Effect of Chronic Insulin Administration on Mouse Parotid and Submandibular Gland Function (43718)

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Abstract. **Chronic (six-day) injection of insulin (im, 50 μM/animal) into BALB/c mice resulted in changes in secretory function, hypertrophy and hyperplasia of the parotid and submandibular glands. There were no significant changes in the flow rate or concentrations of proline-rich proteins, TGF_α, or amylase in saliva when measured against constant protein levels. However, amylase enzyme activity and total saliva protein content were reduced when measured against constant saliva volume. In contrast, EGF synthesis and secretion from the submandibular gland was increased. Both the parotid and submandibular gland showed evidence of gland hypertrophy and increased rates of DNA synthesis as indicated by [^{3}H]-thymidine incorporation in response to insulin treatment. Chronic injection of insulin did not effect the level of receptor in the plasma membrane of either gland.** [P.S.E.B.M. 1994, Vol 205]

Insulin is necessary for the active transport of glucose across the cell membrane. In diabetes, a pathological state reflecting the loss of insulin, glucose entry into the cell is impaired. Loss of the biological function of insulin results in hyperglycemia, glucosuria, polydipsia, weakness, and weight loss (1). Diabetes also effects the general metabolism of protein and fat, leading to complications affecting the function of many organ systems. Thus, our basic understanding of the physiological and biochemical effects of insulin have been derived from studies involving the loss of the hormone. Diabetes has been associated with altered salivary secretion and mouth dryness.

The action of insulin on cell physiology appears to be mediated by its binding to the α subunit of the transmembrane receptor, intrinsic tyrosine kinase activation, receptor autophosphorylation, and association of insulin receptor substrate–1 (2). Additional substrates in the signal transduction cascade include the insulin receptor substrates phosphotidylinositol 3-kinase (3), phospholipase Cγ (4), growth factor receptor–bound protein 2 (5), protein tyrosine phosphatase–2, and 46 and 52 kDa Shc proteins (6). Thus, the activation of many of the same SH-2-containing elements of the tyrosine-kinases previously associated with growth factor receptor activation of cell proliferation, suggest insulin may also play a role in tissue homeostasis (7). Insulin also activates additional cellular kinases and phosphatases through specific serine/threonine (ser/thr) phosphorylation mechanisms (7–9). The level of phosphorylation of the ser/thr kinases and phosphatases regulate cellular metabolism such as the cAMP response in a variety of cells and tissues (7–9).

The effects of insulin on salivary gland function have been detailed by chemically inducing diabetes in animal models with either alloxan or streptozotocin followed by insulin supplementation (10–12). In the rat, the loss of insulin resulted in decreased salivary gland growth rates and secretory enzyme levels (10). Treatment of rats with exogenous insulin following chemically induced diabetes results in increased protein synthesis in both the parotid gland and pancreas (13–15). Treatment of submandibular gland dispersed cell cultures with insulin has been shown to stimulate protein synthesis in a rapid dose dependent fashion (16). More specifically, it has been observed that the

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rate of synthesis of the secretory protein, amylase, is dramatically altered by the level of insulin both *in vivo* and *in vitro* (13–15). Recently it has been shown that the insulin response of amylase gene expression in pancreatic acinar cells is directly related to chromosomal regulatory DNA sequences activated by the presence of insulin (17).

In the present investigation, we have examined the effects of chronic high doses of insulin supplementation on the function of the salivary glands of normal mice. We present evidence showing that increased insulin does not affect the general protein profiles of saliva or stimulated flow rates of saliva from the salivary glands, while reducing the overall concentration of protein in saliva in animals receiving chronic stimuli. The synthesis and concentration in saliva of amylase (an acinar cell product of the parotid gland) were reduced while EGF levels (a duct cell product of the submandibular gland) were increased following insulin treatment. Additionally, increased insulin concentrations in mice resulted in hypertrophy and hyperplasia of the parotid and submandibular glands.

Materials and Methods

Materials. Male BALB/c mice six months of age were purchased from the Department of Pathology, University of Florida breeding colony. All animals weighed between 15 and 20 g. Insulin, dl-isoproterenol HCl, and pilocarpine were from Sigma Chemical Co. (St. Louis, MO). Chemicals for SDS-polyacrylamide gels and prestained molecular weight standards were obtained from Bio-Rad (Richmond, CA). Radiolabeled [^{125}I]-protein A was purchased from Amersham (Chicago, IL) and [^{3}H]-thymidine (specific activity 20 Ci/mmole) from New England Nuclear (Boston, MA). Antibody to α-amylase was purchased from The Binding Site Co. (Birmingham, UK). Polyclonal antibody to TGF_{α} and rat acidic proline-rich protein were generous gifts from Dr. Gregory Schultz, Department of Obstetrics and Gynecology, University of Florida, Gainesville, FL and Dr. David Castle, Department of Anatomy and Cell Biology, University of Virginia Charlottesville, VA, respectively (18, 19). All other reagents were of ultrapure quality and obtained through commercial sources. Radioimmunoassay of plasma insulin levels was performed in the Department of Pathology, University of Florida.

Animal Preparation. Animals were given *ad libitum* exposure to food and water. Consumption rates of each were measured after a 24-hr period. Toxic insulin effects were monitored by observation of animal behavior as well as by dietary intake rates, in addition to measuring blood glucose levels in response to hormone administration by using Boehringer Mannheim Diagnosis (Indianapolis, IN) ChemStrip bG reagent strips. Peripheral blood was obtained from the tail 1 hr following insulin administration. Separate groups of animals ($n = 6$) were given either twice daily intramuscular injections of 0.1 ml containing saline or insulin (50 μ*M*/animal) for one to six days. The animals were killed 15 min following the last injection by cervical dislocation of the spine. The parotid and submandibular glands were subsequently identified by gross morphology, quickly trimmed of connective tissue and lymph nodes and dispersed into 2.0 ml of 10 m*M* Tris buffer pH 8.0 containing 1.0 μ*M* leupeptin and 100 μ*M* PMSF. Since plasma insulin levels average 1.0 ng/ml (25 U/mg) for rodent species (20), the bolus injection of insulin would be equivalent to a 2000-fold increase in hormone concentration based upon unit equivalents. The insulin level achieved in blood plasma was 100-fold greater than untreated animals (data not shown).

