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Plasma Immunoreactive Glucagon Fractions in Four Cases of Glucagonoma: Increased "Large Glucagon – Immunoreactivity"*

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Summary. Immunoreactive glucagon (IRG) fractions from plasma of 8 normal subjects and 4 patients with glucagon secreting tumors were studied by gel filtration techniques on Bio Gel P–30 and Sephadex G–50 columns. The pancreatic glucagon specific anti serum (30K) of Unger was utilized to measure IRG. Columns were calibrated with labelled albumin, proinsulin, insulin and glucagon. Four peaks were defined in normal and tumor bearing patients: peak I (>20000 mol. wt.), peak II (primarily 9000 mol. wt.), peak III pancreatic glucagon (3500 mol. wt.) and peak IV small glucagon (<3500 mol. wt.). Glucagonoma patients differed from our normal and reported normal subjects in that peak II contained most of the circulating IRG. The percent of IRG associated with peak II was 9.5–31.5% in normals and 39.1–61.2% in glucagonomas. Glucagon-like biological activity in an isolated hepatocyte system was demonstrated for all peaks. However, relative to immunoreactivity, peak II showed reduced activity (25–33%). Immunoassay of dilutions of all peaks revealed the probability of immuno determinants identical with porcine pancreatic glucagon. The presence of heterogeneous IRG peaks with biological glucagon-like activity suggest that the larger molecules may be prohormones. Further, it is possible that specific elevation of peak II may be a diagnostic feature of glucagonomas.

Key words: Glucagonoma, increased plasma "Large glucagon-immunoreactivity".

The availability of "specific" radioimmunoassays for measurement of pancreatic glucagon (IRG) in plasma and tissue has permitted the documentation of an increasing number of glucagon secreting tumors [1, 2, 3, 4, 5, 6]. A glucagonoma syndrome has emerged which is characterized primarily by the presence of diabetes, a skin rash described as bullous necrolytic migratory erythema and a pancreatic islet cell neoplasm (most often adenocarcinoma) containing α–2 granules [4]. We have studied 4 patients with documented glucagon secreting tumors. All had strikingly elevated plasma IRG levels, weight loss, diabetes or glucose intolerance, and only one had a classical skin rash. The purpose of these studies was to characterize the circulating glucagon components and to determine their biological activity relative to their immunoreactivity compared with standards of porcine pancreatic glucagon. In addition, studies were carried out with the circulating glucagon components observed in normal human subjects.

Materials and Methods

Patients Studied

Table 1 describes pertinent features of the glucagonoma patients. Eight normal, ambulatory subjects were also studied. Normal subjects on normal diets and receiving no medication showed fasting plasma glucagon levels of < 0.20 ng/ml with a mean of 0.15 ng/ml. Blood samples were obtained in chilled tubes containing EDTA and Trasylol (500 U per ml of blood). Tubes were rapidly centrifuged in the cold, and the plasma immediately frozen and stored at –20°C. Unless otherwise indicated, overnight fasting samples were utilized.

Gel Filtration Studies

Plasma IRG components were separated in the cold (4°C) by gel filtration on columns measuring 45 × 1.5 cm. Columns were calibrated with ^{125}I-glucagon, ^{131}I-

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Table 1. Features of glucagonoma subjects studied

	Symptoms and signs						
	Age	Sex	Rash	Diabetes	Other	Plasma IRG	Documentation
H.C.	60	M	0	+ insulin Rx	Wt. loss, abd. pain, metastases to liver	3–5 ng/ml	Pancreatic islet cell tumor, E.M. α granules; sudden death 10 yrs. p dx.
C.S.[a]	52	M	+	+ insulin Rx	Wt. loss, abd. pain, metastases to liver	5–13 ng/ml	Pancreatic islet cell tumor, E.M., 2 types granules including α–2, immunofluorescent glucagon in tumor, tumor IRG 14 ng/gm, good response to streptozotocin Rx
L.L.	68	F	0	glucose intolerance	Abd. pain, abd. mass, metastases to liver	2.35 ng/ml	Pancreatic islet cell tumor, E. M. α granules, good response to 1st course of streptozotocin, remission 1st year, relapse
M.H.[b]	53	F	0	+ insulin Rx insulin resistance	Wt. loss, hepatomegaly	2.30 ng/ml	Pancreatic islet cell tumor E.M. 2 types granules including α tumor IRG 0.78 µg/g G–I bleeding, sudden death

