Heterogeneity of Plasma Glucagon

CIRCULATING COMPONENTS IN NORMAL SUBJECTS AND PATIENTS WITH CHRONIC RENAL FAILURE

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ABSTRACT Plasma immunoreactive glucagon (IRG) concentrations were measured in 36 patients with chronic renal failure (CRF) and 32 normal subjects. In addition, the components of circulating IRG were analyzed by gel filtration in the fasting state and after physiological stimuli. Fasting IRG was elevated ($P <$ 0.001) in CRF patients (534±32 pg/ml) compared with the levels found in healthy subjects (113±9 pg/ml). Oral glucose suppressed plasma IRG in CRF patients from a basal level of 568±52 to a nadir of 354± 57 pg/ml (120 min). This degree of suppression (38%) was comparable to that found in normal subjects (basal = 154±20 to 100±23 pg/ml) at 120 min (35%). Intravenous infusion of arginine (250 mg/kg) resulted in a 71% rise in IRG in CRF patients and a 166% increase in normal subjects.

Gel filtration of fasting plasma from CRF patients showed three major peaks. The earliest (A) was found in the void volume (mol wt > 40,000) and constituted 16.5±4.7% of the elution profile. The middle peak (B) eluted just beyond the proinsulin marker (approximately 9,000 mol wt) and constituted the largest proportion of the elution profile (56.5±3.4%). The third peak (C) coincided with the standard glucagon and [^{125}I]glucagon markers (3,485 mol wt) and comprised 27.0±4% of the IRG profile. In contrast, only peaks A and C were found in fasting plasma of normal subjects (53.6±10.4% in A and 46.4±10.4 in C). After oral glucose, glucagon immunoreactivity in the 3,500 mol wt peak (C) was markedly suppressed, while the B peak in patients with CRF declined to a lesser extent. The A peak in both groups was unchanged. After an arginine infusion only the C peak increased in both groups of subjects.

Gel filtration of plasma in 3 M acetic acid gave similar profiles to those obtained in glycine albumin buffer. Exposure of serum to trypsin indicated that the B and C peaks were digestible, while the A peak was resistant to the action of the enzyme. In one sample, peak C increased after a 2-h exposure of serum to trypsin.

We conclude that circulating IRG in normal subjects and patients with CRF is heterogenous. The hyperglucagonemia of renal failure is largely due to an increase in IRG material of approximately 9,000 mol wt, consistent with proglucagon, although the 3,500 mol wt component is also considerably elevated (threefold). The significance of circulating IRG levels should be interpreted with caution until the relative biological activity of the three components is established.

INTRODUCTION

Chronic renal failure (CRF) [1] is accompanied by elevated plasma immunoreactive glucagon (IRG) levels (1–6). In a preliminary communication (7) we have shown that circulating IRG in this condition is heterogenous, and that a significant amount of glucagon immunoreactivity is present in a peak with a molecular weight of approximately 9,000, consistent with descriptions of proglucagon (8–14). Studies by other investigators have

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[1] *Abbreviations used in this paper:* CRF, chronic renal failure; IRG, immunoreactive glucagon.

indicated that IRG may exist in several forms in plasma of normal and diabetic subjects (15, 16) and of experimental animals (17, 18). However, the responses of the various plasma IRG species to physiological stimuli have not been defined in detail, and their significance is uncertain. Valverde et al. (16) showed that arginine stimulated only the IRG of 3,500 mol wt ("true" glucagon) in normal man. In another report (17) similar results were noted in pancreatectomized dogs. In addition these authors pointed out that an infusion of somatostatin caused a reduction of both the 3,500 and 9,000 mol wt material (17). In the present study we have investigated the effects of oral glucose and intravenous arginine administration on the various IRG fractions in the blood of normal and CRF subjects, and have analyzed the large molecular weight fractions by gel filtration in acidic buffers and by limited tryptic digestion. In addition, the hyperglucagonemia and characteristic gel-filtration patterns of plasma IRG in patients with CRF have been induced in rats after nephrectomy.

METHODS

Subjects. Fasting plasma was obtained from 36 stable CRF patients (15 females, 21 males; age 44.6±2.2 yr) for measurement of glucagon, glucose, and creatinine. All patients were on chronic hemodialysis and had glomerular filtration rates of less than 5 ml/min. Their serum creatinine averaged 15.0±0.8 mg/100 ml, and none were overtly diabetic. 32 fasting healthy subjects (21 males and 11 females within 15% of ideal body weight; age range 19–43 yr), including laboratory staff and medical students, provided blood for control plasma glucagon determinations. All patients were studied on nondialysis days (oral glucose) or immediately before dialysis (intravenous arginine). Medications were omitted on the morning of the test. All subjects ate at least 250 g carbohydrate for 3 days before the study. Written, informed consent to participate in the study was obtained from each patient.