Saliva was collected from mice following the removal of laboratory chow for 18 hr as previously described (21). Briefly, total saliva was collected from the oral cavity by micropipette following stimulation of secretion using 0.20 mg isoproterenol and 0.05 mg pilocarpine/100 g body wt dissolved in saline and injected (0.1 ml volume) ip into control and experimental mice. The saliva was placed into chilled 1.5-ml microcentrifuge tubes. Volume was calculated based on weight change of the preweighted tubes using a Mettler analytical balance. Saliva was collected over a 15-min period.

Total Protein and Amylase Content of Saliva. For each analysis, all samples were assayed at one time and in duplicate to minimize error. Total protein was measured by the method of Bradford using bovine serum albumin as standard (22). Amylase activity was determined by activity assays (21, 23) using starch as standard substrate. Saliva was diluted 1000-fold for these assays in PBS. One unit of amylase was defined as the amount that hydrolyzed 1 mg starch min^{-1} mg $protein^{-1}$ at 37°C.

Submandibular Gland and Saliva Levels of EGF. For EGF estimation, the volume of total saliva was brought to 1.0 ml with sterile water. Fifty-eight microliters of glacial acetic acid were added to obtain a 1 *N* solution. The solution was cooled on ice for 30 min and then centrifuged at 4°C for 30 min in an Eppendorf centrifuge. The supernatant was transferred to a new tube, frozen at −80°C and then lyophilized. The remaining proteins were resuspended in 200 μl sterile water. One hundred microliters of clarified saliva or submandibular gland lysate were added to 100 μl of human placental microvillus membranes and [^{125}I]-labeled human EGF. After incubation at 37°C for 2 hr, the membrane mixture was diluted in 3.5 ml PBS, centrifuged for 20 min at 7000*g*, and the radiolabel associated with the membrane determined in a Beckman gamma counter (24). This method of binding is independent of the species of the EGF source (21, 25).

Polyacrylamide Gel Electrophoresis. Ten micrograms of whole saliva protein or 20 μl of saliva volume were subjected to electrophoretic separation on a 1.5-mm thick 10% or 15% SDS-polyacrylamide gel system as described by Pugsley and Schnaitman (26). The gels were fixed and stained by protocols described previously for mouse saliva (21, 27).

For Western blot analysis of salivary proteins, 30 μg of whole saliva protein was separated by SDS-polyacrylamide gel and transferred to nitrocellulose at 17 V for 12 hr (28). The nitrocellulose filters were blocked for 1 hr in PBS containing 3% BSA and 0.1% Tween-20 (Bio-Rad). The filters were then incubated for 12 hr at room temperature with a 1:500 dilution of antibody monospecific to the proline-rich protein, amylase, or TGF_{α}. Following repeated washing, the nitrocellulose was incubated for 2 hr with blocking buffer containing [^{125}I] protein-A, washed repeatedly and finally placed at −80°C for 72 hr for autoradiography using Kodak XAR-5 film.

The analysis of the insulin receptor was performed using 50 μg of plasma membrane prepared from the parotid and submandibular glands as described by Arvan and Castle (29). Polyclonal antibody to the α subunit of the insulin receptor was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Incorporation of [^{3}H]-Thymidine into DNA. The rate of *in vivo* DNA synthesis of the parotid and submandibular gland was determined by the incorporation of [^{3}H]-thymidine into TCA precipitable counts. Control and experimental animals were injected with 0.05 μCi [^{3}H]-thymidine 4 hr prior to killing. Following removal of the gland, wet weight was determined by an analytical balance. Following homogenization using a polytron tissue dispenser and Dounce homogenization in 10 m*M* Tris HCl pH 8.0 containing 100 μ*M* PMSF and 1.0 μ*M* leupeptin, 100 μl were removed to determine radiolabel incorporation as described previously (30).

Statistical Analysis. The Bonferroni paired comparisons test was used to determine significant differences among means after a significant analysis of variance (ANOVA) test. $P < 0.05$ was considered to be significant difference. The number of animals per experimental time point was $n = 6$ unless otherwise indicated. All values are expressed as the mean ± standard error.

Results

Physiological Responses of BALB/c Mice to Insulin Injection. The effects of chronic insulin injection on mouse physiology were measured by analyses of body weight and changes in food consumption. As indicated in Table I, intramuscular bolus injections of insulin, at 2000-fold concentrations of blood levels for the hormone, did not significantly ($P > 0.05$) alter total body weight or consumption of food and water during the course of the experimental protocol.

Salivary Gland Hypertrophy and Hyperplasia. The parotid and submandibular glands were analyzed for the effects of chronic insulin treatment on gland weight. Changes in gland weight were recorded per 100 g body wt. As shown in Figure 1A, the parotid gland exhibited a 1.6-fold increase in wet weight 24 hr after the injection of insulin into mice ($P < 0.01$). No further increase in gland size was observed for the six days of injection. There was a smaller increase in wet weight for the submandibular gland. A gradual increase out to four days of chronic treatment is evident in Figure 1B. However, this increase was not statistically different from control animals until Day 3 of treatment ($P < 0.05$). In both the parotid and submandibular glands, the levels of total protein in cell lysate increased 1.3- and 1.4-fold respectively 24 hr after the

Table I. Effects of Chronic Insulin on Mouse Physiology

Insulin treatment	Body weight[a]	Water consumption[b]	Food consumption[c]	Gland weight[d]		Blood glucose[e]	Gland protein[f]	
				PAR	SMX		PAR	SMX
CTL	16.6 ± 0.85	23 ± 4	3.3 ± 1.7	0.20 ± 0.03	0.37 ± 0.03	180	14.8 ± 2.6	18.2 ± 1.7
1 Day	16.5 ± 1.06	25 ± 3	3.1 ± 0.8	0.32 ± 0.05	0.38 ± 0.02	120	19.0 ± 1.7	25.4 ± 2.8
2 Day	16.8 ± 0.83	22 ± 4	3.4 ± 1.0	0.31 ± 0.04	0.43 ± 0.04	120	25.2 ± 1.1	27.8 ± 2.1
3 Day	16.7 ± 0.89	25 ± 4	3.8 ± 0.9	0.33 ± 0.03	0.45 ± 0.03	80	26.2 ± 1.7	28.9 ± 2.3
4 Day	17.1 ± 0.88	23 ± 4	3.3 ± 1.1	0.31 ± 0.04	0.47 ± 0.05	80	30.2 ± 3.5	26.3 ± 1.9
5 Day	16.5 ± 1.11	23 ± 4	3.6 ± 0.7	0.34 ± 0.05	0.48 ± 0.05	80	28.5 ± 1.7	26.1 ± 2.3
6 Day	17.0 ± 1.50	21 ± 5	3.2 ± 1.2	0.30 ± 0.01	0.45 ± 0.05	80	25.6 ± 4.8	26.5 ± 2.5

[a] All values expressed a mean g ± SE.
[b] Values expressed as ml/24-hr period ± SE.
[c] Values expressed as mean g chow consumed/24 hr ± SE per animal.
[d] Values expressed as mean mg ± SE.
[e] Blood glucose values are expressed as mean mg/dl ± SE as per manufacturers instructions.
[f] Values expressed as mean mg cell protein ± SE.
$n = 6$ for all experimental groups.