[a] Clinical findings and plasma IRG responses to streptozotocin to be reported elsewhere [7].
[b] Previously reported case [6].

albumin, ^{125}I-insulin and ^{125}I-proinsulin. Each column was calibrated with all markers prior to filtration of plasma. Columns were only used for a 7 day period since, after that time, the columns were more tightly packed and some variation in peak tubes for markers occurred. To provide an internal control for all samples, ^{125}I-glucagon was included with each sample run. One to 4 ml of plasma were placed on Bio-Gel P–30 columns, prepared and eluted with 0.2 M glycine buffer containing 0.25% human albumin and 1% normal sheep serum (pH 8.8), as described by Valverde et. al. [8, 9]. The flow rates were 0.13 ml/min. Fractions of 1.5 ml were collected in 500 U Trasylol and assayed directly for IRG by the procedure of Unger et al. [10] using his 30K anti serum, specific for pancreatic glucagon. The assay measures 0.02 ng/ml of glucagon with reproducibility. Recovery of glucagon on Bio-Gel columns ranged from 60–125%. The total IRG appearing in the volume of distribution of ^{125}I-albumin was used to calculate peak I or big IRG. (In most instances, this included tubes No. 11–17). The volume of distribution of ^{125}I-glucagon was used to calculate peak III or "pancreatic" IRG (usually tubes 28–38). The IRG peak(s) appearing between albumin and glucagon were totalled for peak II. This fraction was primarily composed of material in the elution volume of proinsulin, but obviously included more than one molecular weight species. Complete separation of these peaks was not always possible. Peak IV represented the IRG appearing beyond the ^{125}I-glucagon standard. From these data, the percent IRG in each peak was calculated. Studies of immunologic behavior of these peaks were carried out utilizing the tube having the highest concentration of IRG. In peak II, this tube usually coincided with the proinsulin peak.

Plasma samples, 50–80 ml, from each glucagonoma subject and from normal subjects were extracted with acid alcohol by the Davoren procedure [11]. This procedure was carried out through the alcohol-ether step. These extracts (utilizing the equivalent of 8–10 ml plasma per column) were then subjected to gel filtration on Sephadex G–50 superfine columns (45 × 1.5 cm), equilibrated and eluted with 0.02 M $(NH_4)_2CO_3$, pH 8.8. Filtration was carried out at 4°C and flow rates were 0.175 ml/min. Fractions (2 ml) were collected and aliquots were lyophilized from each tube for assay. Four IRG peaks were separated as noted above and identified by use of labelled albumin and glucagon markers. Addition of labelled glucagon to plasma prior to acid alcohol extraction and gel filtration revealed a 75% recovery of the glucagon with an elution volume on G-50 Sephadex similar to that observed when ^{125}I-glucagon was added subsequent to acid-alcohol extraction. Occasionally when an older (presumably partially degraded) ^{125}I-glucagon marker was used, a small ^{125}I peak appeared in the void volume as well as in the expected location. This could be seen either on Sephadex or Bio-Gel

columns. Subsequently, the IRG peaks were pooled and lyophilized prior to assay for biological activity. Proinsulin was iodinated by the procedure of Hunter and Greenwood [12]. ^{125}I-glucagon was obtained from Nuclear Medical Laboratories, ^{125}I-insulin from New England Nuclear and ^{131}I-albumin from Abbott Laboratories. Porcine glucagon for use as standards was kindly provided by Dr. Mary Root of Eli Lilly Co. Bio-Gel was purchased from Bio Rad Laboratories and Sephadex from Pharmacia, Inc.

