 57,000 and 73,000 daltons. The former glycoprotein is thought to be amylase. The latter glycoprotein corresponds to a secretory protein observed by Walther and co-workers (1974) in differentiating pancreatic rudiments. It was not identified in terms of enzymatic activity, nor was any indication given that it was a glycoprotein. As was noted earlier, Ronzio and colleagues have observed a similar glycoprotein in zymogen granule contents from adult rat pancreas labeled *in vitro* with radioactive glucosamine.

A third soluble induced glycoprotein species is tentatively identified as procarboxypeptidase A or lipase solely on the basis of molecular weight calculations from its mobility on SDS-polyacrylamide gels. It was not, however, apparent in the electrophoretic profiles of anti zymogen granule content (antiserum) precipitates of soluble proteins from labeled pancreatic rudiments.

Other soluble glycoproteins present in pancreatic rudiments appear to undergo induction primarily in terms of glycosylation rather than protein synthesis. All are lower molecular weight glycoproteins. A 34,000-38,800 dalton species is labeled only by radioactive fucose. A 29,800-34,000 dalton species labels with radioactive fucose and glucosamine. A third induced glycoprotein, molecular weight 26,600-29,800 daltons, labels with radioactive

N-acetylmannosamine as well as the two other glycoprotein precursors. This lowest molecular weight species is thought *not* to correspond with the similarly migrating induced protein of pancreatic rudiments tentatively identified as chymotrypsinogen/trypsinogen. Slight variations in mobility prevent absolute assignment of this glycoprotein species as one of these secretory proteins, although Ronzio et al. (to be published) have observed a similar glycoprotein in adult rat pancreas zymogen granule contents. Because these three glycoproteins are induced only with respect to glycosylation, the implication is that they also must be the substrates for induced glycosyltransferase activities. The possibility that these minor induced glycoproteins are actually minor secretory proteins, the enzymatic natures of which have not been determined, cannot be eliminated. Alternatively, they are non-secretory proteins involved in the differentiation process.

Data obtained by radioimmunoassay for zymogen granule membrane-like glycoproteins in differentiating pancreas suggests that zymogen granule membrane glycoproteins, in general, do not undergo induction during the secondary transition period. This is evidenced by the presence of a glycoprotein similar to and suspected to be the major zymogen granule membrane component, glycoprotein GP-2, in the protodifferentiated as well as the differentiated pancreatic rudiment and in immunoprecipitates of membrane proteins from protodifferentiated and differentiated rudiments. This seems to contradict the observation that a GP-1-like particulate glycoprotein appears to undergo differentiation-dependent induction with respect to glycosylation. SDS-polyacrylamide gel electrophoresis analysis of the anti zymogen granule membrane (IgG) immunoprecipitates of particulate fractions from embryonic pancreas indicates, however, the presence of a large

number of non-induced glycoprotein species, not all of which are zymogen granule membrane-like glycoproteins. Thus, it is suggested that the induced glycosylation of a single zymogen granule membrane glycoprotein species during differentiation may have remained undetected by the radioimmunoassay technique utilized.

Results of radioimmunoassays for zymogen granule content-like proteins in soluble fractions from differentiating pancreatic rudiments were also indicative of the presence of one or more major differentiation-induced secretory glycoproteins. The absence of such glycoproteins in rudiments cultured in the presence of 5-BrdU and labeled with [^3H]glucosamine is further evidence for their involvement in the secondary transition events leading to the differentiated pancreas.

Glycosylation of Pancreatic Secretory Glycoproteins

To date, characterization of rat pancreas secretory proteins has not provided evidence for their glycosylation. Strong evidence for glycosylation of rat pancreas secretory proteins, however, is available from gas-liquid chromatographic analysis of the rat pancreas zymogen granule content proteins (Ronzio et al., submitted for publication). Evidence for secretory protein glycosylation has been provided by *in vitro* labeling of rat pancreas with radioactive glycoprotein precursors and subsequent observation of either glycosylated secretory granule content proteins on SDS-polyacrylamide gels (Ronzio et al., submitted for publication) or the release of glycosylated secretory products in response to stimulation of the labeled tissue with secretagogues (Völkl et al., 1976; Kronquist et al., 1977). Furthermore, evidence exists from studies of the homologous enzymes from other animal species and other tissues for

glycosylation of pancreatic secretory proteins. For example, amylase has been isolated from porcine pancreas and human parotid and submandibular glands as mixtures of glycosylated and non-glycosylated forms (Beauport-Abadie et al., 1973; Watanabe and Keller, 1974). Ribonucleases isolated from bovine and porcine pancreas have been shown to be highly glycosylated (Plummer and Hirs, 1963; Reinhold et al., 1968). Porcine pancreatic lipases have also been found to be glycosylated (Garner and Smith, 1972). Interestingly enough, in the case of porcine ribonuclease, enzyme activity does not seem to be affected significantly by the presence or absence of the carbohydrate moiety.

