PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 179, 356–364 (1985)

Characterization of a Heat Stable, 12,000 Da, Glucagon-like Immunoreactive (GLI) Peptide from Porcine Ileum That Stimulates Hepatic Glucose Production *in Vivo* and *in Vitro*[1] (42109)

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Abstract. Glucagon-like immunoreactivity (GLI) was extracted from porcine ileal mucosa with boiling 2 *M* HAc followed by elution on DEAE A-50 and fractionation on Sephadex G-50 F. Intact GLI was isolated from mesenteric blood by fractionation of several plasma samples from a mesenteric vein of a dog on Sephadex G-50 M followed by refractionation of the pooled GLI from these columns on G-50 F. Analysis of the semipurified mucosal and plasma GLI on Sephadex G-50 SF, eluted with 0.1 *M* Tris/HCl + 8 *M* urea, pH 7.5, demonstrated a single, sharp peak of GLI with a relative molecular mass (M_r) between 12,000 and 13,000. Electrophoresis on PAGE gels at acid pH with 2 *M* urea demonstrated a single charged GLI species in both the plasma and mucosal fractions. Stimulation of the release of GLI from a loop of ileum isolated *in situ* in an anesthetized dog following removal of the known sources of glucagon (stomach, pancreas, and duodenum) resulted in an immediate and sustained increase in hepatic glucose production. The isolated GLI from ileal mucosa (5×10^{-8} *M*) stimulated gluconeogenesis from 10 m*M* [^{14}C]alanine in hepatocytes isolated from fed rats. The stimulation was equal to 25% of the maximal stimulation observed with 10^{-8} *M* glucagon. These experiments demonstrate that the ileum synthesizes and secretes a GLI peptide with a M_r of approximately 12,000 that stimulates hepatic glucose production *in vivo* and *in vitro*.

The determination of the sequence of several mammalian pancreatic islet mRNAs coding for glucagon (1–3) has established the primary structure of preproglucagon, a protein of 180 amino acids, composed of a hydrophobic signal peptide followed by glicentin (4) and ending with 2 carboxyl terminal glucagon-like peptides (GLP 1 and GLP 2). The exact series of post-translational cleavages that result in the liberation of glucagon in the mammalian pancreas have not been defined yet but experiments (5–9) indicate that following removal of the signal sequence, a 10,000 mol wt C-terminal peptide is released, presumably leaving glicentin which is subsequently cleaved to yield the 4500 mol wt proglucagon fragment (10) and the 3445 mol wt glicentin related pancreatic polypeptide (GRPP) (11). An 8 amino acid C-terminal extension is removed from the 4500 mol wt peptide to produce glucagon.

The relationship of the pancreatic glucagon precursors to intestinal glucagon-like immunoreactive (GLI) materials is not clear. Glicentin was isolated from porcine small intestine (12) and the 4500 mol wt peptide identified in commercial glucagon preparations (10) has also been isolated from intestinal mucosa and is referred to as enteroglucagon II (13). Glucagon itself, however, has not been isolated from the distal intestine where GLI is concentrated as would be expected if GLI functioned as a proglucagon.

Experiments conducted to date attempting to characterize GLI have established that the distal small intestine contains the highest concentration of GLI (14, 15), that this material is released into the circulation upon intraluminal stimulation by various agents (16–23), and that the relative molecular mass (M_r) of the majority of the GLI is larger than 3485 (16, 17, 21). This paper describes the characterization of a GLI peptide with a M_r of approximately 12,000 extracted from the mucosa of porcine terminal ileum under conditions that minimize proteolytic degradation. The peptide is secreted in response

[1] This work was supported in part by grants from the Sinai Hospital General Research Fund and the American Diabetes Association, Michigan Affiliate.

0037-9727/85 $1.50

to intraluminal glucose and stimulates glucose production by the liver both *in vivo* and *in vitro*. Previous experiments have proven that the stimulation of release is stereospecific (18) and is inhibited by somatostatin, but not by insulin (21).

