Tissue Distribution of Avian Pancreatic Polypeptide-Degrading Activity (42930)

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Abstract. **Degradation of avian pancreatic polypeptide (APP) by subcellular fractions from homogenates of chicken kidney, liver, and brain was characterized in this study. Chicken kidney cytosol exhibited the highest degrading activity of all kidney subcellular fractions studied including nuclear, mitochondrial, and microsomal. The cytosolic kidney APP-degrading activity was inhibited in a dose-dependent manner by bacitracin, serine protease inhibitors, and dithiothreitol, and eluted in the void volume of a Sephadex G-100 column, indicating that it is a soluble, serine protease-like activity with a M_r > 100,000 kDa and with some dependence on disulfide bonds. Soluble cytosol fractions from chicken liver, kidney, and brain all exhibited greater APP-degrading activity than that of corresponding membrane fractions and, furthermore, were similar in activity between one another. It is concluded that APP degradation by tissue homogenates occurs via a soluble, cytosolic protease which is inhibited by selected serine protease inhibitors; the activity does not differ among liver, kidney, and brain, three tissues which show different receptivity for APP.** [P.S.E.B.M. 1989, Vol 191]

Specific ^{125}I-avian pancreatic polypeptide (APP) binding sites have been described in a number of chicken tissues (1–5). The APP binding sites on chicken adipocytes and brain membranes are of sufficiently high affinity to be consistent with their being hormonal receptors and, indeed, evidence is favorable that those of chicken adipocytes are biologically relevant receptors (1). In our survey of chicken tissue membrane APP binding sites, kidney membranes were notable in that they did not bind ^{125}I-APP specifically and yet they actively degraded the peptide (5). The *in vivo* binding studies of Kimmel and Pollock (2) in chickens as well as extraction studies in humans and dogs (6, 7) also have suggested the kidney to be of prime importance in the metabolism of pancreatic peptide (PP). Determination of those organs responsible for metabolic clearance of PP is necessary for a more complete understanding of PP's physiologic significance. The purpose of this study was to characterize the chicken kidney APP-degrading activity in terms of subcellular distribution and susceptibility to inhibition by antiproteases and to quantitatively compare its activity and subcellular distribution with the APP-degrading activity present in chicken brain and liver. Such studies have not been reported to date and are a necessary prelude to purification and characterization of the enzyme system(s) responsible for APP degradation.

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Materials and Methods

Animals. Single-comb white Leghorn chickens (6–8 weeks old) of either sex were obtained from Rich-Glo, El Campo, TX, and were maintained at 21–22°C under a 12-hr light/12-hr dark cycle with free access to food and water. Chickens were allowed 3 days of acclimation before being used in experiments.

Chemicals and Hormones. The following were obtained from Sigma Chemical Co., St. Louis, MO: Tris; bovine serum albumin, fraction V (BSA); chloramine-T; bacitracin; aprotinin; benzamidine; tosyl arginine methyl ester (TAME); and dithiothreitol. Lima bean trypsin inhibitor was obtained from Worthington Biochemical Co., Freehold, NJ.

Avian pancreatic polypeptide from chickens (lot nos. II-286 and II-216-217) and turkeys (lot nos. Joy III-84 and TCT-NB-1) was a gift from Dr. J. R. Kimmel, Kansas University Medical School, Kansas City, KS, and the VA Hospital Kansas City, MO. APP was iodinated using chloramine-T (8, 9). Specific activities of 73–313 μCi/μg were obtained, the iodinated peptide was 90–97% precipitable with trichloroacetic acid (TCA), and there was an average of less than one iodine/mole of APP. The ^{125}I-APP used in these studies was probably iodinated at the C-terminus TYR-36 as shown by other workers for oxidative PP iodination

(10). However, the preparation was neither monoiodinated nor carrier free.

