Large Glucagon Immunoreactivity in Extracts of Pancreas*

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SUMMARY

The glucagon immunoreactivity present in canine pancreatic extracts can be separated by gel filtration into two fractions; one fraction, comprising over 90% of the immunoreactivity, is similar in molecular size to the glucagon-¹³¹I marker, while the other appears to be at least twice as large. Incubation of this large glucagon immunoreactivity (LGI) in solutions such as 1 M acetic acid, 8 M urea, 0.1 M mercaptoethanol, or 6 M guanidine hydrochloride, which favor the dissociation of noncovalent protein complexes, fails to change its molecular size as determined by gel filtration, suggesting that LGI is not glucagon noncovalently bound either to itself or to another protein. However, tryptic hydrolysis of LGI results in the disappearance of most of the immunoreactivity and in a reduction in the molecular size of the remaining immunoreactivity to approximately that of glucagon-¹³¹I. Neither LGI nor its tryptic product, both of which resemble glucagon immunologically, has glycogenolytic activity in the perfused rat liver. It is possible that LGI includes the immunoreactive sequence of glucagon or of an immunologically similar peptide.

METHODS AND MATERIALS

Pancreatic Extraction Procedure—Pancreatic tissue was resected from mongrel dogs immediately after their death from Nembutal overdose. The pancreases were frozen promptly in Dry Ice and were then stored at -20°. Within 1 month they were extracted by the acid-alcohol method of Kenny (3). Extracts were lyophilized and stored in a vacuum at 4° until the time of use within 2 months. At that time they were reconstituted in 0.05 M NH₂HCO₃ solution at pH 8.8 so as to give an initial concentration ranging from 20 to 50 mg of extract, dry weight, per ml of solution. After at least 2 hours of grinding with a glass rod and overnight dialysis against 0.025 or 0.05 M NH₂HCO₃ solution at pH 8.8, the reconstituted extract was centrifuged at 2000 rpm for 20 min, and the sediment was discarded. The extract solution was subjected to chromatography immediately after. The specific activity of these crude extracts averaged 1 μg of glucagon immunoreactivity per mg of protein.

Gel Filtration Procedures—The initial chromatography of the reconstituted extract was carried out on Bio-Gel P-10 (100 to 200 mesh) columns in siliconized glass cylinders, 5 × 115 cm. They were equilibrated in 0.05 M NH₄HCO₃ and adjusted with NaOH to pH 8.8. Following calibration of each column with blue dextran 2000, ribonuclease, insulin-¹³¹I, and glucagon-¹³¹I, 30 ml of the extract solution, containing 120 to 180 mg of protein (Lowry) and a trace of insulin-¹³¹I and glucagon-¹³¹I, were subjected to column chromatography at 4°. A flow rate of approximately 1.2 ml per min was maintained and fractions of 20 ml were collected in an automatic fraction collector and stored at -20°.

Rechromatography of eluates was carried out as follows: each pool of eluates containing LGI or its tryptic derivatives was concentrated by lyophilization and then dialyzed overnight at 4° against 0.001 M NH₄HCO₃ adjusted to pH 8.8 with NaOH, and lyophilized. Bio-Gel P-10 (100 to 200 mesh) columns in siliconized glass cylinders, 0.9 × 115 cm, were equilibrated with one of the following solutions: 0.05 M NH₄HCO₃ at pH 8.8, 1 M acetic acid at pH 2.2, 8 M urea at pH 8.1, 0.1 M mercaptoethanol in 8 M urea at pH 7.0, or 6 M guanidine hydrochloride at pH 3.7. A flow rate of from 0.12 to 0.21 ml per min was maintained. Columns were calibrated with blue dextran, glucagon-¹³¹I, and insulin-¹³¹I. The lyophilized pools of eluates were reconstituted and incubated at 25° in one of the above solutions at a concentration of 17 mg of protein per ml. After the addition of insulin-¹³¹I and glucagon-¹³¹I in tracer amounts as molecular weight markers, the pools were subjected to rechromatography at room temperature. In order...
to obtain optimal resolution no more than 10 mg of protein in a volume of 0.6 ml were applied to a column. Fractions of 1 ml were collected in an automatic fraction collector.