Materials and Methods. *Materials.* All chemicals used were reagent grade. [^{125}I]-Glucagon and [^{14}C]alanine were from New England Nuclear (Boston, Mass.), collagenase (CLS III) from Worthington Biochemicals (Freehold, N.J.), aprotinin and hyaluronidase, from Sigma Chemical Company (St. Louis, Mo.), indocyanin green from Hynson, Wescott and Dunning, Inc. (Baltimore, Md.), and Sephadex chromatographic gels from Pharmacia Fine Chemicals (Uppsala, Sweden). Ubiquitin was a gift of Dr. G. Goldstein, Ortho Pharmaceuticals (Raritan, N.J.).

Extraction procedure. Segments of porcine terminal ileum (30 cm) were obtained from the local abattoir at the time of slaughter and placed in iced saline. The tissue was flushed with tap water and the mucosa scraped into liquid N_2 within 15 min. The frozen material was pulverized in a Waring blender and 300 g of the powder was put into 500 ml of boiling 2 M acetic acid (HAc) + 1 m*M* benzamidine hydrochloride (BzCl) and boiled for 5 min to inactivate proteolytic enzymes. The extract was immediately cooled in an ice bath, centrifuged at 20,000*g* for 30 min, and the pH of the supernatant adjusted to 7.0 with concentrated NH_4OH. The resulting precipitate was removed by centrifugation at 20,000*g* for 30 min and $(NH_4)_2SO_4$ was added to the supernatant to saturation. This was centrifuged at 20,000*g* for 60 min and the pellet resuspended in 0.05 *M* phosphate buffer, pH 6.8, reprecipitated with $(NH_4)_2SO_4$ at 95% saturation, centrifuged, and the pellet resuspended in 0.05 *M* phosphate buffer, pH 6.8, and frozen in aliquots. All steps up to this point were conducted on the same day in a cold room at 4°C.

Aliquots of the extract were desalted on Sephadex G-25 *M* (2.6 × 30 cm) eluted with 0.05 *M* phosphate buffer, pH 6.8. The protein peaks (abs 280 nm) from the desalting columns were pooled and applied to a column of DEAE A-50 (2.6 × 25 cm) eluted with 0.05 *M* phosphate buffer, pH 6.8, followed by a linear gradient of NaCl (0.0 to 1.0 *M*) in the same buffer. The fractions (4.5 ml) from this column were assayed for GLI and those containing significant immunoreactivity were pooled and precipitated with 95% $(NH_4)_2SO_4$. After centrifugation at 20,000*g*, the pellet was resuspended in 0.05 *M* NH_4HCO_3 + 1 m*M* benzamidine hydrochloride, pH 7.0, and applied to a column of Sephadex G-50 F (2.6 × 90 cm) eluted with the same buffer. The fractions (4.5 ml) from this column were assayed for GLI using a 1:2500 dilution.

Circulating GLI. Five hundred milliliters of whole blood were collected from a mesenteric vein draining a 40-cm loop of ileum isolated *in situ* in an anesthetized male mongrel dog 30 min after the introduction of 50 ml of a 5% dextrose solution into the lumen. EDTA and BzCl were added to the blood to 0.01 *M* and 0.001 *M* concentrations, respectively, and the sample was immediately chilled on ice and centrifuged for 20 min at 3000*g*. The plasma was removed, frozen in 20-ml aliquots, and stored at −20°C until used.

The aliquots were fractionated on Sephadex G-50 M (2.6 × 90 cm) eluted with 0.05 *M* NH_4HCO_3 + 0.001 *M* BzCl + 0.01 *M* EDTA, pH 8.6, at a flow rate of 75 ml/hr. The fractions (4.5 ml) were assayed for GLI and all those with activity were pooled, and precipitated with 95% $(NH_4)_2SO_4$ and centrifuged. The pellet was resuspended in 0.05 *M* NH_4HCO_3, pH 8.6, and fractionated on Sephadex G-50 F (2.6 × 90 cm) eluted with the same buffer (20 ml/hr). The fractions (4.5 ml) from this column were assayed for GLI and the peak tubes were pooled, lyophilized, and stored at −20°C until analyzed.

