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METHODS PAGE

Measurement of pancreatic protein synthesis.

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Introduction

Different methods have been used to measure protein synthesis involving administration of isotopically labeled amino acids with subsequent measurement of the incorporation of label into protein. For animal studies this is usually a radioactive isotope due to cost and ease of measurement while human studies have usually used stable isotopes (16, 17). Protein synthesis in the exocrine pancreas has been analyzed in studies involving pancreatic stimulation by diet (6, 11, 25), hormones (insulin, CCK) (1, 9, 10, 12, 19, 21, 24, 26-28) as well as in different models of acute pancreatitis (4, 5, 13, 15, 18, 22). Most of the early studies were done using different ways of administering the labeled amino acid as a tracer and some were lacking an accurate assessment of the specific radioactivity of the precursor amino acid at the site of protein synthesis. As indicated with all these studies, the amino acid labeled analysis can be performed using pancreatic tissue obtained *in vivo* or *in vitro* with isolated pancreatic cell fragments or lobules. While total protein synthesis is the usual parameter measured, the same principles apply to determination of the synthesis of individual proteins after isolation by immunoprecipitation or gel electrophoresis.

Measurement of pancreatic protein synthesis *in vivo*. The Flooding Dose technique.

For *in vivo* experiments, the use of readily accessible compartment pools, such as the intracellular free amino acids or plasma pools, to estimate precursor labeling is based on the assumption that the experimental treatment does not alter the relationship between the labeling of the sampled pool and that of the aminoacyl-tRNA, the direct precursor of protein synthesis. However, experimental conditions have the potential to alter precursor enrichment either by affecting the amount of labeled amino acid entering the cell or by affecting the contribution of unlabeled amino acids derived from protein degradation to the charging of aminoacyl-tRNA. In these cases, the problem of accurately determining precursor enrichment may be minimized by the flooding dose technique (3).

With the flooding dose technique, the labeled amino acid is injected, not as a tracer, but contained in a large (i.e., much larger than the endogenous free amino acid pool) bolus of unlabeled amino acid, making the specific activities in all free amino acid compartments more alike than if the labeled compound is given as a tracer dose. Thus the labeling of aminoacyl-tRNA is less likely to be affected by experimental manipulations. In addition, the large amount of

amino acid injected ensures that the specific activity in the free pools remains almost constant for a certain period of time after injection (2). Although various amino acids have been used, we use L-[^{3}H]-phenylalanine as a radioactive tracer, because Sweiry and co-workers (29) demonstrated that phenylalanine transport across the basolateral membrane of the pancreatic acinar cells is not a rate-limiting factor for protein synthesis and because facilities to quantitate [^{3}H] by liquid scintillation counting (LSC) are readily available. [^{3}H] is inexpensive compared to [^{14}C] or [^{35}S] and safer for the user. Alternatively, stable isotopes can be used with derivatization and quantitation by gas chromatography-mass spectrometry (GC-MS). Finally, the flooding dose technique is advantageous not only because it is reliable, but also because it can be used in unrestrained and unanesthetized animals (2) and has been well validated in muscle (20), liver (14), and pancreas (29).

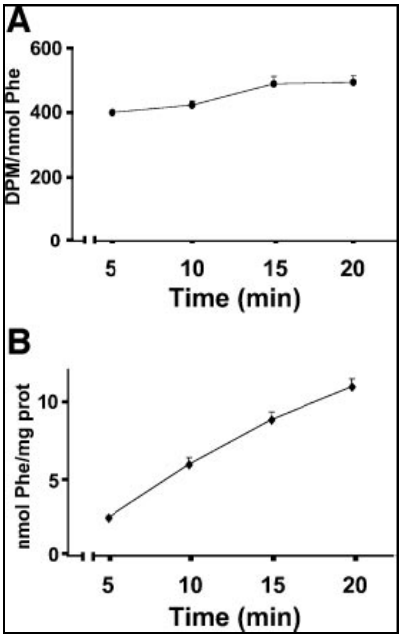

Figure 1. Time course of specific radioactivity (*A*) and L-[^{3}H]phenylalanine (Phe) incorporation into pancreatic protein *B*), in ICR mice. Values are expressed as disintegrations per minute per nanomole of Phe in the pancreatic acid soluble pool in ***A*** and as nanomoles of incorporated Phe per milligram of protein in the acid precipitable pool in ***B***. All data points are means ± SE of 10–12 mice per group (23).