Biological Activity Studies

The assay procedure used involved the measurement of cAMP generated by isolated rat liver cells in the presence of respective IRG peaks. Liver cells were isolated from fed Sprague-Dawley rats by the collagenase digestion method of Zahlten and Stratman [13, 14]. Collagenase was obtained from Worthington Biochemical. Standard curves with crystalline porcine glucagon were run simultaneously. The incubations were carried out at 37°C in 95% O_2–5% CO_2 in 1.5 ml Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.5% human albumin (Cutter) with 3.75×10^6 cells. After 45 minutes of preincubation in a Dubnoff shaking incubator, 0.5 ml of glucagon standards (containing a total of 1.5–5.0 ng) or samples of IRG peaks were added. Theophylline was present in a final concentration of 2 mM. Triplicates of all samples and quadruplicates of standard glucagon were assayed. Incubation was terminated at exactly 1 minute with addition of 1 ml cold 10% trichloroacetic acid. After sonication and centrifugation, the supernatant was placed on Dowex 50×8 (H^+) 200–300 mesh columns, washed with 0.1 NHCl and eluted with 8 ml water. The water eluate was then placed on AG 1×2 (Cl^-) 100–200 mesh columns, washed with water and eluted with 1 NHCl. These columns were prepared in Pasteur pipettes (0.5×6.5 cm). The eluates were lyophilized and assayed for cAMP by the method of Gilman et al. [15]. Corrections were made for the recovery of ^{3}H-cAMP (Schwarz-Mann) from each ion exchange column. Cyclic AMP increments of 30 ± 7 pmoles were observed with 1.5 ng glucagon, 45 ± 5 for 2.5 ng glucagon and 70 ± 6 for 5 ng glucagon. These values represent the mean ± SEM.

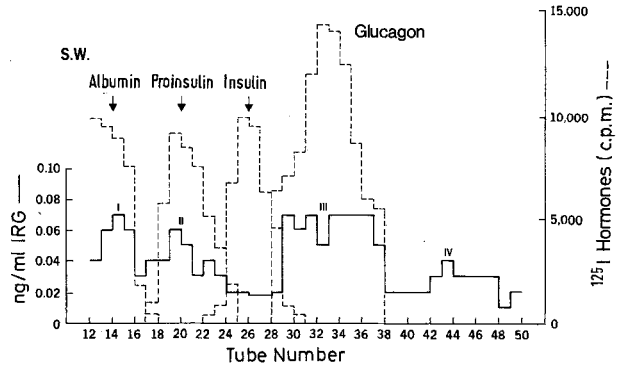

Fig. 1. Normal plasma IRG pattern on a Bio-Gel P-30 column. The positions of marker radioactive hormones are indicated. Four peaks can be seen. Peak II is predominantly a 9000 M. W. species

Table 2. Distribution of plasma IRG in bio-gel column peaks

		% Total IRG in respective peaks[a]			
		Peak I	Peak II	Peak III	Peak IV
Normal subjects:	S.B.	45.4	9.5	35.0	10.1
	P.H.	58.2	10.8	18.8	12.2
	B.B.	45.8	13.3	27.4	13.5
	E.B.	37.0	21.8	29.6	11.6
	S.W.	12.2	17.1	39.8	30.9
	M.K.	10.8	14.2	47.0	28.0
	A.W.	14.7	16.1	47.1	22.1
	E.F.	7.7	31.5	50.8	10.0
Mean		29.0	16.8	37.0	17.2
Glucagonoma subjects:	L.L.	19.8	46.1	24.6	9.4
	C.S.	7.5	61.2	19.2	12.2
	M.H.	19.3	39.1	22.8	18.8
	H.C.[b]	8.7	40.4	40.1	10.8
Mean		13.8	46.7	26.7	12.8
Glucagonoma with Rx:	C.S.	28.0	25.0	38.0	7.0
Diabetic subject on diet:	P.R.	9.3	29.4	47.8	13.4

[a] Data obtained from Bio-Gel P–30 columns. The calculations are based on the total IRG of each column and the IRG associated with each peak. Column recoveries varied as indicated in the text.
[b] Data obtained from G–50 Sephadex column.

Results

Normal Plasma Glucagon Fractions

Figure 1 shows the IRG pattern on an Bio-Gel P-30 column utilizing basal plasma from a normal subject (S. W.) The dotted lines indicate the location of molecular markers on this column. Four IRG peaks may be seen similar to those previously described by Valverde et al. [8]. Peak I (high molecular weight "big glucagon") present in the void volume of the column, peak II corresponding with 9000 molecular weight (elution volume of proinsulin), designated as "proglucagon" by Valverde et al. [9], peak III 3500

