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Glucagon from Avian Pancreatic Islets: Purification and Partial Characterization of a 9000-Dalton Species with Glucagon Immunoreactivity[1,2] (39542)

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Studies of glucagon biosynthesis have shown that labeled amino acids are incorporated into islet proteins ranging in molecular weight from 69,000 to 6000 daltons and, therefore, are larger than glucagon itself (3500 daltons (2-8)). Although these proteins may represent proglucagon molecules, relatively little has been done to purify them for biochemical characterization. Using isolated pigeon islets, we observed the incorporation of ^{3}H-labeled tryptophan into a 69,000-dalton (Peak I) and a 9000-dalton component (Peak II) (5, 6). Both fractions reacted with glucagon antisera (AGS), and Peak II was significantly more immunoreactive than Peak I. Immunoreactive forms of similar sizes have been found in extracts of canine pancreas and mouse islets (9) and in human plasma (10). This paper describes additional attempts to extract and purify proteins with glucagon immunoreactivity from pigeon islets.

Methods and materials. Islet preparation. Pigeons (Mogul Ed Company, Oshkosh, Wis.), fasted for a week before the experiments, were sacrificed with an overdose of sodium pentobarbital. The islets were prepared using a modification of the method of Lacy and Kostianovsky (11) as follows: After disrupting the exocrine tissue with Hanks' buffer (12) injected into the pancreatic ducts, the pancreata were removed and cut in half, and each half was placed in a glass vial containing 0.1 ml of a collagenase solution (Type III, Sigma Chemical Co., St. Louis, Mo., 0.4 mg/ml). The tissue was minced for 10 min, shaken for 10 min at 37°, and minced for an additional 10 min. Minced samples from five or six birds were pooled, suspended in 250 ml of ice-cold Hanks' buffer in a 500-ml beaker, and further disrupted by repeated passage through a 30-ml glass syringe (20 times without a needle and 15 times with a No. 15 needle). The beaker containing the suspension was allowed to rest on ice for 2 min, and the islet-containing supernatant was decanted and placed on ice for 45 min. The islets were removed from the bottom of the beaker by means of a Pasteur pipet and placed in a test tube. An aliquot of about 0.2 ml of the islet suspension was removed with a Pasteur pipet and was replaced by an equal volume of Hanks' buffer, and the suspension was again stirred vigorously and allowed to stand 2 min before removing the next aliquot. Islets collected within a 45-min period were centrifuged at 200 rpm for 1 min using a tabletop clinical centrifuge. The pellet was washed six times with 10 ml of ice-cold Hanks' buffer. Trichloroacetic acid (TCA, 30%, w/v) was added to the islet suspension to a final concentration of 15%. The suspension was centrifuged at 2000 rpm for 3 min. The TCA-precipitated islets were stored at −20° for subsequent extraction with acid ethanol (13). Alternately, the islets were treated with a mixture of 1 ml of 30% TCA and 1 ml of 1 m*M* benzamidine hydrochloride (Aldrich Chemicals, Milwaukee, Wis.) in 1 *M* acetic acid or with 2 ml of 15% TCA containing 500 kiu of aprotinin solution (Trasylol, FBA Pharmaceuticals, Inc., New York, N.Y.). These proteolytic enzyme inhibitors were added to reduce the degradation of glucagon-related proteins during the purification procedure (14). Since aprotinin, a 6000- to 7000-dalton protein, would have interfered with the chro-

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matographic procedures while benzamidine hydrochloride can be removed by dialysis and by gel filtration, we used the latter for most of the preparations described in this paper.

Extraction of islet proteins and gel filtration. TCA-precipitated islets were homogenized in acid ethanol using a Potter–Elvehjem homogenizer (13) and extracted by stirring overnight at 4°. In a typical large-scale experiment, islets from 20 birds were extracted in 40 ml of acid ethanol containing 10% of 1 m*M* benzamidine hydrochloride (15) in 1 *M* acetic acid. The acid alcohol-soluble fractions, obtained by centrifuging the homogenate at 2000 rpm for 15 min in a refrigerated centrifuge, were lyophilized, reconstituted in acetic acid, and chromatographed on a Sephadex G-50 column (Pharmacia Fine Chemicals, Piscataway, N.J.) that had been equilibrated in 1 *M* acetic acid at 4°. Absorbance at 275 nm was determined using a Gilford uv spectrophotometer and aliquots were used for glucagon radioimmunoassay. Appropriate fractions were pooled, lyophilized in the presence of benzamidine-HCl, and stored at −20° for further fractionations.