The flooding dose technique, was originally described by Garlick and co-workers for heart (3) and later validated by Sweiry and co-workers (29) in rat pancreas. We have adapted the protocol of Lundholm and co-workers (14) described to measure liver protein synthesis to total pancreas protein synthesis in mice (23, 25) and rats (26), and measured the uptake of radioactive phenylalanine into pancreas as a function of time. The time course for the specific activity (dpm/nmol of L-Phe) of the pancreatic intracellular non-protein pool indicated that the precursor labeling in the pancreas pool does not change within the time range from 5 to 20 min after the injection of the tracer (Fig.1). The incorporation of L-[^{3}H]-Phe into pancreatic protein as a function of time shows a linear increase from 5 to 20 min (Fig.1). Ten min was chosen as the optimal time to determine protein synthesis in mice and 15 min in rats, because incorporation of ^{3}H into protein was increasing linearly at this time point (25, 26).

1. Materials

1.1 Animals

Pancreas samples are typically obtained from mice (about 20-30 g body weight) or rats (about 150 g body weight). Other animal species can be used.

1.2 Reagents and equipment

1. L-phenylalanine was from Sigma (St. Louis, MO) and L-[2,3,4,5,6- ^{3}H]-phenylalanine from Amersham Pharmacia Biotech (Piscataway, NJ); (126 Ci/mmol).
2. Perchloric acid (PCA), Potassium Hydroxide (KOH) and Sodium Hydroxide (NaOH) were also from Sigma (St. Louis, MO).
3. Derivatizing reagents were from Waters AccQ*Fluor Reagent Kit (Millipore, Milford, MA)
4. Scintillation liquid was Bio-Safe II, from Research Products International (Mount Prospect, IL); it is biodegradable and non-

hazardous, has a high flash point and is non-flamable. It also has high quantum efficiency quench resistant fluors in a non-ionic emulsifier system which accommodates a wide range of samples. The choice of scintillation fluid may be determined by institutional policy.

5. Liquid Scintillation Analyzer. We use a TRI-CARB 2100TR from Packard Bioscience (Meriden, CT), but any modern instrument able to perform quench correction can be used.
6. A polytron-type rapid homogenizer for sample homogenization. We use a PowerGen700 from Fisher Scientific (Pittsburg, PA).
7. HPLC Chromatographic Workstation (Millennium^{32} v3.2 Chromatographic Workstation, Waters, Milford, MA) and HPLC pump (Waters, Analytical HPLC Pump 510) as well as vials and their different parts from National Scientific Company (Rockwood, TN)

2. Method

2.1 Administration of radioactive tracer into animal

0.4 µCi/g of L-[^{3}H]-phenylalanine mixed with unlabeled L-phenylalanine (1.5 µmol/g) is injected by the intraperitoneal route in a volume of 300 µl for a 30 g mouse, and up to 1.5 ml in rats. For rats, the administration of tracer and unlabeled amino acid (2 ml for a 200 g rat) is by the i.v. route into the tail vein because it is rapidly administered and distributed. Administration into the tail vein of mice is possible but IP injection is easier and gives satisfactory results.

2.2 Collection and processing of pancreatic samples

1. Following administration of L-[^{3}H]-phenylalanine, blood is drawn by cardiac puncture into heparinized syringes, and the pancreas is rapidly removed, frozen in liquid nitrogen and kept at -80 ^{o}C until analysis.
2. Blood is centrifuged at 4 ^{o}C and plasma samples are mixed (1:1) with cold 1.2 N Perchloric Acid (PCA), left precipitating for 1 h on ice, and centrifuged at 10,000 *g* for 15 min. The resulting supernantant is neutralized with KOH before analysis for ^{3}H and L-phenylalanine. These supernatant samples are considered to be the free amino acid pools for calculations of specific activity. The tip of the polytron must be thoroughly rinsed between samples.
3. Frozen pancreases are subsequently homogenized in a 16 ml plastic tube using 10 volumes of 0.6 N PCA by using a polytron with a small generator, keeping the samples on ice all the time.
4. Homogenated samples are also left precipitating for 1 h on ice and centrifuged at 10,000 *g* for 15 min. After centrifugation, the supernatants are removed and neutralized with KOH before analysis for ^{3}H and L-phenylalanine. The pellet (protein fraction) is kept and washed three times with 0.6 N PCA and resuspended in 100-200 µl of 0.3 M NaOH for determination of ^{3}H and protein concentration.
5. The addition of KOH to plasma and pancreas supernatant solutions neutralizes them and creates a precipitate that needs to be removed. These samples are centrifuged again at 14,000 g for 15 min and the clear supernatant (intracellular, non protein pool, fraction) is used for HPLC and radioactivity (^{3}H) analysis. The flooding dose technique keeps the specific activity of the radiolabeled amino acid constant in the plasma and intracellular precursor pool.