Peptide characterization. Semipurified GLI from both mucosal extracts and plasma from the stimulated gut experiments were fractionated on a column of Sephadex G-50 SF (1.5 × 90 cm) eluted with 0.1 *M* Tris/HCl + 8 *M* urea, pH 7.5. The column was run at 25°C, at a flow rate of 2 ml/hr and 1.1 ml fractions were collected. This column was calibrated with blue dextran, ribonuclease A, insulin, and ^{125}I-glucagon before and after each run and all samples were run with ^{125}I-glucagon as an internal standard.

Electrophoresis was performed on 7.5% polyacrylamide disc gels containing 2 *M* urea

and 5% HAc using a 5% HAc running buffer. Gels were preelectrophoresed at 120 V for 1 hr. The samples were dissolved in 5% HAc + 10 *M* urea, applied to the gels, and electrophoresed at 1.5 mA/gel for 60 min. The gels were either stained overnight with Coomassie brilliant blue R-250 in 15% TCA or were sliced into 4-mm sections and eluted in 0.5 ml of Veronal buffer, pH 8.6, with 1000 kIU aprotonin/ml.

Glucose production in vivo. Male, mongrel dogs, fasted overnight, were anesthetized with iv pentobarbital sodium (50 mg/kg) and barbituric acid (100 mg/kg) and the stomach, pancreas, and duodenum rapidly removed using clamps to close off blood vessels, esophagus, and intestinal lumen. Catheters were placed into the hepatic vein through the right jugular, the portal vein through a splenic vein and into the femoral artery and were kept open with a solution of heparin (0.02 U/ml) given at a rate of 0.25 ml/min. Indocyanin green solution 0.075 mg/M^2/min was infused with a Harvard pump via the left jugular vein, using an angiocath to avoid interruption of jugular blood flow.

Insulin was infused into the portal vein via a splenic vein at an initial rate of 250 μU/kg/min using a Harvard pump. Blood samples were taken every 5 min from the femoral artery and analyzed for glucose and the insulin infusion was adjusted to maintain the blood glucose between 80 and 120 mg/dl. After the glucose levels had stabilized for at least 30 min the experiment was begun. Blood samples (5 ml) were taken simultaneously from the hepatic vein, portal vein, and femoral artery every 5 min for 20 min, at which time 50 ml of a 5% glucose solution was instilled into a 30-cm loop of ileum isolated *in situ.* Samples were taken every 5 min for another 45 min and the experiment was then terminated. All samples of arterial blood were checked for hematocrit. The blood was immediately placed on ice, allowed to chill, and centrifuged.

Indocyanin green levels were determined on the same day by reading the absorption of the plasma at 805 nm. The samples were then frozen and stored at −20°C until assayed for glucose. Glucose production was estimated by artero-venous (A-V) difference, using the method of Leevy *et al.* (24) for estimation of hepatic blood flow. Compensation for decreased portal flow, due to removal of the stomach, pancreas, and duodenum was made assuming a 60% contribution by the portal vein to hepatic blood flow, instead of 70% which is normally used. The product of blood flow by the A-V difference in glucose was considered equal to the net hepatic glucose production (25).

Glucose production in vitro. Hepatocytes were isolated from fed, male, Sprague–Dawley rats by the method of Zahlten *et al.* (26) with modifications (27). The liver was perfused for 20 min with 0.018% collagenase and 0.080% hyaluronidase in Ca^{2+} free Krebs-Henseleit bicarbonate buffer containing 1.0% BSA + 5 m*M* pyruvate + 5 m*M* glutamate + 11 m*M* glucose and 13% aged, washed human erythrocytes. The perfusate was gassed constantly with O_2/CO_2 (95%/5%). The liver was removed, minced, shaken with 20 ml of perfusate for another 15 min, and filtered through a nylon mesh. The cells were washed three times with Krebs-Henseleit bicarbonate buffer containing 1.5% gelatin and resuspended in the same buffer containing 10 m*M* uniformly labeled [^{14}C]alanine for incubation. The final suspension contained 0.75 to 1.25 $\times 10^6$ cells/ml. Incubations were carried out in plastic vials at 37°C for 40 min with constant shaking in an atmosphere of O_2/CO_2 (95%/5%). The experiment was terminated by the addition of perchloric acid to 0.33 *N*. The samples were centrifuged and the cell pellet and supernatant stored at −20°C.