Oral glucose tests. 100 g glucose (Glucola; Ames Co., Elkhart, Ind.) was administered orally after an overnight fast to five CRF patients and eigth control subjects. Blood was obtained at 0, 30, 60, 90, 120, 180, and 240 min and analyzed for glucose and IRG.

Arginine tests. Five CRF patients and five normal subjects received an intravenous infusion of 250 mg/kg body weight arginine hydrochloride over 30 min after an overnight fast. Eight normal subjects also received a dose of 500 mg/kg body weight. Blood was obtained at 0, 15, 30, 45, 60, and 90 min and processed for glucose and IRG.

Assay procedures. IRG was measured by a double antibody radioimmunoassay (7) using a specific α-cell glucagon antibody (30K) obtained from Dr. R. Unger, Dallas, Tex. Plasma glucose and creatinine were measured with the Technicon AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y.).

Gel filtration. 1 or 2 ml plasma was gel-filtered on 50 × 1-cm Biogel P-30 columns (Bio-Rad Laboratories, Richmond, Calif.). The samples were eluted under gravity at room temperature with 0.2 M glycine buffer, pH 8.8, containing 0.25% human serum albumin, 1% lamb serum, and 500 U/ml Trasylol (FBA Pharmaceuticals, Inc., New York). 1-ml fractions were collected and the total volume assayed. For the acetic acid studies, 1.5 ml plasma was gel filtered on 58 × 2-cm Biogel P-30 columns and eluted with 3 M acetic acid. 3-ml fractions were collected and evaporated *in vacuo,* reconstituted in 1.0 ml glycine buffer and assayed. The columns were callibrated using ^{125}I-γ-globulin, porcine proinsulin (9,033 mol wt), porcine insulin (5,777 mol wt), glucagon (3,485 mol wt), or ^{125}I-glucagon, and ^{125}I-Na.

Trypsin digestion. Tryptic hydrolysis was carried out as follows: 1.0 ml CRF plasma (three samples) was incubated with diphenyl carbamyl chloride-treated trypsin at an enzyme protein ratio 1: 100 for 2 and 6 h at room temperature with gentle mixing. The reaction was stopped by cooling and the addition of soybean trypsin inhibitor at an inhibitor/trypsin ratio of 2: 1. Control and trypsin-treated samples were then gel filtered on Biogel P-30 columns. Pooled eluates from the 9,000 mol wt zone of a gel filtered CRF plasma sample were also subjected to tryptic hydrolysis and rechromatographed as described above.

Rat studies. In an attempt to reproduce the hyperglucagonemia of renal failure and to study its gel filtration characteristics, bilateral nephrectomy was carried out in six rats. Six other rats were sham operated (renal decapsulation). Both groups of animals remained fasting after the operative procedure. 24–48 h postoperatively, blood was collected by aortic puncture. Plasma samples were asssayed for IRG and analyzed by gel filtration as described above.

Statistical methods. Results are presented as means± SEM. The statistical significance of changes in individual subjects was assessed by analysis of paired differences and that of differences between group means by the Student's *t* test. *P* values less than 0.05 were considered significant.

RESULTS

Fasting IRG levels. The mean plasma IRG level in the CRF patients was 534±32 pg/ml (range 230–1,050 pg/ml). This was significantly higher ($P < 0.001$) than the concentration in the healthy subjects (113±9 pg/ml). In only 3 of the 36 CRF patients, the plasma IRG level fell within the normal range of 40–266 pg/ml.

Glucose and IRG responses to oral glucose and arginine infusion. Plasma IRG and glucose responses to oral glucose are shown in Fig. 1. In the CRF patients the mean fasting plasma IRG (568±52 pg/ml) was suppressed by 38% to 354±57 pg/ml at the nadir (120 min). A similar degree of suppression of IRG (35%) was found in normal subjects, i.e., 154±20 pg/ml fasting to 100±23 pg/ml at 120 min. Glucose tolerance in the renal failure patients was less than in the normal controls. The immunoreactive insulin concentrations at 0, 30, 60, 120, 180, and 240 min were 20.4±4.7, 134± 19.9, 139.6±21.1, 131.6±32.6, 85.0±20.3, and 56.0±17.0 μU/ml, and 9.3±0.6, 88.5±13.8, 103.0±8.5, 73.3±13.4, 28.0±18.0, and 15.0±8.0 μU/ml in the patients with CRF and the controls, respectively.

Plasma IRG responses to intravenous arginine infusion are shown in Fig. 2. In normal subjects the standard 500-mg/kg dose led to a mean increment of

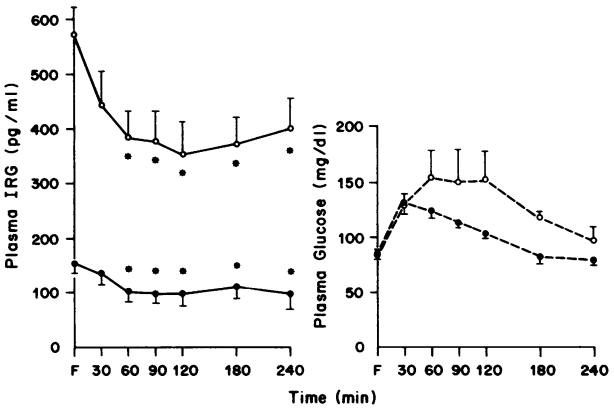

FIGURE 1 Plasma IRG and glucose responses (mean± SEM) to 100 g oral glucose load in five CRF patients (○) and eight normal subjects (●). Significant difference from fasting level ($P < 0.05$) as assessed by paired t test (*).