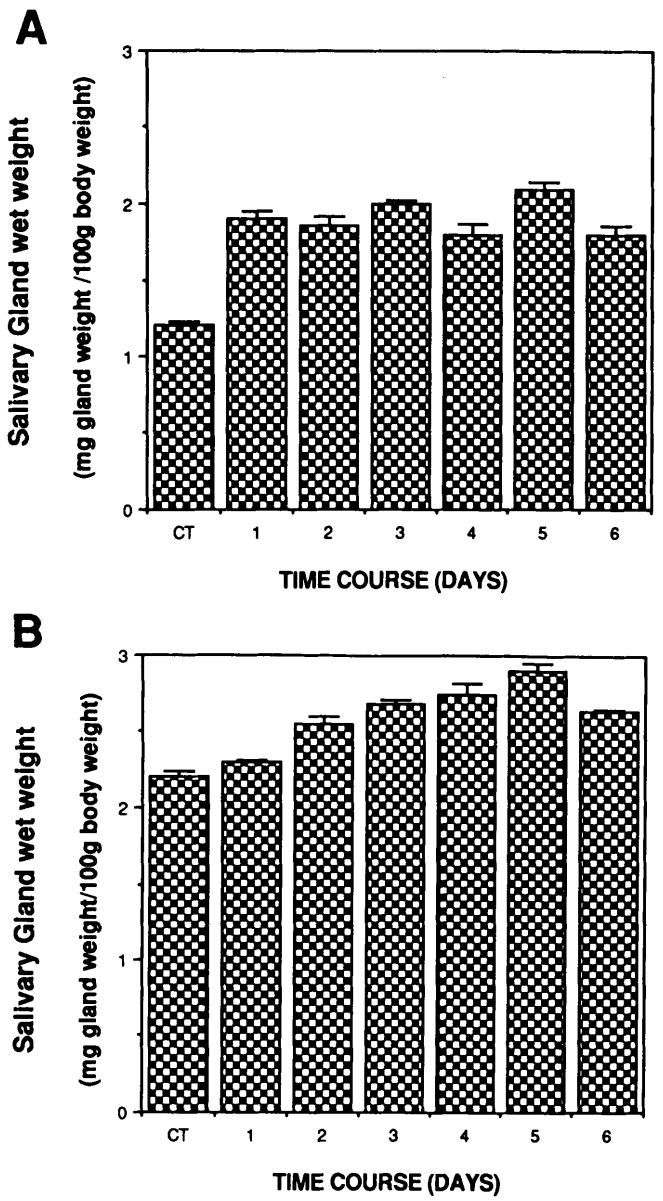

Figure 1. Histogram of salivary gland wet weight following chronic insulin treatment of BALB/c mice. Panel A represents values for the parotid gland while Panel B represents values for the submandibular gland. All values are the mean tissue weight of six experimental animals ± SE/100 g body wt.

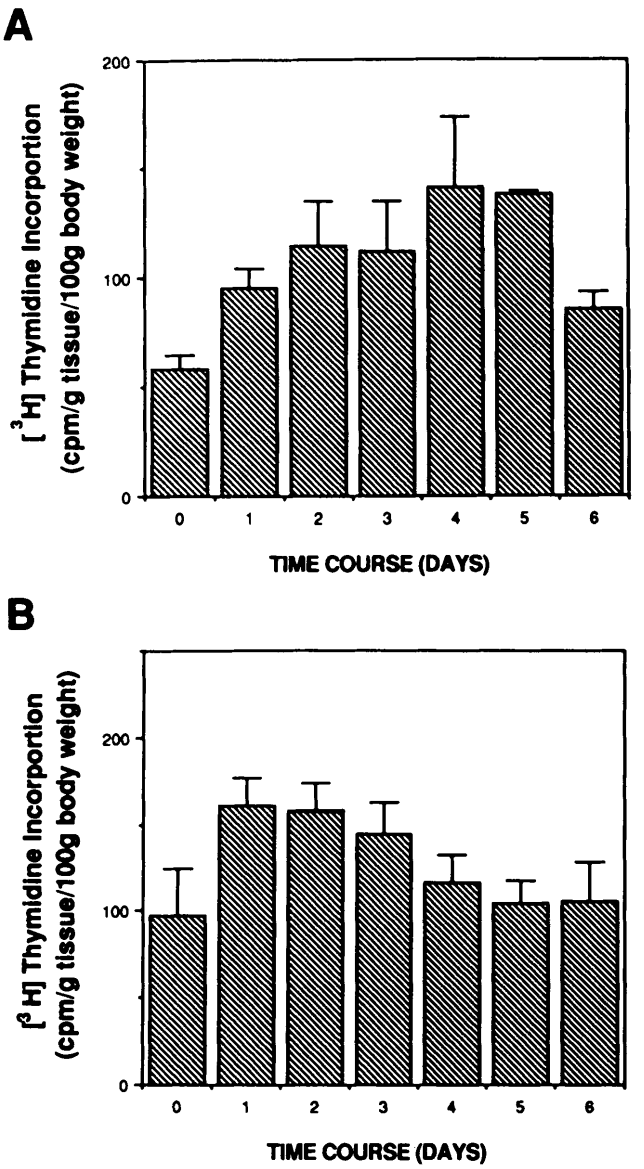

Figure 2. Histogram of the rates of DNA synthesis following chronic insulin treatment of the parotid gland (Panel A) and submandibular gland (Panel B). DNA synthesis was measured by the rate of incorporation of [^{3}H]-thymidine into TCA precipitable counts. All values are expressed as mean cpm ± SE/g tissue/100 g body wt. n = 6 animals per group.

initial injection of insulin (Table I; $P < 0.05$). There was a further increase at 48 hr, although there was not any appreciable changes in the following days of hormone administration.