Ion-exchange column chromatography. Diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman Co., supplied by Reeve Angel, Clifton, N.J.), equilibrated in 0.01 *M* Tris–HCl, pH 8.7, followed by 0.01 *M* Tris–HCl, 3 *M* urea (charcoal-treated) (Sigma Chemical Co., St. Louis, Mo.), was packed in 12.5 × 0.90-cm columns (Pharmacia Fine Chemicals, Piscataway, N.J.), which had been washed with Tris–HCl–urea buffer for 10 hr. A 2-ml sample of protein solution in Tris–HCl–urea was applied to the column and eluted with 20 ml of the buffer containing 0.01 *M* NaCl, followed by 20 ml of the same buffer containing 0.1 *M* NaCl. Fractions of 1 ml were collected. Urea and salt were removed by dialyzing the fractions at 4° against three changes of distilled water (3 liters, 48 hr each), using Spectrapor membrane tubings (Spectrum Medical Industries, Inc., Los Angeles, Calif.) with a molecular weight cutoff of 3500 daltons. The dialyzed material was lyophilized and stored at −20° for further analysis.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed at pH 3.5 using 7.5% gels according to Moller and Chrambach (16). The dimensions of the gel were 7 × 75 mm; running time was 10 hr at 5 mA/gel. The gels were stained for protein with amido black and destained in 7% acetic acid.

Amino acid analysis. Protein fractions were hydrolyzed in 6 *N* HCl and amino acid analyses were performed by L. H. Ericson of the AAA Laboratory, Seattle, Wash., using a Durrun analyzer, Model D-500. Two separate samples were analyzed.

Radioimmunoassay for glucagon. Radioimmunoassay for glucagon was performed using a method previously described (17), except that dextran-coated charcoal was used to separate free from antibody-bound hormone (18). Aliquots of 0.2 ml from consecutive gel-filtration fractions were pooled and lyophilized with 2000 kiu of aprotinin, redissolved in 0.7 ml of sodium barbital–albumin buffer, pH 8.7, cleared by centrifugation, and assayed for glucagon.

Aliquots of 0.1 ml, from ion-exchange fractions, were dialyzed against distilled water in the presence of 1000 kiu of aprotinin, lyophilized, and dissolved in 0.5 ml of sodium barbital–albumin buffer for radioimmunoassay.

Results. Fractionation of acid ethanol-soluble islet proteins with a Sephadex G-50 (fine) column yielded three peaks with absorbance at 275 nm (Fig. 1A). Peak I eluted at the void volume, Peak II eluted as a component of ≃9000 daltons, and Peak V eluted at the salt volume. Radioimmunoassay indicated that the bulk of the immunoreactive material was located in the glucagon region (III and IV), with smaller amounts eluting as Peaks I and II (Fig. 1B). Fractions corresponding to Peak II were pooled, lyophilized, reconstituted in 1 *M* acetic acid, and rechromatographed on a Sephadex G-50 column, eluted with 1 *M* acetic acid. Figures 2A and B show that, upon rechromatography, Peak II appears as a major immunoreactive component. In addition, protein components with glucagon immunoreactivity eluted in the glucagon region and beyond. However, when islets were extracted in the presence of inhibitors of proteolysis, more than 90% of the immu-