Preparation of Subcellular Fractions. In the first series of experiments, four chickens were sacrificed and portions of their kidneys were removed and homogenized in 10 volumes of 0.25 *M* sucrose for 45 sec using the Polytron at a setting of 7. Subcellular fractions were obtained by differential centrifugation (11, 12) with minor modifications. Homogenates were centrifuged at 600*g* for 10 min, after which the supernatant was centrifuged at 12,000*g* for 30 min. The resulting supernatant was adjusted to 0.1 *M* NaCl and 0.1 m*M* $MgSO_4$ and centrifuged at 45,000*g* for 60 min. This final supernatant was saved. The three pellets (600*g*, P_1; 12,000*g*, P_2, 45,000*g*, P_3) were washed once in 25 m*M* Tris (pH 7.4) and then resuspended in the same buffer. Aliquots of the original homogenates, as well as the membrane fractions and final supernatant, were stored at −32°C until assayed.

In the second series of experiments, three chickens were sacrificed and brain, liver, and kidney were removed and homogenized individually as described above. The homogenates were centrifuged at 480*g* for 10 min and the resulting pellets were discarded. The supernatant from this centrifugation step was then centrifuged at 105,651*g* for 60 min. The resulting pellet (P) was washed once in 0.25 *M* sucrose and then resuspended in sucrose and stored at −32°C, as was the 105,651*g* supernatant (cytosol) and aliquots of the original homogenate.

In all cases, protein concentrations were determined according to the method of Lowry *et al.* (13) after TCA precipitation and NaOH solubilization. BSA was used as a standard.

Assay of ^{125}I-APP-Degrading Activity. For Series I experiments, chicken kidney homogenate, particulate fraction, or cytosol was diluted in the appropriate medium to a protein concentration of 800 μg/ml. Fifty microliters were added to 150 μl of 25 m*M* Tris, 10 m*M* $MgCl_2$, and 0.5% BSA (pH 7.4) containing ^{125}I-APP at 0.1 ng/ml final concentration. The reactions were conducted in glass 12- x 75-mm test tubes at 25°C in a Dubnoff shaking incubator and were terminated by placing the tubes in an ice-water bath and adding 2 ml of 10% TCA and 0.5 ml of 2% BSA. The tubes were kept at 4°C until they were centrifuged at 3000 rpm for 15 min (4°C). The supernatants were aspirated and the pellets, representing nondegraded ^{125}I-APP, were counted in a Nuclear-Chicago model 1185 gamma counter. Counts were converted to absolute disintegrations using the formula of Eldridge and Crowther (14).

^{125}I-APP-degrading activity in Series II experiments was determined similarly, except that a citrate phosphate buffer (pH 7.4) containing 0.5% BSA was used. Fifty microliters of homogenate, membrane pellet, or cytosol diluted in 0.25 *M* sucrose to contain 40 μg of protein were added to 150 μl of buffer containing ^{125}I-APP (final concentration 0.2 ng/ml) to initiate the reaction.

Degradation of ^{125}I-APP is expressed as the percentage of TCA-precipitable disintegrations present at time 0 rendered soluble in TCA. In both Series I and Series II, control incubations containing the appropriate diluent in place of tissue were conducted in order to assess the extent of nonenzymatic degradation of ^{125}I-APP.

Results

Series I. Subcellular Distribution of ^{125}I-APP-Degrading Activity in Chicken Kidney. As seen in Figure 1, membrane fractions as well as soluble supernatants from chicken kidney homogenates exhibited time-dependent ^{125}I-APP-degrading activity. The soluble supernatant (45,000*g* supernatant) possessed by far the greatest APP-degrading activity per unit protein of all subcellular fractions studies. Initially, while the ^{125}I-APP degradation was linear with respect to time, the soluble supernatant degraded 5–10 times more APP than did the membrane fractions and twice as much as did the whole homogenate. At later time points, when the rate of degradation was no longer linear, cytosol ^{125}I-APP degradation remained 2- to 5-fold above that of membrane fractions. Since soluble supernatant protein accounted for 42.8 ± 3.3% of total homogenate protein, recovery of total APP degrading activity was also greatest in the soluble supernatant. Membrane fractions exhibited very low APP-degrading activity relative to the soluble fraction, especially at early incubation times (e.g., up to 15 min) where it approximated or marginally exceeded the nonenzymatic APP degradation. The latter was less than 4% over the entire time course, irre-