Chronic insulin injection into mice caused a transient increase in hyperplasia as indicated by the increase in [^{3}H]-thymidine incorporated into DNA (Fig. 2). In the parotid gland, thymidine incorporation increased from 55 cpm/g tissue/100 g body wt to reach a maximum incorporation at Day 4 of 165 cpm/g tissue/100 g body wt ($P < 0.01$). Following the maximum rate of [^{3}H]-thymidine incorporation, DNA synthesis declined on Day 5 and 6 (Figure 2A). For the submandibular gland, the greatest rate of DNA synthesis was observed after two days of chronic insulin treatment. The rate of [^{3}H]-thymidine incorporation increased from 96 cpm/g tissue/100 g body wt to 172 cpm/g tissue/100 g body wt ($P < 0.05$). By Day 5 of insulin injection, the rate of DNA synthesis returned to control values (Figure 2B).

The decline in hypertrophy and hyperplasia under conditions of chronic insulin injection was further investigated by examining the level of receptor present in plasma membranes from the parotid and subman-

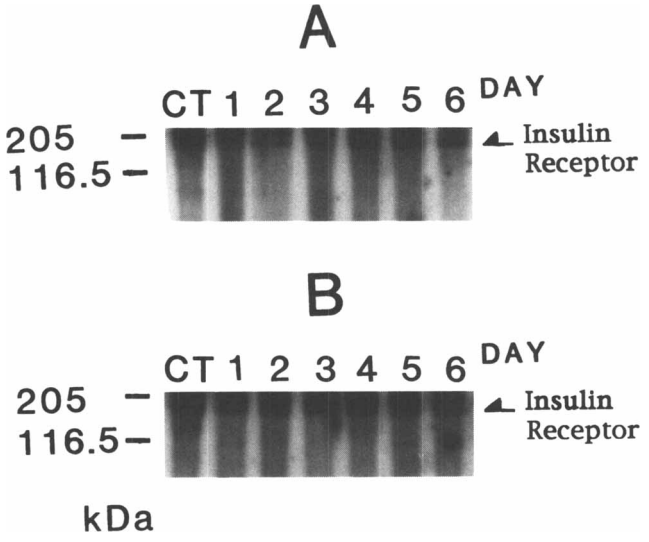

Figure 3. Autoradiography of insulin receptor levels in the parotid (Panel A) and submandibular gland (Panel B) following insulin injection into BALB/c mice. Fifty μg of plasma membrane protein were separated on 10% SDS-polyacrylamide gels prior to Western blot analysis using [^{125}I]-protein A. Each membrane sample represents a pool of six animals per day of insulin injection. Prestained molecular weight standards are: myosin, 205,000 Da; β-galactosidase, 116,500 Da; bovine serum albumin, 80,000 Da; and ovalbumin, 49,500 Da. Lane 1 (C), control, saline injected mice; Lane 2, one day of insulin treatment; Lane 3, two days of insulin treatment; Lane 4, three days of insulin treatment; Lane 5, four days of insulin treatment; Lane 6, five days of insulin treatment; Lane 7, six days of insulin treatment.

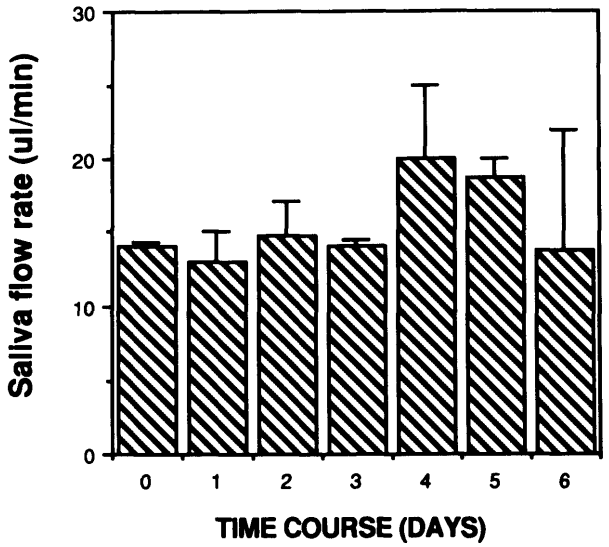

Figure 4. Histogram of stimulated saliva flow rates from insulin treated BALB/c mice. Whole saliva was collected following stimulation of secretion by a combined injection of isoproterenol and pilocarpine. All values are expressed as mean μl/min ± SE. n = 6 animals per time point.

dibular glands. As shown in Figure 3, the level of a 185 kDa protein (presumably the insulin receptor) remained unchanged in membrane preparations for these tissues. The identification of the insulin receptor in both glands also serves to suggest that the effects on gland physiology by the hormone are most likely mediated by direct binding to cells of the salivary glands rather than indirectly through a product of insulin metabolism.

Saliva Flow Rate and Composition. Changes in the flow rate of stimulated saliva of animals receiving insulin injections were measured following stimulation of secretion by a combined injection of isoproterenol and pilocarpine (21). As shown in Figure 4, while fluctuations in saliva flow were observed for Day 4 and 5, there was no significant difference in the volume of saliva collected over the 15-min time course.

The protein content of saliva was examined by SDS-polyacrylamide gel electrophoresis. Similar staining patterns of the total proteins present were observed (Figure 5A) when evaluated using constant protein per experimental group. If, however, a constant volume of saliva was analyzed on the gels, the Coomassie blue staining pattern showed that there was a decrease in the total protein content of stimulated saliva with chronic insulin treatment (Figure 5B). The major protein at approximately 56 kDa is amylase. An analysis of enzyme activity using a constant volume of saliva from the experimental groups again showed the same pattern in the loss of enzyme activity from saliva. By Day 6 of chronic treatment with insulin, amylase activity was reduced by 82% compared with saline injected controls ($P < 0.01$; Figure 6A). Changes in saliva amylase levels were also reflected in a decrease in glandular levels of enzyme activity. Parotid gland lysates showed a progressive loss of amylase activity with increasing days of insulin treatment (Figure 6B). Following five days of chronic insulin treatment, enzyme activity in the gland was reduced by 76% ($P < 0.01$).