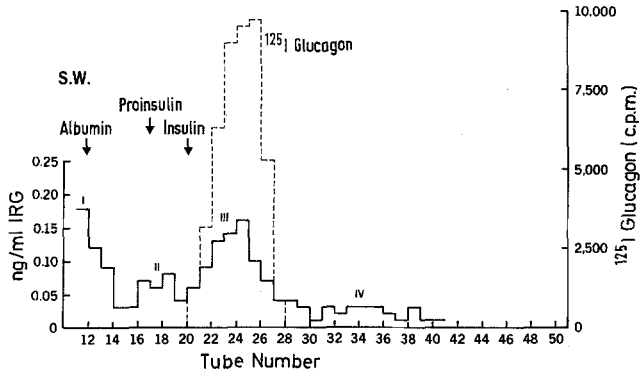

Fig. 2. Acid alcohol extract of normal plasma filtered on a Sephadex G-50 column. The peak position of labelled albumin, proinsulin and insulin are shown by arrows. The location of ^{125}I-glucagon coincides with peak III. Four peaks can be seen as in Bio-Gel columns

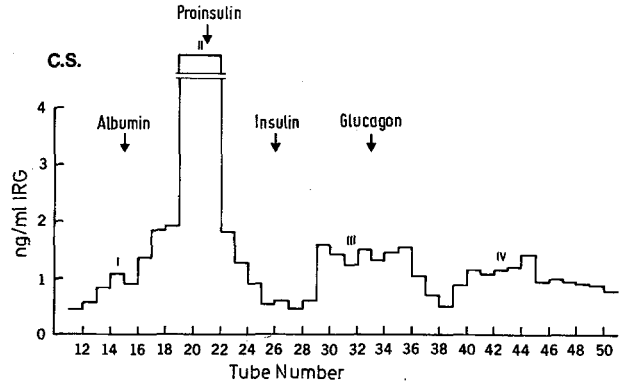

Fig. 3. Bio-Gel column-IRG pattern obtained with plasma from patient C. S. with a documented glucagonoma. Peak II is markedly increased (M.W. 9000)

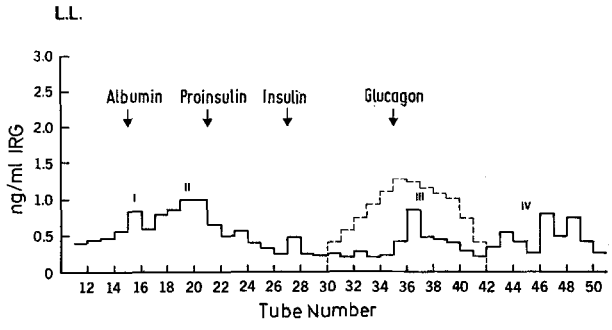

Fig. 4. Bio-Gel column- IRG pattern obtained with plasma from patient L.L. with a documented glucagonoma. Note increase in peak II IRG. Dotted lines indicate location of labelled glucagon peak

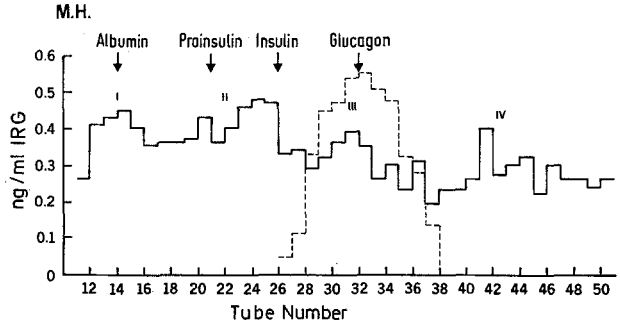

Fig. 5. Bio-Gel column-IRG pattern obtained with plasma from patient M.H. with a documented glucagonoma. Peak II is prominent and appears to be heterogeneous with a marked 7000 M. W. component

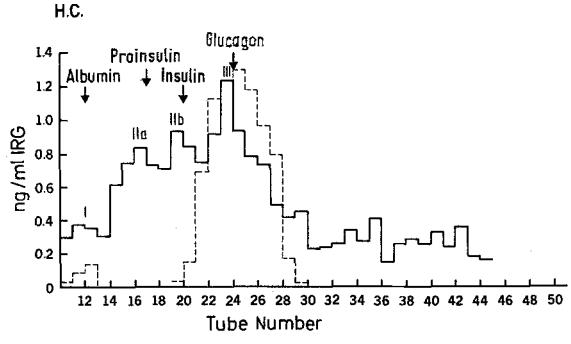

Fig. 6. Sephadex G-50-IRG pattern obtained with an acid alcohol extract of plasma from patient H.C. with documented glucagonoma. The two components of peak II are labelled a (9000 M.W.) and b (7000 M. W.)

molecular weight (elution volume of pancreatic glucagon) and peak IV <3500 molecular weight. The patterns obtained in the 8 normal subjects studied were similar to those reported by Valverde et al. [8]. There was considerable variation in the proportion of IRG in each of the peaks. In no instance was there a preponderance of peak II. Table 2 quantifies the basal IRG patterns in these subjects. The percent of total column IRG present in each of the peaks is shown. It should be noted that peak II represented 9–31.5% of total IRG.

