Specific Biologic Effects of Intestinal Glucagon-Like Materials

RAUL A. GUTMAN, GLORIA FINK, NANCY VOYLES, HELENA SELAWRY, JUAN C. PENHOS, ALBERT LEPP, and LILLIAN RECANT

From the Veterans Administration Hospital, Washington, D. C. 20422 and Departments of Medicine and Physiology, Georgetown University, Washington, D. C. 20007

ABSTRACT It has been demonstrated that gastrointestinal extracts contain substances which react immunologically with antibodies prepared to pancreatic glucagon. These extracts have been termed intestinal GLI for glucagon-like immunoreactivity, or enteroglucagon. To determine whether GLI has *specific biological effects*, studies were designed using the criterion of effect with antiglucagon antibodies. These antibodies did not cross-react with either secretin or pancreozymin.

Rat intestinal extracts were prepared and filtered on Sephadex G-50 columns eluted in 0.02 M ammonium carbonate buffer pH 8.8. Two peaks of GLI (I, II) were consistently found, and the in vitro effects of these peaks on two biological systems were tested: (*a*) immunoreactive insulin (IRI) release by rat pancreas pieces, and (*b*) free fatty acid (FFA) release and 3′, 5′-cyclic adenosine monophosphate (cAMP) levels in adipose tissue. Both GLI peaks increased IRI release in the absence of glucose and also enhanced the glucose effects. Antiglucagon antibody suppressed only peak II GLI activity. Both peaks increased FFA release and cAMP levels in adipose tissue. Only peak II GLI activity was suppressed by antibody.

These findings support a *specific* IRI-releasing and lipolytic action for Peak II GLI. Hypotheses are presented concerning the structure and possible physiologic role of peak II GLI.

INTRODUCTION

Sutherland, Cori, Haynes, and Olsen in 1949 (1) and Makman and Sutherland in 1963 (2) demonstrated that gastrointestinal extracts contained a substance which had certain biological properties in common with pancreatic glucagon. Unger, Eisentraut, Sims, McCall, and Madison (3) and Samols, Tyler, Megyesi, and Marks (4) subsequently reported that intestinal extracts cross-reacted with pancreatic glucagon antibodies. As a result, such extracts were termed intestinal GLI for glucagon-like immunoreactivity, or enteroglucagon.[1] Intestinal GLI of canine, bovine, and porcine origin have since been partially characterized on gel filtration (5) and ion exchange chromatography (6). In addition GLI has been shown to be present in serum and to be released into the circulation under a variety of circumstances (5, 7–9).

The metabolic role of intestinal GLI is, however, not as yet understood. Unger, Ohneda, Valverde, Eisentraut, and Exton (7) reported that the injection of intestinal GLI in dogs caused an increase in immunoreactive insulin (IRI) release accompanied by a drop in blood sugar. These results suggested the hypothesis that GLI may be the intestinal signal responsible for the greater IRI response to orally given glucose as compared to glucose given intravenously (10). The same authors, however, later disclaimed this physiological role of GLI on IRI release since they observed that although several monosaccharides given intraduodenaly raised circulating GLI, this was not accompanied by a significant increase in plasma IRI levels (11).

"In vitro" studies on the effects of GLI on IRI release rendered contradictory results. Buchanan, Vance, and Williams (12) failed to see any effect of intestinal GLI on IRI release from isolated islets.

Abstract presented at IV International Congress of Endocrinology, Washington, D. C., June, 1972.

Received for publication 12 October 1972 and in revised form 4 January 1973.

[1] *Abbreviations used in this paper:* AG-GPS, antiglucagon guinea pig serum; cAMP, 3′,5′-cyclic adenosine monophosphate; GLI, glucagon-like immunoreactivity; IRI, immunoreactive insulin; N-GPS, normal guinea pig serum.

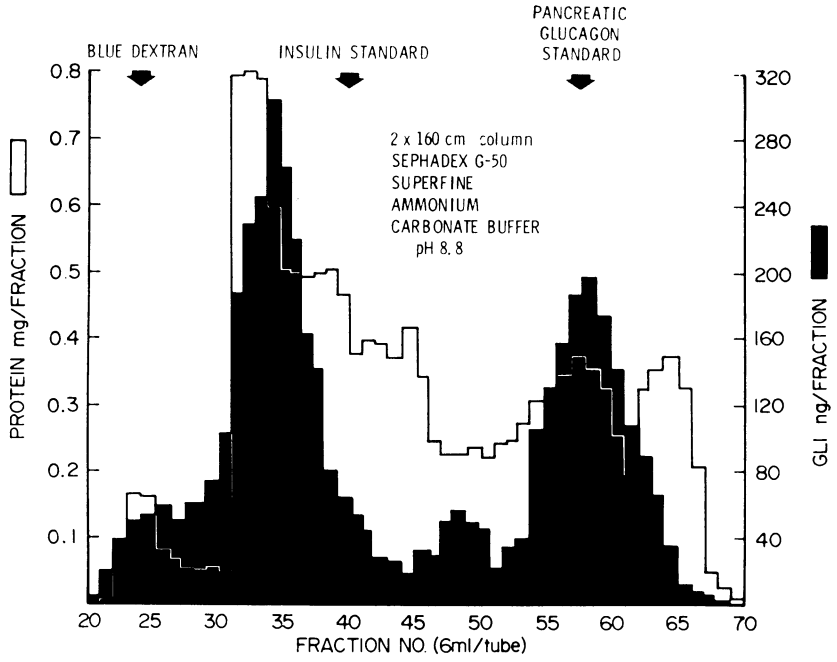

FIGURE 1 GLI and protein patterns of an acid-alcohol extract of rat small intestine. Two major GLI peaks are noted. Peak I elutes as a larger molecule, while peak II elutes in the area where pancreatic glucagon elutes.

Moody, Markussen, Schaich, Freis, Steenstrup, and Sundby (6) showed that several porcine intestinal fractions containing GLI produced IRI release. They indicated, however, that their data did not permit them to assess whether material possessing GLI is only associated with, or is, in fact, responsible for IRI release. Unknown, as well as known, intestinal hormones such as secretin and pancreozymin may contaminate intestinal GLI extracts. Both secretin and pancreozymin appear to release IRI under certain circumstances (13).