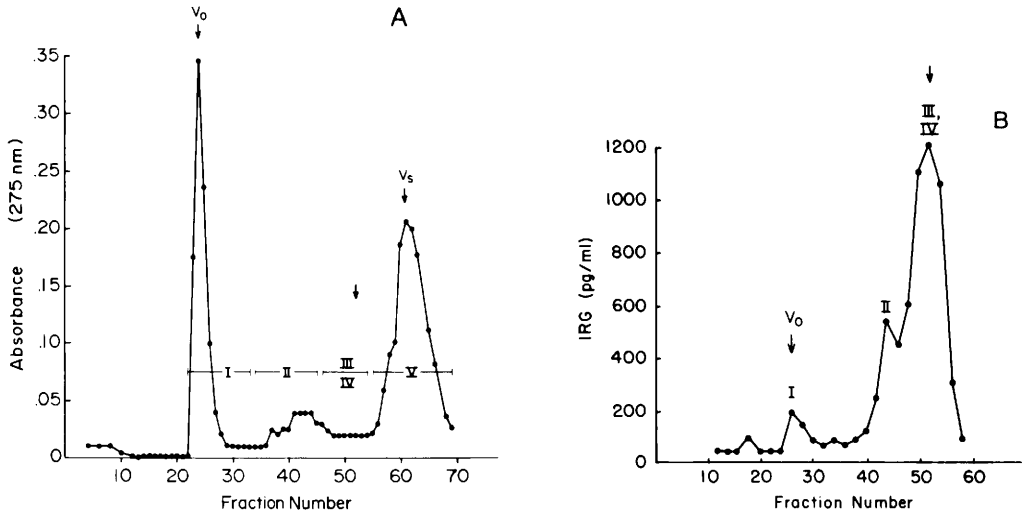

FIG. 1. (A) Sephadex G-50 gel filtration of acid ethanol-soluble islet proteins. Column size, 80 × 2.5 cm. Fraction volume, 2 ml. TCA-treated islets from 16 birds were extracted with acid ethanol. The soluble fraction was lyophilized, taken up in 5 ml of 1 *M* acetic acid and 0.5 ml of glacial acetic acid and chromatographed. V_0, void volume. V_s, salt volume. ↓, position of ^{125}I-labeled porcine glucagon. (B) Aliquots of 0.2 ml from consecutive fractions were pooled and lyophilized with 2000 kiu of aprotinin. Each lyophilized fraction was dissolved in 0.7 ml of sodium barbital–albumin buffer, pH 8.7, insoluble materials were removed by centrifugation and the supernatant was assayed for glucagon. IRG, concentration of immunoreactive glucagon per assay sample.

noreactivity was eluted as Peak II (Fig. 2C). These results suggest that Peak II was degraded by proteases present in the extracts, resulting in products immunologically related to glucagon.

Fractions corresponding to Peak II were pooled, lyophilized, dissolved in Tris–HCl–urea buffer, and applied to a DEAE-cellulose column. When the column was eluted with 0.01 *M* NaCl, a small amount of immunoreactivity (DEAE-IIa) appeared. When eluted with 0.1 *M* NaCl, a second peak (DEAE-IIb) containing more than 90% of the immunoreactivity was obtained (Fig. 3). Fractions 30 to 33 corresponding to the peak of DEAE-IIb were pooled, dialyzed against distilled water, lyophilized, and stored at −20° for subsequent gel-electrophoresis and gel-filtration analyses.

Polyacrylamide-gel-electrophoresis and gel-filtration analysis of glucagon immunoreactive protein purified on DEAE-cellulose. The homogeneity of DEAE-IIb was assessed by polyacrylamide disc gel electrophoresis in urea-containing gels, at pH 3.5, and a single amido black-stained band was obtained (Fig. 4). Because of scarcity of material, we estimated the molecular size of the purified protein by labeling it with ^{125}I (19) and chromatographing it on a Sephadex G-50 column, eluted with 1 *M* acetic acid. Figure 5 shows a major radioactive peak of approximately 9000 daltons. Minor components, appearing at the void and at the salt volume, probably represent damaged labeled peptides adsorbed to serum albumin and free iodide, respectively.

Amino acid analysis of DEAE-IIb protein. The amino acid analysis of the DEAE-IIb fraction obtained from the islets of 350 birds revealed the presence of 76 residues.