The conversion of alanine to glucose was determined by the method of Exton and Park (28). Rates of incorporation of ^{14}C-labeled substrate into glucose are expressed as micromoles of alanine converted to glucose per gram of liver minus basal conversion observed in control tubes, assuming 9.8×10^7 cells/g (26).

Glucagon assay. Glucagon-like immunoreactivity was determined using antiglucagon serum AGS 10 as previously described (29) except that dextran-treated charcoal (30) was used to separate bound and free labeled hormone. AGS 10 reacts with the entire glucagon molecule, but not with its 1–12, 13–17, 17–29, and 18–29 segments (S. Pek, personal communication), providing an assay

sensitive to proteolytic cleavage of the glucagon sequence.

Results. Extraction of the ileal mucosa in boiling 2 *M* HAc yielded a significant amount of semipurified GLI (1.1 μg/g of wet tissue). Extraction of ileal mucosa in 0.05 *M* NH_4HCO_3 + 0.001 *M* BzCl + 0.01 *M* EDTA, pH 8.6, at 4°C gave a much lower yield and contained several peaks of immunoreactivity of smaller mol wt (not shown) indicating the need for the severe extraction conditions to limit artifact due to proteolytic activity. Care was taken during the extraction with boiling 2 *M* HAc to cool the extract rapidly and immediately adjust the pH of the supernatant to 7.0 following centrifugation at 4°C.

The GLI eluted after the major peak of absorbance (280 nm) in the wash of the DEAE columns and very little GLI was eluted by NaCl (not shown). The pooled GLI fractions from the DEAE column eluted on G-50 F as a single peak (Fig. 1). Analysis of this peak on G-50 SF, eluted at a slow flow rate (2 ml/hr) in the presence of 8 *M* urea, demonstrated a single, sharp peak of GLI with a M_r of approximately 12,000 (Fig. 2). Examination of the G-50 F peak by acid electrophoresis in the presence of 2 *M* urea demonstrated a single band of GLI following elution of the gel slices in Veronal buffer containing aprotonin (Fig. 5).

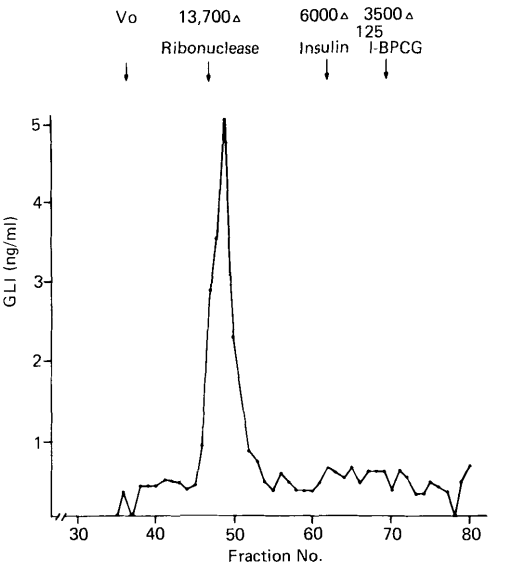

FIG. 2. Fractionation of peak GLI from Fig. 1 on Sephadex G-50 SF (1.5 × 90 cm) eluted with 0.1 *M* Tris/HCl + 8 *M* urea, pH 7.5 (2 ml/hr). Fraction size 1.2 ml.