In order to determine whether GLI has specific biological effects on pancreas or adipose tissue, we designed studies using the criterion of suppressibility of GLI biological action by antiglucagon serum. This serum did not cross-react with either secretin or pancreozymin. These studies indicate that GLI may be a causative agent in the release of IRI.

METHODS

Intestinal GLI extraction procedure

Male, Sprague-Dawley rats (300–350 g) were used as intestine donors. Under Amytal anesthesis (66 mg/kg) the small intestine was rapidly dissected from a point 3 cm below the angle of Treitz to the cecum. After being flushed with cold saline, the mucosa was scraped and immediately extracted in the cold, by the acid-alcohol procedure of Davoren (14). Pooled mucosa (wet weight 63–65 g) obtained from 12 fed rats was routinely processed as a single batch with 1000 U Trasylol/10 g mucosa. As previously reported by Heding (8), the use of lower than standard concentrations of alcohol resulted in higher GLI yields. However, we observed that a 50% increase in GLI recovery was accompanied by an unwanted eightfold increase in the final protein concentration. We, therefore, decided to use the standard 77% acid-alcohol concentration for the first step of the extraction. Extractions were then carried to the ammonium acetate step, a point at which recoveries of pancreatic glucagon were found to be of the order of 50%. Similar yields were obtained with [^{125}I]-glucagon added to carrier intestinal mucosa. The ammonium acetate precipitate was then resuspended in ammonium carbonate buffer, the pH readjusted to 8.8, and after spinning down the insoluble proteins, the supernatant was subjected to Sephadex filtration.

Gel filtration was carried out at 4°C on a 2 × 160 cm column packed with Sephadex G-50 superfine equilibrated in 0.02 M ammonium carbonate buffer pH 8.8. When columns and glassware were coated with Siliclad (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.), recoveries of pancreatic glucagon from the column were found to be 70%. Fractions of 6 ml were collected. Aliquots were lyophilized and the GLI and protein patterns determined (Fig. 1). GLI peaks were then separately pooled and aliquots were taken from each pool for GLI and protein determinations. Pools were subsequently lyophilized and kept at 4°C for less than 2 wk before being used for biological studies. By utilizing this procedure, variations in GLI or protein content per gram of wet weight of mucosa were minimal (Table I). Over a 2 wk period, it was shown that the immunological, biological, and gel filtration properties of both GLI peaks remained stable.

Radioimmunoassays

Glucagon-like immunoreactivity. GLI was determined by using L-67 antiglucagon guinea pig serum (AG-GPS) which

had a broad spectrum of immunoreactivity. L-67 cross-reacted with intestinal extracts 10 times as well as Unger's 30K antibody (Table I). Pure secretin and pancreozymin in concentrations up to 10μ g/ml were unable to displace [^{125}I]glucagon bound to L-67 antibody. The assay procedure was similar to the Morgan-Lazarow double antibody method (15), using a glycine buffer pH 8.8 to which Trasylol (1,000 U/ml) was added. Fig. 2 shows a pancreatic glucagon standard curve in which a final concentration of 1:88,000 of L-67 antibody was used. Good reproducibility of samples was obtained. Pancreatic glucagon added to known amounts of intestinal GLI was recovered quantitatively. However, dilution curves of intestinal extracts behaved somewhat differently from dilution curves of pancreatic glucagon, indicating a lack of immunological identity. Similar findings with canine GLI peaks have been previously reported (7). The following limitations were consequently imposed on the results obtained with the use of the L-67 antibody: (*a*) quantitative results of different samples were compared only when the same dilution factors were applied to the samples, and (*b*) results were considered as nanogram immunoequivalents of pancreatic glucagon rather than absolute values.

Pancreatic glucagon determinations. Immunoreactive pancreatic glucagon (IRG) assays were carried out by using the method described by and the antibody (30K) provided by Dr. R. Unger.

Immunoreactive insulin. IRI was assayed by the Morgan-Lazarow procedure (15).

Biological studies on GLI

After determination of the GLI and protein content of the pools of peaks, volume aliquots were used to obtain the various GLI concentrations used for the biological studies.

IRI release from pancreas pieces. Incubation medium consisted of Krebs-Henseleit bicarbonate buffer with 200 mg/100 ml of gelatin and 1,000 U Trasylol/ml. Buffer was gassed with $O_2 + CO_2$ (95:5) for 10 min to achieve a pH of 7.4. GLI or other experimental materials to be tested were added to 1 ml of the medium and preincubated for 30 min at 37°C before the tissue was added. Pancreatic tissue was obtained from fed rats, cut into 100–120 mg pieces and, after a 20 min "wash-out" period in 60 mg/100 ml of glucose, transferred to the vessels containing the experimental medium. The actual experimental incubation consisted of three sequential incubation periods of 30 min each at 37°C in a Dubnoff shaker. No glucose was added to the medium in the first incubation period. In the second and third period glucose was added to a concentration of 100 and 300 mg/100 ml, respectively. At the end of each period, the medium was sampled for IRI. Each point of observation consisted of three to six vessels. Glucose controls were run with each experiment. An experiment was carried out to determine whether glucagon remains intact under the conditions described above. [^{125}I]-glucagon (50 ng/ml) was incubated as above for 60 min. The Sephadex filtration pattern before and after incubation remained essentially unchanged.

Studies on rat epididymal fat pad. All experiments were paired and run in duplicate, with one fat pad (cut in three pieces and placed in three vessels) as a control and the other fat pad (three vessels) as the experimental series. As described in the studies with pancreas pieces, GLI was preincubated for 30 min at 37°C in the incubation medium before the tissue was added to the vessel.

(*a*). Studies on FFA release were carried out as previously described (16). (*b*) Studies on tissue 3′, 5′-cyclic adenosine monophosphate (cAMP) levels were carried out by incubating fat pads for 10 min in a Krebs-Henseleit bicarbonate buffer pH 7.4 containing no albumin. Incubation was stopped by dropping the tissue into liquid nitrogen. Tissues were then extracted in 5% cold TCA, the supernatant extensively

TABLE I

Substances Quantitated in Sephadex Peaks of Intestinal GLI

	Total yield of GLI	
	Peak I	Peak II
Acid-alcohol extract of pooled small intestine of 12 rats, *ng GLI*	2,070±156	1,353±102
Concentration of:		
Protein, *μg/100 ng GLI*	193	159
Immunoreactive insulin (IRI), *μU/100 ng GLI*	2.7	1.2
Immunoreactive Pancreatic Glucagon* (IRG), *ng/100 ng GLI*	10.7	12.5
Pancreozymin,‡ *concentration units/100 ng GLI*	Undetectable	0.020753
Secretin‡, *concentration units/100 ng GLI*	Undetectable	0.003059

* As assayed with Unger's 30K antibody, specific for pancreatic glucagon.

