6-1986

The Parotid Salivary Glands. I. Potential Role of a Parotid Factor Modulating Pancreatic β-Cell Function. II. An Immunohistological Study to Identify Parotid Hormone Secreting Cell(s)

Denise S. Cook

Abstract

THE PAROTID SALIVARY GLANDS: I. POTENTIAL ROLE OF A PAROTID FACTOR MODULATING PANCREATIC β-CELL FUNCTION II. AN IMMUNOHISTOLOGICAL STUDY TO IDENTIFY PAROTID HORMONE SECRETING CELL(S)

The physiologic role of the parotid glands is more complex than its recognized salivary function. Various investigators have suggested an intriguing relationship between the parotids and the pancreas, both endocrine and exocrine in nature.

We studied in rats the contribution of salivary glands in the regulation of β-cell function in response to an acute intra-arterial glucose challenge in parotidectomized, submandibulariectomized and totally sialoadenectomized animals. Fifteen days post-parotidectomy or total sialoadenectomy, the β-cell response, at 2 minutes post-glucose infusion, diminishes and stabilizes at about 40% of the preoperative value. Concurrent with the depressed insulin secretion a correspondingly higher glucose titer was observed at 6 and 10 minutes post-glucose infusion.

In addition, the role of age on the effect of long-term sialoadenectomy and the effect of different concentrations of glucose on the β-cell response were examined in rats. The decreased sensitivity of the β-cells following parotidectomy is not dependent on the age at which the animals are operated. Even though there was less insulin available in the parotidectomized young rats, their growth was not affected. β-cell function and glucose clearance in parotidectomized animals show a progressive incompetence in the ability to cope with an increasing

glucose challenge. Results indicate a rise in fasting plasma immunoreactive insulin (IRI) and glucose values in both parotidectomized and sham-operated animals over time. Animals which have been parotidectomized for 80 days have higher fasting plasma IRI and glucose levels than sham-operated controls.

Immunohistological techniques were employed in an attempt to identify the particular cell(s) in the parotid gland responsible for production of parotid hormone. Even though our data were not conclusive, they suggest that cells associated with connective tissue may be the source of parotid hormone.

LOMA LINDA UNIVERSITY

Graduate School

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by

Denise S. Cook

A Dissertation in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Field of Physiology

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June 1986

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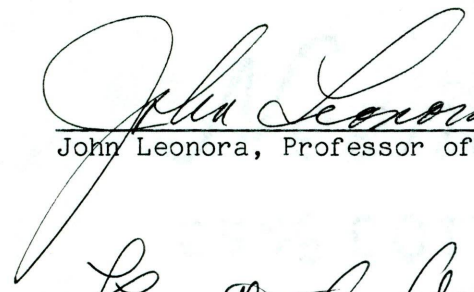

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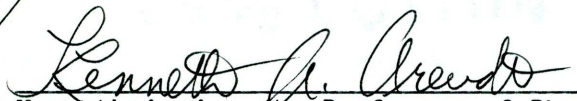

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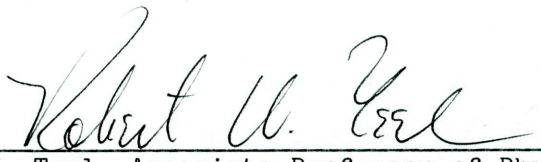

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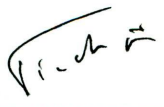

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ACKNOWLEDGMENTS

I wish to express my deep gratitude to Dr. John Leonora for his excellent guidance, support and countless hours of help in the perform-formance of various procedures, and also to Dr. Jean-Marc Tieche for his skillful collaboration. A warm appreciation goes to Dr. Kenneth Arendt, Dr. Raymond Hall, and Dr. Robert Teel, members of my Guidance Committee, for their constructive criticisms.

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CHAPTER I

INTRODUCTION

Salivary Glands as Endocrine and Exocrine Organs

For many years evidence has accumulated which suggests that the salivary glands function as endocrine as well as exocrine organs (32, 53, 60, 71, 83, 115, 123, 133).

As early as 1886 Hardin reported bilateral enlargement of the parotid glands during pregnancy (43) and Phillips (92) noted the enlargement of these glands during lactation.

Most of the early work concerned with the hormonal influence of salivary glands has been published by Japanese investigators. Takizawa (122, 123) reported data on the trophic effect of salivary internal secretions upon the development of bone tissue. Katagiri (60) reported that histologic changes appeared in the bones, hypophysis, parathyroid and thyroid glands of rats in which the parotid ducts were ligated and the submandibular and sublingual glands have been removed. In addition, the testis, thymus, and spleen atrophied but the adrenal cortex and uterus hypertrophied.

An extensive amount of work has been done on a protein named parotin, isolated from the bovine parotid gland by Ogata (88, 89). It has been shown in the rabbit to cause growth stimulation in hard tissues, a marked dimunition in serum calcium levels, a decrease in the number of circulating leukocytes and also a strong calcification effect on rat teeth and the hard tissues around them (53).

There have been reports of various forms of parotin-like substances

from horse and bovine parotids, and human salivary glands (54, 61, 86). Other investigators who attempted to repeat the Japanese studies, they were not able to show growth enhancement (30) or a reduction in serum calcium in the rabbit (98). This could suggest that the observed biological effects are more likely the result of the interaction of several factors, rather than a single factor.

Lacassagne made significant contributions in attempting to relate salivary glands and endocrine organs. In 1940 he demonstrated that there were histological differences between the male and female mouse submaxillary glands and that if male sex hormones were administered to a female mouse, its submaxillary glands assumed the characteristics of those of the male (66, 67, 68).

Buillard et al. (15) reported atrophic changes in the salivary glands following castration. Raynard (94) has shown that the injection of testosterone into the submandibular glands of mice resulted in a marked hypertrophy of the tubules. This was interpreted as evidence of a direct action of androgen on the gland.

Much investigation has been done in connection with those changes occurring in salivary glands that appear related to the pituitary and thyroid glands. Arvy et al. (1) along with Shafer et al. (109) and Gabe (33) reported that thyroidectomy induces atrophy of the submaxillary glands which can be reversed by administration of thyroxin to thyroidectomized or hypophysectomized animals. However, Eartly et al. (23) did not confirm these findings. They reported that the administration of thyroxine alone to a hypophysectomized animal did not restore the submandibular gland to its normal state, although thyroxin would restore

the gland in thyroidectomized animals, and thus suggested that the granular tubules of the submandibular gland might be under direct control of a hypophyseal factor.

Fawcett et al. (28) proposed that one important function of the salivary gland is to control the level of thyroxin in the blood by deiodinating thyroxin and recycling the iodine to the thyroid via the saliva and digestive tract. Piatnek et al. (93) and Mason et al. (80) reached similar conclusions; however, Ruegamer (101) has disputed this evidence and has suggested that salivary glands play no active role in the metabolism of thyroid analogues.

Wase et al. (133) presented evidence that sialoadenectomy reduces thyroid activity, which they attributed to changes in iodine uptake and turnover via a fall in circulating TSH. Taurog et al. (126) found concentrated inorganic ^{131}I in mouse submandibular gland after its intraperitoneal injection. This iodine-concentrating capacity of submandibular gland tissue was not impaired by hypophysectomy nor by administration of TSH to either normal or hypophysectomized mice. They concluded that the submandibular iodine pump, unlike the thyroid iodine pump, is not affected directly by the presence or absence of TSH.

Salivary Glands and Carbohydrate Metabolism

Of particular interest to this study are observations that suggest a relationship between the salivary glands and carbohydrate metabolism.

In 1892 de Renzi et al. reported the occurrence of diabetes in dogs following surgical removal of the salivary glands and duodenum (20). However, Minkowski (82) found only a slight transient glucosuria after

removing only the salivary glands and concluded that diabetes observed by de Renzi *et al.* was due to damage inflicted on the pancreas by the removal of the duodenum. He attributed the slight glucosuria to stress caused by the procedure.

In 1911 Farroni produced extracts of bovine salivary glands which reduced glycosuria induced by epinephrine administration in rabbits (27).

Goljanitzki found that extirpation of both parotid and submaxillary glands led to glycosuria in rabbits, followed by emaciation and death (40, 41, 42). He also found that epinephrine-induced glycosuria was prevented by parotid duct ligation, while a human patient showed improvement of diabetic symptoms following parotid duct ligation.

Mansfeld postulated that parotids produce a substance which stimulates pancreatic function. He observed some reduction in blood sugar, following ligation of the parotid ducts (77, 78, 79). Seelig reported improvement in some diabetic patients following ligation of their parotid ducts (105, 106). In addition, he observed in dogs that parotidectomy produced hypertrophy of the islets of Langerhans and a subsequent increase in liver glycogen content. Zimmerman reported that glycosuria induced in intact dogs could be ameliorated by parotid duct ligation. He also noted, in support of the findings of Mansfeld and Seelig that the duct ligation improved glucose tolerance (137). In the next decade, Birnkrant (3) reported a transient drop in basal blood sugar which lasted several days following extirpation of the parotids. However, if the gland removal was incomplete, a transient hyperglycemia resulted. The hyperglycemia became pronounced if ligatures were placed around the remaining parotid fragments. Gault (35) found that bilateral

parotidectomy did not effect the titer of the fasting blood sugar.

Birnkrant et al. (4) produced a bovine parotid extract which in their experiments, caused hyperglycemia in rats. Bubyakina et al. (12) observed in rabbits effects similar to those seen by Zimmerman and other workers previously cited. They reported that parotidectomy resulted in a reduced glucose response to epinephrine. The parotidectomized animals were more sensitive to insulin than intact controls, showing a more rapid, deeper glucose clearance response to an exogenous insulin challenge, and a more rapid return to baseline. However, if parotidectomized rabbits were subjected to a glucose tolerance test, they required more time to return to baseline.

In the 1960s Godlowski et al. began to focus attention on the submandibular and sublingual glands, apparently because these glands are histologically more similar to the pancreas than the parotids (36, 37, 38) Despite histologic similarity Godlowski did not propose a functional similarity. He reported 3 cases of complete remission of maturity onset diabetes mellitus following the surgical ablation of the submaxillary gland (39). He postulated that the submaxillary glands produce an insulin antagonist which if removed, results in an increased hypoglycemic response to circulating insulin. This hypothesis was based upon the demonstration in dogs of increased insulin sensitivity during insulin tolerance tests following bilateral extirpation of the submaxillary glands (38).

However, Garcia et al. (34) found no effect on either blood glucose or plasma insulin by intravenous glucose tolerance tests performed on dogs with the submandibular glands extirpated. Steinberg et

al. (116) found no significant changes in tolerance to either oral glucose or intravenous insulin following removal of the submandibular gland in dogs.

The work of Hoshino et al. (48) supports the findings of Godlowski et al. Working with normal mice, they found that ligation of the submandibular duct produced a reduction in fasting blood sugar, which could be potentiated with parotid duct ligation; the latter by itself was without effect. Similar results were found in spontaneously-diabetic mice or mice made diabetic by streptozotocin. It should be noted that the significant changes observed in blood sugar following ligation of the glands, were accompanied by substantial weight loss.

Asymptomatic enlargement of the parotids has been associated not only with diabetes mellitus but also with hypothyroidism, menopause, pregnancy, lactation, testicular atrophy, malnutrition, alcoholism with liver disease and obesity (9, 13, 16, 22, 55, 58, 59, 76, 81, 87, 100, 102, 104, 131, 134).

Pathologists have described similarities in architecture and cellular detail between the pancreas and the parotid and physiologists have revealed the responsiveness of both glands to similar stimulants as well as common characteristics of exocrine secretion (21). The fact that in diabetics a transitory or permanent enlargement of the parotids is not uncommon, has been considered suggestive of a compensatory activity in this organ (4, 29).

Davidson et al. (18) reported 16 patients with asymptomatic parotid enlargement, fourteen of whom were considered to have diabetes. This association was examined by Levine (75) in the Pima, an American Indian

tribe with a high incidence of diabetes mellitus. Sixty-one percent of the cases were associated with asymptomatic parotid enlargement. Godlowski observed three cases of enlarged parotids and with mildly diabetic glucose tolerance profiles (36).

Studies suggesting evidence for immunoreactive insulin-like substance from the parotid or submandibular glands are of interest (62, 70, 85, 110, 111, 114). Lawrence et al. (70) extracted an immunoreactive insulin-like material from rat and rabbit parotid gland homogenates. These investigators had previously reported the existence of a large glucagon-like immunoreactive substance which was concentrated primarily in the submandibular glands of rodents and man. Small amounts were also identified in the parotid and sublingual glands (71). Perez-Castillo et al. also reported significant amounts of glucagon and glucagon-like immunoreactivity in the salivary glands and established that biosynthesis was in situ (91). Murakami et al., found that extracts of rat and human parotid glands contained insulin-like immunoreactivity. They suggested that the parotid glands may be an extrapancreatic source for insulin, and that insulin biosynthesis does occur in extrapancreatic tissues (85). Smith et al. (114), using radioimmunoassay and immunocytochemical techniques, reported that the parotid glands of male rats possess a population of cells that contain an insulin-like substance. These cells were situated mainly in groups along the intercalated ducts, or less frequently as isolated cells dispersed throughout the acini. Shubinkova et al. (111) presented evidence for the synthesis of insulin-like protein (ILP) in the submandibular glands of male mice, rats, hamsters, cats, rabbits, pigs, bulls

and humans. Immunofluorescence studies showed that the ILP was located in the granular duct cells of mice and rats. In the submandibular glands of animals which do not contain granular ducts, ILP was found in the striated tubules.

In summary, various investigators have suggested an intriguing relationship between the parotids and the pancreas. Bilateral enlargement of the parotid glands has been associated with diabetes mellitus and has been considered suggestive of a compensatory activity in response to decreasing insulin secretion. Some investigators have suggested that removal of the parotid and submandibular glands causes glycosuria, while others report a transient drop in basal blood sugar following parotidectomy. Induced glycosuria in intact animals has been shown to decrease with either parotid duct ligation or administration of salivary gland extracts. In contrast, it has also been reported that administration of parotid gland extract causes hyperglycemia. Finally, it has also been reported that parotidectomy has no effect on blood sugar. The inherent weakness in these studies is that the β-cell function was evaluated indirectly by monitoring changes in blood glucose. We now know that a variety of factors, both neural and hormonal, can have a profound effect on blood glucose, which could limit the significance of these observations.