2.3 Analysis of Phenylalanine by HPLC

1. L-Phenylalanine is measured on the intracellular fraction samples by HPLC on a C18 reverse phase column after

precolumn derivatization to produce a stable fluorescent derivative of L-phenylalanine: Fluorescent amino acid derivatives are prepared by reacting 1µL of sample with 79 µL of a pH 8.8 borate buffer and 20 µL of fluorescent derivatizing agent (AccQ*Fluor, Waters, Milford, MA) at room temperature. After one min of incubation the 100 µL aliquot is transferred to a low volume (300 µl) insert and heated at 55° C for 10 min to complete reaction prior to injection on the column.

2. *High Performance Liquid Chromatography* (Note1): Phenylalanine in standard solutions and unknowns are separated from other amino acids by reverse-phase liquid chromatography in the Chromatographic Workstation. A two-pump gradient system delivers 10 mM sodium acetate trihydrate (pH 5) (mobile phase A) and 60% acetonitrile in HPLC grade water (mobile phase B) at 1 ml/min for 48 minutes. The gradient (described as % of mobile phase B) is as follows: 10% at 0 mins, 35% at 33 mins, 100% 34-37 min, and 10% 38-48 mins. Separation is performed on a 4.6 mm $\times$ 150 mm, 3.5 µm column (Waters, XterraMS C18) with an identical 3.9 mm $\times$ 20 mm guard column, both controlled at 36°C (Brinkmann Eppendorf, Westbury, NY, CH-500 integrated heater/controller) (Note2). Standards, blanks, and samples are injected by means of a Waters, 717Plus Autosampler. Fluorescent peak height and area is evaluated at an excitation of 250 nm and an emission of 395 nm (Waters, 2475 Multi-wavelength Fluorescence Detector) and used to calculate the amount of amino acid. This system can be optimized to separate almost all amino acids and used to quantitate values of individual amino acids in plasma.

2.4 Radioactivity analysis and total protein concentration

1. One hundred µl aliquots from the intracellular fraction and from the PCA precipitate are placed in 5 ml of scintillation fluid and analyzed in a scintillation counter for ^{3}H for 1-5 min being sure to accumulate 10,000 cpm for statistical precision. 100 µl aliquots of 0.3 M NaOH are used in the blank samples, which also serve to control for chemiluminescence. The machine is set for quench correction and reports calculated d.p.m.
2. Protein concentration from the PCA precipitate samples is performed by using the BioRad protein detection reagent.

3. Calculations

Protein synthesis is calculated from the amount of radioactive L-phenylalanine incorporation into pancreatic protein using the specific radioactivity (DPM/nmol Phe) of pancreatic PCA-soluble L-phenylalanine as representative of the precursor pool and expressed as nanomoles of L-phenylalanine per milligram of protein.

4. Notes

Note1: This protocol for separation of amino acids was developed at the University of Michigan by Dr. Louis G. D'Alecy and Steve E. Whitesall.

Note2: Although an extremely small amount of radioactivity is injected, all column effluent solutions must be treated as radioactive. Because the effluent contains organic solvent, check with your safety people to how they want it collected.

Measurement of pancreatic protein synthesis *in vitro* by isolated pancreatic acini

Measuring protein synthesis *in vitro* in isolated pancreatic acini is similar to the techniques used for isolated lobules and any cell type in culture (7, 8, 19). In these *in vitro* systems, a radioactively labeled (with ^{35}S or ^{3}H) amino acid (usually methionine and leucine) is used, flooding the extracellular space with an excess of amino acid. The cell media is sampled as the precursor pool as it is the same for all samples and is readily accessible.