Following acid alcohol extraction of plasma and gel filtration on G-50 Sephadex, 4 similar peaks can be identified (Fig. 2). Although the apportionment of IRG seen in these peaks differs from that observed on Bio-Gel columns, this was not always the case. In most instances, similar proportions were noted. Rechromatography of these peaks on Sephadex G-50 reveals no change in elution of IRG.

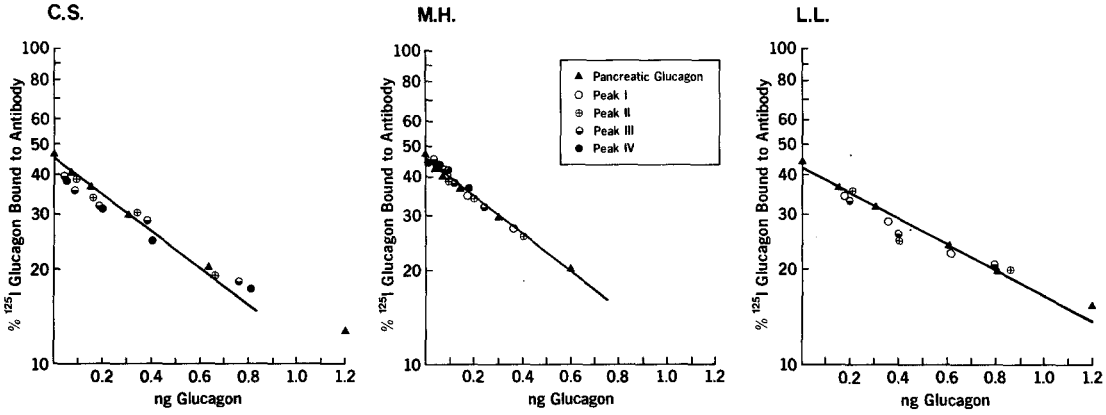

Fig. 7. Peak IRG tubes from Bio-Gel columns obtained with plasma of 3 patients with glucagonoma (C.S., M.H., L.L.) were diluted, IRG assayed and compared with standard pancreatic glucagon. All IRG fractions appear to have similar immuno determinants

Plasma Glucagon Fractions in Patients with Glucagonoma

Figures 3, 4 and 5 show Bio-Gel P-30 patterns of plasma IRG observed in 3 subjects with documented glucagonoma, while Figure 6 shows the IRG pattern observed after acid alcohol extraction in the fourth glucagonoma patient (H.C.). Calculations of percent IRG in each peak are shown in Table 2. Patients L.L., M.H. and H.C. showed patterns grossly similar to normal. However, peak II contained 39.1–61.2% of IRG present in plasma. Patient C.S. differed from the others, and the normals in that peak II was extraordinarily elevated representing > 60% of the total IRG. Following a course of streptozotocin, his plasma glucagon fell to 0.55 ng/ml [7], and peak II diminished (25% of IRG) so that the IRG pattern qualitatively and quantitatively could not be differentiated from normal (Table 2).

Dilution Curves of IRG Peaks

Peak tubes were obtained from Bio-Gel P–30 columns and IRG determined. Tubes were then diluted 1:2, 1:4, 1:8 and re-assayed. The values are plotted on semilogarithmic paper as percent ^{125}I-glucagon bound to antibody versus the presumed IRG concentration (ng/ml), based on the assay of undiluted material. Figure 7 shows that dilution of all peaks results in parallel lines to dilutions of standard pancreatic glucagon.

Biological Activity of IRG Peaks in Patients with Glucagonoma

Acid alcohol extracts of plasma from the 4 glucagonoma subjects were placed on Sephadex G-50. The pooled peaks were lyophilized for bioassay. The Sephadex columns showed IRG patterns essentially similar to the Bio-Gel columns noted above with striking elevation of peak II in C.S. The results of bioassay are shown in Table 3.