Leonora et al. have demonstrated the endocrine potential of the parotids (73, 74). These investigators have proposed the existence of a hypothalamic-parotid endocrine axis which is involved in the regulation of fluid movement through teeth. The parotid glands secrete a parotid hormone (PH) which stimulates the dental fluid transport (DFT) mechanism

in rat teeth, whereby dental lymph is pumped through the dentin and onto the surface of the enamel. Studies have shown an inverse relationship between the incidence of dental caries and the functioning of the DFT mechanism. Furthermore, by maintaining DFT with dietary supplements, cariogenesis can be significantly inhibited even in the presence of a cariogenic diet. However, the cariostatic effects of the dietary additives were without effect in parotidectomized rats (73, 74, 117, 118, 129). This suggests that systemically the parotids and a functioning DFT mechanism are part of an important defense mechanism against caries. Other studies support the concept of a systemic mechanism involved in caries control. A significant increase in the incidence of caries is associated with insulin insufficiency which can be corrected by controlling the diabetic state with insulin therapy (5-8, 24, 25, 44, 72, 121, 136). Collectively, these studies suggest that the induction of dental caries may in part be related to glucose intolerance and that the parotids and the pancreas have a systemic role in maintaining normal dental health.

In view of the potential linkage between the parotids and the pancreas, we have investigated the role of the parotid glands as they may affect β-cell function. By quantitating the immunoreactive insulin response to glucose infusion in intact and parotidectomized rats, we have attempted to elucidate the effects of: (1) short and long-term parotidectomy, (2) age, at the time of parotidectomy and (3) different concentrations of infused glucose.

CHAPTER II

MATERIALS AND METHODS

Materials

Anti-porcine insulin guinea pig serum M8170, Novo Research Institute, Copenhagen, Denmark.

Bacteriostatic sodium chloride, USP Invenex Laboratories, Chagrin Falls, OH.

Bovine serum albumin: (BSA), Sigma Chemical Company, St. Louis, MO.

Brevital sodium, Eli Lilly & Co., Indianapolis, Indiana.

Ethylenediamine tetraacetic acid: (EDTA), Sigma Chemical Company, St. Louis, MO.

Glucose analyzer reagent, Lancer--Division of Sherwood Medical, St. Louis, MO.

Glucose, Sigma Chemical Company, St. Louis, MO.

Goat anti-guinea pig globulin serum, Arnel Products Co., Inc., New York, NY.

Normal guinea pig serum, Arnel Products Co., Inc., New York, NY.

Phosphoric acid, Baker Chemical Co., Phillipsburg, NJ.

Polyethylene tubing (PE-50), Clay Adams--Division of Becton Dickinson & Co., Parsippany, NJ.

Polypropylene Micro-centrifuge tubes (500 µl), American Scientific Products, McGaw Park, IL.

Polystyrene tubes (12 x 75mm), Sarstedt, West Germany.

Radioactive Insulin (^{125}I iodoinsulin), ICN Radiochemicals, Irvine, CA.

Rat Insulin, Novo Research Institute, Copenhagen Denmark.

Sialastic tubing, Dow Corning Corporation, Midland, MI.

Sodium azide, American Scientific Products, Irvine, CA.

Sodium chloride: (NaCl), VWR Scientific Inc., San Francisco, CA.

Sodium heparin, Elkins-Sinn Inc., Cherry Hill, NJ.

Sodium phosphate dibasic, Baker Chemical Co., Phillipsburg, NJ.

Unassayed control serum, Beckman Instruments Inc., Fullerton, CA.

Surgical Procedures

Removal of Submandibular and Sublingual Glands

Rats were anesthetized with Brevital (5 mg/100g body weight). The glands were removed through an incision along the ventral midline of the neck. In the rat, submandibulariectomy involves removal of the sublingual glands because they are embedded in the posterior end of the submandibular glands where their respective salivary ducts and blood vessels enter. The glands were separated by blunt dissection from connective tissue surrounding them, and a ligature was placed around the ducts and blood vessels. The vessels and ducts were then cut and the glands removed. The incision was closed with 3.0 surgical suture. The animals were given 5% glucose drinking water and rested for 5 days before starting experiments.

Removal of Parotid Glands

Rats were anesthetized as previously described. An incision was made on each side of the neck, beginning slightly behind the ear and extending just behind the mandible to the ventral midline of the neck. The parotid tissue was carefully removed by blunt dissection. Care was

taken to avoid rupture of any sizeable blood vessel in this well vascularized area, and also, to remove tags of glandular tissue attached to the blood vessel. The incision was sutured and the animals were given 5% glucose drinking water and rested.

Removal of Parotid, Submandibular and Sublingual Glands

Totally sialoadenectomized rats were prepared as for parotidectomy, with slightly longer incisions on each side of the neck, allowing the removal of all salivary glands. Rats were given 5% glucose drinking water and rested.

Sham Operations

To provide controls for the above experimental procedures, sham operated animals were prepared as for the total sialoadenectomy, except that the glands were only gently manipulated with blunt forceps. Rats were given 5% glucose drinking water and rested.

Catheterization

Rats were anesthetized and weighed. The left common carotid artery was exposed by blunt dissection, the distal end was ligated and the proximal end was temporarily clamped. The isolated arterial segment was incised and the beveled tip of an 18-inch piece of polyethylene tubing (PE-50) was inserted toward the heart. After unclamping the proximal end of the artery, the catheter was positioned so that the tip reached the aorta. Placement of the catheter into the artery to reach the aorta was initially developed on a body weight basis by trial and error.

Subsequently, we determined a ratio of body weight to catheter insertion length, which provided a 90-95% functionally successful catheterization rate. Inserting the catheter 2.0 cm was appropriate for a 250g rat. It was necessary to insert the catheter an additional 0.1 cm for each 25g increment in body weight to 350g. Beyond this weight, the catheter was inserted 0.1 cm further for each 10g increase in body weight. The tubing was then secured by a ligature attached to adjacent tissue. The distal end of the catheter was carefully tunnelled under the skin around the shoulder and was exteriorized dorsally between the shoulders. The tubing was protected by inserting it into a 12-inch metal coil soldered at right angle to a 2.5 x 4 cm metal plate which was sutured over the catheter opening in the animal's skin. The animals were rested 5 days before experimentation.

Maintenance

Rats were kept in individual raised metal cages 18 x 24 x 17 cm. A dark plastic lid with a midline slot in its long axis was fitted to the cate, allowing the animal complete freedom of movement. Cages were positioned so that all catheter manipulations could be done without allowing the animals to see the investigator. Catheters were flushed twice daily with heparinized sterile saline (20 U/ml) and filled with more concentrated solution (300 U/ml). The animals had access to water and laboratory chow ad libitum. Their weight and feed intake were monitored daily.

Glucose Tolerance Test

Rats were weighed and fasted 24 hours before an experiment was performed. The glucose tolerance test was always conducted at 2 p.m., under strictly controlled conditions. The bottles of sterile heparinized saline and glucose solution were equilibrated in a water bath at 37°C. Before a blood sample was taken, the saline solution remaining in the catheter was withdrawn with a 1 cc disposable tuberculin syringe. This syringe was replaced with a 500 µl gas-tight syringe which was used to draw all blood samples. A 420 µl baseline blood sample was drawn at a constant rate of 2 µl/second over 3.5 minutes.

After the fasting sample had been obtained, approximately 0.2 ml of warmed glucose solution in saline (15mg/100g body weight) was infused from a disposable tuberculin syringe through the catheter over a 15-second period. After flushing and removing the saline from the catheter, blood sampling was started exactly 30 seconds after glucose infusion, and continued for 1.5 minutes at a constant rate of 5 µl/second. Blood samples collected at 3, 6 or 10 minutes, were drawn over a twenty-second period at a rate of 17.5 µl/second. After each blood sample was drawn an equal volume of warmed saline was infused over a 20-second period. Blood samples were placed in 500 µl polypropylene microcentrifuge tubes and kept in an ice bath until centrifuged.

When sample collection was completed, the animal was allowed to eat, and the samples were centrifuged for 10 minutes at 3000 RPM. From each blood sample, 50 µl of plasma were aliquoted in triplicate in 12 x 75 mm polystyrene tubes, which were then capped and kept frozen (-50°C) until processed in the insulin radioimmunoassay (RIA). The remaining

plasma was used for glucose determination.

Immediate and Long-Term Effects of Sialoadenectomy on β-Cell Function

The immediate and long-term effects of sialodenectomy on the acute response of the β-cell to an intra-arterial glucose load were studied. Adult (300g) Sprague-Dawley derived male rats were chronically catheterized and kept in individual cages as previously described. The animals were divided into 4 groups and between days -15 and 0, five glucose tolerance tests were done at 3-day intervals in the awake animals. The IRI and plasma glucose levels were obtained before glucose infusion, and then at 2, 6, and 10 minutes post-infusion. On day 0, each group of rats was either parotidectomized, submandibulariectomized, parotidectomized and submandibulariectomized, or sham-operated. The glucose tolerance test was repeated in each animal on days 5, 8, 12, 15, 19, 22, 26, 29, and 33, post-surgery. Because it is difficult to maintain the catheter patent for more than 40 days, 2 more groups of rats were either parotidectomized or sham-operated. Eighty days later, they were catheterized and the glucose test was carried out on day 85, 89, 92, 96 and 100.

The Role of Age in the Effect of Long-Term Sialoadenectomy on β-Cell Function and Blood Glucose Titer

The long-term effect of parotidectomy on β-cell function was assessed in young male rats operated when 22-25 days old and weighing 50-60g, and in adult male rats weighing 275-300g. Control animals were

sham operated. Both groups were catheterized 80 days after surgery, and the responsiveness of the β-cell to the glucose challenge was tested on days 85, 89, 92, 96, and 100 post-parotidectomy.

The Effect of Different Concentrations of Glucose on the β-Cell Response in Parotidectomized Rats

The capacity of the β-cell to respond to a high, medium, and low glucose challenge was tested in parotidectomized and sham operated adult rats. The concentrations of glucose were 45, 15 and 7 mg/100g body weight respectively. The glucose tolerance test was administered to 3 groups of adult rats on days 15, 11, 8, 4 and 1 before surgery; and after parotidectomy or sham surgery on days 15, 19, and 22. Since the insulin and glucose response had returned to baseline by 6 minutes post-glucose infusion, blood titers were determined at 2, 3, and 6 minutes.

Plasma Glucose Determination

Plasma glucose determinations were made on a Beckman Glucose Analyzer, which utilizes a glucose oxidase method (84). Blood glucose determinations, each requiring 10 µl of plasma, were done in triplicate. Calibration of the standard was done with a ± 3% error. Quality controls consisted of two levels of Beckman's unassayed control serum, which had been standardized by the clinical lab (LLUMC), allowing determination of unknowns with a ± 6% error.

Plasma IRI Determination

A modified double antibody procedure derived from Novo RIA kit (Novo

Research Institut, Denmark) was used. One incubation buffer was used for diluting insulin standard, unknown samples, labeled antigen and antiserum. Its composition was 0.051 M sodium phosphate dibasic, 0.154 M NaCl, 0.004 M EDTA, 0.3 (w/v) bovine serum albumin, 1.43% (v/v) normal guinea pig serum, 0.02% NaH_3 titrated to pH 7.4 with H_3PO_4. The incubation was carried out in 12 x 75 mm polystyrene tubes with a total volume of 0.8 ml. Unknowns (50 µl) or insulin standards (rat insulin, 25pg-800pg), 0.5 ml incubation buffer, 0.1 ml first antibody (anti-porcine insulin guinea pig serum at a dilution to bind 20-25% of the labelled antigen without unlabelled hormone) were pre-incubated 8 hours at 22°C. This was followed by the addition of 0.1 ml [^{125}I]-iodoinsulin (usually 10,000 cpm) and incubation at 4°C for 24 hours. The separation of bound from free antigen was achieved by precipitation with 0.1 ml of a titered solution of goat anti-guinea pig gamma globulin serum in normal saline. Incubation for 6 hours at 4°C was followed by centrifugation at 1,700 x g for 15 minutes at 4°C, and removal of the supernatant by aspiration.

Sensitivity of the assay was equal to or less than 0.15ng insulin/ml plasma. Quality controls consisted of pooled rat plasma collected from fasted, non-fasted and glucose stimulated rats. Insulin concentrations in each of the three plasma pools were then determined against the insulin standards.

Triplicate quality controls from each dose were included in each assay. The intra-assay coefficient of variation of the plasma estimates at 1.1, 3.0, and 6.0 ng/ml were 6.5%, 4.4%, and 3.7% respectively. The interassay coefficients of variation at the same levels were 10%, 5.6% and 8.8% respectively.

Statistical Analysis

Statistical analysis and reduction of the RIA data were done according to Rodbard (97). The insulin and glucose data were expressed as percent change x 10^{-2} for the statistical analysis. For each post-infusion data point, the difference was calculated from the pre-infusion basal level. For each animal, means and standard errors were determined for these values when the identical experiment had been repeated over successive days. Means and standard errors were also determined between animals within the same group for each successive day. Statistical significance was assessed by the student's paired or unpaired "t" test, as applicable.

CHAPTER III

RESULTS

Immediate and Long-Term Effects of Sialoadenectomy on β-Cell Function

There are no significant differences in insulin response among the 4 groups of rats before surgery (Fig. 1A). During the first 15 days post-surgery, the parotidectomized rats show at 2 minutes a progressive, significant impairment in insulin secretion, relative to the sham group. In contrast, the β-cell response in the submandibulariectomized animal is not different from the sham control group. After 15 days, the insulin response stabilizes and remains at about 40% of the preoperative value. At the 2 minute mark, between days 19-33, the sham controls and submandibulariectomized animals were statistically higher than parotidectomized and totally sialoadenectomized rats at $p < 0.001$. The same impairment in insulin secretion was evident in rats parotidectomized for 85-100 days. By 6 and 10 minutes post infusion, there is no significant difference in insulin level among the 4 groups, for this reason the data from the submandibulariectomized and totally sialodenectomized animals at 6 and 10 minutes in Fig. 1A were omitted to improve graphic clarity.

The effect of sialoadenectomy on the clearance of blood glucose is summarized in Fig. 1B. At 2 minutes post-infusion, the glucose level is uniformly elevated in all groups both prior to and after surgery. At 6 minutes a significant difference in glucose management becomes apparent between groups. After surgery, the glucose titer in the parotidectomized rat is indistinguishable from that of the totally sialoadenectomized animals, and both are significantly higher than the sham control

and submandibulariectomized rats. The statistical differences between the sham control and parotidectomized groups at 6 minutes were at the following p levels: $p < 0.01$ between days 19 and 33, $p < 0.02$ between days 85 and 100. At 10 minutes post-infusion, the level of statistical significance between parotidectomized and sham intact groups were: $p < 0.05$ between days 19 and 33 and $p < 0.01$ between days 85 and 100.