Western blot analysis of the proportion of proline rich proteins, amylase, and TGF_α in mouse saliva following chronic insulin treatment was performed using constant protein as was done for data shown in Figure 5A. The results shown in Figure 7, suggest the concentration of all three of the proteins remained constant as would be predicted from the Coomassie blue stained saliva protein profiles. The antibody to amylase detected a single peptide of approximately 58,000 Da (Panel A). Two proteins were detected in saliva with the antibody to the proline-rich proteins with molecular masses of approximately 40,000 and 20,000 Da (Panel B). A single protein species of approximately 6000 Da was identified with antibody to TGF_α which comigrated with purified human TGF_α as previously shown (18). The lower migrating protein species may be a degradation product of the growth factor (Figure 7, panel C).

Receptor binding assays for EGF levels in saliva and the submandibular gland indicated an increase in

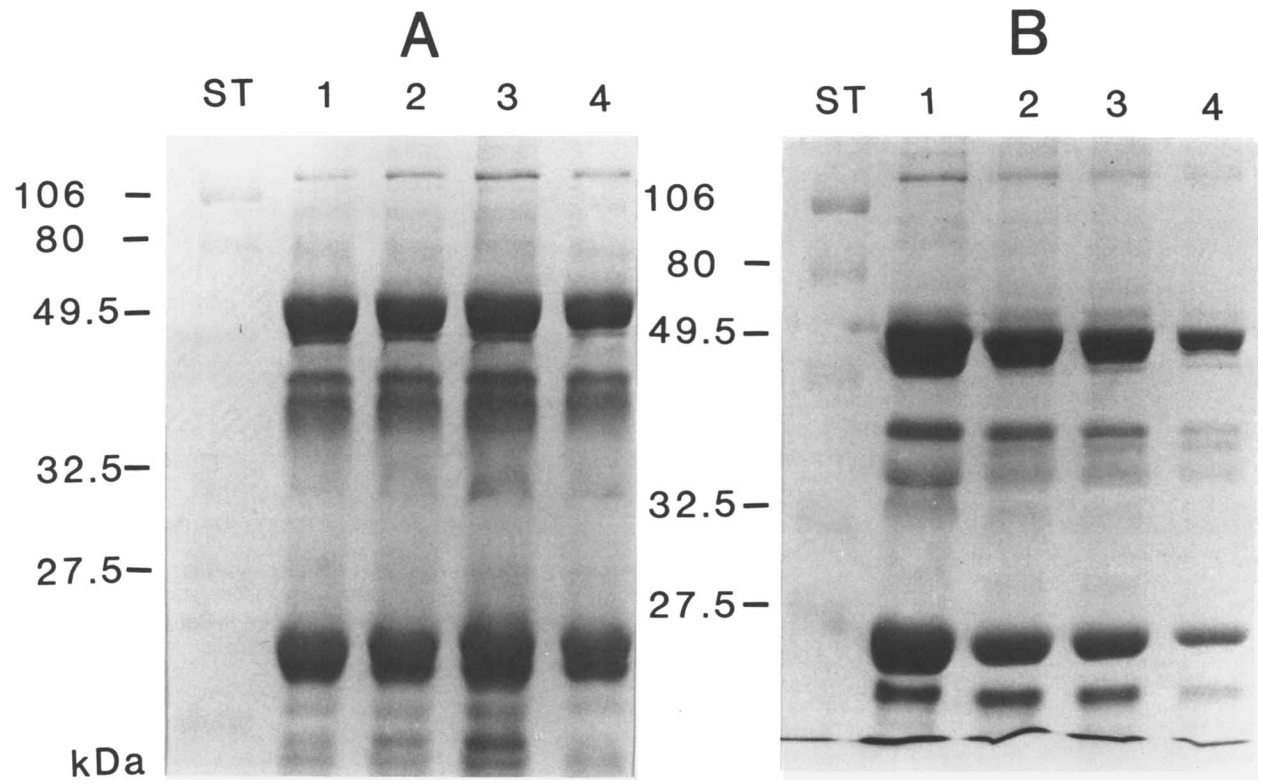

Figure 5. SDS-polyacrylamide gel profile of saliva proteins following chronic insulin treatment of BALB/c mice. Panel A, whole saliva profile following electrophoresis of constant protein per time point (10 μg). Panel B, protein profile of saliva following electrophoresis of a constant volume (20 μl) of saliva. Each time point represents a typical profile observed for six individual animals in each group. Prestained molecular weight standards were: phosphorylase B, 106,000 Da; bovine serum albumin, 80,000 Da; ovalbumin, 49,500 Da; carbonic anhydrase, 32,500 Da; and soybean trypsin inhibitor, 27,500 Da. Lane 1, molecular weight standards; Lane 2, control, saline injected mice; Lane 3, two days of chronic insulin injection; Lane 4, four days of chronic insulin injection; Lane 5, six days of chronic insulin injection.

the presence of the growth factor with chronic insulin administration. Unlike the synthesis of amylase by the acinar cells of the parotid gland, duct cell synthesis of EGF in the submandibular gland increased when expressed per unit protein (Table II) during the initial three days of insulin injection. Synthesis by Day 6 was reduced from the peak day of synthesis but was still above control values ($P < 0.005$). The levels of EGF in saliva increased throughout the injection regimen when expressed as concentration per unit volume. Saliva values for EGF as well as those for the gland are similar to previously reported concentrations based upon radioimmunoassay (31, 32).

Discussion

In the present investigation, we have examined the role of elevated levels of insulin on the normal physiology and biological function of the salivary glands of mice. To accomplish this, normal BALB/c mice (i.e., fully functional pancreatic insulin producing islet cells) were given twice daily supplemental injections of 2000-fold greater International Units of insulin than present in normal blood hormone levels. While animal behavior such as food and water consumption remained normal, the increased insulin produced dramatic decreases in blood glucose concentrations. Too, there was no evidence of alterations in the physical appearance of the animals or total body weight over the six-day injection regimen.