Serum samples (20 ml) were eluted on G-50 M at a very high flow rate to quickly separate the GLI from the bulk of the proteolytic activity which would elute in the void volume. These columns yielded diffuse peaks of GLI which were pooled, reprecipitated with $(NH_4)_2SO_4$, and fractionated on

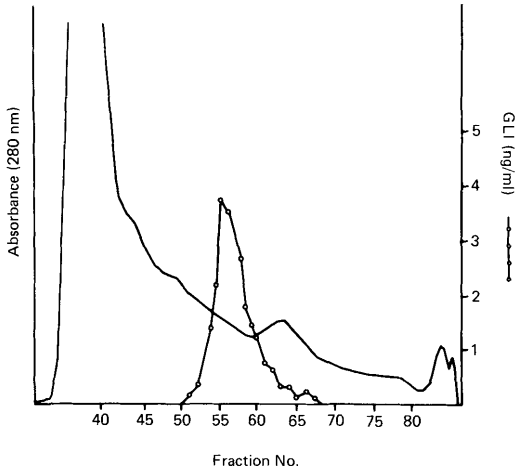

FIG. 1. Fractionation of GLI from DEAE A-50 column on Sephadex G-50 (2.6 × 90 cm) eluted with 0.05 *M* NH_4HCO_3 + 0.001 *M* benzamidine hydrochloride, pH 8.6. Fraction size 4.5 ml.

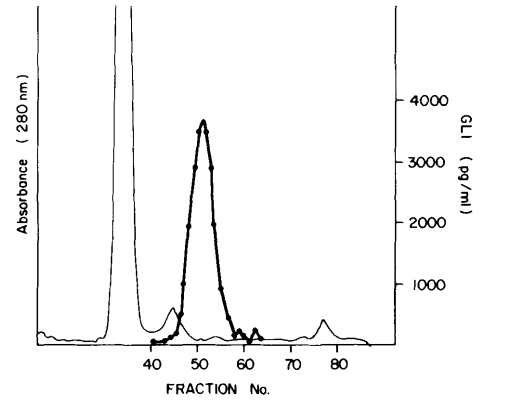

FIG. 3. Pooled GLI from Sephadex G-50 M fractionation of plasma samples from a stimulated gut experiment refractionated on Sephadex G-50 F (2.6 × 90 cm) eluted with 0.05 *M* NH_4HCO_3, pH 8.6 (15 ml/hr). Fraction size 4.5 ml.

G-50 F giving a single GLI peak (Fig. 3). The GLI from this column was analyzed on G-50 SF using a slow flow rate (2 ml/hr), in the presence of 8 *M* urea and only a single, very sharp peak of GLI was obtained with a M_r of approximately 13,000 (Fig. 4). Acid electrophoresis of this material demonstrated a single band of GLI following elution of the gel slices in Veronal buffer (Fig. 5).

Introduction of a 5% dextrose solution into the lumen of the ileum *in situ* following the removal of the known sources of glucagon caused a rapid and sustained increase in the release of glucose by the liver (Fig. 6), concomitant with a 195 ± 50% increase of GLI over basal as previously demonstrated (22). The incubation of hepatocytes isolated from fed rats with the partially purified GLI (5×10^{-8} *M* immunoequivalents) caused a significant increase in gluconeogenesis over basal levels which amounted to approximately 25% of the maximal stimulation observed with 10^{-8} *M* glucagon (Fig. 7).