The Role of Age in the Effect of Long-Term Sialoadenectomy on β-Cell Function and Blood Glucose Titer

The severity of the depressed β-cell response to a glucose load, resulting from parotidectomy, was assessed in young rats operated when weighing 50-60g and in adult rats weighing 275-300g. The age at which the rats were parotidectomized does not alter the β-cell response to the glucose challenge (Fig. 2). A significant decrease in insulin secretion, at 2 minutes relative to the control, and a corresponding increase in plasma glucose at 6 minutes is observed in both age groups, as in the previous experiment.

The Effect of Different Concentrations of Glucose on the β-Cell Response in Parotidectomized Rats

The insulin and glucose responses for the intact control group before and after sham surgery is not different from the pre-surgery response of the parotidectomized animals (Fig. 3). This suggests that the effects of time and surgical trauma are negligible in all groups. Consequently, with each animal serving as its own control, comparisons can be made between the post- and pre-parotidectomy states, the latter serving as control.

Insulin secretion in control animals before and after sham surgery was not altered; however, in the parotidectomized animals, the response was significantly diminished (Fig. 3A). At 2 minutes the decrease in insulin secretion is readily apparent for the high, medium and low glucose doses. However, at 3 and 6 minutes, only the high dose shows a significant drop in insulin titer from the pre-surgical state. The β-cell response of the parotidectomized animals at 2 minutes to the 3 glucose loads shows a progressive inability to cope with the increasing glucose challenge as indicated by the slope difference between the pre- and post-parotidectomized state, and by the sustained difference in IRI observed in the high glucose group at 3 and 6 minutes. Insulin clearance over the 6 minute period following each glucose challenge is essentially the same with each treatment group before surgery; however, after parotidectomy the clearance rate is significantly lower than in the pre-parotidectomized animals as indicated by the slope difference.

Monitoring the plasma glucose levels to all 3 challenges shows that at 2 minutes there is no distinguishable difference between the pre- and post-parotidectomy state of the animals (Fig. 3B). However, at 3 and 6 minutes post-infusion, the plasma glucose level after parotidectomy is significantly higher at all three levels of glucose challenge. After 2 minutes with identical glucose loads, the glucose clearance rate over the 6 minute period is slower in the post-parotidectomy state. In addition, following the low glucose challenge, the glucose titer in the parotidectomized animals remained elevated over the entire 6 minute interval.

Effect of Sialoadenectomy on Feed Intake

Feed intake was assessed in rats whose insulin and glucose data are summarized in Fig. 2 (Fig. 4). There was no significant difference in feed intake between control and sialoadenectomized animals, nor was there a significant difference in feed intake between groups between days 70-100.

Effects of Sialoadenectomy on Body Weight

Body weights of animals whose insulin and glucose data are shown in Figs. 1 and 2 are shown in Figs. 5 and 6. For graphic clarity the data from submandibulariectomized animals were omitted since they are not different from those of the sham-operated controls. Group I (Fig. 5) represents body weights of adult rats weighing 275-300g twenty days before surgery. There was a significant decrease in body weight in all rats following catheterization ($p < 0.02$); however, in each case the animals' body weight before glandular surgery, 17 days later, were not significantly different from before catheterization. Following surgery only rats with total sialoadenectomy had a significant decrease in body weight ($p < 0.05$). These rats continued to lose weight over the remainder of the experiment ($p < 0.02$).

In the second group (Fig. 5) adult rats weighing 275-300g were either sham-operated or parotidectomized on day zero, then 80 days later they were catheterized. Seventy days post-surgery the body weights of the parotidectomized rats were not significantly different from the intact controls. Both groups experienced a significant drop in body weight following catheterization ($p < 0.05$) however, by day 100 there was no significant difference from the pre-catheterization body weight.

Figure 6 summarizes the body weights of young rats weighing 60-70g that were either parotidectomized, parotidectomized and submandibulariectomized or sham operated, then 80 days later catheterized. There was no significant difference in body weight between parotidectomized rats and sham operated control rats before catheterization on day 70; however the rats subjected to total sialoadenectomy had body weights which remained significantly lower than the sham operated controls ($p < 0.001$). All rats experienced a decrease in body weight following catheterization. Parotidectomized and sham control rats recovered their weight loss by day 100. Their body weight did not differ significantly from their pre-catheterization weight.

Fasting Plasma IRI and Glucose Values

The effect of age on plasma IRI and glucose titers, after 24 hours fasting in sham-operated control and parotidectomized rats, is shown in Figs. 7-8 respectively. In group I, rats were catheterized when approximately 63 days old and sham operated or parotidectomized when approximately 82 days old. Group II represents rats sham operated or parotidectomized at approximately 63 days and catheterized 80 days later when 142 days old.

The results indicate that with age there was a progressive rise in the fasting titer of both plasma IRI and glucose. Between days 68-81 there was no significant difference in either plasma IRI or glucose values. Between days 85-95 there was a marginally significant rise in both insulin and glucose values in the control and parotidectomized rats ($p < 0.05$). The plasma IRI and glucose values between days 99-113

remained significantly above the values at days 68-81 ($0.001 < p < 0.02$).

Basal plasma glucose and IRI values in group II are significantly higher between days 153-168 than those in group I between days 68-81 ($p < 0.001$). It is also interesting to note that in group II the rats which had their parotids removed for 80 days have higher basal plasma IRI ($p < 0.001$) and glucose ($p < 0.001$) titers than the sham-operated controls.

Fig. 1. Short and long-term effects of the removal of salivary glands on the acute response of the β-cell to an intra-arterial glucose infusion. parotidectomized (★), submandibulariectomized (●), parotid and submandibulariectomized (□), or sham-operated (☆). (A): Plasma insulin was monitored at 2, 6 and 10 min post-glucose infusion. Values are means of 4 to 6 animals ± standard error. (B): Plasma glucose concentration was concurrently monitored.

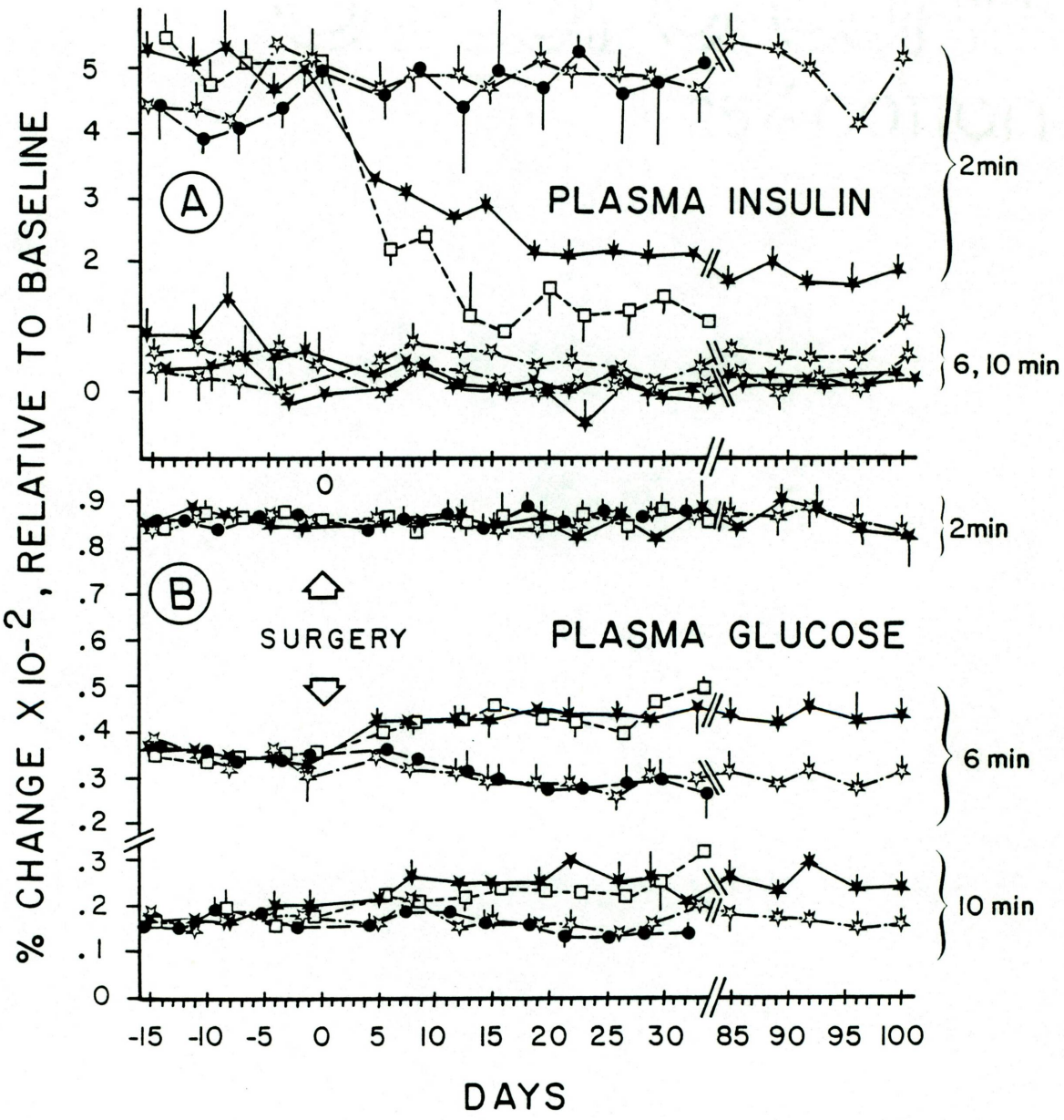

% CHANGE X 10-2, RELATIVE TO BASELINE
A
PLASMA INSULIN
2min
6, 10 min
0
2min
B
SURGERY
PLASMA GLUCOSE
6 min
10 min
-15 -10 -5 0 5 10 15 20 25 30 85 90 95 100
DAYS

Fig. 2. Long-term effect of parotidectomy on β-cell function in response to a glucose challenge: effect of age at time of surgery. Each bar represents the pooled mean titer ± standard error of 6 animals intact, sham operated; P, parotidectomized rats; 21, group operated at 21 days of age; AD, group operated when adult.

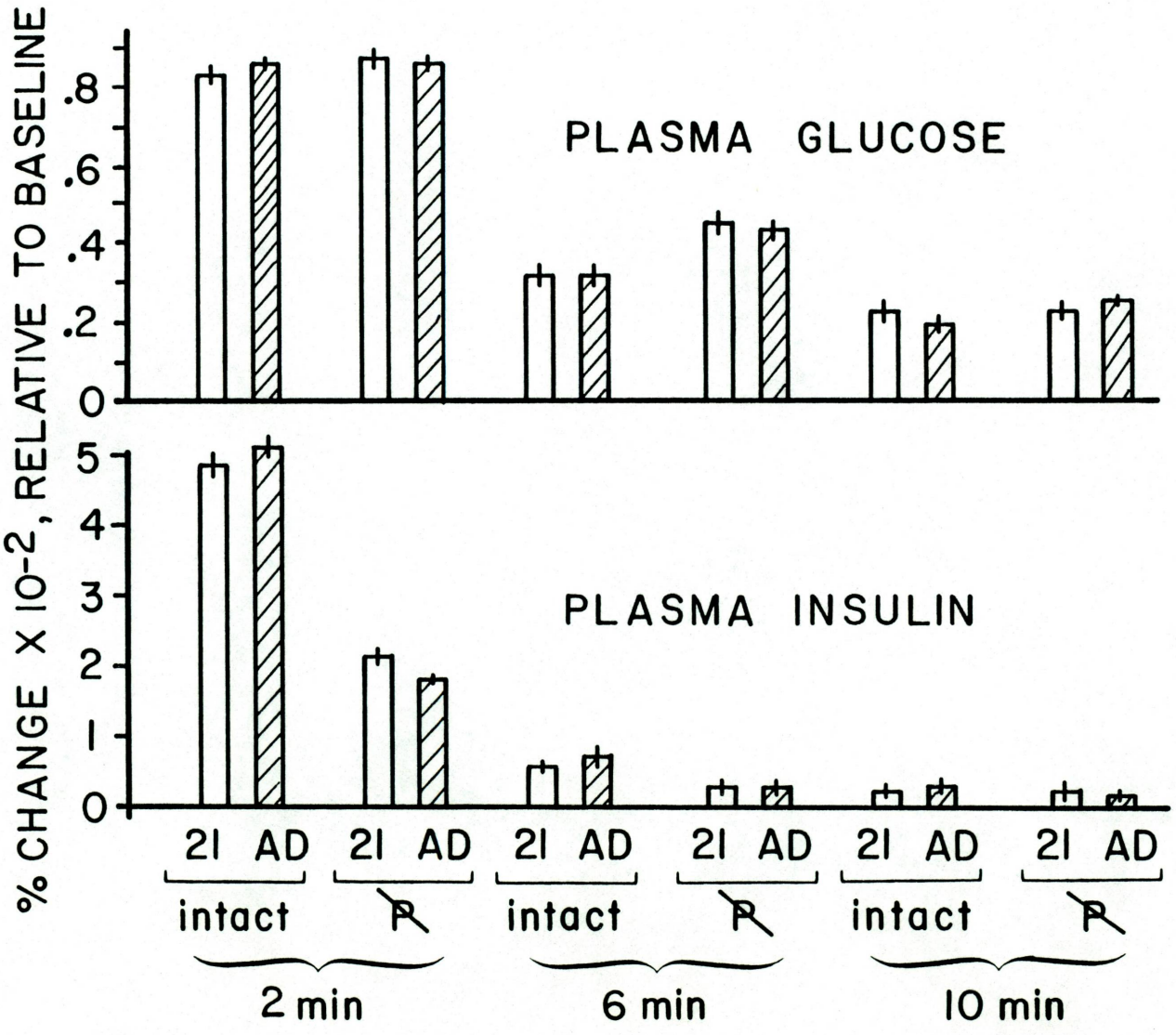

% CHANGE X 10-2, RELATIVE TO BASELINE
PLASMA GLUCOSE
PLASMA INSULIN
0
.2
.4
.6
.8
1
2
3
4
5
21 AD
21 AD
21 AD
21 AD
21 AD
21 AD
intact
intact
intact
2 min
6 min
10 min

Fig. 3. Effects of high, medium and low levels of glucose challenge on β-cell function in parotidectomized (P) or sham operated (intact) rats. Pairs of bars represent several experiments in the pre- (left bar) and post- (right bar) surgery state in the same animals. Values are the pooled mean titers ± standard error of 6 animals. Statistical differences between the pre- and post-surgery state is indicated by stars as follows: 1, 2, 3, and 4 stars correspond to $p < 0.05$, $p < 0.02$, $p < 0.01$, and $p < 0.001$, respectively.