‡ Bioassay.

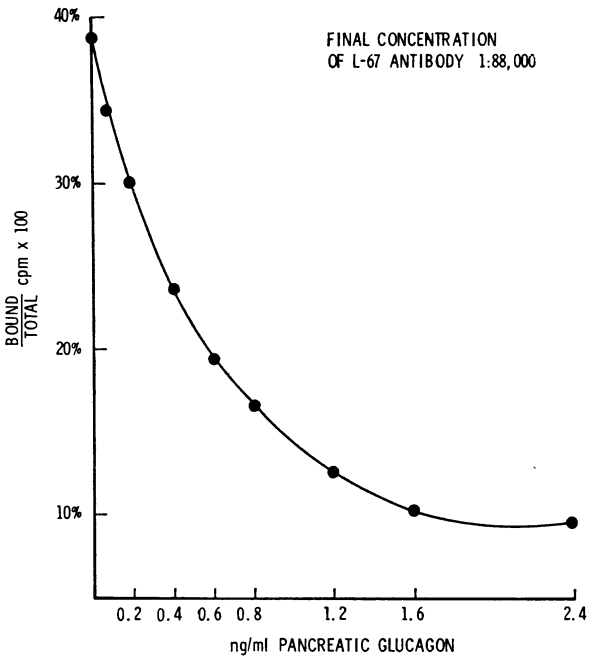

FIGURE 2 L-67 antiserum was produced in guinea pigs using pancreatic glucagon as an antigen. This is a relatively non-specific antibody since it cross-reacts with GLI. The immunoassay curve utilizing ^{125}I-labeled pancreatic glucagon is shown.

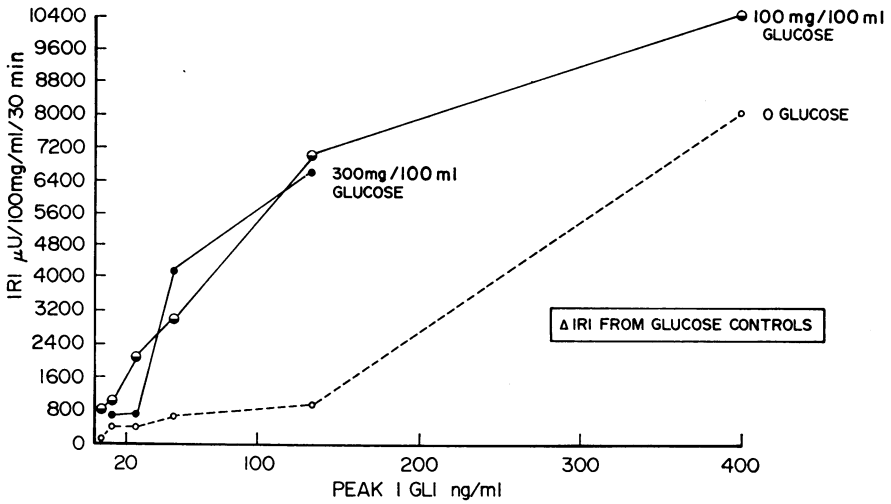

FIGURE 3 The in vitro effect of peak I GLI on insulin release from rat pancreas pieces is shown as the increment over controls.

washed with ether and finally lyophilized. cAMP determinations were carried out by the method of Gilman (17).

Antibody suppression studies. The effect of the addition of L-67 AG-GPS was studied upon the release of IRI or fat pad cAMP in the presence and absence of GLI, secretin, or pancreatic glucagon. Three types of vessels were prepared and preincubated for 30 min at 37°C before adding the tissues to the vessels: (*a*) the hormone or extract alone, (*b*) the hormone or extract plus L-67 AG-GPS, and (*c*) the hormone or extract plus normal (nonimmunized) guinea pig serum (N-GPS). The final antiserum concentration used was 1:250 since approximately 75% of 50 ng/ml of GLI were bound to the antibody under the experimental conditions above mentioned.

Liver perfusions. Closed circuit liver perfusions were carried out using overnight fasted rat livers as previously described (18). The perfusate was Krebs-Henseleit bicarbonate buffer pH 7.4 with 1% human albumin and 3% Dextran (T-40). After a 30 min equilibrium period, eluates containing peak I or peak II of intestinal GLI or pancreatic glucagon were added to the perfusate. 5 min was allowed for mixing purposes. The perfusate was then sampled at various times over a 25 min period for GLI to determine the effect of the liver on these materials.

Other methods

Secretin and pancreozymin content of the intestinal extracts was kindly determined by Dr. T. Scratcherd (Case, Harper, and Schratcherd [19]) using biological assays. The assay involves use of the isolated saline perfused cat pancreas. Water and electrolyte secretion are measured (19). Theophylline (10^{-4} M) was added to the perfusate. This increased the sensitivity of the preparation two- to fourfold. With this method the minimal detectable secretin is 250 pg. Secretin, prostaglandins E_1, E_2, and possibly $F_{2\alpha}$ as well as gastrin are the only known naturally occurring substances which will stimulate this preparation.

With regard to the pancreozymin assay, other than cholecystokinin and pancreozymin, gastrin and cholinergic stimuli can also stimulate the perfused pancreas. The assay can measure accurately less than 0.13 Crick, Harper, Raper units of pure pancreozymin.

Protein content of samples was assayed by the method of Lowry, Rosebrough, Farr, and Randall (20). Bovine albumin was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, and human albumin from the Cutter Laboratories, Berkeley, Calif. [^{125}]glucagon was obtained from Cambridge Nuclear, Cambridge, Mass. Pancreatic glucagon was a gift from the Eli Lilly and Company, Indianapolis, Ind. Sephadex and dextran were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. Y. Pure secretin and pancreozymin were obtained from Dr. J. E. Jorpes (Karolinska Instutet, Stockholm, Sweden). Tritium-labeled cAMP was obtained from Schwarz/Man Div., Becton, Dickinson & Co., Orangeburg, N. Y.