**Polyacrylamide Gel Electrophoresis**—Disc electrophoresis was carried out by the method of Davis (4) on 10% polyacrylamide gel at room temperature in Tris-glycine buffer at pH 8.3, with the use of a Canalco model 12 apparatus. From 20 to 40 µl of sample containing 100 µg of protein were applied. A constant current of 4 mA per tube was maintained with a Spinco Duostat power supply. Gels were either stained with 1% Amido black or divided into 5-mm sections for glucagon assay. Each section was placed in a tube containing 0.5 ml of assay diluent (0.2 M glycine at pH 8.8 with 0.25% sodium bicarbonate and 1:100 normal rabbit serum). After thorough grinding of the gel with a glass rod for at least 2 hours, the mixture was centrifuged at 2000 rpm for 30 min, and the supernatant fluid was assayed for glucagon immunoreactivity. About 95% of the applied glucagon immunoreactivity could be recovered.

**Tryptic Hydrolysis**—Tryptic hydrolysis was carried out as follows: a lyophilized pool of eluates containing LGI2 dissolved in 0.5 ml of 0.05 M NH4HCO3 at pH 8.8 and trypsin at a concentration of 1 mg per ml of water was added so as to give an enzyme to protein ratio of 1:100. After gentle stirring at room temperature for 15 min, an aliquot of the mixture was removed and stored at -20°C until the time of glucagon assay, and the remainder was subjected to immediate rechromatography.

**Determination of Biological Activity**—Glycogenolytic activity of various samples in vitro was examined by measuring glucose production by the perfused rat liver. The perfusion system and method was modified only slightly from that used by Mortimore (5). The perfusate consisted of a 20% suspension of outdated human red cells, washed twice in 0.9% NaCl and twice in Krebs-Ringer phosphate buffer at pH 7.4 and then suspended in Krebs-Ringer phosphate buffer at pH 7.4 with 3% bovine albumin (Cohn Fraction V). A perfusion apparatus with a Harvard peristaltic pump was used. Fed Sprague-Dawley rats weighing between 120 and 150 g were prepared for liver perfusion. After a 30 min equilibration period the sample to be tested, dissolved in 1 ml of assay diluent, was introduced into the drum reservoir for 60 to 80 min of perfusion. The perfusion was conducted at a constant rate of 7 ml per min. One-milliliter aliquots of perfusate were withdrawn for glucose analysis from the tubing on the portal side of the liver at 5- and 10-min intervals throughout the perfusion.

Hyperglycemic activity in vivo was determined by measuring venous glucose concentration after the endoporal injection of the sample in an anesthetized dog, as previously described (6).

**Analytical Methods**—Eluates were assayed for protein by means of absorbance at 280 mµ in a DU spectrophotometer or by the method of Lowry et al. (7), or both. Glucagon immunoreactivity of eluates was determined by the previously described radioimmunoassay (8), in which antiserum highly specific for pancreatic glucagon were used. Eluates containing urea, acetic acid, or guanidine hydrochloride, were either dialyzed at 4°C for 48 hours against 0.025 M NH4HCO3 at pH 8.8 or diluted to concentrations which could not influence the assay. Eluates containing mercaptoethanol were lyophilized. Elution patterns of the 125I-labeled markers were determined by assay of eluates for radioactivity.

Glucose concentration of samples of liver perfusate and of canine plasma was determined by the method of Hoffman (9) with the use of the Technicon autoanalyzer.

**RESULTS**

**Chromatography of Canine Pancreatic Extracts**—The elution pattern of extractable glucagon immunoreactivity of the canine pancreas from Bio-Gel P-10 column is shown in Fig. 1A, which is representative of eight such experiments. More than 90% of the immunoreactivity appeared in the zone of the glucagon-125I marker, indicating a molecular weight in the 3500 range. A small fraction of immunoreactivity, less than 10% of the total, appeared before the insulin-125I marker, indicating a molecular size at least twice that of glucagon.

To determine whether spontaneous dissociation of LGI occurs under these conditions, a concentrated pool of eluates containing 6 mg of protein and 150 to 300 mcg eq of LGI in 0.6 ml was rechromatographed on a Bio-Gel P-10 column in NH4HCO3 solution at pH 8.8, as described under "Methods and Materials." Almost all the immunoreactivity remained in its original zone, eluting before the insulin-125I marker. Fig. 1B illustrates the elution pattern of rechromatographed LGI obtained in each of two such experiments.

![Fig. 1. A (upper), elution pattern of glucagon immunoreactivity in extracts of canine pancreas. A typical pattern showing a small early appearing peak of immunoreactivity, LGI. B (lower), elution pattern of immunoreactivity after the rechromatography of the LGI-containing eluates. No shift in elution volume is noted.](http://www.jbc.org/)
To determine whether self-aggregation of glucagon under these conditions might account for the small fraction of large glucagon immunoreactivity, crystalline beef-pork glucagon was subjected to rechromatography in the same manner. Rechromatography of 4 mg of glucagon in 0.5 ml of NH₄HCO₃ solution disclosed no evidence of aggregation, all of the immunoreactivity remaining in the glucagon-¹³¹I zone.