303±31 pg/ml (383%) in plasma IRG. The 250-mg/kg dose produced a significant increment ($P < 0.001$) in plasma IRG of 189±21 pg/ml (166%). Only 250 mg/kg arginine was infused in the CRF patients to avoid aggravating azotemia and acidosis. The mean increment of plasma IRG (420±85 pg/ml) was 2.2 times greater than that of control subjects given the same dose, but represented only a 71% rise from basal values.

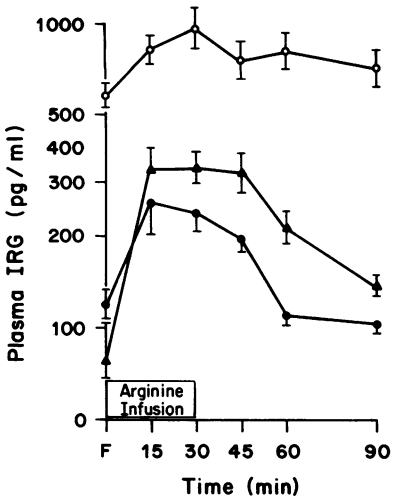

FIGURE 2 Plasma IRG response (mean±SEM) to arginine infusion (250 mg/kg) in five CRF patients (○) and five normal subjects (●). Values in eight normal subjects (▲) given 500 mg/kg arginine are also shown.

Gel filtration

Elution profiles of plasma IRG in normal and CRF subjects. Recovery of endogenous IRG in 20 CRF plasmas was 109.2±3.2%, while it was 79.3±6.4% in 16 samples from normal subjects. The lowest recoveries

TABLE I

Plasma Glucagon Responses and Distribution of Glucagon Immunoreactivity in the Fasting State and after Oral Glucose in Five Patients with CRF and Four Normal Subjects

	Fasting				After oral glucose				
		IRG in fraction					IRG in fraction		
Subject	IRG	A	B	C	Time	IRG	A	B	C
		pg/ml			*min*		*pg/ml*		
CRF									
1	480	20	263	257	180	220	24	206	12
2	740	52	499	267	180	520	40	442	85
3	580	67	523	158	180	400	59	311	40
4	440	155	179	55	180	400	158	190	0
5	600	62	358	253	180	310	32	295	60
Mean	568	71.2	364.4	198		370	62.6	288.8	39.4
SE	52.4	22.5	66.3	40.8		50.2	24.5	45.1	15.5
Normal									
1	180	60	0	128	90	135	76	0	0
2	250	85	0	79	120	180	105	0	47
3	120	8	0	59	90	80	5	0	18
4	155	34	0	79	60	54	34	0	0
Mean	176.3	46.8	—	86.3		112.3	55.0	—	16.3
SE	27.5	16.6	—	14.7		28.2	22.1	—	11.1