TABLE II

Concentrations of Peak I and Peak II GLI in Rat Duodenum and Stomach of Fed Rats (GLI ng/g of wet weight)*

	Duodenum	Stomach
Peak I GLI	13.2	5.9
Peak II GLI	19.9	14.4
Ratio peak I/peak II	0.66	0.41

* Data obtained on at least two experiments with six rats used in each.

RESULTS

Sephadex filtration patterns of rat intestinal extracts. Fig. 1 shows the typical and reproducible GLI pattern on Sephadex of acid-alcohol extracts of rat small intestine. Two major GLI peaks were seen. The first peak (peak I GLI) eluted in the 7,000 mol wt zone, and the second (peak II GLI) eluted as did the pancreatic glucagon standard (3500 mol wt). A similar GLI pattern was obtained whether gel filtration was carried out in 1 M acetic acid or in 0.02 M ammonium carbonate. The protein pattern followed a somewhat different distribution when compared to the GLI pattern.

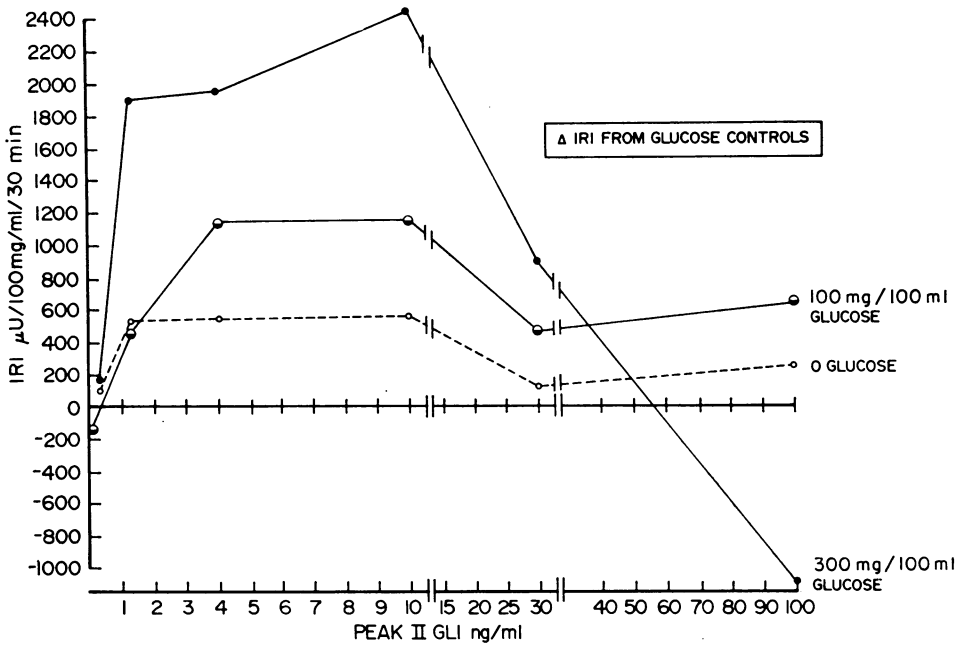

FIGURE 4 The in vitro effect of peak II GLI on insulin release from rat pancreas pieces is shown as the increment over controls.

Fractions with high concentration of protein were found to have low GLI content and vice versa. In the small intestine (excluding the duodenum) approximately 60% of the GLI was found to elute as peak I and 40% as peak II (Table I and Fig. 1).

Extracts of mucosa from duodenum and stomach showed on gel filtration different concentrations of both GLI peaks. On mucosal wet weight basis peak I GLI was diminished in the duodenum and strikingly diminished in the stomach as compared to the small intestine. The concentrations of peak II GLI also decreased, but these changes were much less pronounced. As a result, the ratio peak I GLI/peak II GLI fell from 1.52 in the small intestine to 0.66 in the duodenum and 0.41 in the stomach (Table II).

The nutritional status of the animal affected the small intestine concentrations of both GLI peaks. Table III shows that although both peaks were diminished, starvation preferentially affected peak I GLI. Consequently, the ratio peak I GLI/peak II GLI fell to 1.33 and 0.91 at 24 and 48 h of fasting, respectively.

Table I lists the concentration of measured components in each GLI peak. Secretin and pancreozymin as estimated by bioassay were undetectable in the peak I GLI pool and were present in very small amounts in the pool of peak II GLI.

GLI effect on IRI release from pancreas pieces. Both GLI peaks stimulated IRI release from pancreas pieces in the absence of glucose. In addition, they also potentiated the effect of either 100 or 300 mg/100 ml of glucose (Figs. 3 and 4, Table IV). Peak I GLI produced an increasing IRI-releasing effect when concentrations between 10 and 400 ng/ml GLI were used at any of the three glucose concentrations tested. Peak II GLI showed what seemed to be a dose-dependent IRI release between 0.3 and 4 ng/ml. The IRI responses were plateaued when doses between 4 and 10 ng GLI/ml were tested. This IRI-releasing pattern was similar at any glucose concentration. From 30 to 100 ng GLI/ml, an inhibitory effect was seen on the IRI release, particularly that induced by 300 mg/100 ml of glucose.

50 ng/ml of pancreatic glucagon had no effect on IRI release at any glucose concentration tested (Table V). Up to 3 U/ml of pure pancreozymin did not stimulate IRI release at 0 or 100 mg/100 of glucose. When

TABLE III

Effect of Fasting on Peak I and Peak II GLI Concentrations in Rat Small Intestine (GLI ng/g of wet weight)*

	Fed	24 h fasting	48 h fasting
Peak I GLI	32	25.1	13.4
Peak II GLI	21	18.8	14.7
Ratio peak I/Peak II	1.52	1.33	0.91

* Data obtained with at least 2 experiments utilizing the intestine of four or more rats in each of the fasting experiments and 25 experiments utilizing six rats in each experiment in the fed group.