1. Materials

1.1 Suspension of pancreatic acini

Isolated pancreatic acini are typically obtained by collagenase digestion from mouse (about 20-30 g of body weight) or rat (about 150 g of body weight) pancreas. Preparation of isolated pancreatic acini is described elsewhere in The Pancreapedia.

1.2 Reagents and equipment

1. The radioactive tracer for *in vitro* studies is, most often, L-[^{35}S]-methionine (1,175 Ci/mM); from NEN Life Science Products (Boston, MA), because of its higher specific activity and because it has been shown that other amino acids that were usually used for these type of experiments (i.e. leucine) can stimulate the synthetic machinery (26) and this could potentially confound the results. A mixture of ^{35}S cysteine and methionine can also be used.
2. Trichloroacetic acid (TCA) and Sodium Hydroxide (NaOH) are from Sigma (St. Louis, MO)
3. Collagenase from Serva (NB8) or from Worthington (CLSPA).
4. The same scintillation liquid as for the *in vivo* samples.
5. A probe –type sonicator is used to homogenize acini samples and a Liquid Scintillation Analyzer, TRI-CARB 2100TR from Packard Bioscience (Meriden, CT).

2. Method

2.1 Methinonine incorporation into pancreatic protein

1. Pancreatic acinar suspension is prepared as described by Williams et al. (30) by collagenase digestion (for the preparation of pancreatic acini suspension it is important that the collagenase has a very low level of clostripain activity), and pre-incubated for 30 or 60 min in Hepes Ringer Buffer (HRB) (29) followed by a incubation period with the specific secretagogues that can also range from 30 to 60 min, as described by Sans et al. (24).
2. Fifteen min before the end of the incubation period 2 µCi/ml (final concentration) of [^{35}S]-methionine are added to the incubation media. The concentration of methionine in DMEM for example is 30 mg/L (166.5 µM) which is somewhat higher than the usual plasma values (around 40 µM during fasting, and 125-130 µM after re-feeding; Sans and Williams, unpublished results) (Note3)**.**
3. At the end of the incubation time, samples are collected and added to plastic tubes on ice, already containing 2 ml of ice-cold 154 mM NaCl, and centrifuged at 300 rpm for 5 min at 4^{o} C to remove supernatant.
4. The samples are rapidly washed twice with 2 ml of ice-cold NaCl and after discarding the last wash supernatant, 600-650 µl of ice-cold distilled water are added.

2.2 TCA precipitation

1. Samples are sonicated for 7 sec on ice and the same volume (600-650 µl) of ice-cold 20% TCA is added. TCA precipitation is for 30 min on ice, followed by

centrifugation at 10,000 rpm for 10 min at 4° C.

2. The supernatant is removed and pellet is washed twice with ice-cold 10% TCA and dissolved in 100-200 µl (depending of the pellet size and original acini concentration) of 0.1 N NaOH.

2.3 Radioactivity analysis and total protein concentration

1. For the analysis of incorporated radioactivity into TCA precipitable protein, 75-100 µl of the pellet re-suspended in NaOH are added to scintillation liquid in a scintillation vial and analyzed in a scintillation counter for 3 min. All samples contain an equal amount of water and NaOH to ensure equal quenching. Background samples contain NaOH to control for chemiluminiscence.
2. Protein concentration from the TCA precipitate samples is performed by using the BioRad protein detection reagent.

3. Calculations and results

Radioactivity obtained as cpm are used to express the amino acid incorporated into protein and differences among groups are expressed as percent change from control values. In these *in vitro* studies we do not use the specific activity of the intracellular pool and/or media for calculations, because the tracer concentration in the media is the same for all the samples. As an example of hormonal stimulation, the effects of CCK to stimulate total protein synthesis in pancreatic acini in a dose dependent manner, showing a biphasic response (Fig.2).