In view of the current ideas on post-translational processing of secretory proteins, in particular as proposed in the "signal" hypothesis of Blobel and Sabatini (1971; also, Blobel and Dobberstein, 1975), one might speculate that glycosylation of secretory proteins may also provide a transient tag by which secretory proteins are recognized for packaging. Devillers-Thiery and co-workers (1975) have found that a number of dog pancreas secretory proteins, in particular trypsinogen, are synthesized in an *in vitro* translation system as pre-proenzymes containing an amino terminal extension of sixteen amino acid residues. This has been called the signal sequence of the secretory proteins and is speculated to serve in guiding the secretory proteins into the cisternae of the rough endoplasmic reticulum for eventual shuttling into the Golgi and finally into packaging vesicles. Secretory protein glycosylation begun in the rough endoplasmic reticulum and completed in the Golgi stacks might be seen to serve as a further signal mechanism for the sequestration and packaging of secretory proteins.

That isolated rat pancreas secretory proteins have gone unremarked as glycoproteins may not be too surprising. Just as the signal peptide is hypothesized to be removed from the pre-proenzyme during passage into the cisternae of the rough endoplasmic reticulum, so might a signal carbohydrate moiety be removed from the proenzymes or amylase prior to or during concentration in secretory vesicles or during secretion. However, no evidence for such a signal carbohydrate moiety on secretory proteins is presently available.

Less hypothetical explanations may be invoked, however, for the unremarked glycosylation of rat pancreas secretory proteins. The presence of active glycosidases such as amylase in pancreatic tissue may have resulted in loss of carbohydrate from secretory proteins during secretory protein isolation. The influence of diet on the carbohydrate content of secretory proteins has not been investigated. It is known that the composition of the diet, be it high in protein or high in carbohydrate, determines the relative rates of synthesis of chymotrypsinogen and amylase (Reboud et al., 1964; Reboud et al., 1966).

The low levels of fucosylation observed for zymogen granule content proteins from adult tissue labeled *in vitro* with [³H]fucose in contrast with fucosylation of proteins of embryonic pancreas is a puzzle. An altered glycosylation of adult pancreatic secretory proteins relative to embryonic secretory proteins is possible. The synthesis and/or turnover of the carbohydrate moieties of secretory proteins of non-stimulated adult pancreas may be slow compared to the rate of synthesis of glycosylated secretory proteins of differentiating pancreas. A reduced rate of fucose transport into the adult tissue or the presence of a large intracellular pool of

fucose might also explain the low levels of incorporation of radioactive fucose into secretory proteins of the adult pancreas.

Metabolism of Glycoprotein Precursors

One criticism which may be made of these experiments is that sugar-labeled rudiments were not analyzed for conversion of the labeling sugars into other glycoprotein precursors or intermediates of glycolysis. It has been found that when radioactive L-fucose is administered to rats, 2% or less of the radioactivity appears in labeled CO₂, approximately 30% of the radioactivity appears as free fucose in the urine, and the remainder is found free as fucose, fucose-1-P, or GDP-fucose, or is bound to protein in various tissues. No conversion of fucose to other glycoprotein precursors has been observed (Coffey et al., 1964; Bekesi and Winzler, 1967).

Radioactive labeling of cells and tissues with glucosamine has generally resulted in the incorporation of radioactive N-acetylglucosamine, N-acetylgalactosamine, or N-acetylneuraminic acids into glycoprotein. In the adult rat pancreas, it has been observed that 70-94% of the protein-bound radioactivity resulting from labeling of the tissue *in vitro* with [³H]- or [¹⁴C]glucosamine is recoverable as glucosamine (Ronzio, unpublished observations). One might expect the incorporation of glucosamine by embryonic pancreatic rudiments in culture to be similar to the incorporation into glycoproteins by adult tissue. The question of metabolic reutilization of the glucosamine through conversion to glycolytic intermediates, in this case, cannot be answered.

N-Acetylmannosamine is a very proximal precursor of sialic acids; it is not incorporated itself directly into glycoproteins.