TABLE IV

GLI-Induced IRI Release from Rat Pancreas Pieces (μU IRI/100 mg per ml)

	GLI (ng/ml)	No glucose Av	SEM	No. exp.	100 mg/100 ml glucose Av	SEM	No. exp.	300 mg/100 ml glucose Av	SEM	No. exp.
Controls	—	345±52		15	940±103		21	3664±307		21
Peak I GLI	400	8475±2112 (*b*)*		3	11520±1406 (*a*)		3			
	135	1215±228 (*b*)		3	7721±841 (*b*)		3	10298±497 (*b*)		3
	50	1055±170 (*b*)		6	3820±1230 (*a*)		6	7796±958 (*b*)		6
	25	716±94 (*b*)		3	3076±324 (*b*)		3	4161±467 (*b*)		3
	10	732±81 (*b*)		6	1949±474 (*f*)		6	4200±672 NS		6
	5	452±111 NS		3	1748±133 (*b*)		3			
Peak II GLI	100.0	589±129 (*d*)		3	1580±188 (*a*)		3	2612±247 (*e*)		3
	30.0	578±76 (*e*)		6	1433±167 (*b*)		6	4547±627 NS		6
	10.0	878±49 (*b*)		9	2095±166 (*b*)		9	6085±419 (*b*)		9
	4.0	896±86 (*b*)		12	2072±99 (*b*)		12	5623±258 (*b*)		12
	1.3	896±72 (*b*)		6	1411±150 (*e*)		6	5564±600 (*c*)		6
	0.3	452±91 NS		3	789±164 NS		3	3836±373 NS		3

**P* values designated by the letters (*a*) through (*f*): (*a*) <0.001; (*b*) <0.0025; (*c*) <0.005; (*d*) <0.01; (*e*) <0.02; (*f*) <0.025; NS, not significant.

pharmacologic doses were used and only in the presence of glucose was pancreozymin able to release IRI. Pure secretin stimulated IRI release with characteristics similar to that of pancreozymin (Table V).

The presence of L-67 antiglucagon serum (AG-GPS) in the incubation medium had no effect on the capacity of peak I GLI to release IRI (Table VI). In contrast, it significantly inhibited the IRI release produced by peak II GLI. 53%, 67%, and 51% inhibition of the peak II GLI effect was seen at 0, 100, and 300 mg/100 ml of glucose, respectively. The presence of L-67 AG-GPS did not affect secretin-induced IRI release.

GLI effect on epididymal fat pad (Fig. 5 and Tables VII and VIII). Both GLI peaks increased tissue cAMP levels and FFA release from epididymal fat pad (Table VII). Increasing concentrations of peak II GLI between 32 and 672 ng/ml resulted in a progressive and parallel increase in both tissue cAMP levels and FFA release into the incubation medium (Fig. 5). In contrast, 900 ng/ml of peak I GLI were required to obtain a minimal response (Table VII). 100 ng/ml of pancreatic glucagon elicited a clear tissue cAMP and FFA response. 60–70% of the effects of peak II GLI on tissue levels of cAMP were inhibited by the addition

TABLE V

Effects of Pancreozymin, Secretin, and Pancreatic Glucagon on IRI Release from Rat Pancreas Pieces (μU IRI/100 mg/ml per 30 min)

	Dose	No glucose Av	SEM	No. exp.	100 mg/100 ml glucose Av	SEM	No. exp.	300 mg/100 ml glucose Av	SEM	No. exp.
Controls	—	345±52		15	940±103		21	3664±307		21
Pancreozymin, *U/ml*	1.0	314±30 NS		6	931±35 NS		6			
	3.0	395±66 NS		6	1024±158 NS		6			
	10.0	386±13 NS		6	1031±131 NS		6	5048±333 (*b*)*		6
Secretin, *U/ml*	3.0	399±49 NS		6	919±106 NS		6	6656±688 (*b*)		6
	10.0	387±67 NS		6	1585±362 (*a*)		6	9358±872 (*b*)		6
Pancreatic glucagon, *ng/ml*	50.0	417±159 NS		6	814±147 NS		6	3558±371 NS		6

* *P* values designated in the footnote of Table IV.

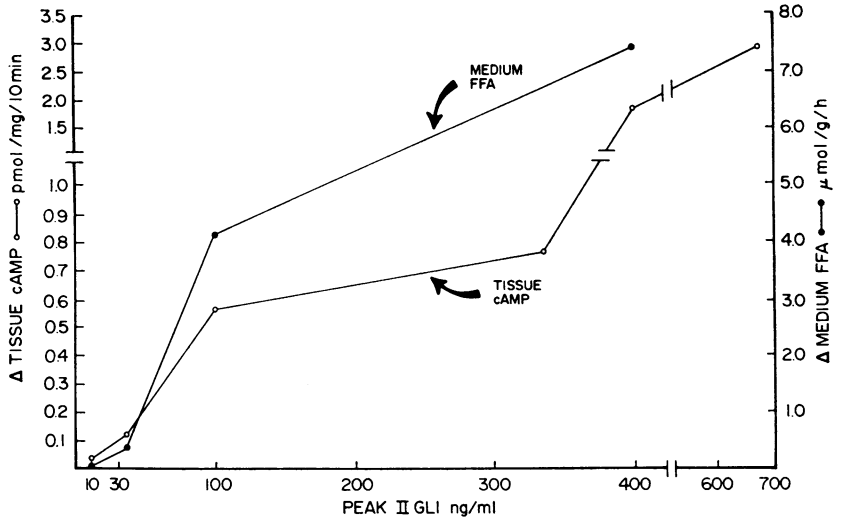

FIGURE 5 In vitro effect of peak II GLI on rat epididymal fat pad. Peak II GLI produces an increase in levels of cAMP in adipose tissue and increases FFA release into the medium. The increment over control tissue or medium levels is plotted.

of L-67 AG-GPS (Table VIII). The same antiglucagon serum suppressed most of the FFA-releasing effects of 100 ng/ml of pancreatic glucagon (Table IX). Peak II GLI had no significant effect on the basal or insulin-stimulated [^{14}C] glucose conversion to $^{14}CO_2$ (data not shown).