Discussion. The results of these studies demonstrate that the ileum synthesizes and

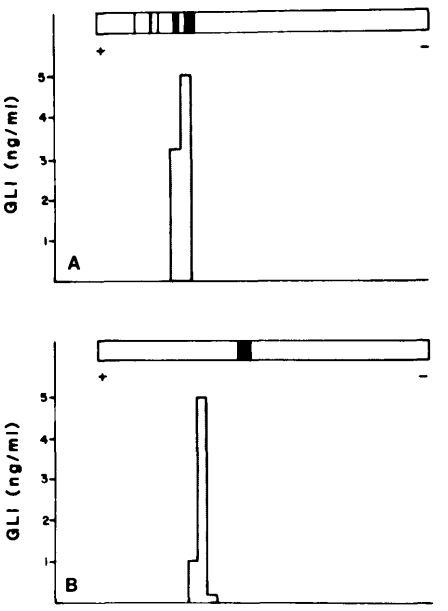

FIG. 5. (A) Electrophoresis on PAGE gels, with 2 *M* urea, pH 2.5, of semipurified GLI from Fig. 1. GLI measured following elution of gel slices in Veronal buffer. Parallel gel stained with Coomassie brilliant blue in 15% TCA. (B) Electrophoresis on PAGE gels, with 2 *M* urea, pH 2.5, of plasma GLI from Fig. 3. GLI measured following elution of gel slices in Veronal buffer. Stained gel is ubiquitin (mol wt 8428, p*I* 6.9) run in parallel and stained with Coomassie brilliant blue in 15% TCA.

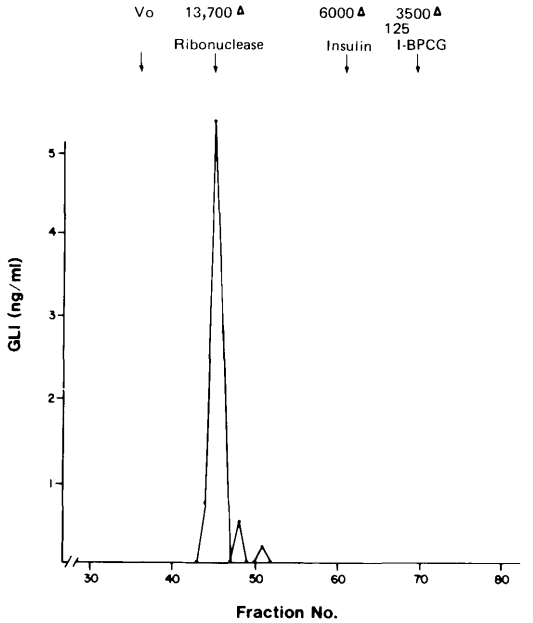

FIG. 4. Fractionation of peak GLI from Fig. 3 on Sephadex G-50 SF (1.5 × 90 cm) eluted with 0.1 *M* Tris/HCl + 8 *M* urea, pH 7.5 (2 ml/hr). Fraction size 1.2 ml.

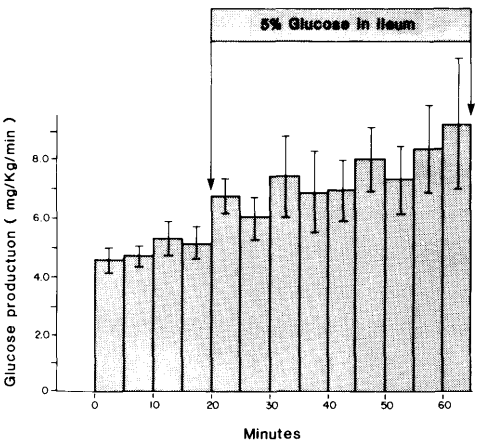

FIG. 6. Effect of GLI release on glucose production in the liver. Hepatic glucose production in anesthetized dogs (n = 12) with stomach pancreas and duodenum removed in response to instillation of 50 ml of 5% glucose solution into an isolated loop of ileum (*in situ*).