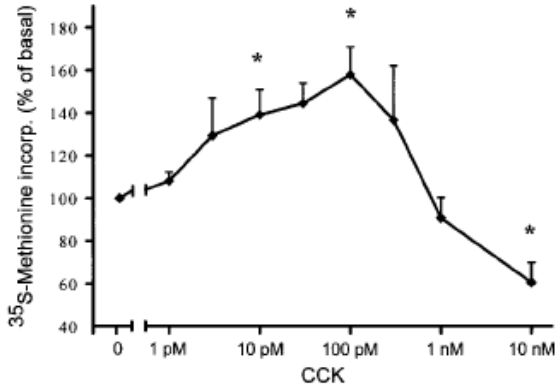

Figure 2. Effect of CCK octapeptide on L-[^{35}S]methionine incorporation into acinar protein. Rat pancreatic acini were incubated for 45 min with varying concentrations of CCK octapeptide before the addition of 2 µCi/ml of the labeled amino acid for 15 min. Incorporation was measured into TCA precipitable protein. Values are expressed as percent change from control values and are means ± SE of 4–8 experiments, each assayed in duplicate. *P 0.05 vs. control (basal group) (24).

4. Notes

Note3: If it is desired to separate individual protein on a gel (27) or by immunoprecipitation (19) S_{35} is used and it is usually necessary to increase the amount of radioactivity to 25-50 µCi/ml or to reduce the amount of carrier methionine. However, leaving out all carrier will change the intracellular precursor concentration.

5. References

1. **Bragado, MJ, Groblewski, GE and Williams, JA.** Regulation of protein synthesis by cholecystokinin in rat pancreatic acini involves PHAS-I and the p70 S6 kinase pathway. *Gastroenterology* 115: 733-742, 1998. PMID: 9721171

2. **Davis, TA, Fiorotto, ML, Nguyen, HV and Burrin, DG.** Aminoacyl-tRNA and tissue free amino acid pools are equilibrated after a flooding dose of phenylalanine. *Am J Physiol* 277: E103-109, 1999. PMID: 10409133

3. **Garlick, PJ, McNurlan, MA and Preedy, VR.** A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [3H]phenylalanine. *Biochem J* 192: 719-723, 1980.
4. **Gilliland, L and Steer, ML.** Effects of ethionine on digestive enzyme synthesis and discharge by mouse pancreas. *Am J Physiol* 239: G418-426, 1980. PMID: 6159794
5. **Kern, HF, Adler, G and Scheele, GA.** Structural and biochemical characterization of maximal and supramaximal hormonal stimulation of rat exocrine pancreas. *Scand J Gastroenterol Suppl* 112: 20-29, 1985. PMID: 3859914
6. **Kern, HF, Rausch, U and Scheele, GA.** Regulation of gene expression in pancreatic adaptation to nutritional substrates or hormones. *Gut 28 Suppl:* 89-94, 1987. PMID: 3319815
7. **Kimball, SR, Yancisin, M, Horetsky, RL and Jefferson, LS.** Translational and pretranslational regulation of protein synthesis by amino acid availability in primary cultures of rat hepatocytes. *Int J Biochem Cell Biol* 28: 285-294, 1996. PMID: 8920637
8. **Korc, M.** Effect of lanthanum on pancreatic protein synthesis in streptozotocin-diabetic rats. *Am J Physiol* 244: G321-326, 1983. PMID: 6299118
9. **Korc, M, Bailey, AC and Williams, JA.** Regulation of protein synthesis in normal and diabetic rat pancreas by cholecystokinin. *Am J Physiol* 241: G116-121, 1981. PMID: 6791509
10. **Korc, M, Iwamoto, Y, Sankaran, H, Williams, JA and Goldfine, ID.** Insulin action in pancreatic acini from streptozotocin-treated rats. I. Stimulation of protein synthesis. *Am J Physiol* 240: G56-62, 1981. PMID: 6161541
11. **Lahaie, RG.** Dietary regulation of protein synthesis in the exocrine pancreas. *J Pediatr Gastroenterol Nutr* 3 Suppl 1: S43-50, 1984. PMID: 6209378
12. **Lahaie, RG.** Translational control of protein synthesis in isolated pancreatic acini: role of CCK8, carbachol, and insulin. *Pancreas* 1: 403-410, 1986. PMID: 2436215
13. **Lampel, M and Kern, HF.** Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch A Pathol Anat Histol* 373: 97-117, 1977. PMID: 139754
14. **Lundholm, K, Ternell, M, Zachrisson, H, Moldawer, L and Lindstrom, L.** Measurement of hepatic protein synthesis in unrestrained mice-evaluation of the 'flooding technique'. *Acta Physiol Scand* 141: 207-219, 1991. PMID: 2048407
15. **Machovich, R, Papp, M and Fodor, I.** Protein synthesis in acute pancreatitis. *Biochem Med* 3: 376-383, 1970. PMID: 5523414
16. **O'Keefe S, J, Lee, RB, Li, J, Zhou, W, Stoll, B and Dang, Q.** Trypsin and splanchnic protein turnover during feeding and fasting in human subjects. *Am J Physiol Gastrointest Liver Physiol* 290: G213-221, 2006. PMID: 16123201
17. **O'Keefe, SJ, Lemmer, ER, Ogden, JM and Winter, T.** The influence of intravenous infusions of glucose and amino acids of pancreatic enzyme and mucosal protein synthesis in human subjects. *JPEN J Parenter Enteral Nutr* 22: 253-258, 1998. PMID: 9739026
18. **Ogden, JM, Modlin, IM, Gorelick, FS and Marks, IN.** Effect of buprenorphine on pancreatic enzyme synthesis and secretion in normal rats and rats with acute edematous pancreatitis. *Dig Dis Sci* 39: 2407-2415, 1994. PMID: 7525167
19. **Okabayashi, Y, Moessner, J, Logsdon, CD, Goldfine, ID and Williams, JA.** Insulin and other stimulants have nonparallel translational effects on protein synthesis. *Diabetes* 36: 1054-1060, 1987. PMID: 3301474
20. **Otsuki, M, Okabayashi, Y, Ohki, A, Suehiro, I and Baba, S.** Dual effects of hydrocortisone on exocrine rat pancreas. *Gastroenterology* 93: 1398-1403, 1987. PMID: 2445619
21. **Perkins, P and Pandol, S.** Cholecystokinin-induced changes in polysome structure regulate protein synthesis in pancreas. *Biochim Biophys Acta* 1136: 265-271, 1992. PMID: 1381613
22. **Saluja, A, Saito, I, Saluja, M, Houlihan, MJ, Powers, RE, Meldolesi, J and Steer, M.** In vivo rat pancreatic acinar cell function during supramaximal stimulation with caerulein. *Am J Physiol* 249: G702-710, 1985. PMID: 2417493
23. **Sans, MD, Dimagno, MJ, D'Alecy, LG and Williams, JA.** Caerulein-induced acute pancreatitis inhibits protein synthesis through effects on eIF2B and eIF4F. *Am J Physiol Gastrointest Liver Physiol* 285: G517-528, 2003. PMID: 12773302