Big glucagon and pancreatic glucagon in C.S. are as biologically active as predicted from the immunoassay. Peak II (the major peak) showed only 32% of the activity predicted from immunoreactivity, while the small glucagon appeared more active than predicted. In 3 cases, the 3500 mol wt. peak was as active, or more active, than predicted by immunoassay. In L.L., peaks I and III were as active or more so than predicted, while peak II had reduced activity (33% of immunoreactivity). Patient H.C. showed active peak III, but reduced activity of peak II (25% of predicted value). In M.H., a combination of peaks I and II revealed reduced activity (23% of predicted value), while peak III was slightly more active than predicted. Since a combined pool of peaks I and II showed less activity than predicted, while peak I alone was active in 3 instances, the possibility was considered that peak II might contain materials which inhibit cAMP formation. This was tested in C.S. Standard amounts of pancreatic glucagon were added to peak II. Bioassay revealed good recovery of added glucagon.

Discussion

At least four different molecular weight components of immunoreactive glucagon have been described in normal human plasma [8]. Similar components are present in normal canine plasma [9]. Nothing is known of the nature nor biological importance of the molecules which are larger or smaller than the 3500 molecular weight component. The latter is thought to be identical with pancreatic glucagon. The site of origin of these substances has not been defined since all

Table 3. Biological activity of circulating IRG components

	Pooled peaks[a]	IRG ng	Bioassay – ng glucagon equivalents[b]	Ratio Bio/IRG	Molecular size by markers
C.S.	I	1.2	1.4	1.16	>20000
	II	7.7	2.5	0.32[c]	9000
	III	3.9	4.1	1.05	3500
	IV	1.1	3.6	3.28	< 3500
L.L.	I	1.4	3.9	2.79	>20000
	II	3.0	1.0	0.33	9000
	III	2.13	2.5	1.17	3500
H.C.	II a + b	4.0	1.0	0.25	6–9000
	III	4.0	4.0	1.0	3500
M.H.	I + II	5.8	1.1	0.23	7000 –> 20000
	III	2.3	4.0	1.74	3500

[a] Peaks obtained from acid-alcohol extracts of plasma filtered on G-50 Sephadex columns.
[b] Calculated from cAMP accumulation in rat hepatocyte assay.
[c] Addition of pancreatic glucagon to peak II resulted in recovery of bioactivity of added glucagon.

components are present in depancreatized dog plasma [9] as well as in eviscerated rats lacking pancreas and gut for 72 h [16].

The availability of plasma from four patients with glucagon secreting tumors prompted this investigation. Since a variety of endocrine tumors appear to secrete increased amounts of larger molecular species such as proinsulin [17], big-big insulin [18] and big-big gastrin [19], it was of interest to examine the patterns of IRG secretion in patients with glucagonomas. In addition, it seemed possible that information concerning the biological activity of these plasma components could be obtained by virtue of the availability of large amounts of IRG.

The IRG patterns in our 8 normal subjects were similar to those previously reported. In the glucagonoma patients, peaks similar in elution position to those observed in normal subjects could be identified. These peaks included peak I (> 20000 mol. wt.), peak II (9000 mol. wt. primarily, but also probably 7000 size), pancreatic glucagon, peak III (3500 mol. wt.) and small glucagon, peak IV (< 3500 mol. wt.). Despite this similarity of pattern to normal, calculation of the distribution of IRG in the four peaks revealed that peak II contained most of the IRG in H.L., M.H. and C.S. In H.C. peaks II and III had the highest IRG content. In patient C.S., peak II was dramatically increased. It should be noted that in normal subjects, despite the variation in the percent of IRG associated with the respective peaks, *in no instance* was peak II the largest peak. In fact, in all cases, peak II represented a smaller percentage of IRG than peak III, with a mean value of 16.8% of the total IRG. In the glucagonoma patients, peak II represented 39.1% to >60% of IRG, with a mean of 46.7%. The unusual pattern observed in patient C.S. assumes greater significance in that following treatment with streptozotocin, previously shown to be effective in two cases of glucagonoma [5] [20], not only did the total plasma IRG fall [7], but peak II diminished to 25% of IRG. It is of special interest that of the glucagonoma subjects, only C.S. manifested the "classical" dermatitis described in the glucagonoma syndrome. It is tempting to speculate 1) that elevation of peak II IRG is a concomitant of glucagonomas and perhaps diagnostic, and 2) that the clinical syndrome may be dependent upon the particular IRG moieties secreted in great excess.