Effects of perfusion of intestinal GLI and pancreatic glucagon through isolated rat liver (Fig. 6). Glucagon immunoreactivity of native pancreatic glucagon disappeared from the liver perfusate with a $t_{\frac{1}{2}}$ of approximately 4 min. In contrast, immunoreactivity from either peak I or peak II GLI was essentially unaffected

TABLE VI

Effect of Antiglucagon Antiserum (AG-GPS) on GLI-Induced and Secretin-Induced IRI Release from RAT Pancreas Pieces (μU IRI/100 mg/ml per 30 min)

		No glucose			100 mg/100 ml glucose			300 mg/100 ml glucose	
	GLI (ng/ml)	Av	SEM	No. exp.	Av	SEM	No. exp.	SEM	No. exp.
Controls		345±52		15	940±103		21	3664±307	21
Peak I GLI	25 + N-GPS	717±94		3	3076±324		6	4161±467	3
	25 + AG-GPS	830 ± 115 NS*		3	2960±383 NS		3	5063±611 NS	3
Percent inhibition		0			0			0	
Peak II GLI	4	896±86		12	2072±99		12	5623±258	12
	4 + N-GPS	938±67		3	1980±102 NS		3	5888±307 NS	3
	4 + AG-GPS	606±111 (*f*)		3	1316±200 (*b*)		3	4659±231 (*e*)	3
Percent inhibition‡		53			67			51	
Secretin	7.5 + N-GPS	357±82 NS		3	960±122 NS		3	7075±620	3
	7.5 + AG-GPS	398±28 NS		3	932±64 NS		—	7460±775	3
Percent inhibition								0	

* *P* values designated in the footnote of Table IV.

‡ The GLI effect was calculated as the increment in IRI over the respective glucose control. The percent inhibition of the GLI effect by AG-GPS was then calculated as follows:

$$100 \times \frac{(\text{GLI effect}) - (\text{GLI effect in AG-GPS})}{(\text{GLI effect})}.$$

TABLE VII

GLI Effects on Rat Epididymal Fat Pad

		Tissue, cAMP levels				Medium FFA Levels			
		Control pair		Experimental pair		Control pair		Experimental pair	
	GLI	Av	SEM	Av	SEM	Av	SEM	Av	SEM
	ng/ml	*pmol/mg per 10 min*				*μmol/g per h*			
Peak I GLI	900	0.109±0.041		0.307±0.028 (*a*)*		1.27±0.09		2.95±0.23 (*b*)	
	300	0.180±0.051		0.206±0.017 NS					
Peak II GLI	672	0.206±0.081		3.096±0.375 (*b*)					
	400	0.218±0.034		2.047±0.345 (*c*)		0.82±0.03		8.30±1.10 (*c*)	
	336	0.233±0.071		0.986±0.366 (*d*)					
	100	0.186±0.021		0.706±0.110 (*c*)		0.81±0.03		4.98±1.11 (*c*)	
	32	0.163±0.026		0.280±0.034 (*d*)		0.44±0.07		0.87±0.03 (*a*)	
	10	0.126±0.028		0.143±0.014 NS		0.68±0.08		0.61±0.07 NS	
Pancreatic glucagon	100	0.175±0.049		0.384±0.012 (*c*)		0.22±0.04		4.66±0.97 (*c*)	
	50					0.22±0.06		0.26±0.04 NS	

* *P* values designated in the footnote of Table IV.

by the liver over 25 min. To rule out the possibility that some factor present in the intestinal extracts affected the ability of the liver to destroy GLI, [^{125}I]-glucagon was added to an intestinal extract and then subjected to liver perfusion. It was seen that [^{125}I]-glucagon was destroyed to a comparable extent in the presence or in the absence of intestinal proteins.

DISCUSSION

Effect of GLI on IRI release. Peak I GLI and peak II GLI actively stimulated IRI release in the absence as well as in the presence of glucose. When the features of the IRI-releasing effects of each peak were compared, four major differences emerged. First, peak I GLI produced optimal IRI release at 100 mg/100 ml of glucose while 300 mg/100 ml seemed to be optimal for peak II. This was clearly evidenced when the increments in IRI over the glucose controls were plotted (Fig. 4 and 5). A second difference was found in the characteristics of the respective dose-response curves. Peak I GLI produced a progressive increase in IRI release with increasing GLI concentrations. In contrast, the results obtained with peak II GLI suggested that at concentrations between and above 4–10 ng/ml, an inhibitor was present in the extract. Two known inhibitors of IRI release, serotonin (21) and epinephrine (22), are present in gastrointestinal mucosa and could have been contaminating our extracts. Whatever the nature of this inhibitor, it could explain why the GLI of some intestinal extracts was reported to have no IRI-releasing activity (23). It could also account for the fact that no quantitative correlation was found

TABLE VIII

Effects of Antiglucagon Serum (AG-GPS) on GLI-Induced Elevations of cAMP in Fat Pad

Peak II GLI		No. exp.	Tissue cAMP	Net GLI effect on cAMP	Effect of AG-GPS	
					Δ	Inhibition
ng/ml			*pmol/mg per 10 min*			%
0	Controls	26	0.161±0.013	—	—	—
70	+N-GPS*	3	0.540±0.069	+0.379	−0.227	60
	+AG-GPS‡	3	0.313±0.024 (*a*)§	+0.152		
100	+N-GPS	3	0.830±0.085	+0.669	−0.459	70
	+AG-GPS	3	0.371±0.082 (*e*)	+0.210		

* N-GPS, normal guinea pig serum at 1:250 dilution.

‡ AG-GPS, guinea pig antiglucagon serum at 1:250 dilution.

§ *P* values designated in the footnote of Table IV.

by Moody et al. (6) between the GLI content and the IRI-releasing potency of some of their fractions. The third difference noted between peak I and II GLI was the potency of their IRI-releasing effects. Peak II GLI appeared to be active at lower concentrations than peak I at any glucose concentration tested, although the quantity of insulin released by peak I was greater than that elicited by peak II. The final and most interesting difference between peak I and peak II GLI related to the glucagon antibody suppression experiments. While antiglucagon serum had no noticeable effect on the peak I action, it significantly suppressed most of the IRI-releasing effects of peak II GLI. These results provide direct evidence that the GLI material present in peak II is indeed the causative agent of most of the in vitro IRI-releasing action associated with this peak. The lack of complete antibody suppression of the effects of peak II suggests that either some GLI material remained unbound and thus, still active, or that some other material accompanying peak II also had IRI-releasing effects. This latter material is unlikely to be either secretin or pancreozymin, because 1.3 ng/ml of peak II GLI, the lowest active concentration, contained about 0.2 mU of pancreozymin and 0.03 mU of secretin. No effect on IRI release could be attributed to such small amounts of secretin or pancreozymin. Most authors (12, 6, 23) including this laboratory (16) failed to show in vitro IRI release with pure secretin or pancreozymin even when doses of magnitude greater than that present in our extracts were tested. It also seems unlikely to be the rat intestinal polypeptide preparation of Turner and Marks which appears to require glucose for insulin-releasing activity (24).