Though mannose is a precursor in glycoprotein synthesis, the existence of enzymes for the conversion of N-acetylmannosamine to mannose has not been reported.

Biogenesis of Zymogen Granule Membranes

Current theories of membrane biogenesis, elaborated by Morré and colleagues (1974), involve two concepts, that of membrane flow and that of membrane differentiation. It is generally considered that membrane of the endoplasmic reticulum with its complement of proteins and glycoproteins is gradually transported to the Golgi stacks via transporting vesicles which fuse with the outermost cisternae of the cis side of the Golgi. Subsequently, membranes of secretory vesicles, zymogen granule membranes, appear to derive from the last trans cisternae of the Golgi. Each membrane compartment of the cell, nevertheless, maintains a unique protein-glycoprotein composition (Meldolesi and Cova, 1972), though it appears that, in keeping with the membrane flow hypothesis, certain constituents characteristic of each type of membrane are carried over to the next type of membrane in the biogenic pathway. Evidence has appeared that additional protein synthesis and insertion into membranes does occur at the Golgi apparatus through the activity of Golgi-associated polysomes in rat liver (Elder and Morré, 1976).

Indications that intracellular membranes of the rat exocrine pancreas undergo a similar biogenic process are derived from the work of Völkl and co-workers (1976) and Ronzio and colleagues (work submitted for publication). The former group has shown, using electron microscopic techniques, the transport of membrane vesicles from the endoplasmic reticulum to the cis face of the Golgi and the

formation of condensing vacuoles (presumably zymogen granule precursors) at the trans face of the Golgi. SDS-polyacrylamide gel electrophoresis analysis of membrane subfractions from rat pancreas by Ronzio and co-workers has shown the distinct protein-glycoprotein character of each of the intracellular membranes but, in addition, has suggested the existence of at least one zymogen granule membrane-like glycoprotein in Golgi membrane. Immunochemical studies by Ronzio and Mohrlok (1977) have provided evidence for zymogen granule membrane glycoprotein precursors in the Golgi which serve as endogenous substrates for a Golgi-associated galactosyltransferase.

Studies of the cross-reactivity of anti zymogen granule membrane (IgG) with rough and smooth microsomal membranes presented in this report supply further evidence for the similarity of the intracellular membranes of the rat exocrine pancreas. SDS-polyacrylamide gel electrophoresis of anti zymogen granule membrane (IgG) immunoprecipitates of particulate glycoproteins from differentiating rat pancreas indicate not only the presence of glycoproteins similar to the two major components of zymogen granule membranes, GP-1 and GP-2, but also the presence of a number of other high molecular weight membrane-bound glycoproteins. Presumably these are glycoprotein constituents of rough and smooth microsomal membranes (including Golgi membranes), and though direct evidence is not available to the effect, it may also be that plasma membrane glycoproteins are present as well.

Glycosyltransferase Induction During Pancreatic Differentiation

Differentiation-dependent induction of glycoprotein synthesis in the rat pancreas appears to be of two types. In some cases

glycosylation is coordinate with induced protein synthesis. In other cases, induced glycosylation occurs with non-induced protein species as substrates, thus implying the induction of specific glycosyltransferase activities.

Direct involvement of glycosyltransferases as differentiation-induced enzymes of the rat pancreas is supported by the work of Carlson and colleagues (1973) and Marvel and Ronzio (1976). Carlson has found that a forty-fold increase occurs in the specific activity of a pancreatic N-acetylgalactosamine-protein:galactosyltransferase during the period from 11 to 12 days *in utero* to birth. 5-BrdU, however, does not inhibit the differentiation-dependent rise in the galactosyltransferase activity, suggesting that galactosyltransferase synthesis and secretory protein synthesis are regulated differently during pancreatic development. Similarly, a two-fold increase in the specific activity of a pancreatic N-acetylglucosamine-protein:galactosyltransferase has been observed by Marvel and Ronzio to occur during the secondary transition period of rat pancreas differentiation.

In the case of those soluble glycoprotein species of the embryonic pancreas, thought to be secretory proteins, it is not possible, on the basis of the experiments described in this report, to assess the induction of specific glycosyltransferases associated with the processing of the newly synthesized proteins. No evidence exists to indicate that *simultaneous* induction of glycosyltransferase activities and endogenous substrates in the form of secretory proteins does not occur. In the event that the concentrations of endogenous substrates are so low as to be rate limiting in

glycosylation reactions, it is an apparent induction of transferases that will be observed, however.