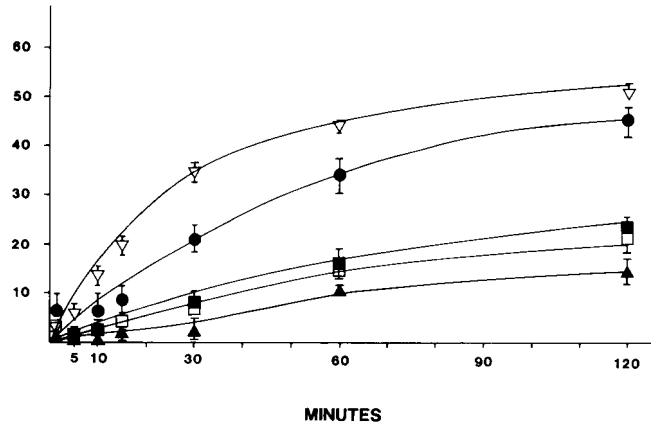

Figure 1. Time course of ^{125}I-APP degradation by chick kidney. Chicken kidney homogenates (●) were separated by differential centrifugation into a 600*g* pellet (P_1, ▲), a 12,000*g* pellet (P_2, ■), a 45,000*g* pellet (P_3, □), and the resulting cytosol supernatant (▽). Forty micrograms of protein from each of these fractions were assayed for ^{125}I-APP degradation over a time course from 2 to 120 min. Degradation was assessed by plotting the increase in TCA-soluble radioactivity, as a percentage of the total, versus incubation time. Data are shown as mean ± SE for preparations from four different chickens.

spective of the diluent tissue fractions in which they were present (i.e., 0.25 *M* sucrose, 0.25 *M* sucrose + 0.1 *M* NaCl and 0.2 m*M* $MgSO_4$, or 25 m*M* Tris, pH 7.4). At later incubation times (i.e., 30–120 min), ^{125}I-APP degradation by the membrane fractions proceeded fairly linearly with the 12,000*g* and 45,000*g* pellets showing slightly greater APP-degrading activity than did the 600*g* pellet (Fig. 1). It is possible, however, that all of the degrading activity associated with the pellets was due to trace amounts of adhering, contaminating soluble fraction.

Effects of Protease Inhibitors on ^{125}I-APP Degradation by Chicken Kidney-Soluble Fraction. The data presented in Figure 1 indicated that the predominant ^{125}I-APP-degrading activity in chicken kidney was cytosolic. This cytosolic activity was studied for its susceptibility to inhibition by selected antiproteases. Antiproteases were co-incubated with kidney cytosol and ^{125}I-APP for 30 min at 25°C, and the TCA-precipitable disintegrations per minute were determined. The results are shown in Figures 2–4. As seen in Figure 2, bacitracin but not benzamidine (a trypsin inhibitor reported to inhibit glucagon degradation (15)) inhibited ^{125}I-APP degradation, with complete inhibition seen at a concentration of 500 μM. Similarly, the serine protease inhibitors aprotinin and lima bean trypsin inhibitor inhibited ^{125}I-APP degradation by chicken kidney cytosol in dose-dependent manners, as seen in Figures 3 and 4. For both of these antiproteases, complete inhibition of degradation occurred at concentrations of 1 m*M*. In contrast, the artificial trypsin substrate TAME had essentially no inhibitory effect (Fig. 3) while the reductant dithiothreitol was capable of some inhibition of ^{125}I-APP degradation (Fig. 4). However, at maximally inhibitory concentrations of 0.5 to 1 m*M*,