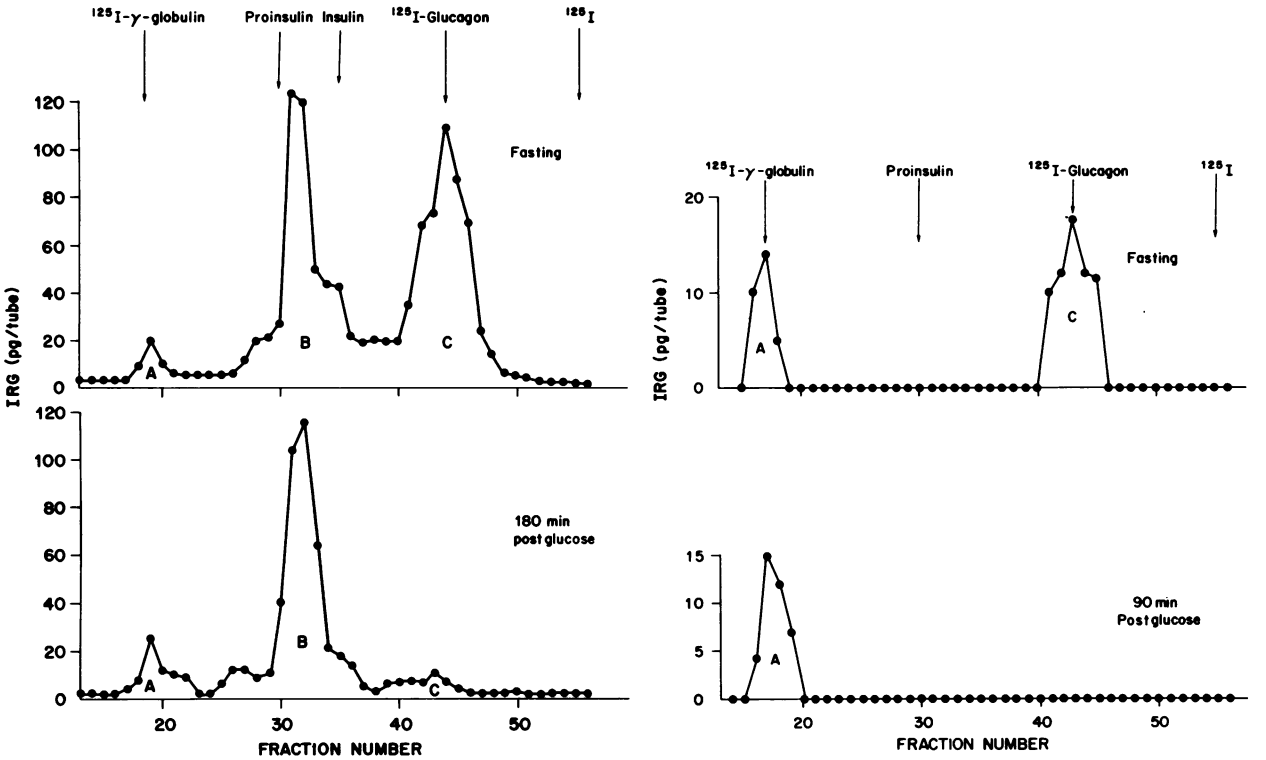

FIGURE 3 Representative elution profiles of plasma IRG on 1 × 50-cm Biogel P-30 columns before and after 100 g oral glucose in a patient with CRF (left) and in a healthy subject (right).

were found in normal samples with low concentrations of total IRG. The recovery of known amounts of purified beef-pork glucagon dissolved in buffer was 95%, which was similar to the result obtained when the same standard was added to plasma and then gel filtered. Both elution profiles were comparable. Aliquots of a pooled plasma sample run twice on the same column and on different columns gave essentially similar profiles.

Representative gel filtration profiles of plasma samples from fasting CRF and normal subjects are shown in Figs. 3 and 4, respectively. In the CRF patients (10 samples), three major peaks were detected (Tables I and II). The earliest peak (A) was found in the void volume (mol wt > 40,000) and constituted 16.5±4.7 of the eluted IRG. The second peak (B) eluted in the region of the proinsulin marker (9,000 mol wt) and represented the largest proportion of the elution profile (56.5±3.4%). The third peak (C) coincided with the standard glucagon and [^{125}I]glucagon markers (3,485 mol wt) and comprised 27.0±4.0% of the eluted IRG material. It is of interest that this value approximates the percentage suppression of basal IRG by oral glucose (38%, *vide supra*). In contrast to these findings, only peaks A (53.6±10.4%) and C (46.4±10.4%) were found in normal subjects. If the percentages of IRG eluting in peak C (27 and 46% in CRF and normal subjects, respectively) were arbitrarily used to estimate the true glucagon level in all subjects studied, the mean values in the 36 CRF patients (144 pg/ml) would still be considerably higher than in the 32 controls (52 pg/ml).

The elution profiles of plasma samples from CRF patients and normal subjects after glucose administration are shown in Fig. 3, and the distribution of glucagon immunoreactivity in the various peaks is shown in Table I. IRG in peak A was similar at both sampling times in the two groups of subjects. In contrast, IRG in peak C was significantly suppressed by oral glucose ($P < 0.05$), while in the CRF patients a small decline in peak B material also occurred, which bordered on statistical significance ($0.1 > P > 0.05$).

The elution profiles of plasma IRG before and after an arginine infusion are shown in Fig. 4, and the distribution of IRG in peaks A, B, and C is shown in Table II. Only peak C changed significantly ($P = < 0.05$) in both groups of subjects.

Acetic acid studies. Gel filtration of CRF plasma samples on Biogel P-30 columns equilibrated in 3 M acetic acid resulted in IRG profiles similar to those