Elution Pattern of Pancreatic Glucagon Immunoreactivity in Other Species—To determine whether pancreatic extracts of other species also contain a large sized fraction of glucagon immunoreactivity, whole pancreas of duck and beef, obtained immediately after death, a human pancreas obtained at autopsy, and rat islets of Langerhans, isolated by the technique of Lacey and Kostianovsky (10), were extracted. The elution pattern of immunoreactivity of each species is shown in Fig. 2. In each case a small but significant quantity of immunoreactivity appeared in or before the zone of the insulin-¹³¹I marker. Although these represent only single experiments for each species, they suggest that large sized immunoreactivity is not confined to the dog.

Separation of Large Glucagon Immunoreactivity from Unidentified Proteins—As shown in Fig. 1A, the protein peak of the pancreatic extract is located very near the region of large glucagon immunoreactivity, raising the possibility that the high protein concentration, rather than true immunoreactivity, might have given rise to nonspecific readings in the radioimmunoassay. In order to eliminate this possibility, separation of the immunoreactivity from most of the protein was attempted by disc electrophoresis on polyacrylamide gel. The results, illustrated in Fig. 3, indicate that the immunoreactivity was not present in the sections of gel containing the most protein, Sections 1 and 2, but was present in the sections containing little or no protein, Sections 3 and 4. Large glucagon immunoreactivity does not, therefore, appear to be a nonspecific consequence of the high protein concentration of eluates in which it appears.

Biological Activity of Large Glucagon Immunoreactivity—In order to determine if LGI has biological activity, its glycogenolytic effect was determined in the perfused rat liver system of Mortimore (5). In every one of 19 experiments, perfusion of approximately 20 mpg of either crystalline beef-pork glucagon or dog glucagon was associated with an unequivocal glycogenolytic response as compared to the buffer control. Yet in each of four experiments the perfusion of 40 mpg eq of LGI failed to elicit a significant glycogenolytic response; in three additional experiments 100 mpg eq of LGI were also found to be inactive (Fig. 4). (This lack of response cannot be attributed to contaminating insulin, since the perfusion of insulin alone did not reduce glucose production in this liver system; furthermore, the 100 mpg doses of LGI had been first treated with mercaptoethanol to destroy the insulin.) To assess its biological activity in vivo 1 ug eq of LGI was injected endoportally in an anesthetized dog. As shown in Fig 5, LGI caused no rise in glucose, in contrast to 1 ug of crystalline beef-pork glucagon which caused a rise of 20 mg per 100. However, this LGI-containing eluate pool also contained 10 units of insulin, thus accounting for its hypoglycemic effect; it seemed possible, therefore, that a hyperglycemic effect of LGI might have been nullified by the insulin. To evaluate this possibility,
1 µg of crystalline glucagon containing 10 units of insulin was also administered. The results in Fig. 5 indicate that this amount of insulin does not prevent the initial hyperglycemic activity of crystalline glucagon.

These findings suggest that large glucagon immunoreactivity is devoid of glycojenolytic activity in vitro and in vivo.

Effect upon Large Glucagon Immunoreactivity of Solutions Favoring Dissociation of Noncovalent Protein Complexes—It seemed possible that, under the conditions prevailing during column chromatography, the LGI molecule might consist of a molecule of glucagon (mol. wt. 3485) bound noncovalently either to itself or to another protein in such a way as to render it biologically inactive. To test this possibility LGI was incubated with and rechromatographed in several solutions which favor the dissociation of protein complexes.

If LGI consisted of glucagon in complex with a basic protein, the complex should dissociate at low pH. Therefore, eluates containing 176 µg of LGI were incubated at 25° in 1 M acetic acid at pH 2.2 for 1 hour. The elution volume of the immunoreactivity from the zone preceding the insulin-131I marker was found to have shifted and now appeared an average of 8 min after the glucagon-131I peak. A representative elution pattern of the tryptic product of LGI is shown in Fig. 6.

Effect of Urea and Guanidine Hydrochloride on Large Glucagon Immunoreactivity—Both urea and guanidine hydrochloride in high concentration are known to cause dissociation of polypeptide chains held together by noncovalent forces. Thus eluates containing 300 µg of LGI were incubated at 25° for 48 hours in 8 M urea at pH 8.1 (one experiment) and for 24 hours in 6 M guanidine at pH 3.7 (one experiment) and were then rechromatographed in the respective solution. Neither urea nor guanidine incubation caused a shift in the elution volume of the immunoreactivity from the zone preceding the insulin-131I marker.