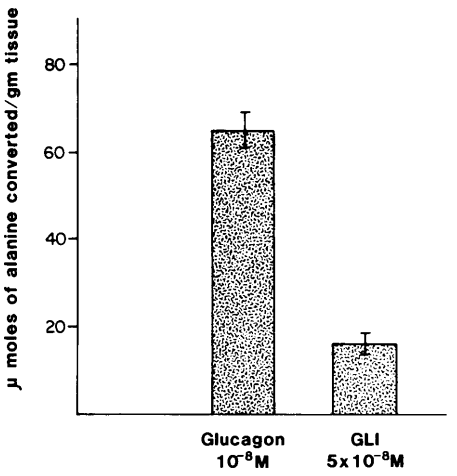

FIG. 7. Stimulation by glucagon vs semipurified GLI of gluconeogenesis from 10 m*M* uniformly labeled [^{14}C]alanine over basal in hepatocytes isolated from fed rats.

secretes a GLI peptide with a M_r of approximately 12,000 to 13,000 and that this peptide causes increased glucose production by the liver. The use of boiling 2 *M* acetic acid, with rapid cooling and adjustment of the pH to 7.0, resulted in a high yield of semipurified GLI (approximately 1.1 μg immunoequivalents/gram of tissue). The identification of the intact peptide in the plasma was facilitated by the rapid separation of the GLI from the bulk of the enzymatic activity. This was accomplished by using aliquots of plasma so that the entire sample could be applied to the column immediately after thawing and by fractionation on G-50 M at 4°C in the presence of EDTA and the proteolytic inhibitor benzamidine hydrochloride, at an extremely high flow rate of 75 ml/hr. This allowed the separation, in 3 hr, of the GLI from the higher mol wt components of plasma where you would expect most of the proteolytic activity to elute. Although the GLI eluted from these columns in a very broad peak, precipitation of the pooled fractions from several columns followed by refractionation on G-50 F produced a single, refined peak (Fig. 3).

Analysis of both mucosal (Fig. 2) and plasma (Fig. 4) GLI on G-50 SF eluted with 8 *M* urea in the buffer at 25°C using a slow flow rate (2 ml/hr) demonstrated a single major peak of GLI with a M_r of approximately 12,000. The apparent slightly larger size of the plasma GLI is most probably due to species variation (dog plasma vs pig mucosa). Electrophoretic analysis on acid gels containing 2 *M* urea demonstrated a single charged species for both plasma and gut GLI (Fig. 5). Again there is an apparent difference in charge further suggesting a slight species difference.

A GLI peptide with a M_r of 12,000 has been identified in extracts of intestine by numerous investigators (6, 15, 17, 31–35). This peak is reactive with N-terminally, but not with C-terminally directed antiglucagon sera. Tager and Markese found that treatment of the 12,000 M_r peptide with trypsin and carboxypeptidase B together resulted in the liberation of C-terminally reactive fragments identical to the fragments obtained by enzymatic digestion of glucagon (33). These experiments indicated that this peptide contains glucagon and Ghatei *et al.* (15) have found this peak to react with antiglicentin antisera R-64, suggesting the presence of the N-terminal sequence of glicentin.

The fact that both glicentin (12) and the 4500 M_r proglucagon fragment termed enteroglucagon II (13) have been isolated from porcine small intestine demonstrates that the intestine synthesizes at least glicentin, if not the entire preproglucagon molecule. The question that remains is whether the 12,000 M_r peptide from the ileum is identical with proglucagon and more importantly if it functions in the GI tract as a prohormone or is a distinct hormonal entity. Experiments identifying a single ileal cell type containing both GLI and glicentin by immunofluorescence have been reported (36, 37) but problems remain with these observations. Glicentin Antisera R-64 was produced prior to 1979 (36) when glicentin preparations were only 30% pure (4).

Glicentin was not the only GLI fraction identified in the original isolation studies demonstrating that there are other GLI peptides present in the intestine. Further, the estimation of its relative molecular mass as 11,625 may have been due to electrostatic interaction with the contaminating peptides. The use of 8 *M* urea in the analytic columns in the experiments reported here would elim-

inate this effect and glicentin should elute in the 8000 range, closer to its actual mol wt.