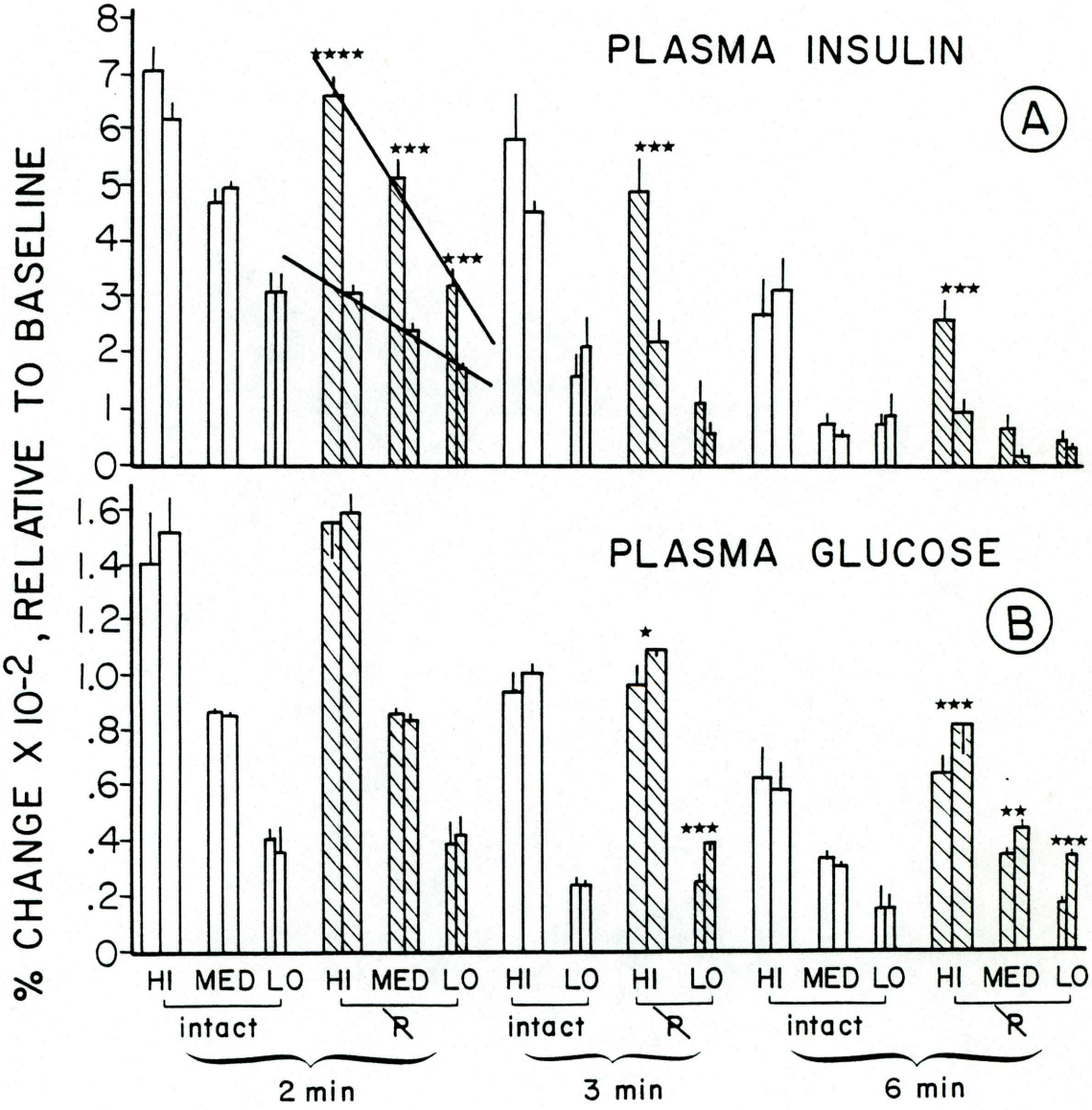

PLASMA INSULIN
A
PLASMA GLUCOSE
B
% CHANGE X 10-2, RELATIVE TO BASELINE
HI MED LO HI MED LO HI LO HI LO HI MED LO HI MED LO
intact R intact R intact R
2 min 3 min 6 min

Fig 4. Twenty-four hour feed intake 70-100 days post-surgery
Values are means of 4-6 animals ± standard error.

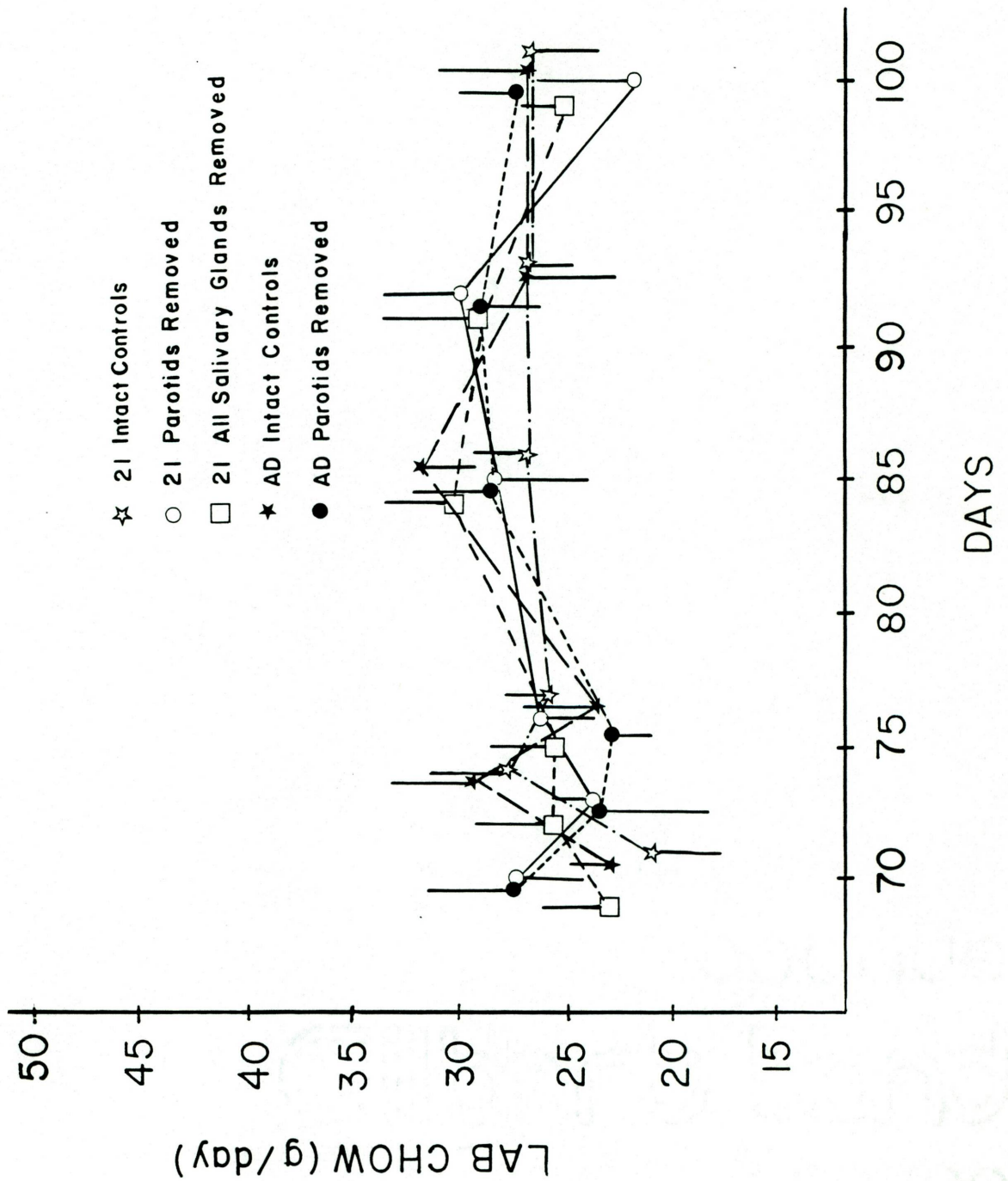

☆ 21 Intact Controls
○ 21 Parotids Removed
□ 21 All Salivary Glands Removed
★ AD Intact Controls
● AD Parotids Removed
LAB CHOW (g/day)
50
45
40
35
30
25
20
15
70
75
80
85
90
95
100
DAYS

Fig. 5. Changes in body weight in surgically treated adult rats. Values are means of 4-6 rats ± standard error. Group I represents body weights of adult rats weighing 275-300g twenty days before surgery. Group II represents adult rats weighing 275-300g sham-operated or parotidectomized on day zero, then 80 days later catheterized.

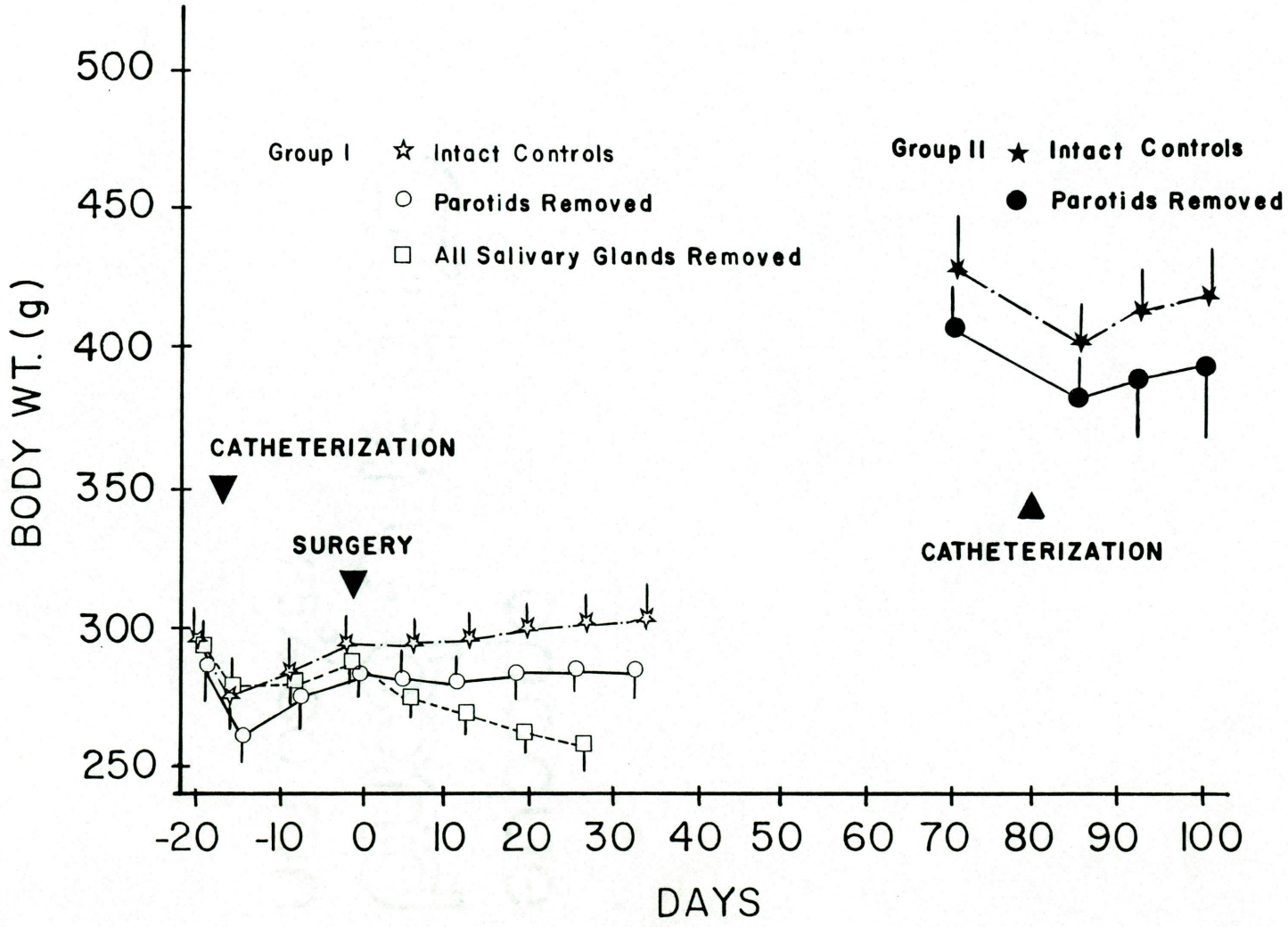

500
450
400
350
300
250
BODY WT.(g)
Group I
Intact Controls
Parotids Removed
All Salivary Glands Removed
Group II
Intact Controls
Parotids Removed
CATHETERIZATION
SURGERY
CATHETERIZATION
-20
-10
0
10
20
30
40
50
60
70
80
90
100
DAYS

Fig. 6. Changes in body weight of surgically treated young rats 70-100 days post-surgery. Values are means of 6 rats ± standard error.