Pancreatic Glycoprotein Synthesis and the
Model for Pancreatic Differentiation

Of major concern to these studies was determining whether or not induced glycoprotein synthesis occurs synchronously with secretory protein synthesis during pancreatic differentiation and can, thus, be accounted for by the current model described by Rutter and colleagues for pancreatic differentiation (Figure 1). The basic problem involves the number and timing of regulatory events required for the induction of glycoprotein synthesis during the secondary transition from the protodifferentiated to the differentiated state.

The results of the studies reported here suggest that several secretory proteins, among them amylase, are glycosylated. However, the experiments were not designed such that the induction of glycosyltransferase activities responsible for the glycosylation of the secretory proteins could be observed independently of the induction of protein synthesis. Thus, it cannot be ascertained whether these specific glycosyltransferases are constitutive enzymes of the pancreas, are induced in advance of the induction of secretory protein synthesis (thereby indicating independent regulation in the synthesis of glycosyltransferases and the secretory proteins), or are induced simultaneously with the occurrence of increased secretory protein synthesis. In the latter case, additional regulatory events would not be required for initiation of glycosyltransferase synthesis during differentiation.

In the case of glycoproteins which appear to be induced during differentiation solely in terms of glycosylation, the implication is

for induction of specific glycosyltransferases. This could occur synchronously with the induction of secretory proteins. Thus, an additional regulatory event again is not necessitated in the differentiation scheme. This is further supported by the inhibitory effect of 5-BrdU on the induced glycosylation of these proteins.

Increased synthesis of the major zymogen granule membrane component, GP-2, does not appear to occur during the secondary transition period of pancreatic differentiation. Strong evidence is accumulating for the presence of zymogen granule membrane proteins and glycoproteins or their precursors as constituents of the Golgi membrane (and possibly the rough endoplasmic reticulum as well). This suggests that zymogen granule membrane proteins are, unlike the pancreatic secretory proteins, either constitutive proteins of the cell or that the major induction of synthesis of zymogen granule membrane protein precursors occurs before the start of the secondary transition period of pancreatic differentiation. Estimates that three percent or less of the membrane proteins of smooth and rough endoplasmic reticulum can be GP-2 (Ronzio, unpublished), however, suggest that the zymogen granule membrane glycoproteins are probably not constitutive glycoproteins of the pancreas cell. On the other hand, zymogen granule membrane glycoproteins (or their protein precursors) may well be induced during the primary transition period of pancreatic differentiation, the period when low but detectable amounts of the secretory proteins are present in the cell.

The differentiation-induced completion of glycosylation of a zymogen granule membrane GP-1-like glycoprotein may be the differentiation event which signals the conversion of Golgi membrane into zymogen granule membranes, which then begin to bud from the

Golgi in the formation of zymogen granules. This differentiation of Golgi membrane to form zymogen granule membrane potentially requires the induction of a number of glycosyltransferase activities. Once again, the induction of glycosyltransferase activity need not occur independently of the induction of the secretory proteins.

The results of these studies, therefore, are not sufficient to indicate that the current model for pancreatic differentiation does not hold for glycoprotein synthesis. They do suggest that the induction of a GP-2-like zymogen granule membrane glycoprotein must occur before the secondary transition period of differentiation. Whether there is a regulatory event other than the primary transition event of pancreatic differentiation that is required for the induction of this glycoprotein, or the primary transition is actually the major induction period for GP-2 (and possibly other zymogen granule membrane glycoproteins), remains to be determined.

Suggestions for Future Investigations

Now that differentiation-dependent induction of glycoprotein synthesis is known to occur in the rat exocrine pancreas, several very pertinent lines of investigation should be pursued. First, the question of glycosylation of adult rat exocrine pancreas secretory proteins remains to be answered directly. Glycosylation may be of significance with respect to the secretory proteins. Of immense importance in furthering the understanding of the differentiation process in the exocrine pancreas will be studies of the differentiation-dependent induction of glycosyltransferases, in particular fucosyl- and sialyltransferases, and the identification of their specific endogenous substrates. The possible role of

membrane glycosylation in the formation of zymogen granule membranes has been suggested by the results reported here and needs to be studied in detail. Finally, the synthesis of membrane proteins, especially zymogen granule membrane glycoproteins and their precursors, must be investigated in the protodifferentiated pancreas to determine whether or not they undergo a differentiation-dependent induction prior to the secondary transition that requires the inclusion of an additional regulatory event (other than the primary transition event) in the model for exocrine pancreas differentiation.