Chronic injection of insulin resulted in increased rates of DNA and protein synthesis. The abrupt increase in glandular levels of protein could, in part, be responsible for salivary gland hypertrophy. From our measurement of glandular protein levels, it is not possible to conclude the increase is due to *de novo* synthesis or decreased secretion in response to insulin. Insulin has previously been shown to increase the rate of protein synthesis in rat submandibular gland dispersed cell cultures (16). Compared with glandular protein levels, increased DNA synthesis was delayed in the parotid and submandibular gland. The rate of [^{3}H]-thymidine incorporation reached a peak at four days of insulin treatment in the parotid gland versus two days in the submandibular gland. During this time, insulin caused a substantial increase in the level of EGF synthesis in the submandibular gland as well as its secretion into saliva. Insulin signal transduction occurs through tyrosine kinase phosphorylation of pro-

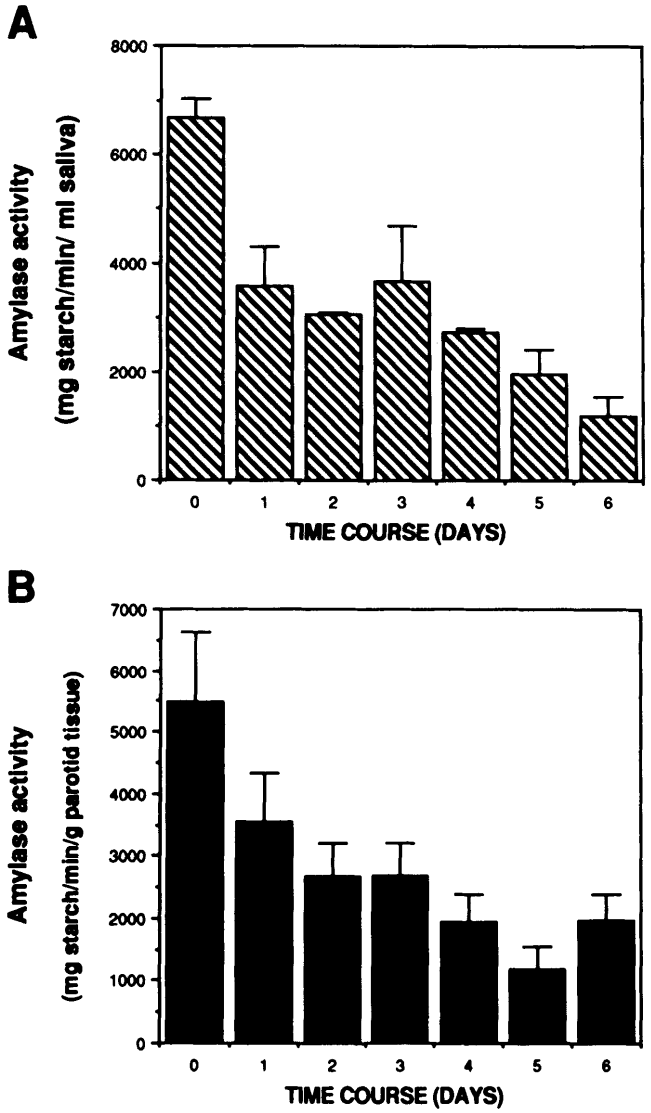

Figure 6. Histogram of α-amylase activity in saliva (Panel A) and parotid gland lysates (Panel B) following chronic insulin treatment of BALB/c mice. Amylase activity was calculated as mean ± SE of mg starch split/min/ml saliva (A) or mg starch split/min/g parotid tissue (B). n = 5 animals for each experimental time point.

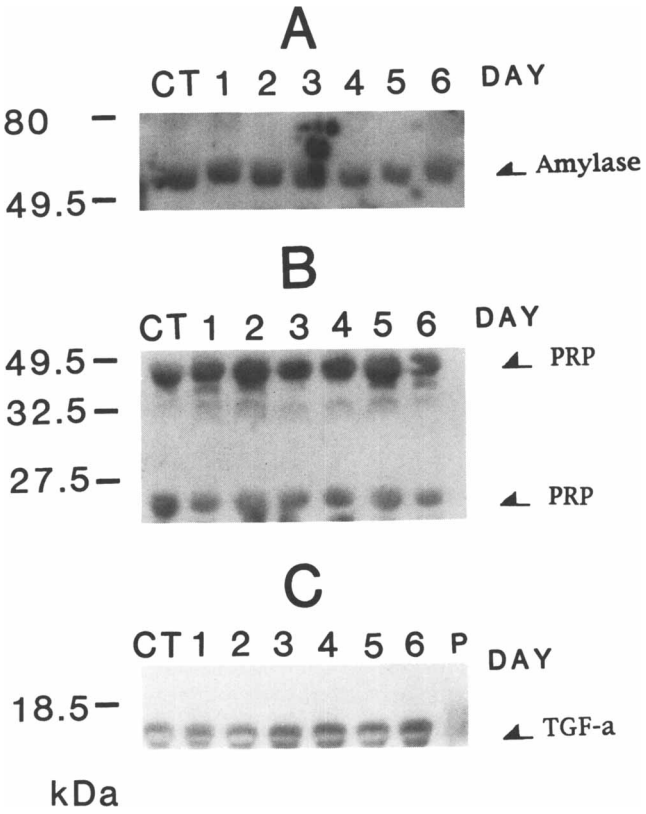

Figure 7. Autoradiographic detection of amylase, proline-rich protein, and TGF_α in saliva from BALB/c mice following chronic insulin treatment. Thirty micrograms of whole saliva protein was analyzed for each experimental group. Prestained molecular weight standards are as indicated above in Figure 3 and 5. Lanes are labeled as in Figure 3, except for Panel C, the last lane on right side (p), which contains human recombinant TGF_α as a positive control. Note the similar gel mobility with mouse saliva TGF_α identified by the antiserum.

tein substrates previously shown to be involved in cell proliferation (33). The observation of acinar cell hyperplasia with chronic insulin treatment of the parotid and submandibular gland suggest similarity to growth stimulation involving components of the tyrosine kinase signaling pathway described for chronic EGF or isoproterenol treatment of rat parotid glands (34–37). The increased synthesis of EGF could contribute to the observed gland hyperplasia by an autocrine growth stimulation of the submandibular gland or paracrine effects from saliva on the parotid gland by systemic dissemination.