The failure of acetic acid, urea, or guanidine hydrochloride to alter the molecular size of LGI makes unlikely the possibility that it represents a glucagon molecule bound noncovalently either to itself or to another protein.

Effect of Mercaptoethanol on Large Glucagon Immunoreactivity—Although glucagon itself contains no cysteine, it seemed possible that LGI might contain cysteine, perhaps as part of an interchain disulfide bridge. To exclude this possibility, a lyophilized pool of eluates containing 800 µg of LGI was incubated overnight in 0.5 ml of 0.1 M mercaptoethanol in 8 M urea and then rechromatographed in the same solution. Again, there was no change in the elution volume of the immunoreactivity, although all of the radioactivity of the insulin-131I marker had shifted to the zone of glucagon-131I, indicating reductive cleavage of its chains.

Effect of Trypsin on Large Glucagon Immunoreactivity—To determine if LGI also is sensitive, a lyophilized pool of LGI-containing eluates, containing 17.5 mg of protein and 1.5 µg eq of LGI, was dissolved in 0.5 ml of 0.05 M NH4HCO3 at pH 8.8 and was incubated with trypsin at an enzyme to protein ratio of 1:100 for 10 min at room temperature. Three such experiments were carried out. In two additional experiments at the same enzyme to protein ratio a pool of eluates containing 400 µg eq of LGI and 11.5 mg of protein was used. Reassay of the five pools after trypsin incubation revealed that 60 to 80% of the original immunoreactivity had disappeared.

From 100 to 700 µg eq of residual glucagon immunoreactivity of the tryptic digests were subjected to rechromatography and the eluates were assayed for glucagon. In each of five experiments the elution volume of the peak of residual immunoreactivity was found to have shifted and now appeared an average of 8 ml after the glucagon-131I marker. Both canine glucagon and crystalline glucagon normally appear an average of 3 ml after the glucagon-131I peak. A representative elution pattern of the tryptic product of LGI is shown in Fig. 6.

Although LGI is sensitive to the action of trypsin, it is clear that limited tryptic hydrolysis brings about a major loss of large glucagon immunoreactivity and a major reduction of its residual immunoreactivity to a molecular size smaller than glucagon-131I.

More prolonged tryptic hydrolysis for 48 hours at a protein to enzyme ratio of 1:1 resulted in disappearance of at least 95% of the immunoreactivity.

Immunological Studies of Large Glucagon Immunoreactivity and Its Tryptic Products—Immunological differences between an anti-

Fig. 5. The effect of the endportal injection of LGI in the dog. Upper, 1 µg of LGI fails to elevate plasma glucose concentration, whereas 1 µg of crystalline glucagon is followed by a 14 mg per 100 ml rise in glucose. The hypoglycemia associated with LGI administration is attributable to the presence of 10 units of insulin in the eluate. Lower, endportal injection of 1 µg of crystalline glucagon with 10 units of added insulin. Plasma glucose rises 24 mg per 100 ml despite the insulin.

Fig. 6. Elution pattern of residual immunoreactivity after incubation of LGI with trypsin.
gen and other substances which cross-react with its antibody may be reflected by a lack of parallelism of their dilution slopes as determined by radioimmunoassay. To determine if immunological differences between crystalline beef-pork glucagon, dog glucagon, dog LGI, and the tryptic product of LGI could be detected, doubling dilutions of each were assayed with an antiserum highly specific for pancreatic glucagon. As shown in Fig. 7, all dilution slopes appear to be parallel. Similar results have been obtained in two other such experiments with an antiserum highly specific for pancreatic glucagon, and in three experiments in which a less specific antiserum was used. There is, therefore, no evidence of immunological dissimilarity between any of the above substances.

Biological Activity of Tryptic Product of Large Glucagon Immunoreactivity—Since the tryptic fragment of LGI was immunologically indistinguishable from glucagon, it seemed of importance to determine if it possessed glucagon-like biological activity in the perfused rat liver system. Four experiments were conducted with 100 m\(\mu\)g eq of the tryptic product of LGI, a dose 5 times that which, in the case of glucagon, consistently gave a striking glycogenolytic response. In none of the four experiments was a significant glycogenolytic response obtained. It is clear, therefore, that this product of LGI is not an intact glucagon molecule.