LIST OF REFERENCES

LIST OF REFERENCES

- Amsterdam, A., and Jamieson, J. D. (1974) *J. Cell Biol.* 63, 1037-1056.
- Beaupoil-Abadie, B., Raffalli, M., Cozzone, P., and Marchis-Mouren, G. (1973) *Biochim. Biophys. Acta* 297, 436-440.
- Bekesi, J. G., and Winzler, R. J. (1967) *J. Biol. Chem.* 242, 3873-3879.
- Bernfeld, P. (1955) In *Methods in Enzymology*, eds. Colowick, S. P., and Kaplan, N. O. (Academic Press, New York), Vol. 1, pp. 149-158.
- Blobel, G., and Sabatini, D. D. (1971) In *Biomembranes*, ed. Manson, L. A. (Plenum Publishing Corp., New York), Vol. 2, pp. 193-195.
- Blobel, G., and Dobberstein, B. (1975) *J. Cell Biol.* 67, 835-851.
- Bonner, W. M., and Laskey, R. A. (1974) *Eur. J. Biochem.* 46, 83-88.
- Carlson, D. M., David, J., and Rutter, W. J. (1973) *Arch. Biochem. Biophys.* 157, 605-612.
- Coffey, J. W., Miller, O. N., and Sellinger, O. Z. (1964) *J. Biol. Chem.* 239, 4011-4017.
- DeCrombrughe, B., Pitt-Rivers, R., and Edelhoch, H. (1966) *J. Biol. Chem.* 241, 2766-2773.
- Devillers-Thiery, A., Kindt, T., Scheele, G., and Blobel, G. (1975) *Proc. Nat. Acad. Sci. USA* 72, 5016-5020.
- Eagle, H. (1959) *Science* 130, 432-437.
- Elder, J. H., and Morré, J. D. (1976). *J. Biol. Chem.* 251, 5054-5068.
- Etzler, M. E., and Branstrator, M. L. (1974) *J. Cell Biol.* 62, 329-343.
- Fraker, P. J., Cicurel, L., and Nisonoff, A. (1974) *J. Immunol.* 113, 791-797.

- Garner, C. W., Jr., and Smith, L. C. (1972) *J. Biol. Chem.* 247, 561-565.
- Geltoskey, J. E., Sui, C., and Lerner, R. A. (1976) *Cell* 8, 391-396.
- GIBCO Catalogue (1976-1977), Grand Island Biological Company, Grand Island, New York.
- Githens, S., Pictet, R., Phelps, P., and Rutter, W. J. (1976) *J. Cell Biol.* 71, 341-356.
- Greenaway, P. J., and LeVine, D. (1973) *Nature (London), New Biol.* 241, 191-192.
- Jamieson, J. D., and Palade, G. E. (1967) *J. Cell Biol.* 34, 577-596.
- Kay, C. M., Smillie, L. B., and Hilderman, F. A. (1961) *J. Biol. Chem.* 236, 118-121.
- Kemp, J. D., Walther, B. T., and Rutter, W. J. (1972) *J. Biol. Chem.* 247, 3941-3952.
- Kleinschuster, S. J., and Moscona, A. A. (1972) *Exp. Cell. Res.* 70, 397-410.
- Krebs, E. (1950) *Biochim. Biophys. Acta* 4, 249-269.
- Kronquist, K. E., Elmahdy, A., and Ronzio, R. A. (1977) *Arch. Biochem. Biophys.* 182, 188-196.
- Laemmli, U. K. (1970) *Nature (London)* 227, 680-685.
- Laskey, R. A., and Mills, A. D. (1975) *Eur. J. Biochem.* 56, 335-341.
- LeVine, D., Kaplan, M. J., and Greenaway, P. J. (1972) *Biochem. J.* 129, 847-856.
- Levy, H. B., and Sober, H. A. (1960) *Proc. Soc. Exp. Biol. Med.* 103, 250-252.
- Lewis, D. S., MacDonald, R. J., Kronquist, K. E., and Ronzio, R. A. (1977) *FEBS Lett.* 76, 115-119.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265-275.
- MacDonald, R. J., and Ronzio, R. A. (1972) *Biochem. Biophys. Res. Commun.* 49, 377-382.
- Marvel, C., and Ronzio, R. A. (1976) *Abstracts of Papers*, 172nd ACS National Meeting, San Francisco, California. Biol. abstract 216.
- Meldolesi, J., Jamieson, J. D., and Palade, G. E. (1971) *J. Cell Biol.* 49, 109-129.