While the level of EGF synthesis and secretion increased in the submandibular gland, amylase synthesis and secretion from the parotid gland was reduced. An analysis of whole saliva by SDS polyacylamide gel profiles revealed that chronic insulin treatment decreased protein content per unit volume. Proline-rich proteins and TGF_α are synthesized by both the parotid and submandibular glands. However, their concentration in saliva and salivary glands were also reduced when evaluated relative to unit protein (data not shown). Thus using specific markers for the parotid and submandibular gland (i.e., amylase and EGF, respec-

Table II. EGF Concentrations in Submandibular Gland and Whole Saliva

Animal treatment[a]	n	Submandibular gland[b]	Saliva[c]
0 Day	6	22 ± 8	29 ± 9
1 Day	6	140 ± 27	456 ± 102
3 Day	6	420 ± 63	970 ± 153
6 Day	6	76 ± 38	2550 ± 368

[a] Animals given twice daily injections of insulin or saline (0 day control).
[b] Values expressed as mean μg/mg gland protein ± SE.
[c] Values for saliva EGF concentrates are expressed as mean μg/ml whole saliva ± SE.

tively) we have shown differential effects on the function of the salivary glands. It should also be considered that the regulation of these proteins is unique in the salivary glands since amylase synthesis appears to be directly regulated by insulin (14, 15) and EGF synthesis by a number of hormones (31, 32), although we found no evidence for insulin effects. Kasayama and Oka (38) have, however, shown genetic and chemically induced diabetic mice to synthesize less NGF in the submandibular gland duct cells than normal mice. Both NGF and EGF are synthesized by the same cells of the submandibular gland (29). Since amylase synthesis has been shown to be responsive to insulin levels (14, 15), we do not have an explanation for the paradoxical behavior for enzyme regulation in a situation governed by high levels of hormone. One possible mechanism may involve the observation that amylase synthesis is down-regulated during acinar cell proliferation (40). EGF and amylase are also the product of different glandular cell subtypes which may influence their response to insulin.

Insulin effects on various cells' metabolism and homeostasis are mediated by protein phosphorylation activities (7). These include not only the tyrosine kinases but a number of ser/thr kinases such as protein kinase A, protein kinase C and the calmodulin-dependent protein kinase II (7, 41). The activation of protein kinase A in response to cAMP has been shown to be involved in β-adrenergic agonist stimulation of protein secretion from salivary gland acinar cells (42, 43). Insulin inhibits a number of cellular cAMP-dependent phosphorylations and alters enzyme activity, but the mechanism by which insulin directly mediates these effects is unclear. In adipocytes and muscle cells insulin decreases isoproterenol-dependent phosphorylation of cAMP dependent protein kinases and regulates the activity levels of phosphodiesterases and protein phosphatases, thus effectively lowering the concentration of cellular cAMP (8, 9, 44). Insulin has been shown to modulate salivary gland cAMP levels (45). Since protein release from salivary acinar cells is cAMP dependent, the lower total protein content of saliva from insulin-treated animals may be a consequence of hormone induced changes in the levels of phosphorylation of proteins necessary for proper secretion. The accumulation of secretory proteins may subsequently result in a feedback down-regulation of synthesis by the cells of the salivary glands. The increase in glandular protein would initially result from the intraglandular accumulation of secretory proteins as well as synthesis of proteins required for DNA synthesis and cell division. Clearly, as we learn more about the phosphorylation patterns of proteins of the salivary glands in response to insulin, a more detailed understanding of the effects on gland function will emerge.

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1. Rossini AA, Mordes JP, Like AA. Immunology of insulin-dependent diabetes-mellitus. Annu Rev Immunol **3**:289–320, 1985.
2. White MF, Maron R, Kahn CR. Insulin rapidly stimulates tyrosine phosphorylation of a M_r-185,000 protein in intact cells. Nature **318**:183–186, 1985.
3. Giorgetti S, Ballatti R, Kowalski-Chauvel A, Tartare S, Obberghen EV. The insulin and insulin-like growth factor–I receptor substrate IRS-I associates with and activates phosphatidylinositol 3-kinase *in vitro*. J Biol Chem **286**:7358–7364, 1993.
4. Backer JM, Myers MG Jr, Shoelson S, Chin DJ, Sun X-J, Miralpeix M, Hu P, Margolis B, Skolkin EY, Schlessinger J, White MF. Phosphatidyl 3-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J **11**:3469–3479, 1992.
5. Mooney RA, Anderson DL. Phosphorylation of the insulin receptor in permeabilized adipocytes is coupled to a rapid dephosphorylation reaction. J Biol Chem **264**:6850–6857, 1989.
6. Pronk GJ, McGlade J, Pelicci G, Pawson T, Bos JL. Insulin-induced phosphorylation of the 46-kDa and 52-kDa Shc proteins. J Biol Chem **268**:5748–5753, 1993.
7. Laurence JC Jr. Signal transduction and protein phosphorylation in the regulation of cellular metabolism by insulin. Annu Rev Physiol **54**:177–193, 1992.
8. Mooney RA, Bordwell KL. Counter-regulation by insulin and isoproterenol of a prominent fat-associated phosphoprotein doubled in rat adipocytes. Biochem J **274**:433–438, 1991.
9. Shibata H, Robinson FW, Soderling TR, Kono T. Effect of Okedaic acid on insulin-sensitive cAMP phosphodiesterase in rat adipocytes. Evidence that insulin may stimulate the enzyme by phosphorylation. J Biol Chem **266**:17948–17953, 1991.
10. Anderson LC. Effects of alloxan diabetes and insulin *in vivo* on rat parotid gland. Am J Physiol **245**:G431–G437, 1983.
11. Anderson LC. Parotid gland function in streptozotocin-diabetic rats. J Dental Res **66**:425–430, 1987.
12. Kim S-K, Cuzzort LM, McKean RK, Allen ED. Effects of diabetes and insulin on α-amylase messenger RNA levels in rat parotid glands. J Dent Res **69**:1500–1504, 1990.
13. Korc M, Iwamoto Y, Sankaran H, Williams JA, Goldfine ID. Insulin action in pancreatic acini from streptozotocin-treated rats I. Stimulation of protein synthesis. Am J Physiol **240**: G56–G62, 1981.
14. Kim S-K, Cuzzort LM, Allen ED. Effects of age on diabetes- and insulin-induced changes in pancreatic levels of α-amylase and its mRNA. Mech Aging Dev **58**:151–161, 1991.
15. Anderson LC, Johnson DA. Effects of alloxan diabetes on rat parotid gland and saliva. Comp Biochem Physiol **B70**:725–730, 1981.
16. Anderson LC. Insulin-stimulated protein synthesis in submandibular acinar cells: Interactions with adrenergic and cholinergic agonists. Horm Metabol Res **20**:20–23, 1988.
17. Johnson TM, Rosenberg MP, Meisler MH. An insulin-responsive element in the pancreatic enhancer of the amylase gene. J Biol Chem **268**:464–468, 1993.
18. Humphreys-Beher MG, Macauley S, Chegini N, van Setten G, Purushotham KR, Stewart C, Wheeler TT, Schultz GS. Characterization of the synthesis and secretion of TGF_α from salivary glands and saliva. Endocrinol (in press).
19. Blair EA, Castle AM, Castle JD. Proteoglycan sulfation and storage parallels storage of basic secretory proteins in exocrine cells. Am J Physiol **263**:C897–C905, 1991.
20. Havrankova J, Schmechel D, Roth J, Brownstein M. Identifi-