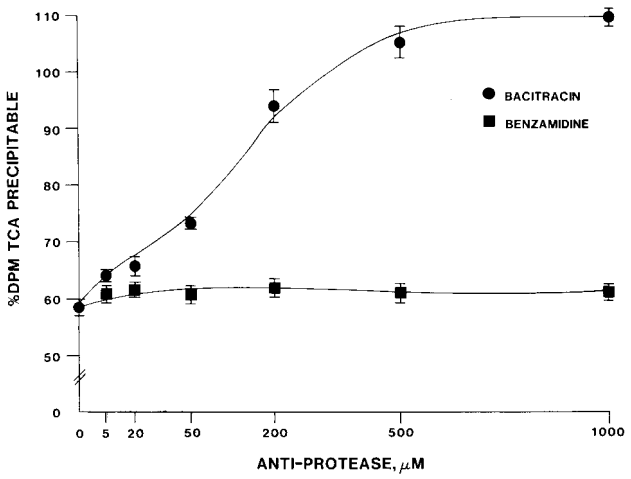

Figure 2. Degradation of ^{125}I-APP by kidney cytosol: effect of bacitracin and benzamidine. Chick kidney cytosol (40 μg of protein) was incubated with ^{125}I-APP and the indicated concentrations of bacitracin (●) or benzamidine (■) for 30 min at 25°C, and the TCA precipitable radioactivity was measured. Degradation is assessed as the percentage of ^{125}I-APP remaining TCA precipitable, which is shown as mean ± SE for cytosols from four chickens.

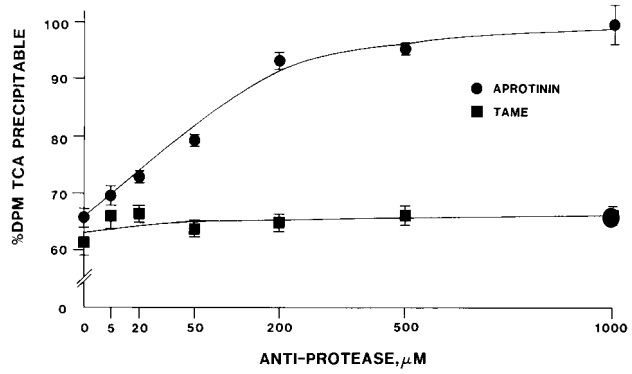

Figure 3. Degradation of ^{125}I-APP by kidney cytosol: effect of aprotinin and TAME. Chick kidney cytosol (40 μg of protein) was incubated with ^{125}I-APP and the indicated concentrations of aprotinin (●) or TAME (■) for 30 min at 25°C, and TCA-precipitable radioactivity was measured. Degradation is expressed as the percentage of ^{125}I-APP remaining TCA precipitable, which is shown as mean ± SE for cytosols from four chickens.

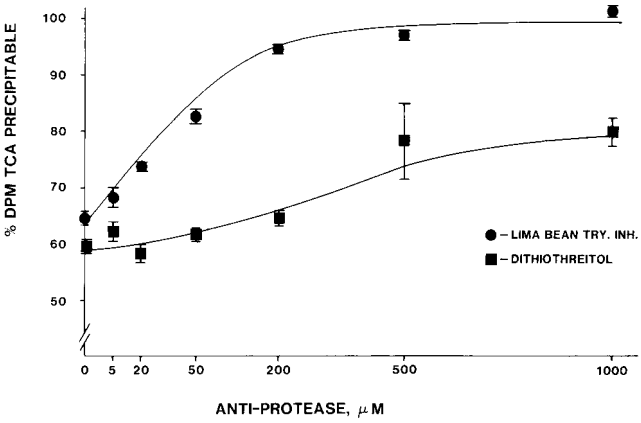

Figure 4. Degradation of ^{125}I-APP by kidney cytosol: effect of lima bean trypsin inhibitor and dithiothreitol. Chick kidney cytosol (40 μg of protein) was incubated with ^{125}I-APP and the indicated concentrations of lima bean trypsin inhibitor (●) or dithiothreitol (■) for 30 min at 25°C, and TCA-precipitable radioactivity was measured. Degradation is expressed as the percentage of ^{125}I-APP remaining TCA precipitable, which is shown as mean ± SE for cytosol from four chickens.

dithiothreitol did not completely inhibit ^{125}I-APP-degrading activity (Fig. 4).