- Meldolesi, J., and Cova, D. (1972) *J. Cell Biol.* 55, 1-18.
- Morré, J. D., Keenan, T. W., and Huang, C. M. (1974) In *Advances in Cytopharmacology*, eds. Ceccarelli, B., Clementi, F., and Meldolesi, J. (Raven Press, New York), Vol. 2, pp. 107-125.
- Ouchterlony, Ö. (1958) *Progr. Allergy* 5, 1-78.
- Patterson, M. S., and Greene, R. C. (1965) *Anal. Chem.* 37, 854-857.
- Pharmacia Fine Chemicals AB (1972) *CNBr-activated Sepharose 4B for Immobilization of Biopolymers, Con A-Sepharose for Affinity Chromatography of Polysaccharides and Glycoproteins*. Uppsala, Sweden.
- Pictet, R. L., Clark, W. R., Williams, R. H., and Rutter, W. J. (1972) *Dev. Biol.* 29, 436-467.
- Plummer, T. H., and Hirs, C. H. W. (1963) *J. Biol. Chem.* 238, 1396-1401.
- Reboud, J. P., Pasero, L., and Desnuelle, P. (1964) *Biochem. Biophys. Res. Commun.* 17, 347-351.
- Reboud, J. P., Marchis-Mouren, G., Pasero, L., Cozzzone, A., and Desnuelle, P. (1966) *Biochim. Biophys. Acta* 117, 351-367.
- Reinhold, V. N., Dunne, F. T., Wriston, J. C., Schwarz, M., Sarda, L., and Hirs, C. H. W. (1968) *J. Biol. Chem.* 243, 6482-6494.
- Roberts, R. C., Makey, D. G., and Seal, U. S. (1966) *J. Biol. Chem.* 241, 4907-4913.
- Ronzio, R. A. (1973a) *Biochim. Biophys. Acta* 313, 286-295.
- Ronzio, R. A. (1973b) *Arch. Biochem. Biophys.* 159, 777-784.
- Ronzio, R. A., and Mohrlok, S. H. (1977) *Arch. Biochem. Biophys.* 181, 128-136.
- Ronzio, R. A., and Rutter, W. J. (1973) *Dev. Biol.* 30, 307-320.
- Rosen, J. M., Woo, S. L. C., and Comstock, J. P. (1975) *Biochemistry* 44, 2895-2903.
- Rutter, W. J. (1967) In *Methods in Developmental Biology*, eds. Wilt, F. H., and Wessells, N. K. (Thomas Y. Crowell Company, New York), pp. 671-683.
- Rutter, W. J., Kemp, J. D., Bradshaw, W. S., Clark, W. R., Ronzio, R. A., and Sanders, T. G. (1968) *J. Cell. Physiol.* 72 Sup. 1, 1-18.
- Sanders, T. G. (1970) Ph.D. thesis, University of Illinois.

- Segrest, J. P., and Jackson, R. L. (1972) In *Methods in Enzymology*, ed. Ginsburg, V. (Academic Press, New York), Vol. 28, pp. 54-63.
- Skutelsky, E., and Farquhar, M. G. (1976) *J. Cell Biol.* 71, 218-231.
- Spooner, B. S., Walther, B. T., and Rutter, W. J. (1970) *J. Cell Biol.* 47, 235-246.
- Truding, R., Shelanski, M. L., Daniels, M. P., and Morell, P. (1974) *J. Biol. Chem.* 249, 3973-3982.
- Vandermeers, A., and Cristophe, J. (1968) *Biochim. Biophys. Acta* 154, 110-129.
- Völkl, A., Bieger, W., and Kern, H. F. (1976) *Cell Tissue Res.* 175, 227-243.
- Walther, B. T. (1972) Ph.D. thesis, University of Washington.
- Walther, B. T., Pictet, R. L., David, J. D., and Rutter, W. J. (1974) *J. Biol. Chem.* 249, 1953-1964.
- Wang, C., and Smith, R. L. (1975) *Anal. Biochem.* 63, 414-417.
- Watanabe, S., and Keller, P. J. (1974) *Biochim. Biophys. Acta* 336, 62-69.
- Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* 244, 4406-4412.
- Weiser, M. M. (1972) *Science* 177, 525-526.
- Weiser, M. M. (1973) *J. Biol. Chem.* 248, 2536-2541.
- Weiser, M. M. (1974) *Univ. Mich. Med. Cent. J.* 40, 168-175.
- Wray, V. P., and Perdue, J. F. (1974) *J. Biol. Chem.* 249, 1189-1197.

MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 03196 0036