cation of insulin in rat brain. Proc Natl Acad Sci USA **75**:5737–5741, 1978.
21. Hu Y, Nakagawa Y, Purushotham KR, Humphreys-Beher MG. Functional changes in salivary glands of autoimmune disease-prone NOD mice. Am J Physiol **263**:E607–E613, 1992.
22. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**:248–254, 1976.
23. Bernfeld P. Amylases, alpha and beta. Meth Enzym **1**:149–158, 1955.
24. Booth AG, Olaniyan O, Vanderpuye OA. An improved method for the preparation of human placental syncytiotrophoblast microvilli. Placenta **1**:327–336, 1980.
25. Simpson RJ, Smith JA, Moritz RL, O'Hare MJ, Rudland PS, Morrison JR, Lloyd CJ, Grego B, Burgess AW, Nice EC. Rat epidermal growth factor: Complete amino acid sequence. Homology with the corresponding murine and human protein; isolation of a form truncated at both ends with full *in vitro* biological activity. Eur J Biochem **153**:629–637, 1985.
26. Pugsley AP, Schnaitman CA. Factors effecting electrophoretic mobility of the outer membrane proteins of *Escherichia coli* in polyacrylamide gels. Biochem Biophys Acta **581**:163–178, 1979.
27. Humphreys-Beher MG, Wells DJ. Metachromatic staining patterns of basic proline-rich proteins from rat and human saliva in sodium dodecyl sulfate-polyacrylamide gels. Appl Biochem **6**:353–360, 1984.
28. Towbin H, Staehlin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA **78**:4350–4354, 1979.
29. Arvan P, Castle JD. Plasma membrane of the rat parotid gland: Preparation and partial characterization of a fraction containing the secretory surface. J Cell Biol **95**:8–19, 1982.
30. Humphreys-Beher MG, Zelles T, Maeda N, Purushotham KR, Schneyer CA. Cell surface galactosyltransferase acts a general modulator of rat acinar cell proliferation. Mol Cell Biochem **95**:1–11, 1990.
31. Perheentupa J, Lakshamanan J, Hoath SB, Beri U, Kim H, Macaso T, Fisher DA. Epidermal growth factor measurements in mouse plasma: Method, antogeny, and sex difference. Am J Physiol **248**:E391–E396, 1985.
32. Fisher DA, Lakshmanan J. Metabolism and effects of epidermal growth factor and related growth factors in mammals. Endocrine Rev **11**:418–442, 1990.
33. Brugge JS. New intracellular targets for therapeutic drug design. Science **260**:918–920, 1993.
34. Nakagawa Y, Purushotham KR, Wang P-L, Fischer JE, Dunn WA, Schneyer CA, Humphreys-Beher MG. Alterations in the subcellular distribution of $p21^{ras}$-GTPase activating protein in proliferating rat acinar cells. Biochem Biophys Res Commun **187**:1172–1176, 1992.
35. Purushotham KR, Dunn WA Jr., Schneyer CA, Humphreys-Beher MG. A novel mechanism for isoprenaline-stimulated proliferation of rat parotid acinar cells involving epidermal growth factor receptor and cell surface galactosyltransferase. Biochem J **284**:767–776, 1992.
36. Wang P-L, Nakagawa Y, Humphreys-Beher MG. Epidermal growth factor and isoproterenol stimulation of the *ras*-guanine nucleotide exchange factor in proliferating rat parotid gland acinar cells. Biochem Biophys Res Commun **192**:860–866, 1993.
37. Purushotham KR, Nakagawa Y, Kurian P, Patel R, Crews FT, Humphreys-Beher MG. Activation of phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase during rat parotid gland acinar cell proliferation. Biochim Biophys Acta (in press).
38. Kasayama S, Oka T. Impaired production of nerve growth factor in the submandibular gland of diabetic mice. Am J Physiol **257**:E400–E404, 1989.
39. Watson AY, Anderson JK, Siminoski K, Mole JE, Murphy RA. Cellular and subcellular colonalization of nerve growth factor and epidermal growth factor in the submandibular glands. Anat Rec **213**:365–376, 1985.
40. Morley DJ, Hodes ME. Amylase expression in human parotid neoplasms: Evidence by *in situ* hybridization for a lack of transcription of the amylase gene. J Histochem Cytochem **36**:487–491, 1988.
41. Scott CW, Mumby MC. Autophosphorylation of rat liver Type II cAMP-dependent protein kinase. Molec Endocrinol **3**:1815–1822, 1989.
42. Quissell DO, Watson E, Dowd FJ. Signal transduction mechanisms involved in salivary gland regulated exocytosis. Crit Rev Oral Biol Med **3**:83–108, 1992.
43. Aub DL, Putney JE Jr. Properties of receptor-controlled inositol triphosphate formation in parotid acinar cells. Biochem J **225**:263–266, 1985.
44. Foulkes JG, Cohen P, Strada SL, Everson WV, Jefferson LS. Antagonistic effects of insulin and β-adrenergic agonists on the activity of protein phosphatase inhibitor–1 in skeletal muscle of the perfused rat hemicorpus. J Biol Chem **257**:12493–12496, 1982.
45. Anderson LC, Bevan CA. Effects of streptozotocin diabetes on amylase release and cAMP accumulation in rat parotid acinar cells. Arch Oral Biol **37**:331–336, 1992.