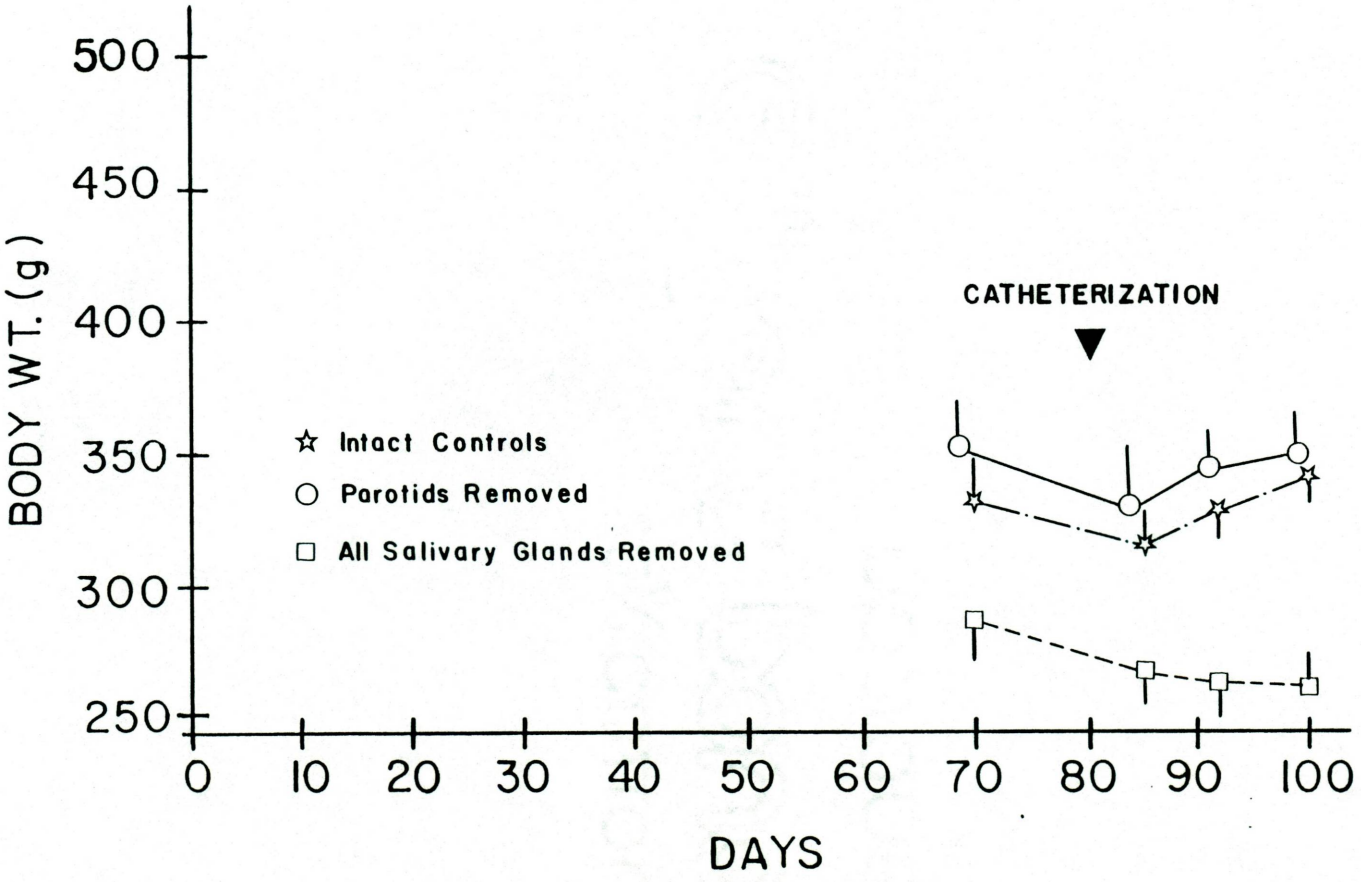

500
450
400
350
300
250
BODY WT. (g)
0
10
20
30
40
50
60
70
80
90
100
DAYS
CATHETERIZATION
Intact Controls
Parotids Removed
All Salivary Glands Removed

Fig. 7. Correlation between fasting plasma IRI and age of the animals. Group I represents parotidectomized or sham-operated control rats from 68-113 days old. Group II represents parotid ectomized or sham-operated control rats from 153-168 days old. Values are means of 4-6 rats ± standard error.

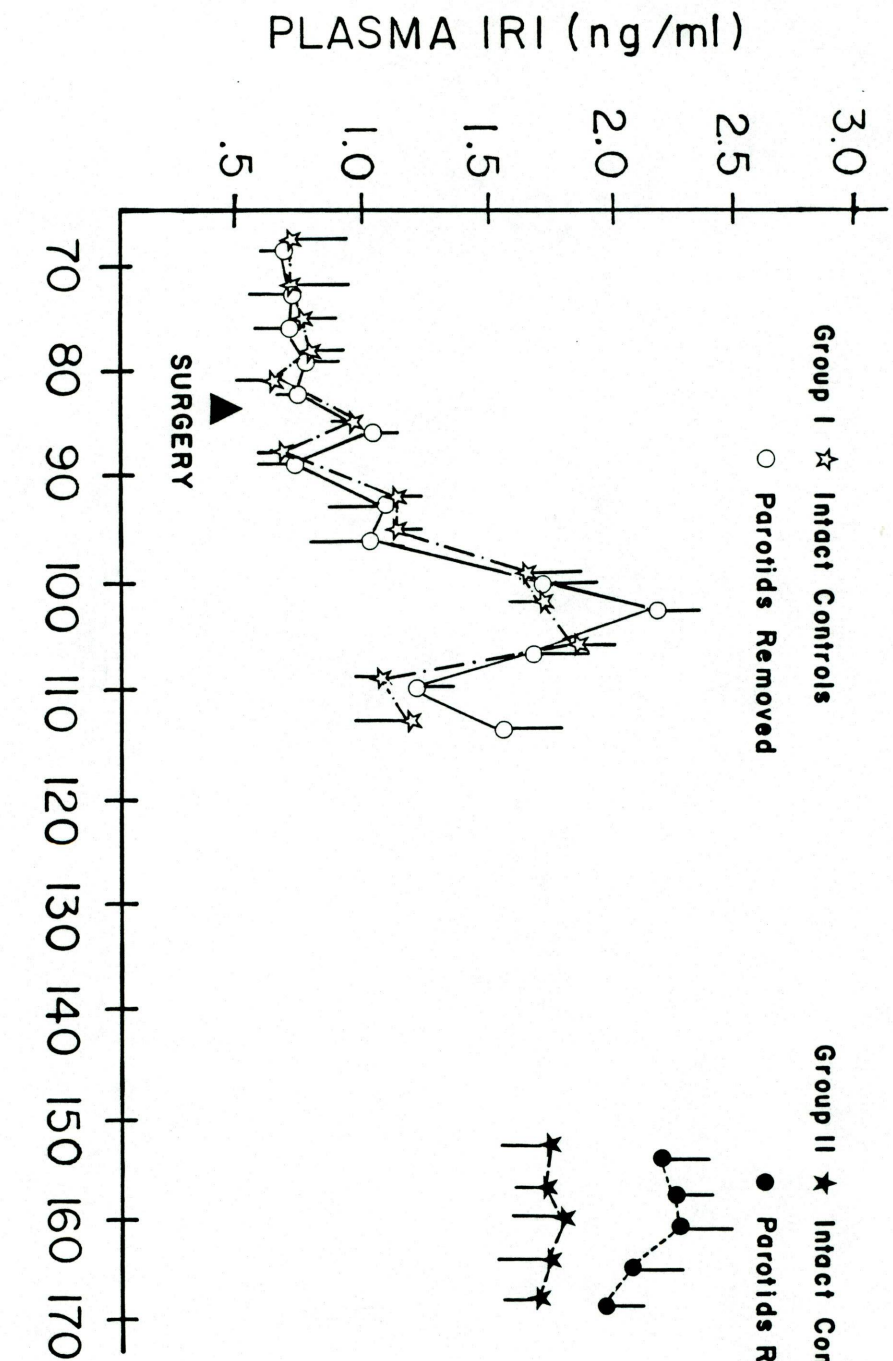
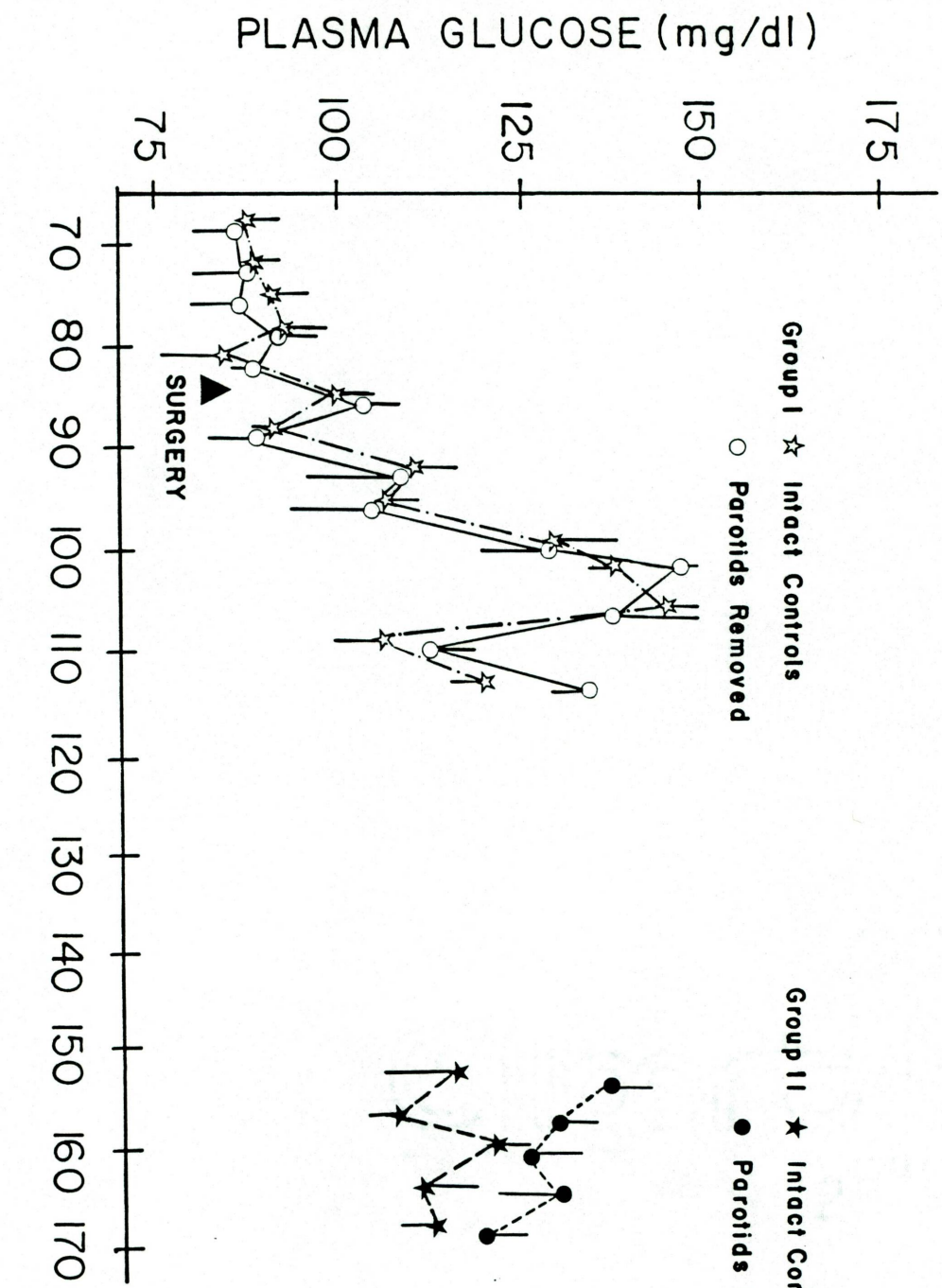

PLASMA IRI (ng/ml)
.5
1.0
1.5
2.0
2.5
3.0
70
80
90
100
110
120
130
140
150
160
170
SURGERY
AGE IN DAYS
Group I ☆ Intact Controls
○ Parotids Removed
Group II ★ Intact Controls
● Parotids Removed

Fig. 8. Correlation between fasting plasma glucose titers and age of the animals. Group I represents parotidectomized or sham-operated control rats from 68-113 days old. Group II represents parotidectomized or sham-operated control rats from 153-168 days old. Values are means of 4-6 rats ± standard errors.


PLASMA GLUCOSE (mg/dl)
75
100
125
150
175
70 80 90 100 110 120 130 140 150 160 170
AGE IN DAYS
SURGERY
Group I ☆ Intact Controls
○ Parotids Removed
Group II ★ Intact Controls
● Parotids Removed

CHAPTER IV

DISCUSSION

The purpose of this study was to examine the contribution of the salivary glands to the regulation of β-cell function in response to an acute intra-arterial glucose challenge.

The results clearly indicate that parotidectomy consistently decreases responsiveness of the β-cell to a glucose challenge (Fig. 1A). In comparison, removal of the submandibular gland appears to be without effect. Other workers have reported that submandibulariectomy in dogs had no effect on blood glucose and insulin levels in response to oral or intravenous glucose tolerance tests (34, 116). Our data suggest that although submandibulariectomy has no effect by itself, it may potentiate the effect of parotidectomy (Fig. 1). Such a contribution is not understood at the present time, and will require further investigation.

Our data indicates that parotidectomy deprives the β-cell of a parotid signal which appears to be essential for optimizing the immediate insulin response to a glucose challenge. Concurrent with the depressed insulin titer at 2 minutes post-infusion in parotidectomized and totally sialoadenectomized rats (Fig. 1A) was a correspondingly higher glucose titer observed at 6 and 10 minutes post-glucose infusion (Fig. 1B). This confirms the findings of other investigators that parotidectomized rabbits subjected to glucose tolerance tests required more time to return to baseline (12).

The progressive decrease in insulin secretion seen between days 5 and 15 post-parotidectomy (Fig. 1A), suggests that parotids exercise a

long-lasting effect on the β-cell. Conversely, the effect of parotidectomy is not transient since no sign of recovery or compensation can be observed between days 15 and 100 post-parotidectomy.

The collaborative function of insulin with other anabolic hormones is essential for optimal growth. We addressed the question of whether the parotidectomy-induced depression of β-cell function would be affected by the age of the animals when surgery is performed. Since the same level of β-cell dysfunction was observed 80 days after parotidectomy in either weanlings or adults, and because there was no significant weight difference between intact and parotidectomized animals, 80 days after surgery, it appears that the amount of insulin available was adequate in meeting anabolic needs of the growing animals.

In our study, sialoadenectomy consistently decreases the responsiveness of β-cells to glucose challenges of markedly different magnitudes (Fig. 3A). Since the glucose challenge was of short duration, the role of the parotids may be directed toward modulating the secretion of preformed insulin. The rate of insulin clearance following each glucose challenge is essentially the same within each treatment; however, after parotidectomy, the clearance rate is significantly lower than in the pre-parotidectomized animals as indicated by the slope difference. In contrast, the rate of glucose clearance decreases with the strength of the glucose challenge in both groups. At identical glucose loads, the glucose clearance rate is slower, after 2 min, in the post-parotidectomy state. However, following the low glucose challenge, the glucose titer in parotidectomized animals remains elevated over the entire 6 minute interval. The latter occurred even though the strength of the stimulus

was sufficient to cause an almost two-fold increase in insulin, which cleared from the circulation at a rate similar to that observed with higher glucose loads. This suggests that in addition to decreasing the β-cell response to a glucose challenge, parotidectomy may have caused peripheral tissue resistance to insulin.