Other experiments, for which data are not presented herein, indicated that chicken kidney cytosolic ^{125}I-APP-degrading activity eluted in the void volume of a Sephadex G-100 column, suggesting that it has a $M_r > 100{,}000$ kDa.

Series II. Comparison of Subcellular Distribution and Activity of ^{125}I-APP Degradation by Chicken Kidney, Liver, and Brain. Interpretation of the studies presented in Series I is complicated by the fact that the fractions were present in different buffers (i.e., sucrose for the homogenate, sucrose + salts for the cytosol, and Tris for the membrane fractions). The reason for these conditions was a desire to utilize an assay buffer identical to that used for studies on ^{125}I-APP binding to chicken tissues. However, it was desirable to remove

the influence of ions and different buffers in order to compare subcellular APP-degrading activity in different chicken tissues. Therefore, in Series II, homogenates from chicken liver, brain, and kidney were separated into two major fractions, cytosol and membrane, and both of these fractions were, like the homogenate, in 0.25 *M* sucrose. A time course of ^{125}I-APP degradation by the tissue cytosols (Fig. 5) revealed that brain and liver, as well as kidney, cytosol avidly degraded ^{125}I-APP. Subsequent determinations of ^{125}I-APP degradation by the homogenate, membrane, and cytosol fractions were conducted for 15 min. The results of these studies are shown in Figure 6. There were no major differences in cytosolic ^{125}I-APP degradation among liver, kidney, and brain, nor did the membrane fractions from any of these tissues degrade ^{125}I-APP to an appreciable extent by 15 min. Thus, as seen previously for chicken kidney, cytosol from chicken liver and brain possessed essentially all of the ^{125}I-APP-degrading activity at pH 7.4. The results with the membrane fraction (Fig. 6) are similar to the results seen in Figure 1, where at 15 min, ^{125}I-APP degradation by the P_1, P_2, and P_3 kidney fractions was at, or just slightly above, nonenzymatic breakdown.

The Series II results thus suggest strongly that ^{125}I-APP-degrading activity is exclusively localized to the soluble cytosol of chicken kidney, liver, and brain and that the activity is equivalent in these three tissues.

All of the assays presented above were conducted at pH 7.4. It is possible that membrane fractions possess

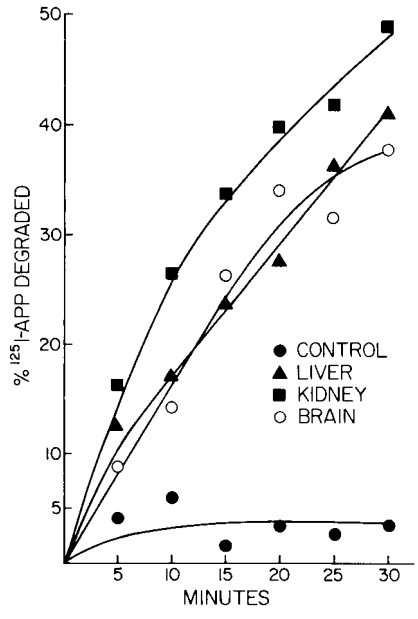

Figure 5. Time course of ^{125}I-APP degradation by chicken tissue cytosol. Cytosol (40 μg of protein) from chicken liver (▲), kidney (■), and brain (○) was incubated with ^{125}I-APP in a citrate phosphate buffer (pH 7.4) at 25°C. The reaction was stopped at the times indicated and the TCA-precipitable radioactivity determined. Control incubations (●) contained diluent in place of cytosol. Data are means of duplicate determinations in a single experiment.