The question arises as to why the biological activity of peak I GLI remained unchanged when L-67 AG-GPS was added to the medium while peak II activity was suppressed. Two possibilities exist: (*a*) the GLI present in peak I is not responsible for the biological effects, or (*b*) the molecule of peak I GLI differs from the molecule of peak II GLI or pancreatic glucagon in that

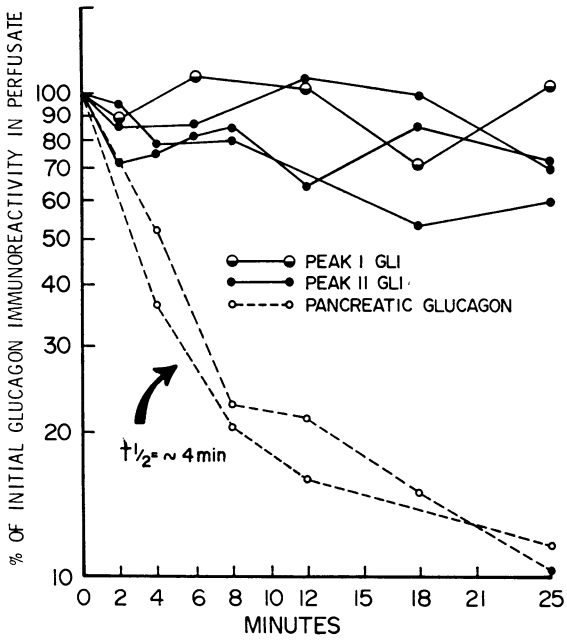

FIGURE 6 Comparative effect of isolated liver perfusion on intestinal GLI and pancreatic glucagon. Perfusion of GLI or pancreatic glucagon through isolated rat liver results in rapid disappearance of immunoreactivity of pancreatic glucagon but not of peaks I or II GLI.

binding of the antibody site does not impair activity of the biological site. The latter could theoretically occur if both sites were either functionally or structurally separated. This type of phenomenon has been shown to be true for various ACTH molecules (25). If peak I GLI is indeed an inactive species, it could conceivably account for the finding that a large variety of nonglucose substances which induce elevations of GLI are not accompanied by plasma IRI elevations (11).

Effects of GLI on epididymal fat pad. Both GLI peaks increased cAMP levels and FFA release from adipose tissue. Peak I GLI, however, was approximately 30 times less effective in this regard than peak

TABLE IX

Effect of Antiglucagon Serum on Pancreatic Glucagon-Induced FFA Release

		Medium FFA levels	No. exp.	Effect of AG-GPS: Δ	Effect of AG-GPS: Inhibition
		μmol/g per h			%
100 ng/ml pancreatic glucagon	+N-GPS*	4.66±0.32	3	−2.82	63
	+AG-GPS‡	1.84±0.15 (*a*)§	3		

* N-GPS, normal guinea pig serum at 1:250 dilution.
‡ AG-GPS, guinea pig antiglucagon serum at 1:250 dilution.
§ $P < 0.001$.

II GLI. A lipolytic effect of dog jejunal GLI peaks has previously been shown (26).

L-67 AG-GPS suppressed 60–70% of the cAMP elevation induced by peak II GLI, indicating that most of the effects on adipose tissue associated with peak II are specifically induced by the GLI present in that fraction. The nonsuppressible activity could be accounted for either by GLI not bound by antibody or by the secretin present in peak II GLI. The latter seems likely since it has been shown (27) that even very low concentrations of secretin (0.1 U/ml) are lipolytic in fat pad. Pancreozymin has been shown to have no effect on FFA release from fat pad (16).

When GLI effects on fat pad were compared to the GLI effects on IRI release, three major differences were evident. In the first place, the threshold for the fat pad response was approximately 10 and 30 times higher for peak I and II, respectively, when compared to the threshold for IRI release. Secondly, no inhibitory phase was evident in fat pad when large doses of peak II GLI were used. Thus, whatever the nature of the inhibitor, it appears to have affected only the IRI response to GLI. Finally, when roughly immunoequivalent amounts of peak II GLI and pancreatic glucagon were compared, they proved to be similarly effective in stimulating cAMP levels and FFA release from adipose tissue. In contrast, as regards IRI release, the biological activity per unit of immunological reactivity was strikingly higher for either GLI peak when compared to pancreatic glucagon. One additional difference between intestinal GLI and pancreatic glucagon was of particular interest. While the liver destroyed the immunoreactivity of pancreatic glucagon with a $t_{\frac{1}{2}}$ of approximately 4 min, it had almost no effect on either GLI peak.

Assan and Slusher (28) have reported information on function-structure, immunoreactivity-structure relationships of pancreatic glucagon and related synthetic peptides. They concluded that the binding sites for the antibody specific for pancreatic glucagon must reside in the 24–29 position of the molecule. The binding sites for the nonspecific antiglucagon antibody (the one binding intestinal GLI) should be present somewhere in the 1–23 peptide. With regard to the biological sites, IRI release and glycogenolysis reside in the 24–29 peptide, while lipolysis is associated with the 19–23 amino acid sequence. The 1–19 peptide was biologically inactive. When our combined immunologic and biologic data on peak II GLI are assessed in terms of Assan's findings, the following possibilities emerge:

(*a*) The mol wt of peak II GLI and pancreatic glucagon are similar suggesting that the number of amino acids in both molecules are similar also, possibly 29.

(*b*) The 24–29 sequence present in peak II GLI must be sequentially different from pancreatic glucagon since the antiglucagon serum specific for pancreatic glucagon reacts very poorly with peak II GLI. Further support for this contention may be found in the fact that immunoequivalent amounts of peak II had much greater IRI-releasing activity than pancreatic glucagon.

(*c*) Sequence 19–23 is probably similar in peak II GLI and glucagon since effects on fat pad were comparable when immunoequivalent doses of both were used.

Our data does not permit us to make any speculations concerning the structure or the biological effects of peak I GLI. On the other hand, a significant physiologic role for peak II GLI as an intestinal signal for IRI release after glucose can be postulated for the following reasons:

(*a*) Fasting results in marked decreases of intestinal content of GLI suggesting that the ingestion of nutrients may regulate GLI.