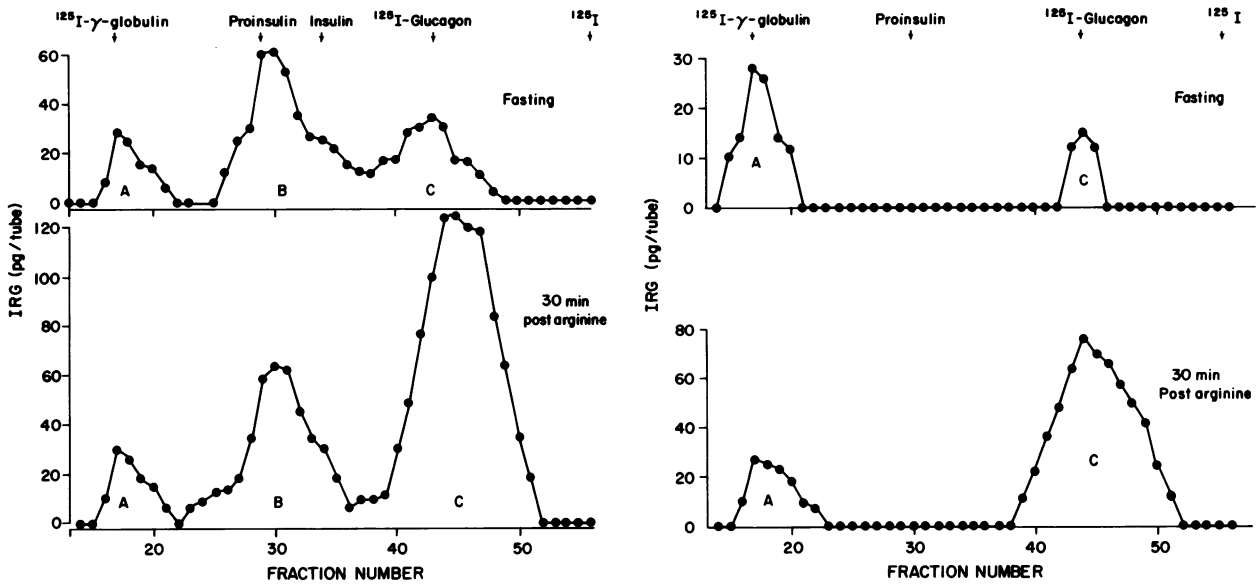

FIGURE 4 Representative elution profile of plasma IRG on 1 × 50-cm Biogel P-30 columns before and after arginine infusion in a patient with CRF (left) and in a healthy subject (right). The arginine was infused over 30 min at a dose of 250 mg/kg in the CRF patient and 500 mg/kg in the healthy subject.

found when the samples were eluted with glycine buffer (Fig. 5).

Trypsin studies. The elution profiles of plasma samples were examined before and after incubation with trypsin for 2 and 6 h (Figs. 6 and 7). In all three plasmas (the results of the third plasma were essentially similar to those shown in Fig. 6), peak A increased slightly at 2 h and been declined, but not to

TABLE II

Plasma Glucagon Responses and Distribution of Glucagon Immunoreactivity in the Fasting State and after Intravenous Arginine in Five Patients with CRF and Four Normal Subjects

	Fasting				After arginine infusion*				
		IRG in fraction					IRG in fraction		
Subject	IRG	A	B	C	Time	IRG	A	B	C
		pg/ml			*min*		*pg/ml*		
CRF									
1	600	42	383	312	30	1,250	74	371	1,233
2	680	91	388	199	30	1,250	99	389	952
3	700	51	663	134	30	1,100	48	580	381
4	500	173	180	56	30	540	135	206	237
6	420	131	247	69	30	700	135	263	449
Mean	580.0	97.6	372.2	154		968.0	98.2	361.8	650.4
SE	53.3	24.6	82.9	47.0		146.9	17.1	64.2	189.0
Normal									
4	95	45	0	20	30	460	63	0	294
5	80	80	0	16	30	420	105	0	375
6	100	56	0	54	45	800	25	0	604
7	50	53	0	0	45	400	36	0	210
Mean	81.3	58.5	—	22.5		520.0	57.3	—	370.8
SE	11.3	7.5	—	11.4		94.2	17.8	—	84.7

* 250 mg/kg was infused in the CRF patients and 500 mg/kg in the normal subjects.

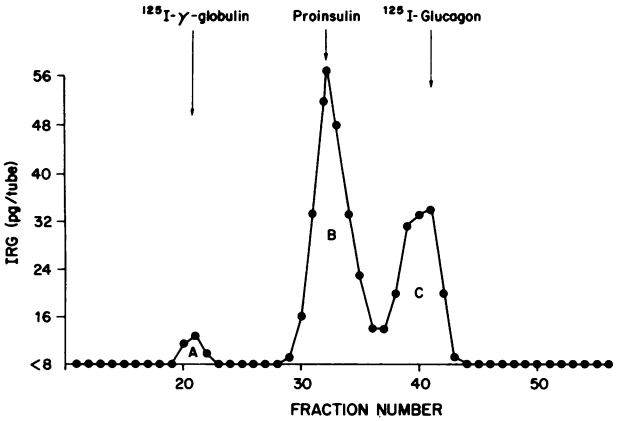

FIGURE 5 Elution profile of plasma IRG from a patient with CRF on 2 × 58-cm Biogel P-30 columns equilibrated in 3 M acetic acid (see text for details).

basal levels, at 6 h: Peak B completely disappeared by 6 h. In one subject, peak C increased substantially at 2 h and remained elevated at 6 h (Fig. 7), while in