Removal of the parotid or submandibular glands does not appear to affect the animal's body weight when compared to the weight of the sham-operated controls (Figs. 5 and 6). However, the removal of all salivary glands when combined with catheterization resulted in a loss of body weight as seen in Fig. 5 group I or a slower weight gain in non-catheterized rats as in Fig. 6. The fact that all animals experienced a decrease in body weight following catheterization suggests that the surgical procedure presents a significant stress. However, any reduction in body weight is transient in animals with intact salivary glands or in animals with only partial sialoadenectomy (Fig. 5). Catheterized rats in group I did not show a significant weight gain beyond their pre-catheterization body weight of 275-300g over a 7-week period. Rats in group II without catheters grew from 275-300g to 395-445g over a 10-week period. These results suggest that catheterization and frequent blood loss may interfere with normal growth.

Our data from intact and parotidectomized rats, show a slow but significant increase in fasting blood glucose and insulin titers with aging (Figs. 7 and 8). This suggests the development of a progressive peripheral insensitivity to insulin. These observations confirm results from other investigators (95, 96). Aging may explain the rise seen in fasting glucose and IRI values over the duration of the experiment, but

it does not explain the higher basal plasma glucose and IRI titers in parotidectomized animals relative to the controls (group II, Figs. 7 and 8). Our data show what appears to be an isolated, accentuated rise in fasting plasma IRI and glucose titers in all groups between days 99-106. This synchronized response was probably due to factor(s) external to our experimental design.

Other investigators have demonstrated the presence of immunoreactive insulin-like material in the parotid tissue and saliva (114). Biosynthesis of insulin was demonstrated in rat and human parotid tissue, and its concentration in rat parotids was 1/6 that of the pancreas (85). However, insulin in human saliva was deduced to be of pancreatic origin (132). There is no current evidence that the parotids secrete insulin into the blood stream, thereby supplementing β-cell secretion. Therefore, the observed inability of the parotidectomized animals to adequately manage a glucose challenge cannot be explained on the basis of depriving the animals of parotid insulin.

In conclusion, the parotids appear to be the source of a principle that may have a significant role in maintaining the β-cells in a state of adequate sensitivity to circulating glucose level, and possibly in keeping the peripheral tissues insulin-sensitive. PH has been isolated from porcine glands, whose function is critical in maintaining a systemic resistance mechanism against dental decay. This PH-mediated mechanism can be suppressed by a cariogenic, high sucrose diet (103, 117, 118). Other studies have shown that poorly controlled insulin-dependent diabetic humans and diabetic rats have a higher incidence of dental caries, which can be corrected with insulin therapy (5-8, 24, 25, 44, 72, 121, 136). From a systemic point of view, cariogenesis,

diabetes and PH secretion have a common denominator: glucose intolerance. Such a coincidental dependance could possibly suggest that PH may be involved in that parotid-β-cell relationship.

CHAPTER V

AN ATTEMPT TO LOCALIZE PAROTID HORMONE SECRETING CELLS BY IMMUNOHISTOLOGICAL TECHNIQUES

Introduction

Pig parotid glands are triangular in shape, unencapsulated and embedded in fatty connective tissue. The apex is ventral to the ear; the anterior border extends onto the masseter muscle and the posterior border onto the cervical muscles. The parenchyma is organized into lobules separated by dense connective tissue, lymph nodes and fat (19, 112).

The parenchymal components are acini, and intercalated, striated, and secretory ducts. They connect in the order named to convey saliva to the oral cavity.

Acini are elongated and branched, with small tortuous lumina. Cells vary from pyramidal to rectangular, and each abuts on the acinar lumen. Low contrast secretory granules give the acinar cell a vacuolated appearance. The rounded nuclei are located at the basal region. In addition to secretory granules, acinar cells contain a moderate amount of visible rough endoplasmic reticulum (RER) which is located mainly basal and lateral to the nuclei (11).

The vacuoles observed in histological sections of acinar cells appear in electron photomicrographs, as electron-lucent secretory granules, a striking departure from the dense serous granules typical of other mammalian parotid glands.

Acini of the pig parotid glands were first classified histochemically as serous (107, 108), but vacuolated acinar cells with electron-

lucent secretory granules and low amylase activity in the saliva led to additional studies which resulted in reclassification as a special serous (10).

Intercalated ducts connect acini with striated ducts, which are then joined by other striated ducts before terminating in the secretory ducts. The segment of the intercalated duct nearest the acinus is lined with elongated, somewhat flattened cells while the segment which connects with the striated ducts is lined with cubodial cells. Secretory granules are more numerous in proximal duct cells near the junction with the acinus (11), which compares with the situation in other mammals (90, 124.)

Mitochondria with tubular or tubulovesicular cristae, which characterize striated duct cells of the pig parotid, have not been described in any other salivary gland (11). This type of mitochondrial structure is usually limited to mammalian cells engaged in steroid metabolism (125). In vitro studies have shown that certain steroids, such as pregnenolone and testosterone, are modified in the pig parotid (57, 113) and that the enzymes for steroid metabolism are associated with ducts (31). These findings indicate that the pig parotid may be involved in steroid metabolism.

It may be concluded that while the architectural plan of the pig parotid resembles that of other mammalian parotid glands, the pig parotid has many ultrastructural features not found in other salivary glands.

The immunoperoxidase technique offers many advantages over other available methods developed for the specific demonstration of cell and

tissue antigens. The evolution of immunocytochemical techniques began with immunofluorescence; however, its value is severely limited by the instability of the stain (63, 127) and because immunofluorescence procedures are generally performed on unfixed cryostat sections, the morphology and histological detail are poor.

Peroxidase-conjugated antibodies are as sensitive as those conjugated to fluorescein-isothiocyanate (FITC). Also peroxidase conjugates give a higher specificity than those seen with fluorescent reagents (45).

The immunoperoxidase technique has become the method of choice in a growing number of studies (127). It has been employed in a wide range of investigations including among others, the identification of steroid and peptide hormones (2, 24, 64, 65) There are also many studies using this technique on the parotid gland, including: detection of parotin in duct cells of human parotid gland (52), staining actin in parotid cells (17), and identification of insulin-like material in the parotid glands of rats (114).

The purpose of the present study was to identify the parotid cells responsible for production of PH, using the immunoperoxidase technique and the specific antiserum against porcine PH developed by Tieche (129).

Materials and Methods

Materials

Ammonium acetate, Baker Chemical Co., Phillipsburg, NJ.

Citric acid, Matheson Coleman & Bell Manufacturing Chemists, Norwood, OH.

Ethanol 100%, U.S. Industrial Chemical Co., Anaheim, CA.

Ethanol 95%, U.S. Industrial Chemical Co., Anaheim, CA.

Goat anti-rabbit antibody, Arnel Products Co., Inc., New York, NY.

Formaldehyde solution (37%), Mallinckrodt Chemical Works, St. Louis, MO.

Freud's adjuvant, Complete, Sigma Chemical Co., St. Louis, MO.

Freud's adjuvant, Incomplete, Sigma Chemical Co., St. Louis, MO.

Hematoxylin, Baker Chemical Co., Phillipsburg, NJ.

Hydrogen Peroxide (H_2O_2), Aldrich Chemical Co., Inc., Milwaukee, WI.

Ketamine, Parke-Davis, Morris Plains, NJ.

Permount, Fisher Scientific Co., Fair Lawn, NJ.

Peroxidase-Anti-peroxidase (rabbit): (PAP), Litton Bionetics Inc., Kensington, MD.

Phenylhydrazine hydrochloride, Baker Chemical Co., Phillipsburg, NJ.

Prep paraffin, Fisher Scientific Co., Fair Lawn, NJ.

Sigmacote, Sigma Chemical Co., St. Louis, MO.

Sodium phosphate dibasic, Baker Chemical Co., Phillipsburg, NJ.

Xylene, Baker Chemical Co., Phillipsburg, NJ.

Tissue Collection

Parotid and submandibular glands, and pancreas tissue were obtained from adult male pigs. The pigs were anesthesized with ketamine and the head and neck region was perfused via the carotid artery with 0.5 - 1 liter of ice-cold saline at 4°C (69), and then with 3 liters of ice-cold 10% buffered formalin (pH 7.3) (56). Pancreatic tissue was excised and fixed along with parotid and submandibular tissue overnight, in ice-cold 10% buffered formalin, dehydrated in ethanol and embedded in paraffin. Sections 6 µm thick were placed on slides coated with gelatin which aids

Incubation With Primary Antiserum

Sections of parotid, submandibular glands, and pancreas tissue were incubated with one of the following primary antisera: rabbit anti-porcine parotid hormone (PHAb) (1:300 dilution; 128), or rabbit anti-porcine parotid saliva (PSAb) (1:250 dilution). Control sections were treated with preimmune serum from the animal in which antisera was prepared, and used at the same dilution. Each primary antiserum diluted with 2% normal goat serum was added to the sections with a 50 µl pipette, making certain that all the tissue was well covered. Incubation was carried out for 24 hours at 22°C in a rocking humidified chamber to enhance binding. After incubation with antiserum was completed, the sections were washed a total of 15 minutes in three changes of buffer.

Incubation With "Link" Antibody

Sections were incubated 1.5 hours with goat anti-rabbit serum at a dilution of 1:20, in the same manner as described for the primary antiserum. The unlabeled immunoperoxidase method depends on the fact that the second antibody has two identical sites that can bind to antigens. Thus, goat anti-rabbit immunoglobulin can bind by one valence to the primary antiserum (rabbit antiserum), and a second valence is available to bind to the antibody against horseradish peroxidase (rabbit antibody to horseradish peroxidase). The primary antibody directed against the particular antigen under

study is linked by the secondary antibody to the histochemical marker (rabbit peroxidase-antiperoxidase).

Addition of Peroxidase-Antiperoxidase

The peroxidase-antiperoxidase reagent, generated in the same specie as the primary antiserum (rabbit), was added with a 50 µl pipette to the sections at a 1:10 dilution and incubated for 1.5 hours as previously described.

Addition of Diaminobenzidine

The color reaction was then developed by exposing the peroxidase-antiperoxidase marker to diaminobenzedine and hydrogen peroxide causing a low contrast, orange-brown immunostain. The diaminobenzidine was freshly prepared and filtered prior to use. A 0.05% solution of diaminobenzidine tetrahydrochloride was prepared in a citric acid-ammonium acetate buffer (pH 5.0) (26, 63, 135). Sections were pre-incubated by immersion in this solution for 20 min to allow equilibration of the tissue pH. Hydrogen peroxide (0.3%) was added to the solution and the reaction was allowed to proceed for 5 minutes after which it was stopped by washing with distilled water (120, 135).

Counterstaining

The sections were counterstained with hematoxylin, then

they were dehydrated through successive changes of alcohol and xylene, and mounted with Permount.

Preparation of Antibody to Porcine Parotid Gland Saliva

Parotid saliva was collected from a catheterized parotid duct in an anesthetized pig. Salivation was induced with 4 mg pilocarpine dissolved in 5 ml saline infused in small increments into the carotid artery. Twelve ml of parotid saliva was collected, dialyzed and then freeze dried. Five male New Zealand white rabbits, weighing 2.0-2.5 kg, were immunized with 180 µg freeze dried saliva, dissolved in 1.0 ml 0.05 M phosphate buffered saline (PBS), pH 7.0. The antigen was emulsified with an equal volume of complete Freund's adjuvant before being injected intradermally at 20 sites on the rabbit's back. All animals were boosted 8 weeks later with half the immunization dose of antigen emulsified in incomplete Freund's adjuvant and administered subcutaneously in the four axillary areas. Subsequent boosters were administered at 5 week intervals. The animals were bled every 14 days from the auricular artery to titer the concentration of antibody.

Parotid Hormone Antibody

A parotid hormone has been isolated from porcine parotid glands (129). A specific antiserum against parotid hormone (PHAb) was raised in rabbits using PH conjugated to human serum albumin (128).

Results

The results of this study are presented as photomicrographs in

Figs. 9-18. The immunological staining of sections of porcine parotid gland, incubated with PHAb, appears to be more intense in the connective tissue between acini than inside the acinar cells. There also is a general lack of staining in the duct cells (Figs. 9 and 10). Control studies include: (1) porcine parotid sections treated with pre-immune serum (Fig. 11), (2) submandibular gland (Fig. 12) and pancreas (Fig. 13) incubated with PHAb. In all cases these sections demonstrated a lack of immunostaining.

The parotid tissue, immunostained with PSAb, (Figs. 14 and 15) demonstrates a positive reaction on the luminal surfaces of striated ducts and around the nuclei of the acinar cells. Staining in the connective tissue is minimal. Immunohistological control sections include: (1) porcine parotid sections treated with pre-immune serum (Fig. 16), (2) submandibular tissue (Fig. 17) and pancreas (Fig. 18) incubated with PSAb. No specific immunostaining is observed in these control sections.

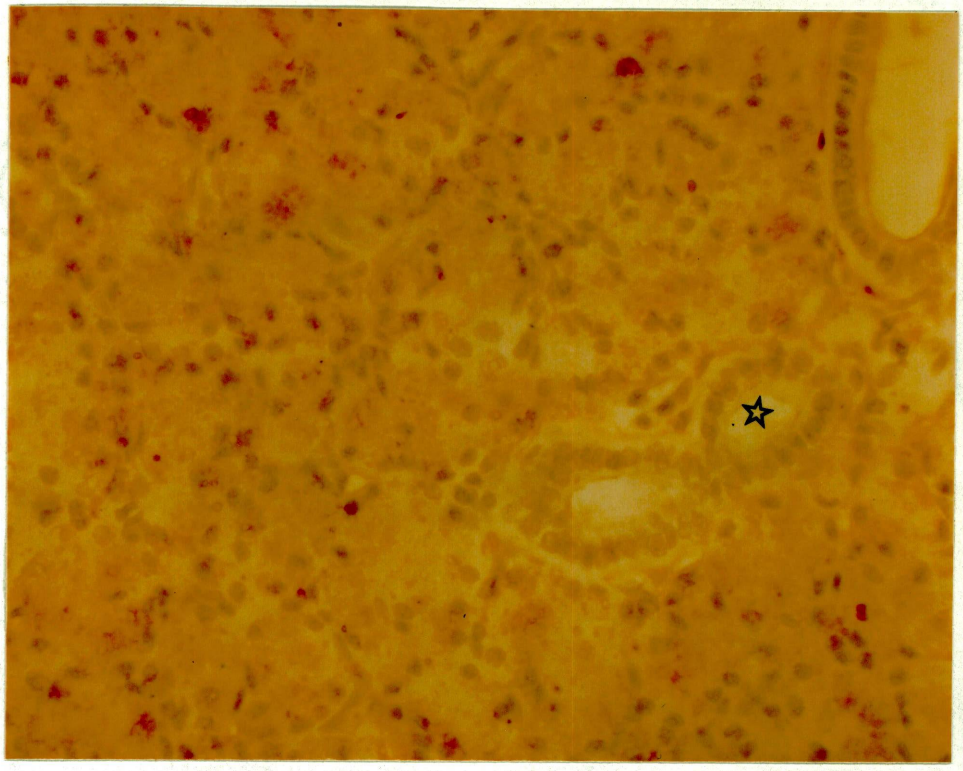
Discussion

The objective for this study was to identify the cell(s) in the parotid glands responsible for hormone production, using a PHAb.