24. **Sans, MD, Kimball, SR and Williams, JA.** Effect of CCK and intracellular calcium to regulate eIF2B and protein synthesis in rat pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol* 282: G267-276, 2002. PMID: 11804848
25. **Sans, MD, Lee, SH, D'Alecy, LG and Williams, JA.** Feeding activates protein synthesis in mouse pancreas at the translational level without increase in mRNA. *Am J Physiol Gastrointest Liver Physiol* 287: G667-675, 2004. PMID: 15117679
26. **Sans, MD, Tashiro, M, Vogel, NL, Kimball, SR, D'Alecy, LG and Williams, JA.** Leucine activates pancreatic translational machinery in rats and mice through mTOR independently of CCK and insulin. *J Nutr* 136: 1792-1799, 2006. PMID: 16772439
27. **Sans, MD and Williams, JA.** Calcineurin is required for translational control of protein synthesis in rat pancreatic acini. *Am J Physiol Cell Physiol* 287: C310-319, 2004. PMID: 15044154
28. **Schick, J, Kern, H and Scheele, G.** Hormonal stimulation in the exocrine pancreas results in coordinate and anticoordinate regulation of protein synthesis. *J Cell Biol* 99: 1569-1574, 1984. PMID: 6208198
29. **Sweiry, JH, Emery, PW, Munoz, M, Doolabh, K and Mann, GE.** Influx and incorporation into protein of L-phenylalanine in the perfused rat pancreas: effects of amino acid deprivation and carbachol. *Biochim Biophys Acta* 1070: 135-142, 1991. PMID: 1751520
30. **Williams, JA, Korc, M and Dormer, RL.** Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. *Am J Physiol* 235: 517-524, 1978. PMID: 215042