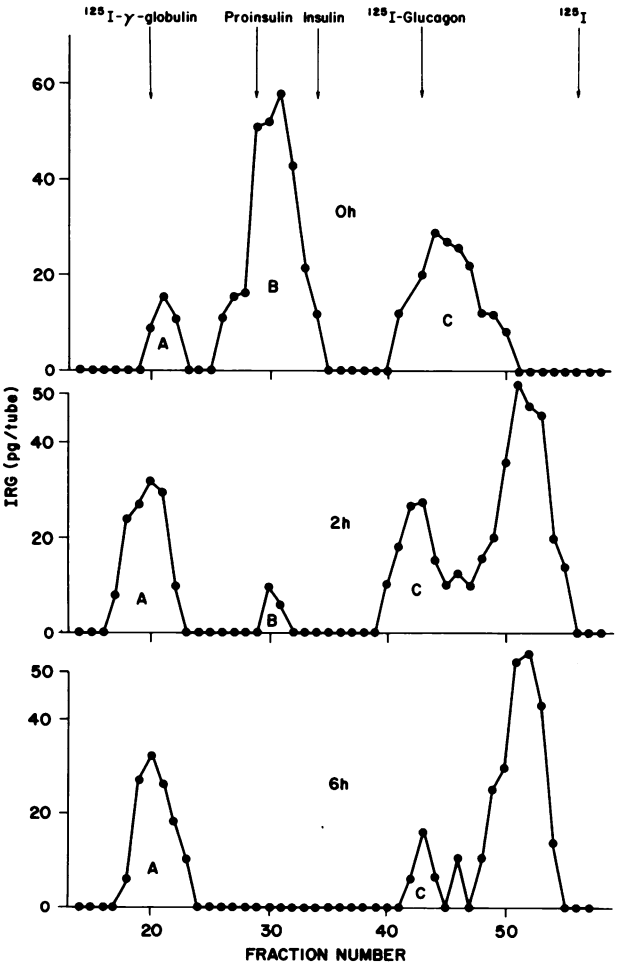

FIGURE 6 Elution profile of plasma IRG from a patient with CRF on 1 × 50-cm Biogel P-30 columns before and 2 and 6 h after trypsin digestion (see text for details).

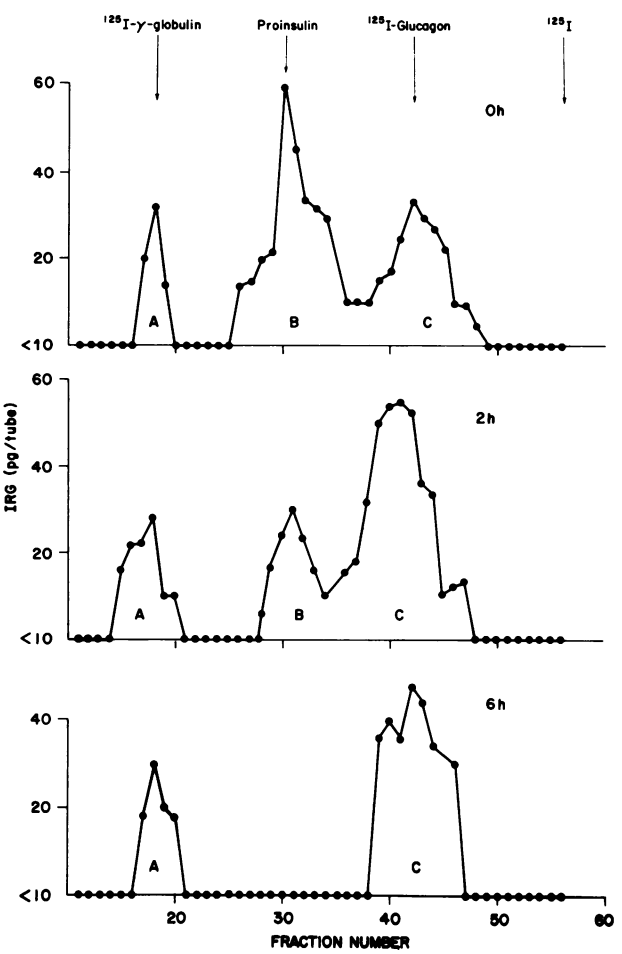

FIGURE 7 Elution profile of plasma IRG from a patient with CRF on 1 × 50-cm Biogel P-30 columns before and 2 and 6 h after trypsin digestion.

the other two subjects peak C diminished with the appearance of an additional, smaller molecular weight peak. The recovery of total IRG at 2 h approximated that of the control sample in all three experiments, but by 6 h only about 50% of the IRG was recoverable.

As shown in Fig. 8, repeat gel filtration of pooled eluates from the peak B region of a CRF patient showed a single peak in the expected zone. After exposure to trypsin for 30 min, IRG material in peak B was converted to substances eluting in the true glucagon and smaller molecular weight zones. The recovery after 30 min trypsin digestion was 80%.

Rat studies. Plasma IRG in the sham-operated rats was 119±12 pg/ml, while in the nephrectomized rats the values rose to 870±146 pg/ml. Gel filtration of plasma samples at 48 h (Fig. 9) showed that peak B was only present in the nephrectomized rats.