Difficulties were encountered in optimizing the immunohistological technique. Parotid tissue fixation proved to be difficult. Various fixatives, such as glutaraldehyde, paraformaldehyde, bouins and buffered formalin were tested. Fixation was carried out either by floating the tissue in the fixatives for various lengths of time at different temperatures, or by perfusion of the fixative through the carotid artery,

Fig. 9. Section of porcine parotid gland immunostained with PHAb. Star indicates lack of staining in a striated duct. Scattered staining generally not associated with nuclei or apex of the acinar cells (250x).

Fig. 10. Section of porcine parotid gland immunostained with PHAb. Star indicates a striated duct cut tangentially, note the lack of specific staining. An area along a blood vessel in the connective tissue, indicated by brackets, shows considerable staining. Elongated nuclei of fibroblasts are evident. Curved arrow indicates the lumen of the capillary (625x).



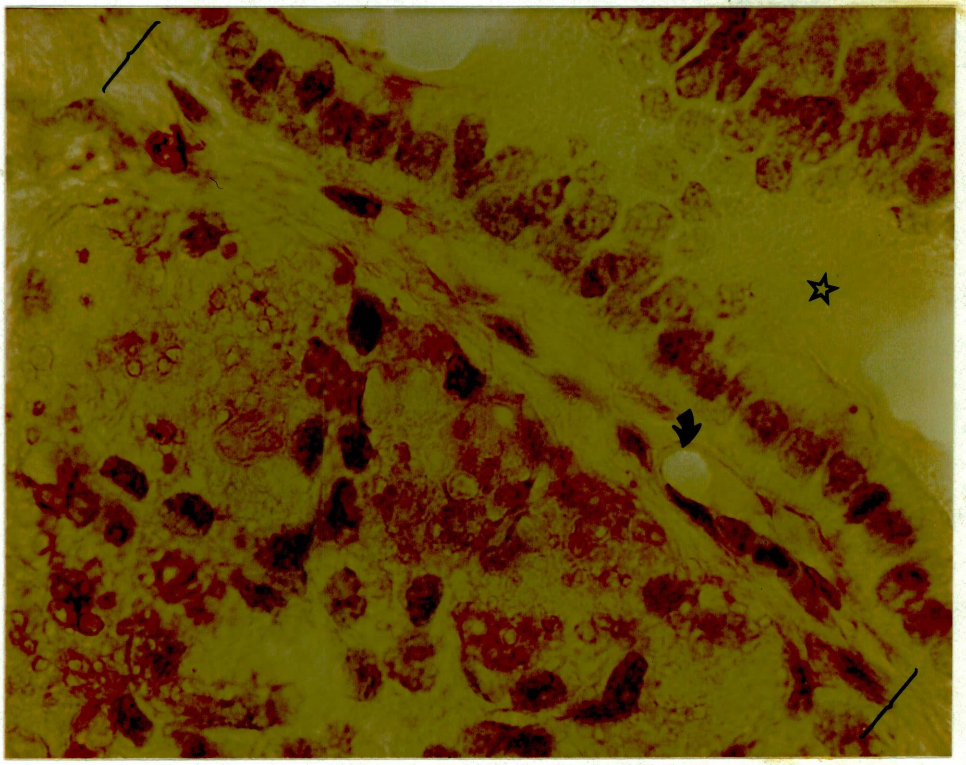
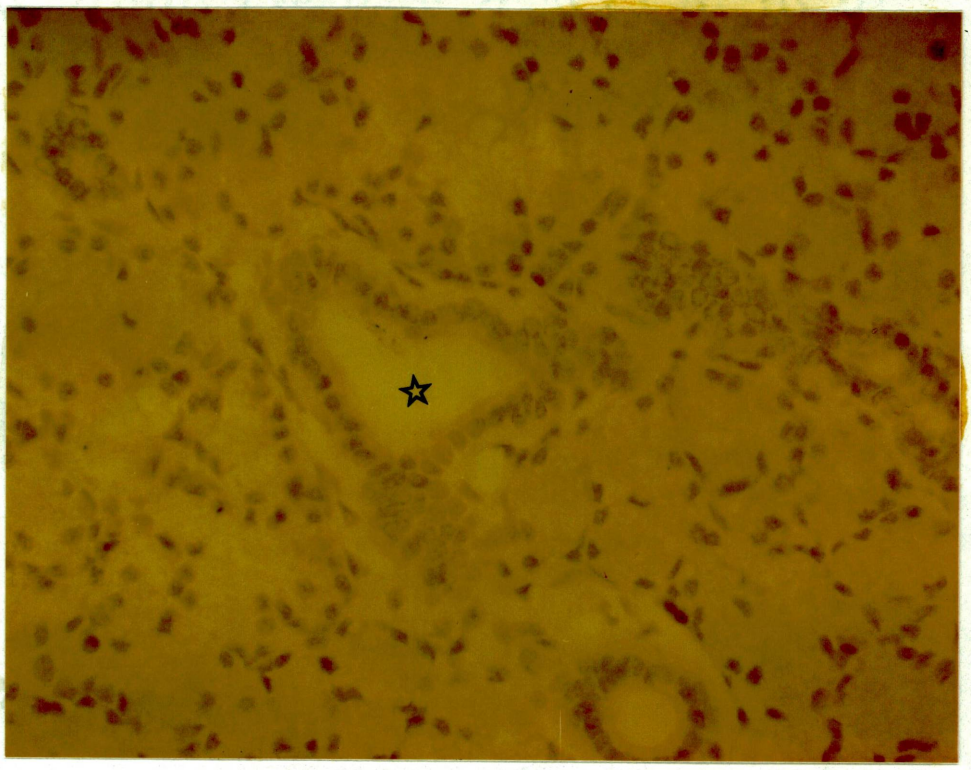


Fig. 11. Immunohistochemical control. Section of porcine parotid gland incubated with pre-immune serum. Star indicates lumen of a striated duct. Note general absence of immunostaining (250x).

Fig. 12. Section of porcine submandibular gland incubated with PHAb. Star indicates a striated duct. Note the absence of immunostaining (250x).



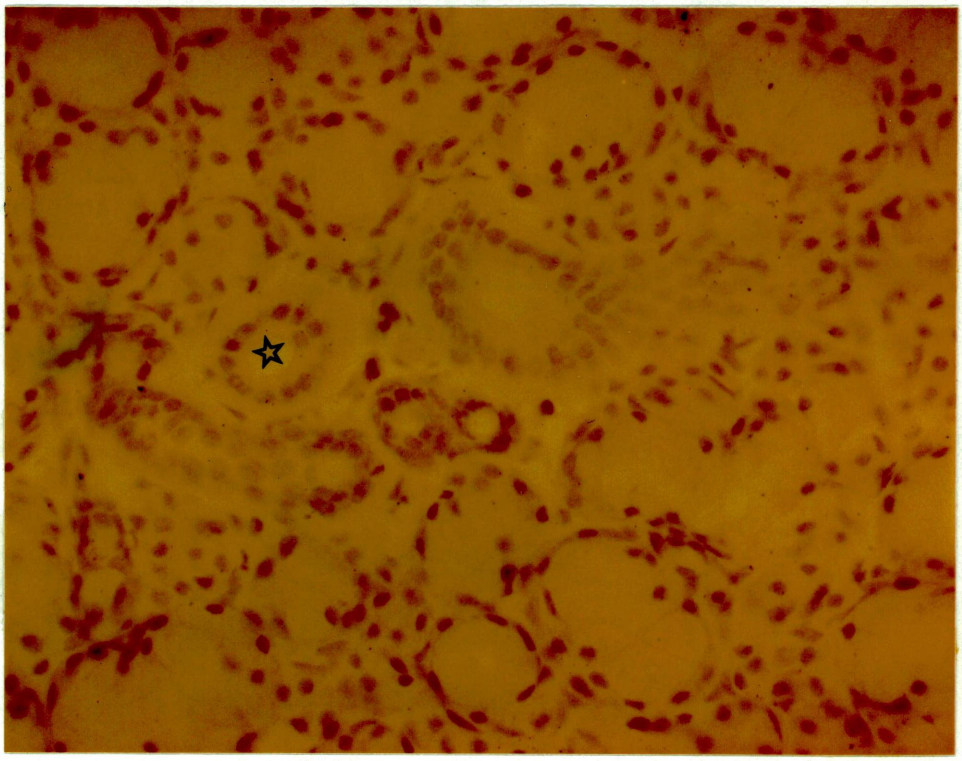
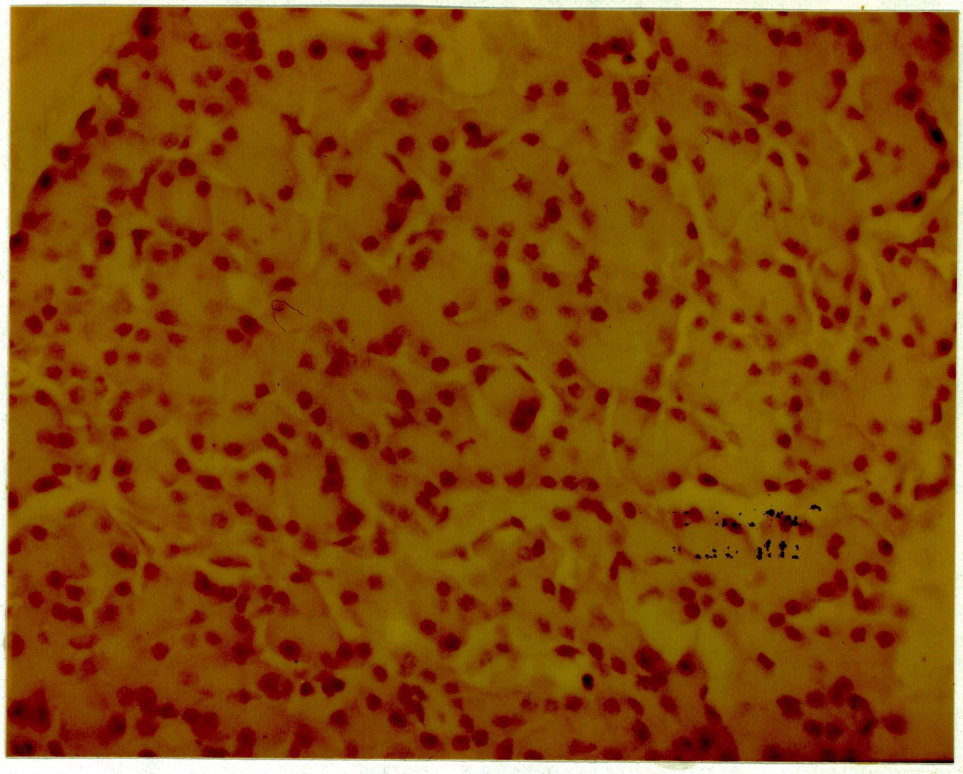


Fig. 13. Section of porcine pancreas incubated with PHAb. Field is filled with pancreatic acini. No specific staining is evident (250x).

Fig. 14. Section of porcine parotid gland immunostained with PSAb. Curved arrow indicates luminal surface of striated duct with immuno-staining. Majority of the scattered staining appears in the apical and basal portion of acinar cells (250x).



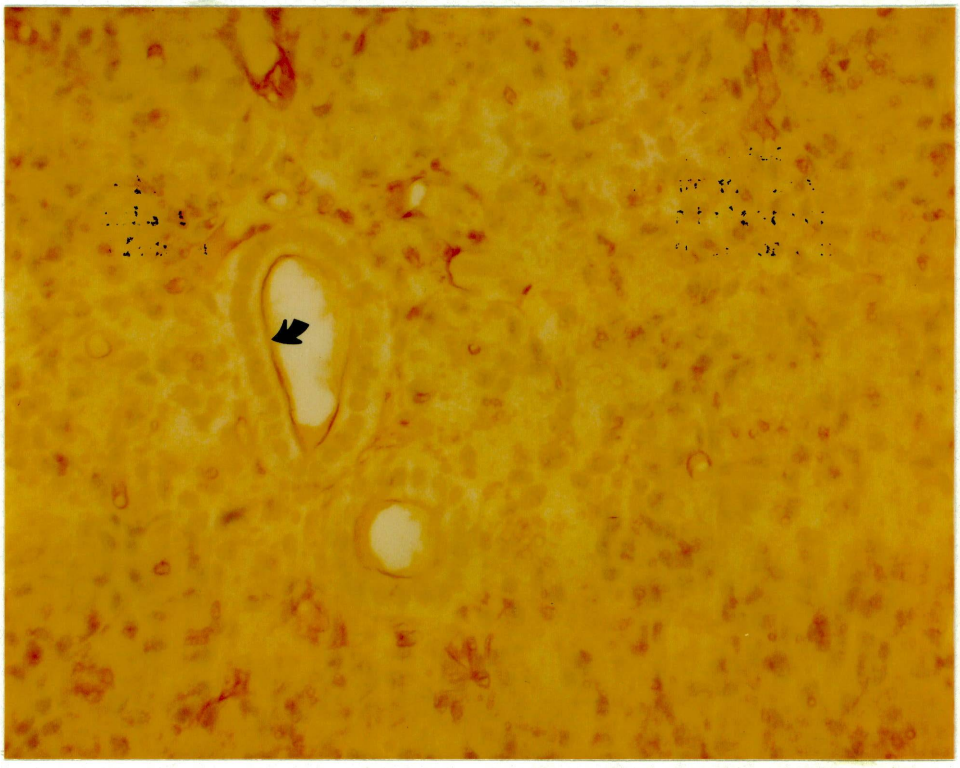
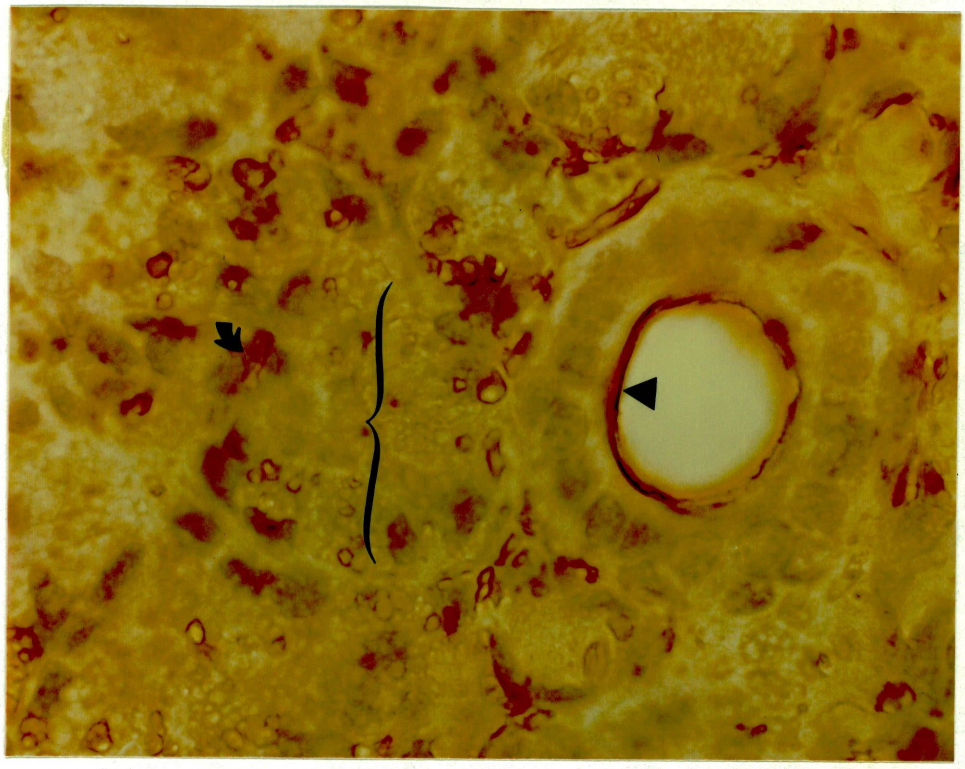