The peptide identified in this study may contain the glicentin sequence but is definitely not glicentin itself. The apparent M_r of approximately 12,000 is much higher than 8128 when eluted on G-50 SF with 8 *M* urea in the eluant. Electrophoresis on acid PAGE gels in the presence of 2 *M* urea, following solution of the sample in 10 *M* urea (Fig. 5) demonstrated that the immunoreactivity migrated well away from ubiquitin, a peptide with the same isoelectric point and molecular weight as glicentin (41). Whether the GLI peptide contains glicentin or not, it is clear from the studies reported here that it is synthesized by the ileum and is secreted into the circulation intact, indicating that it is a distinct hormonal entity.

Further evidence indicating a hormonal function for this peptide was obtained in the biologic activity experiments. It exerts a specific biologic effect on a target organ, the liver. Stimulation of the distal ileum *in situ* with intraluminal glucose resulted in an immediate and sustained increase in glucose production by the liver (Fig. 6) which is concomitant with the 195 ± 50% rise in GLI over basal as previously reported (22). These experiments were conducted in animals that had all the known sources of glucagon (stomach, pancreas, and duodenum) removed. The results of these studies do not rule out the possibility that the hepatic response was due to factors other than ileal GLI, however, and hence the GLI was tested *in vitro* with isolated hepatocytes. The semipurified GLI (5×10^{-8} *M*) stimulated gluconeogenesis from 10 m*M* [^{14}C]alanine in hepatocytes isolated from fed rats to 25% of the maximal response observed with 10^{-8} *M* glucagon (Fig. 7). The gluconeogenic activity of the GLI is probably even greater than is observed in these studies since the fraction of GLI used was in NH_4HCO_3 buffer and ammonium ions are known to inhibit the stimulation of gluconeogenesis by glucagon (26). Further, the circulating levels of GLI are 10 times as high as glucagon and hence the effect of GLI may be comparable to that exerted by glucagon itself.

Glicentin has been observed to cause an increase in glucose production from isolated hepatocytes at 2×10^{-7} *M* although it is not clear whether the studies employed highly purified glicentin or the original impure preparations (42). There was significant proteolytic breakdown of the glicentin immunoreactivity in the hepatocyte incubations in this study and the authors attributed the glucagon-like action to the liberation of smaller glucagon related fragments. Murphy *et al.* observed no stimulation of adenylate cyclase in liver plasma membranes nor inhibition of ^{125}I-glucagon binding using their 12,000 M_r "large glucagon-like immunoreactive" peak (34). The failure of the 12,000 M_r GLI to bind to liver plasma membranes was also observed by Holst (31) and Conlon *et al.* did not observe any increase in glucose production by perfused rat liver in response to a 12,000 M_r GLI peptide from porcine colon (35). All of these investigators, however, found heterogeneity of immunoreactivity in their preparations upon analysis which may explain the failure to identify any glucagon-like biologic activity. I have found this peptide to be quite labile in that the immunoreactivity was decreased by 85% after storage at −20°C for 5 months in the semipurified state. Tissue samples are often reported as frozen on dry ice and stored at −20°C until extracted. In view of the sensitivity of the glucagon biologic activity to proteolytic damage, the absence of activity observed in the studies cited could be due to breakdown during storage. Indeed the decrease in immunoreactivity observed in my preparations of GLI was paralleled by a loss of biologic activity in the hepatocyte studies. The investigations cited, demonstrating a lack of activity, had high levels of glucagon immunoreactivity but this was measured using N-terminal specific antisera whereas it is the C terminus of glucagon that is critical for biologic activity. AGS 10, used in these studies, will not bind the 1–12, 13–17, 17–29, or 18–29 sequences of glucagon but requires the entire glucagon molecule intact (S. Pek, University of Michigan, personal communication) providing an assay that is quite sensitive to proteolytic damage to the glucagon sequence.

The fact that glucose production is stimulated both *in vivo* and *in vitro* gives strong support to the conclusion that one of the functions of the ileal GLI is the modulation of hepatic glucose production. It is clear that

the distal ileum synthesizes and secretes this peptide and that it exerts a biologic action on a target tissue. These observations indicate that this GLI peptide is a distinct hormonal entity rather than a proglucagon molecule.

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Received November 29, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted March 19, 1985.