DISCUSSION

The "glucagon" values measured by direct assay of plasma (IRG) in normal subjects and patients with CRF

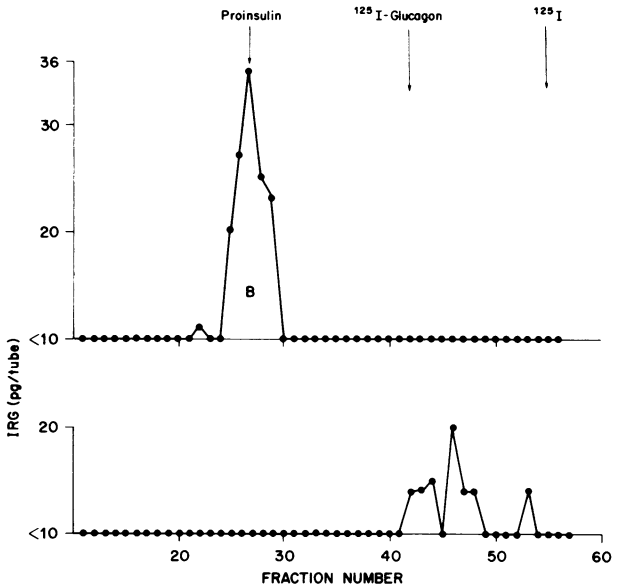

FIGURE 8 Rechromatography of pooled peak B material from a patient with CRF on 1 × 50-cm Biogel P-30 (upper panel). The lower panel represents the elution profile after exposure of pooled peak B material to trypsin for 30 min.

in this study are similar to those previously reported (1–3, 6), the basal IRG levels in CRF patients being considerably higher than in normal subjects. After oral glucose, plasma IRG decreased by between 35 and 40% in both groups. This finding is in accord with that of Bilbrey et al. (1) who measured the fall in IRG after an intravenous glucose infusion. A modified arginine infusion test (250 mg/kg) raised plasma IRG 1.6-fold in controls, while the increase in CRF patients was approximately half as great (71%). Similar results were reported by Sherwin et al. (3) after an alanine infusion, and somewhat smaller increments were found by Bilbrey et al. (1) who used a beef meal to stimulate IRG release. Because we (7) and other investigators (15, 16) have recently shown that circulating IRG in normal subjects and CRF patients is heterogenous, we have considered it of interest to define the relative contributions of the various immunoreactive components to total plasma IRG in fasting plasma as well as during suppression and stimulation of α-cell secretion in these two groups of subjects.

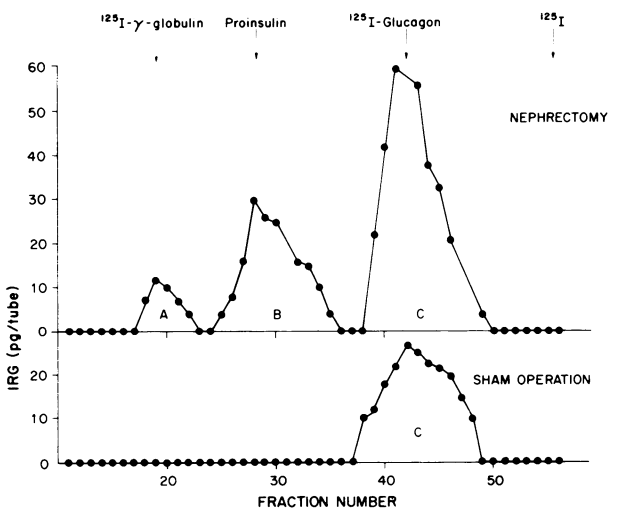

FIGURE 9 Representative elution profiles of plasma IRG on 1 × 50-cm Biogel P-30 columns in a nephrectomized rat (upper panel) and sham-operated rat (lower panel) 48 h postoperatively.

Gel filtration showed that only 46% of the fasting plasma IRG in healthy subjects eluted in the zone corresponding to native glucagon, a finding similar to that of Weir et al. (15). In CRF patients although only 27% of the basal IRG was found in this region of the column, it represented 144 pg/ml, equal to a threefold increase above normal. In contrast to controls, a major fraction of IRG in CRF patients was shown to elute in the 9,000 mol wt region of the column. Since this fraction was not found in the controls, we have postulated that the kidney may be important in its metabolism. Our animal data support this concept, for the 9,000 mol wt material was only found in the plasma of nephrectomized animals. Gel filtration of plasma taken after glucose or arginine administration indicates that the 3,500 mol wt material is physiologically responsive to these agents and points out the potential errors in interpretation which may arise if one does not appreciate the heterogenous nature of plasma IRG.