Discussion. Using inhibitors of proteolysis in the extraction procedure, we have partially purified a "large glucagon" molecule from pigeon pancreatic islets. The protein, purified on DEAE-cellulose appeared homogeneous by polyacrylamide-gel electrophoresis, reacts with anti-glucagon sera and has a size of approximately 9000 daltons. Amino acid analysis showed that this protein resembles angler fish "proglucagon" (20) and contains all the residues of turkey glucagon (21). The material appears to be susceptible to proteolysis, as indicated by its

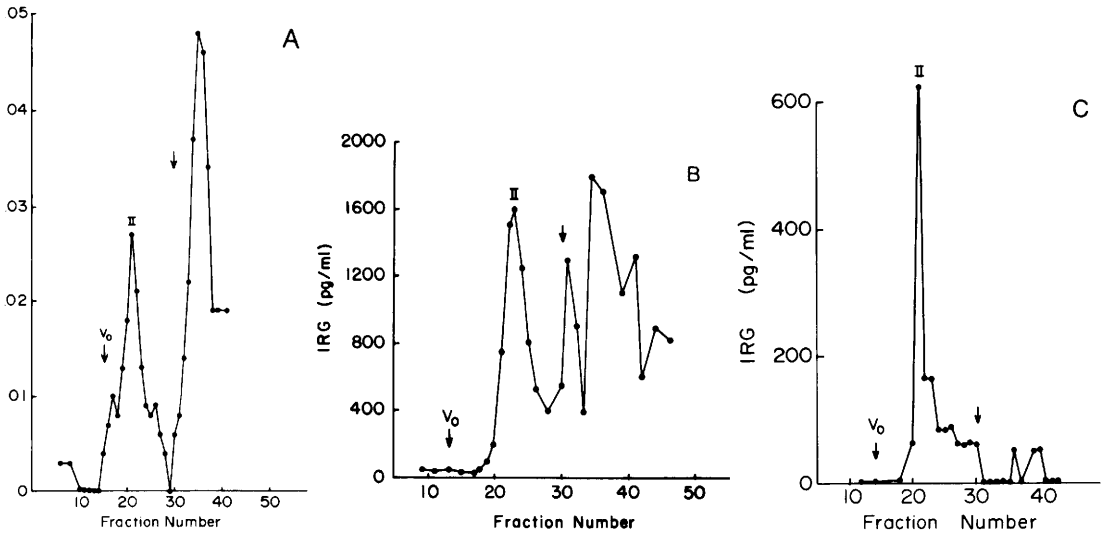

FIG. 2. (A) Rechromatography of Peak II (Fig. 1) on a Sephadex G-50 column (60 × 0.9 cm). Fraction volume, 1 ml. Peak II was obtained as described in the legend of Fig. 1. Results correspond to experiments in which islets were processed without inhibitors of proteolysis. (B) An aliquot of 0.1 ml from each fraction was lyophilized with 0.01 ml of aprotinin solution (1000 kiu). Lyophilized fractions were dissolved in 0.7 ml of sodium barbital-albumin buffer for glucagon radioimmunoassay. (C) Rechromatography of Peak II on a Sephadex G-50 column. Results correspond to an experiment in which islets were processed in the presence of aprotinin.

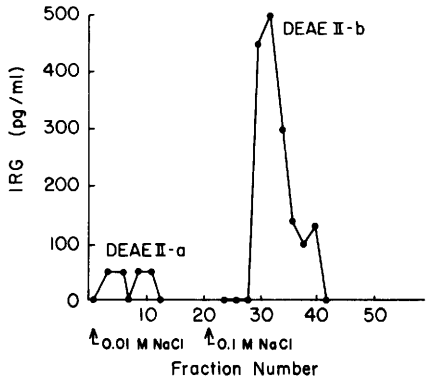

FIG. 3. DEAE-cellulose ion-exchange chromatography of Peak II (Fig. 2) after refractionation on a Sephadex G-50 column. Proteins, extracted from islets in the presence of benzamidine-HCl, were chromatographed. Aliquots of 0.1 ml in 0.1 ml of aprotinin solution (1000 kiu) were dialyzed against distilled water. The material was lyophilized and dissolved in 0.5 ml of sodium barbital-albumin buffer for radioimmunoassay.