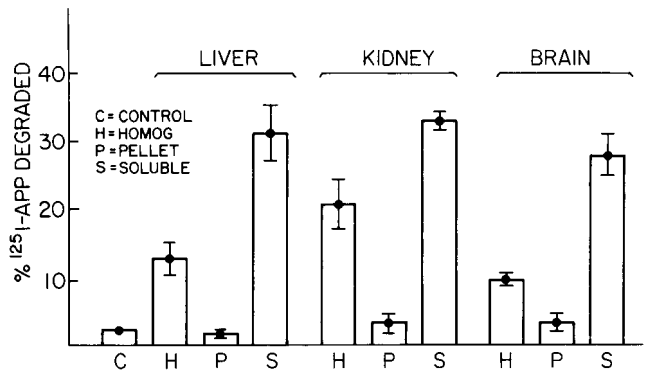

Figure 6. Subcellular distribution of ^{125}I-APP degradation by chicken liver, kidney, and brain tissue. Aliquots of homogenate (H), total membrane pellet (P), and cytosol (S), all containing 40 μg of protein or diluent alone (C), were incubated with ^{125}I-APP in a citrate phosphate buffer (pH 7.4) for 15 min at 25°C, after which the reactions were terminated and TCA-precipitable radioactivity was counted. Data are means of duplicate determinations done on tissue preparations from three different chickens. Vertical bars are ±SEM.

important lysosomal proteases which would only be active at acid pH. Preliminary results indicated that ^{125}I-APP-degrading activity at pH 5 was abolished in tissue cytosols, whereas in the membrane fraction there appeared to exist an APP-degrading activity exceeding the rate of nonenzymatic degradation. However, this membrane acid protease APP-degrading activity, if it exists, is very low, certainly well below the cytosolic APP-degrading activity observed at pH 7.4.

Discussion

In this study, we have made the initial demonstration of tissue and subcellular distribution of APP-degrading activity. The activity (at pH 7.4) appears to be concentrated in the soluble cytosol of chicken brain, liver, and kidney. Similar cytosolic localization of insulin protease activity has been reported (16, 17). Although previous studies utilizing membranes from chicken tissues incubated over prolonged periods with ^{125}I-APP indicated that kidney degraded more ^{125}I-APP than did liver or brain, the present studies utilizing shorter incubation times and subcellular fractionation suggest that cytosolic APP degradation is similar in liver, brain, and kidney, and that over these shorter incubation periods, membrane proteolysis of ^{125}I-APP is minimal compared with that of cytosol.

The studies using antiproteases and kidney cytosol suggest that the APP-degrading activity is serine protease-like, with some dependence on disulfide bonds, and has an M_r > 100,000 kDa based on Sephadex chromatography. Bacitracin inhibits chick kidney APP degradation as shown here and elsewhere (4), as well as chick hepatocyte APP degradation (3) and insulin degradation in a number of systems (18, 19). Interestingly, the inhibitory effect of bacitracin on cellular insulin degradation is associated with cell-specific increase, decrease, or lack of change in insulin binding (19). We

have found that bacitracin is inhibitory to chick liver and cerebellar ^{125}I-APP-specific binding, while inhibiting its degradation by both types of membranes (Adamo and Hazelwood, unpublished observations).

It is of interest that three tissues which show different APP receptivity (4) degrade the hormone to a similar degree. It is possible that significant tissue-specific degradation/alteration of APP occurs without measurable loss of TCA precipitability. Such processing will require high-pressure liquid chromatography methods of analysis. Clearly, the present results do not allow any conclusions as to the specificity of the APP-degrading activity. Our previous membrane studies indicated that unlabeled APP was more effective in inhibiting ^{125}I-APP degradation by kidney than was unlabeled bovine pancreatic polypeptide (4). Also, we have found that *N*-ethylmaleimide inhibits both liver and cerebellar membrane APP degradation and inhibits cerebellar (while stimulating liver)-specific ^{125}I-APP binding (Adamo and Hazelwood, unpublished data). These results, like those with bacitracin, and the fact that cytosolic APP degradation is similar in the three different tissues suggest an uncoupling of APP binding and degradation. It will be necessary in future studies to purify the degrading enzyme(s) from the three tissues and characterize it (them) in order to determine the specificity of the process, relationship to hormone binding, and potential role in regulation of APP action.