Comparison of Tryptic Product of LGI and of Glucagon—The identity of the tryptic product of LGI is obscure. The foregoing results suggest that, if the LGI molecule does contain part or all of the amino acid sequence of glucagon, its tryptic product must consist of the immunoreactive site of glucagon without a functioning biologically active site. Such a molecule has not been shown to exist.

To determine whether an immunologically active but biologically inactive fragment of glucagon could be produced, 115 \(\mu\)g of crystalline beef-pork glucagon was subjected to hydrolysis with 175 \(\mu\)g of trypsin, and was then assayed for immunological and biological activity. In two such experiments 20\% of the initial immunoreactivity remained after incubation with trypsin at this enzyme to protein ratio.\(^1\) On rechromatography this residual immunoreactivity appeared as a single peak 8 ml after the peak of the glucagon-\(^{131}\)I marker, the zone in which the tryptic product of LGI appeared (Fig. 8). This immunoreactive fragment was then tested for biological activity. In four perfusion experiments 100 m\(\mu\)g glucagon eq of the immunoreactivity failed to elicit a glycogenolytic response, excluding the possibility that it was undigested glucagon.

It was concluded that a biologically inactive but immunologically active fragment can be produced by limited trypsin hydrolysis of glucagon.

**DISCUSSION**

The results indicate that extracts of canine pancreas, and probably of duck, beef, rat, and human pancreas as well, contain a polypeptide which reacts like glucagon with glucagon antibodies, and which is at least twice the molecular size of glucagon. Non-specific inhibition of the reaction between glucagon and glucagon antibody by the high protein concentration of the eluates in which the immunoreactivity appears was satisfactorily excluded as the possible cause of a false peak of immunoreactivity.

Large glucagon immunoreactivity, as this fraction has been named, was found to be devoid of glycogenolytic activity in the perfused rat liver and was shown probably not to be glucagon noncovalently bound either to itself or to another protein. Nor does it appear to contain an interchain disulfide bridge. However, as in the case of glucagon, cleavage of its lysine or arginine linkages (or both) by trypsin hydrolysis resulted in loss of its immunoreactivity and a reduction in molecular size of the remaining immunoreactivity to approximately that of glucagon-\(^{131}\)I. Its lack of biological activity proved that the tryptic product could not be glucagon.

If one were to assume that all or part of the amino acid sequence of glucagon forms a part of the LGI molecule, one would then have to assume that this biologically inactive but immunologically active tryptic product of LGI is glucagon with its functional region either unexposed or absent, and that it must, therefore, be either larger or smaller than glucagon. Although resolution in the 3500 molecular weight zone of a Bio-Gel P-10 column is not adequate to permit estimation of molecular size, the consistent recovery of the immunoreactive tryptic product of LGI after the glucagon-\(^{131}\)I marker suggests that it is smaller rather than larger. The existence of at least one immunologically active tryptic fragment of glucagon devoid of glycogenolytic activity was convincingly established by the experiments in which...
crystalline glucagon was subjected to limited tryptic hydrolysis. Bromer (11) has reported that after 2½ hours of tryptic digestion a dodecapeptide 1-12, a pentapeptide 13-17, a dodecapeptide 17-29, and an undecapeptide 18-29 are produced from crystalline glucagon (since the lysyltyrosyl bond is the most sensitive to trypsin (11), it is possible that the heptadecapeptide 13-29 was also present in the more limited 15-min digests of this study). At least one of these fragments must be the source of the immuno-reactivity in the tryptic digest of glucagon. The possibility that the source of immunoreactivity is the tryptic digest of both LGI and glucagon are the same cannot be excluded since (a) they have the same elution volume, (b) they both lack biological activity, and (c) neither LGI nor its tryptic product can be differentiated immunologically from glucagon by means of dilution slope comparisons. Obviously, however, proof of their identity is not provided by this study and the composition of LGI remains to be elucidated. The issue of a precursor-product relationship between LGI and glucagon likewise is not illuminated by this study.

The demonstration of at least two or three immunoreactive peptides (LGI, its tryptic product, and the tryptic product of glucagon) which are devoid of glycogenolytic activity has important implications for the use of the glucagon radioimmunoassay. It can no longer be assumed that all of the plasma glucagon immunoreactivity measured with antisera specific for pancreatic glucagon necessarily represents intact hormone with glycogenolytic activity. Nor is it possible now to differentiate by immunochemical techniques between intact glucagon and these biologically inactive substances. The possibility that biologically inactive immunoreactive glucagon-like substances enter the circulation must now be entertained.

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