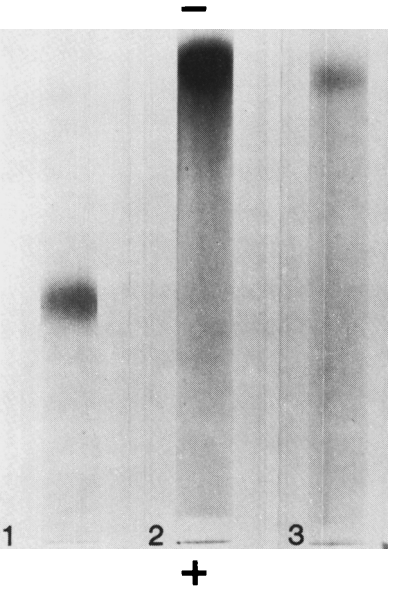

FIG. 4. Polyacrylamide gel-electrophoresis of DEAE-Peak-IIb. Fractions corresponding to DEAE-Peak-IIb (Fig. 3) were pooled and dialyzed against distilled water. The protein recovered by lyophilization was dissolved in 100 μl of 0.01 *M* HCl and subjected to electrophoresis in urea-containing gels, pH 3.5 (12). (1) Pancreatic glucagon; (2) DEAE-Peak-IIa; (3) DEAE-Peak-IIb. +, Anode; −, cathode.

degradation into glucagon and smaller immunoreactive forms during fractionation of the crude extract by gel filtration. These data are consistent with the hypothesis that Peak II contains the glucagon molecule in its

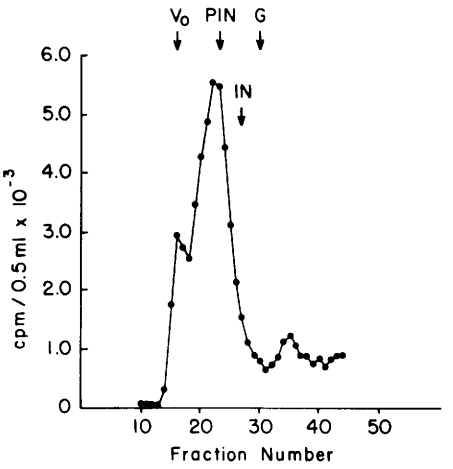

FIG. 5. Sephadex G-50 gel filtration of labeled DEAE-Peak-IIb. Column size, 60 × 0.9 cm. Fraction volume, 1 ml. The protein was iodinated with carrier-free [^{125}I]iodide by the chloramine-T method of Greenwood and Hunter (19). PIN, elution volume of bovine proinsulin (gift of Dr. C. Yip, University of Toronto, Canada); IN, insulin; G, glucagon.

primary structure and that it is not a glucagon polymer.

Recent studies on the biosynthesis of glucagon suggest the existence of biosynthetic precursors or intermediates having molecular weights of 69,000 (5), 18,000 (7), 9000, 6000 (6–8), and 4500 daltons (22). It is not known if these substances are identical to the large glucagon immunoreactive materials found in the crude extracts of pancreas and isolated islets (9, 10); however, it has been suggested that the biosynthesis of pigeon glucagon may proceed through a 9000-dalton intermediate (6). In these studies, labeled Peak II obtained from islets incubated with ^{3}H-labeled tryptophan coeluted with a glucagon immunoreactive fraction of 9000 daltons on a Sephadex G-50 column. Polyacrylamide-gel electrophoresis of the same material showed two radioactive peaks which coincided with two protein bands associated with glucagon immunoreactivity. However, gel-filtration data also revealed a prominent immunoreactive peak of about 6000 daltons, eluting after the labeled Peak II, and electrophoretic data showed an immunoreactive component more cationic than the labeled peak (6). In the present study, using an improved extraction procedure, the purification of Peak II by gel filtration yielded only the 9000-dalton immunoreactive component.

Although these results suggest that the 9000-dalton glucagon immunoreactive protein may be a biosynthetic component, final characterization of this protein as "proglucagon" must await determination of its amino acid sequence.

Summary. Pigeon pancreatic islets were homogenized in acid ethanol containing inhibitors of proteolysis. Purification of the extract, including fractionation on Sephadex G-50 columns, yielded a 9000-dalton protein, which after further purification by DEAE-cellulose chromatography, appeared to be homogeneous and more cationic than porcine glucagon on polyacrylamide disc gel electrophoresis. When the protein obtained by DEAE-cellulose chromatography was iodinated and applied to a Sephadex G-50 column, a single radioactive peak of 9000 daltons was also obtained. The DEAE-cellulose peak is composed of 76 amino acids, including all those of turkey glucagon. Its amino acid composition is similar but not identical to that of angler fish "proglucagon".

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