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1. McCumbee WD, Hazelwood RL. Biological evaluation of the third pancreatic hormone (APP): Hepatocyte and adipocyte effects. Gen Comp Endocrinol **33**:518–525, 1977.
2. Kimmel JR, Pollock HG. Target organs for avian pancreatic polypeptide. Endocrinology **109**:1693–1699, 1981.
3. Cramb G, Langslow DR, Phillips JH. The binding of pancreatic hormones to isolated chicken hepatocytes. Gen Comp Endocrinol **46**:297–309, 1982.
4. Adamo ML, Dyckes DF, Hazelwood RL. *In vitro* binding and degradation of avian pancreatic polypeptide by chicken and rat tissues. Endocrinology **113**:508–516, 1983.
5. Adamo ML, Hazelwood RL. Cerebellar binding of avian pancreatic polypeptide. Endocrinology **114**:794–800, 1984.
6. Boden G, Master RW, Owen OE, Rudnick MR. Human pancreatic polypeptide in chronic renal failure and cirrhosis of the liver: Role of kidneys and liver in pancreatic polypeptide metabolism. J Clin Endocrinol Metab **51**:573–577, 1980.
7. Ishida T, Chou MCY, Chou K, Lewis RM, Hartley CJ, Entman M, Kimmel JR, Field JB. Absence of hepatic extraction of pancreatic polypeptide in conscious dogs. Am J Physiol **245**:E401–E409, 1983.
8. Cuatrecasas P. Insulin-receptor interactions in adipose tissue cells: Direct measurement and properties. Proc Natl Acad Sci USA **68**:1264–1268, 1971.
9. Cuatrecasas P, Hollenberg MD. Membrane receptors and hormone action. Adv Prot Chem **30**:252–261, 1976.
10. Chance RE, Moon NE, Johnson MG. Human pancreatic polypeptide (HPP) and bovine pancreatic polypeptide (BPP). In: Jaffe BM, Behrman HR, Eds. Methods of Hormone Radioimmunoassay. 1st ed. New York: Academic Press, p 657, 1979.
11. Cuatrecasas P. Isolation of the insulin receptor of liver and fat cell membranes. Proc Natl Acad Sci USA **69**:318–322, 1972.
12. Posner BI, Kelly PA, Shiu RPC, Friesen HG. Studies on insulin, growth hormone and prolactin binding: tissue distribution, species variation and characterization. Endocrinology **95**:521–531, 1974.
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
14. Eldridge JS, Crowther T. Determination of ^{125}I in clinical applications. Nucleonics **22**:56–59, 1964.
15. Ensinck, JW, Shepard C, Dudl RJ, Williams RH. Use of benzamidine as a proteolytic inhibitor in the radioimmunoassay of glucagon in plasma. J Clin Endocrinol Metab **35**:463–467, 1972.
16. Duckworth WC. Insulin and glucagon degradation by the kidney. I. Subcellular distribution under different assay conditions. Biochim Biophys Acta **437**:518–530, 1976.
17. Brush JS, Jering H. The importance of proteolysis as the initial step of insulin degradation in rat liver homogenates. Endocrinology **104**:1639–1643, 1979.
18. Roth RA. Bacitracin: An inhibitor of the insulin degrading activity of glutathione-insulin transhydrogenase. Biochem Biophys Res Commun **98**:431–438, 1981.
19. Gansler TS, Smith RM, Jarett L. Cell type-specific variability of bacitracin's effects on insulin binding and intracellular accumulation. Diabetes **35**:392–397, 1986.