Another possibility to be considered is that hyperglucagonemia of any etiology is associated with specific elevation of peak II. This does not appear to be the case since arginine stimulation in man produces a small elevation in peak II but a major increase in peak III [8]. The hyperglucagonemia observed in diabetic dogs [9] was also characterized primarily by increases in peak III. In the one diabetic subject whom we studied, the peak was also peak III. In the glucagonoma subjects, no relationship of distribution of IRG was seen with insulin administration in that L.L. received no insulin and showed 46.1% IRG in peak II, while M.H. was insulin resistant and showed 39.1% IRG in peak II. Further, in no case were glucagon antibodies found.

With regard to the 4 IRG fractions in glucagonoma subjects and in the one normal subject studied, it appears that they probably contain immunodeterminants identical with glucagon. Immunoassay curves of the 4 peaks were identical to standard porcine pan-

creatic glucagon dilution curves. Further, acid alcohol extraction of the plasma, which favours dissociation of noncovalent bonds and antibody complexes, did not alter the gel filtration patterns. In particular, the larger molecular size IRG peaks retained their elution positions. Rechromatography also resulted in no change in elution characteristics. These findings suggest that peaks I and II are not aggregates of pancreatic glucagon, but might well be glucagon moieties incorporated in larger peptide or protein structures, similar to the manner in which the insulin moieties exist in proinsulin [21] and perhaps big-big insulin.

Studies of the biologic activity of these peaks provides data which indicate that all the peaks have some glucagon-like activity, in that they can stimulate the formation of cAMP by isolated rat hepatocytes. Calculation of the ratios of biologic/immuno-activities provides an additional assessment of the nature of these peaks compared with pancreatic glucagon. In 3 of 4 plasmas from patients with glucagon secreting tumors, peak III showed ratios of 1.05–1.74, indicating that the 3500 mol. wt. species appear to be very similar to standard pancreatic glucagon. Peak II, measured separately in 3 of the 4 cases, revealed decreased biologic activity relative to immunoactivity of the order of 23–33% of the expected biologic activity. With regard to the big glucagon (peak I) component, in the 2 cases where the biological activity was tested without additional peaks being present, we recovered highly significant biological activity with biological activity/immunoreactivity ratios of 1.16 and 2.7. In the one instance in which peak IV was studied, biologic activity was greater than expected from the immuno-equivalent activity.

The interpretation of any biologic activity measurements must be made with caution unless one is dealing with "pure" preparations. In the present system, it is not possible to rule out the presence of non-glucagon containing substances which either stimulate or inhibit the assay system. Since added glucagon activity was recovered when tested with peak II of C.S., it was concluded that the decreased activity of this peak was not due to the presence of an inhibitor isolated with that peak. It is obviously desirable to extend these observations to include recoveries of glucagon standards added to each of the peaks, as well as to study the effect of the addition of anti-glucagon anti-serum on the bio-activity. In this way, one could relate biologic activity directly to the glucagon moiety. Such studies are under way but require large amounts of the peak materials.

Unfortunately, studies of bio-activity of normal plasma IRG components were not possible because of the difficulties inherent in obtaining sufficient material. However, it seems reasonable to suggest that the observations concerning biological activity of the four peaks in glucagonomas may well be applicable to normal subjects.

Despite the report of Weir et al. [22] concerning the probability that big glucagon (peak I) represents an interference factor in measurements of plasma IRG, our data would support the likelihood that big glucagon is a biologically active component, possibly a prohormone. This interpretation would be compatible with the tryptic digestion studies of Valverde et al. [8]. Similarly, the finding of biologically active glucagon in peak II supports its potential role as a significant metabolic contributor to the total circulating IRG. This fraction is best termed "large glucagon – immunoreactivity" at this time, though it has also been referred to as proglucagon-like material. In our glucagonoma subject, C.S., considering the bio-activity of peak II at 32% of pancreatic glucagon activity, this component could have represented sufficient active glucagon to produce metabolic effects.

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