Fig. 15. Section of porcine parotid gland immunostained with PSAb. Pointer indicates luminar staining. Bracket indicates limits of an acinus, staining can be seen around the nuclei of the acinar cells. Curved arrow points out staining in an acinar cell (625x).

Fig. 16. Immunohistological control. Section of porcine parotid gland incubated with preimmune serum. Star indicates lumen of striated duct. Note lack of immunostaining (250x).



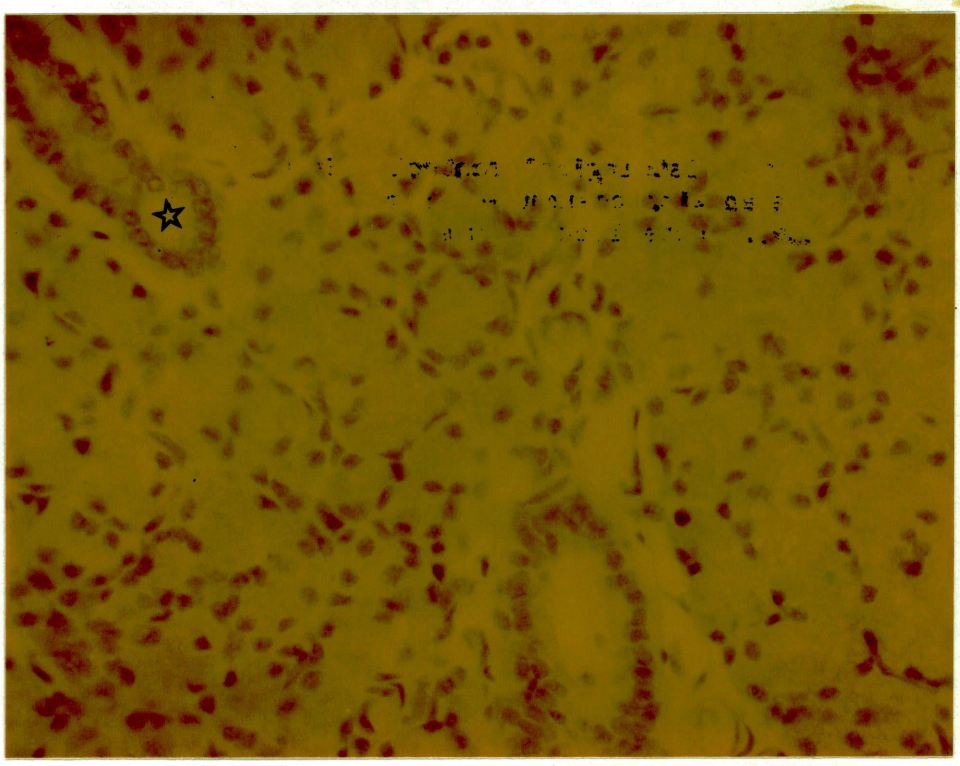
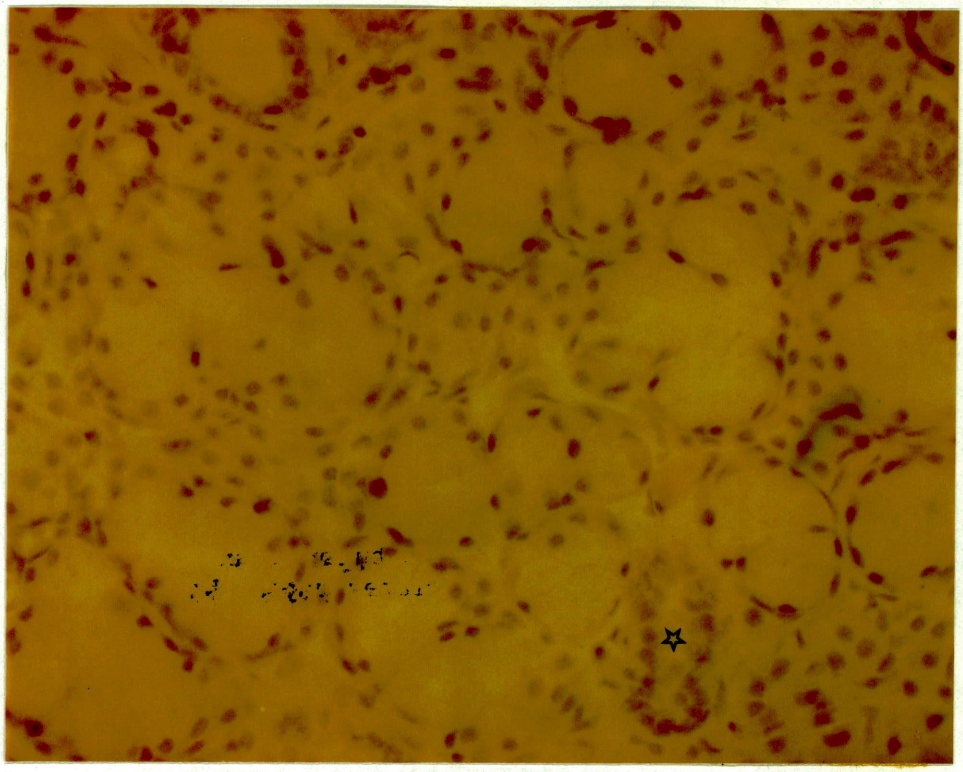
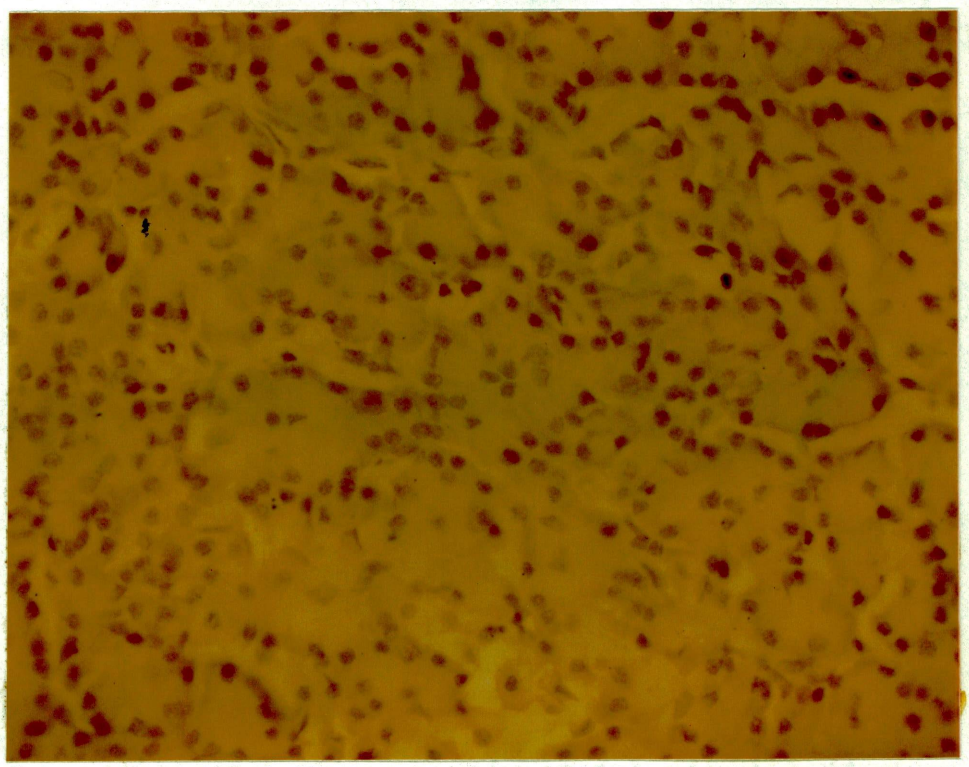


Fig. 17. Section of porcine submandibular gland incubated with PSAb. Star indicates lumen of striated duct. No specific staining is evident (250x).

Fig. 18. Section of porcine pancreas incubated with PSAb. Note the absence of specific immunostaining (250x).





in vivo. It was found that perfused tissue fixed with buffered formalin, had greater immunoreactivity, and tissue morphology was better preserved than with the other fixatives. The proper embedding medium for tissues fixed with buffered formalin is paraffin. A disadvantage with paraffin embedded tissues is that it cannot be sectioned thinner than 6 microns.

Another approach involved a frozen section technique (130); however, this resulted in poor histological detail. Two different immunoperoxidase staining methods were compared: the PAP method (63, 64, 65) and the avidin-biotin complex method (49, 50, 51). The PAP method had higher sensitivity and lower background staining than the avidin-biotin complex method, for this reason the PAP method was employed. There were many attempts to optimize dilutions and incubation times of the primary and secondary antibodies. A long incubation of the primary antibody (24 hrs.) at 1:300 dilution for PHAb and 1:250 dilution for PSAb gave a strong reaction while minimizing background staining. Incubation for 1.5 hours was found to be satisfactory for the secondary antibody.

Electron microscopy was also utilized to identify the hormone producing cell. Staphylococcal protein A, which reacts with the IgG molecules, was labeled with colloidal gold as a marker (99). Either the fixative or the embedding medium interfered with PH immunoreactivity, and these studies were terminated.

To ascertain the distribution on PH secreting cells we divided the gland into 8 different areas and tested many sections from each area. No difference among the 8 areas was evident, suggesting that the hormone secreting cells are evenly distributed throughout the gland.

It is known that the PHAb does have some crossreactivity with parotid saliva (personal communication from Dr. Tieche) which may account, in part, for what appears to be background staining in the parotid sections treated with PHAb. In an attempt to ascertain the difference between the specific reaction to PH and the cross reaction with parotid saliva, a specific antiserum against porcine parotid saliva was raised in rabbits.

PSAb specifically reacts with parotid secretions on the luminal surface of the duct cells and stains less intensely in the connective tissue. There is a strong reaction at the apex and also around the nucleus near the basal portion of the acinar cell. The basal portion is associated with rough endoplasmic reticulum (RER), which is responsible for production of secretory products which are then stored as granules, located at the apical end of parotid acinar cells. This suggests that the immunostaining seen at the basal and apical ends of the cell represent a reaction with the granular constituents of the parotid saliva (Figs. 14 and 15).

In the parotid sections incubated with PHAb, there appears to be a non-uniform stain in the connective tissue. In most cases, the duct cells show an absence of immunostaining. The scattered staining throughout the tissue treated with PHAb does not appear to be associated with acinar nuclei, or the apices of acinar cells. Most polypeptide (protein) hormone-producing cells such as those found in the anterior pituitary, parathyroid glands, pancreas, and calcitonin secreting cells of the thyroid, form their respective hormones in the RER and store them in secretion granules. The fact that in parotid sections, the areas

associated with these structures do not appear to specifically react with PHAb, suggests that the acinar cells do not produce PH. Because the tissue sections are thick, scattered staining may represent surrounding connective tissue.

On the basis of these observations it seems probable that PH may be produced in some unidentified cells lying in the connective tissue surrounding the acini. This could be analogous to the cells of Leydig which lie between the seminiferous tubules in the testis and secrete testosterone. Further studies need to be done to validate these assumptions. However, before further research is attempted in this area, a more specific antibody is required. Thinner tissue sections would also be necessry for definitive interpretation of the immunohistological reaction. In order for this to occur a proper fixation technique is needed that would allow thinner sections (2-3 microns) and yet preserve the immunoreactivity of endogenous PH.

CHAPTER VI

SUMMARY

The contribution of salivary glands in the regulation of β-cell function in response to an acute intra-arterial glucose challenge was studied in rats under well-controlled physiologic conditions. Our data suggest an endocrine relationship between the parotid gland and pancreatic β-cells. A significant decrease in the insulin response to a glucose challenge with a corresponding decrease in glucose clearance was associated with parotidectomy and total sialoadenectomy.

Our study indicated a progressive inability of the β-cells of parotidectomized animals to secrete adequate amounts of insulin in response to an increasing glucose challenge. In addition the β-cells appear to become insensitive to low concentrations of blood glucose with signs of increased peripheral insulin resistance.

We observed the same level of β-cell dysfunction 80 days after parotidectomy in both weanlings and adults, with no significant body weight difference between intact and parotidectomized animals. Thus we concluded that the availability of insulin was adequate in meeting the anabolic needs of growing animals.

We attempted to identify the particular cell in the parotid gland responsible for production of PH using immunohistological techniques. Our data suggest that some unidentified cell(s) lying in the connective tissue surrounding the acini may be responsible for production of PH.

In summary, the parotids appear to be the source of a principle that may have a significant role in maintaining β-cell sensitivity to

circulating glucose levels, and also maintaining the sensitivity of insulin-responsive tissues.

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