It is evident from Fig. 1 that a considerable amount of plasma IRG in both groups of subjects is not suppressed by glucose. This finding is similar to that reported by Bilbrey et al. (1). However, analysis of the gel filtration patterns indicates that the true glucagon component is almost completely suppressed by glucose, and that the nonsuppressible material consists of additional high molecular weight components (Figs. 3 and 4). It would thus seem reasonable to conclude that no impairment in α-cell response to glucose exists in patients with CRF. Further analysis of the α-cell response to arginine indicates that a 4-fold rise in true glucagon occurs in CRF, while the increase in normal subjects given the higher dose (500 mg/kg) was 16-fold. This conclusion differs in a quantitative manner from that obtained by analyzing total plasma IRG concentrations. It should also be noted that glucose suppressed and arginine stimulated mainly the 3,500 mol wt component, suggesting that the higher molecular weight fractions in the plasma do not respond rapidly to physiological maneuvers. The concentration of peak A material was remarkably constant in both groups of subjects before and after glucose or arginine. Nevertheless, the concentration of the 9,000 mol wt material decreased in

four of the five patients with CRF after glucose administration. This result is in agreement with that of Valverde et al. (17), who studied plasma glucagon immunoreactivity in pancreatectomized dogs and showed that infusions of insulin and somatostatin completely suppressed the 3,500 mol wt plasma component and reduced the 9,000 mol wt material to a lesser extent. It thus seems probable that prolonged glucose and arginine infusions, or other stimuli, may modulate the secretion of the higher molecular weight components.

The nature of the high molecular weight components of plasma IRG is at present unresolved. That they are not simple aggregates of 3,500 mol wt glucagon noncovalently bound to plasma proteins is suggested by the similar profiles when samples were gel filtered in either glycine buffer, pH 8.8, or in 3 M acetic acid. The 9,000 mol wt material was digested by trypsin in the three plasma samples studied. In two samples a new substance with a molecular weight smaller than 3,500 was generated. This is similar to the finding of Rigopoulou et al. (8), who found a similar substance after trypsin digestion of the 9,000 and 3,500 mol wt materials extracted from canine pancreas. Digestion of one of our samples, however, showed that the 9,000 mol wt material was converted to a material similar in size to true glucagon, suggesting that this material may be proglucagon. The reason why a fourth peak was not found in this sample, or why 3,500 mol wt glucagon was not detected in the other samples, may be related to differences in the rate of tryptic digestion of the glucagon components in each plasma. Peak A was resistant to trypsin and increased after exposure of plasma to the enzyme for 2 h. Valverde et al. (16) reported similar results in normal plasma. This increase might be due to immunoreactive fragments of glucagon, generated by trypsin, binding to plasma proteins. Another possible explanation is that the trypsin exposed new glucagon immunoreactive sites in this large molecule.

Of the three IRG peaks found in plasma, only the 3,500 mol wt IRG has been shown to be biologically active in studies carried out thus far. Rigopoulou et al. (8) reported that the 9,000 mol wt material in extracts of canine pancreas did not have glycogenolytic activity in the isolated perfused liver preparation. Therefore since both large molecular weight IRG components constitute a significant proportion of total plasma IRG in both normal subjects and CRF patients, caution must be exerted in the interpretation of the biological significance of plasma "glucagon" as measured by radioimmunoassay. This finding is also important in interpreting experiments concerned with the dynamics of plasma IRG, because the presence of high concentrations of immunologically cross-reacting material reduces the calculated magnitude of the response of true plasma glucagon to inhibitors or secretagogues. For instance, the rise in the 3,500 mol wt component after arginine in CRF patients is actually 322 and not 72%, which is the figure calculated from the total plasma IRG values. A further point that must be established concerns the relative immunoreactivity of the material in the A and B peaks compared to the 3,500 mol wt glucagon with different glucagon antisera. The experience with an analogous situation, i.e., proinsulin and insulin, suggests that a considerable variation in reactivity may be found and that these differences may account for the wide range of absolute concentrations for circulating IRG which have been reported. The findings of Bilbrey et al. (1) are important in this regard because these authors found that more than 80% of IRG in a single plasma from a patient with CRF eluted in the 3,500 mol wt zone when assayed with rabbit antiglucagon serum G58. Lesser reactivity of the 9,000 mol wt material with the G58 compared to the 30K antiserum may provide an explanation for the quantitative difference between their result and those in the present study. It should also be stressed that the values for the material in the A and B peaks have been expressed in terms of the 3,500 mol wt glucagon standard. If these components react less well with the 30K antiserum than the 3,500 mol wt glucagon standard, their absolute concentrations will be higher than those reported in this study. Resolution of this problem will necessarily require the availability of highly purified A and B components.

The 9,000 mol wt IRG in CRF plasma resembles material which is present in the pancreas of man and animals and which is now believed to be proglucagon (9–14). Our studies lend support to the possibility that this circulating material might be proglucagon because exposure to trypsin converted it to small molecular weight components, some of which were similar in size to the native hormone. However, other interpretations are possible, and further studies are now required to unequivocally establish its nature. In any event, plasma from CRF patients is a rich source of this material for further biological and chemical studies.

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