(*b*) Both GLI peaks are secreted into the mesenteric vein after the administration of glucose (29). Since the liver did not affect the immunoreactive properties of either GLI peak, it could be assumed that in contrast with pancreatic glucagon, the liver does not degrade GLI. If this assumption is correct, both peaks would be available to peripheral target tissues.

(*c*) And finally, the greater potency of GLI on IRI release compared with the effects on adipose tissue focus upon the pancreas as the major target organ for GLI.

ACKNOWLEDGMENT

This work was supported by Veterans Administration and NIH Grant AM 11578.

REFERENCES

1. Sutherland, E. W., C. F. Cori, R. Haynes, and N. S. Olsen. 1949. Purification of hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. *J. Biol. Chem.* **180**: 825.
2. Makman, M. H., and E. W. Sutherland. 1964. Use of liver adenyl cyclase for assay of glucagon in human gastro-intestinal tract and pancrease. *Endocrinology.* **75**: 127.
3. Unger, R. H., A. M. Eisentraut, K. Sims, M. S. McCall, and L. L. Madison. 1961. Sites of origin of glucagon in dogs and humans. *Clin. Res.* **9**: 53. (Abstr.)
4. Samols, E., J. Tyler, C. Megyesi, and V. Marks. 1966. Immunochemical glucagon in human pancreas, gut and plasma. *Lancet.* **2**: 727.
5. Valverde, I., D. Rigopoulou, J. Marco, G. Faloona, and R. H. Unger. 1970. Characterization of glucagon-like immunoreactivity (GLI). *Diabetes.* **19**: 614.
6. Moody, A. J., J. Markussen, A. Schaich Freis, C. Steenstrup, and F. Sundby. 1970. The insulin releasing activities of extracts of pork intestine. *Diabetologia.* **6**: 135.

7. Unger, R. H., A. Ohneda, I. Valverde, A. M. Eisentraut, and J. Exton. 1968. Characterization of the responses of circulating glucagon-like immunoreactivity to intraduodenal and intravenous administration of glucose. *J. Clin. Invest.* **47**: 48.
8. Heding, L. G. 1971. Radioimmunological determination of pancreatic and gut glucagon in plasma. *Diabetologia.* **7**: 10.
9. Buchanan, K. D., J. E. Vance, T. Aoki, and R. H. Williams. 1967. Rise in serum immunoreactive glucagon after intrajejunal glucose in pancreatectomized dogs. *Proc. Soc. Exp. Biol. Med.* **126**: 813.
10. McIntire, N., C. D. Holdsworth, and D. S. Turner. 1964. New interpretation of oral glucose tolerance. *Lancet.* **2**: 20.
11. Marco, J., G. R. Faloona, and R. H. Unger. 1971. Effect of endogenous intestinal glucagon-like immunoreactivity (GLI) on insulin secretion and glucose concentration in dogs. *J. Clin. Endocrinol. Metab.* **33**: 318.
12. Buchanan, K. D., J. E. Vance, and R. H. Williams. 1969. Insulin and glucagon release from isolated islets of Langerhans. Effect of enteric factors. *Diabetes.* **18**: 381.
13. Unger, R. H., H. Ketterer, J. Dupre, and A. Eisentraut. 1967. The effects of secretin, pancreozymin, and gastrin on insulin and glucagon secretion in anesthetized dogs. *J. Clin. Invest.* **46**: 630.
14. Davoren, P. R. 1962. The isolation of insulin from a single cat pancreas. *Biochim. Biophys. Acta.* **63**: 150.
15. Morgan, C. R., and A. Lazarow. 1963. Immunoassay of insulin: two antibody system. Plasma insulin levels of normal, subdiabetic and diabetic rats. *Diabetes.* **12**: 115.
16. Lazarus, N. R., N. R. Voyles, T. Tanese, S. Devrim, and L. Recant. 1968. Extra-gastrointestinal effects of secretin, gastrin and pancreozymin. *Lancet.* **2**: 248.
17. Gilman, A. G. 1970. A protein binding assay for adenosine 3′: 5′-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 305.
18. Penhos, J. C., J. C. Basabe, N. Lopez, and R. Levine. 1971. Effect of diabetes or hyperglycemia on lipid metabolism and urea formation by the perfused rat liver. *Horm. Metab. Res.* **3**: 10.
19. Case, R. M., A. A. Harper, and T. Schratcherd. 1968. Water and electrolyte secretion by the perfused pancreas of the cat. *J. Physiol. (Lond.).* **196**: 133.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
21. Feldman, J. M., and H. E. Lebovitz. 1969. Inhibition of insulin secretion by serotonin. *Diabetes.* **18**(Suppl. 1): 326.
22. Porte, D., Jr., A. L. Graber, T. Kuzuya, and R. H. Williams. 1966. The effect of epinephrine on immunoreactive insulin levels in man. *J. Clin. Invest.* **45**: 228.
23. Turner, D. S. 1969. Intestinal hormones and insulin release: in vitro studies using rabbit pancreas. *Horm. Metabol. Res.* **1**: 168.
24. Turner, D. S., and V. Marks. 1972. Enhancement of glucose-stimulated insulin release by an intestinal polypeptide in rats. *Lancet.* **1**: 1095.
25. Imura, H., L. L. Sparks, G. M. Grodsky, and P. H. Forsham. 1965. Immunologic studies of adrenocorticotropic hormone (ACTS): dissociation of biologic and immunologic activities. *J. Clin. Endocrinol. Metab.* **25**: 1361.
26. Lefebvre, P. J., R. H. Unger, I. Valverde, A. S. Rigopoulou, A. S. Luyckx, and A. M. Eisentraut. 1969. The effect of dog jejunum GLI material on adipose tissue metabolism. *Horn. Metab. Res.* **1**: 143–144.
27. Raptis, S., J. D. Faulhaber, and K. E. Schroder. 1969. The effect of intestinal hormones upon lipolysis of isolated human fat cells. *Horm. Metab. Res.* **1**: 249.
28. Assan, R., and N. Slusher. 1972. Structure/function and structure/immunoreactivity relationships of the glucagon molecule and related synthetic peptides. *Diabetes.* **21**: 843.
29. Valverde, I., D. Rigopoulou, J. Marco, G. R. Faloona, and R. H. Unger. 1970. Molecular size of extractable glucagon and glucagon-like immunoreactivity (GLI) in plasma. *Diabetes.* **19**: 624.