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*J Clin Invest.* 1976;58(5):1266-1272. <https://doi.org/10.1172/JCI108581>.

## Research Article

The pathogenesis of hyperglucagonemia and of the alterations in the pattern of circulating immunoreactive glucagon (IRG) associated with renal insufficiency was studied in rats in which a comparable degree of uremia was induced by three different methods, i.e., bilateral nephrectomy, bilateral ureteral ligation, and urine autoinfusion. Nephrectomized and ureteral-ligated rats were markedly hyperglucagonemic (575 +/- 95 pg/ml and 492 +/- 54 pg/ml, respectively), while IRG levels of urine autoinfused animals (208 +/- 35 pg/ml) were similar to those of control rats (180 +/- 26 pg/ml), indicating that uremia per se does not account for the hyperglucagonemia observed in renal failure. Similarly, plasma IRG composition in this group of animals was indistinguishable from that of controls, in which 88.2 +/- 5.9% of total IRG consisted of the 3,500-mol wt fraction. The same component was almost entirely responsible (82.6 +/- 4.1%) for the hyperglucagonemia observed in ligated rats, while it accounted for only 57.6 +/- 5.0% of the circulating IRG in nephrectomized animals. In the latter group, 36.8 +/- 6.6% of total IRG had a mol wt of approximately 9,000, consistent with a glucagon precursor. This peak was present in samples obtained as early as 2 h after renal ablation and its concentration continued to increase with time reaching maximal levels at 24 h. These results confirm that the kidney is a [...]

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# Pathogenesis and Characterization of Hyperglucagonemia in the Uremic Rat

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**ABSTRACT** The pathogenesis of hyperglucagonemia and of the alterations in the pattern of circulating immunoreactive glucagon (IRG) associated with renal insufficiency was studied in rats in which a comparable degree of uremia was induced by three different methods, i.e., bilateral nephrectomy, bilateral ureteral ligation, and urine autoinfusion. Nephrectomized and ureteral-ligated rats were markedly hyperglucagonemic ( $575 \pm 95$  pg/ml and  $492 \pm 54$  pg/ml, respectively), while IRG levels of urine autoinfused animals ( $208 \pm 35$  pg/ml) were similar to those of control rats ( $180 \pm 26$  pg/ml), indicating that uremia per se does not account for the hyperglucagonemia observed in renal failure. Similarly, plasma IRG composition in this group of animals was indistinguishable from that of controls, in which  $88.2 \pm 5.9\%$  of total IRG consisted of the 3,500-mol wt fraction. The same component was almost entirely responsible ( $82.6 \pm 4.1\%$ ) for the hyperglucagonemia observed in ligated rats, while it accounted for only  $57.6 \pm 5.0\%$  of the circulating IRG in nephrectomized animals. In the latter group,  $36.8 \pm 6.6\%$  of total IRG had a mol wt of approximately 9,000, consistent with a glucagon precursor. This peak was present in samples obtained as early as 2 h after renal ablation and its concentration continued to increase with time reaching maximal levels at 24 h.

These results confirm that the kidney is a major site of glucagon metabolism and provide evidence that the renal handling of the various circulating IRG

components may involve different mechanisms. Thus, the metabolism of the 3,500-mol wt fraction is dependent upon glomerular filtration, while the uptake of the 9,000-mol wt material can proceed in its absence, as long as renal tissue remains adequately perfused. This finding suggests that the 9,000-mol wt component may be handled by peritubular uptake.

## INTRODUCTION

Plasma immunoreactive glucagon (IRG)<sup>1</sup> is elevated in uremia (1-7), and a significant portion of the glucagon immunoreactivity is present in a fraction with a mol wt of approximately 9,000 (4, 6), consistent with descriptions of proglucagon (8-14). While changes in glucagon secretion do not appear to be responsible for the increments in total IRG (5, 15), the pathogenesis of the alterations in the composition of the circulating hormone is not well understood. It is unclear whether the latter results from changes in the synthesis or secretion of the various IRG components, conceivably due to the uremic environment, or is the consequence of their impaired degradation secondary to the reduction in functioning renal mass. Furthermore, the precise mechanism whereby renal tissue takes up and degrades the various IRG components is unknown. In an attempt to answer these questions we studied changes in plasma IRG levels and its composition in rats with: (a) absent renal tissue (nephrectomized animals), (b) normally perfused but nonfiltering kidneys (rats with bilateral ureteral ligation), and (c) normally filtering renal tissue operating in a uremic milieu

This study was presented in part at the Annual Meeting of the American Federation for Clinical Research, Atlantic City, N. J., 1 May 1976, and published in abstract form in 1976, *Clin. Res.* 24: 399A. (Abstr.)

Dr. Rubenstein is an Established Investigator of the American Diabetes Association.

Received for publication 2 June 1976 and in revised form 21 July 1976.

<sup>1</sup>Abbreviations used in this paper: BUL, bilateral ureteral ligation; BUN, blood urea nitrogen; GFR, glomerular filtration rate; IRG, immunoreactive glucagon; Nx, bilateral nephrectomy; UA, urine auto-infusion.

(animals with intact kidneys rendered uremic by autoinfusion of urine).

The results demonstrate that the elevated IRG in uremia is due to the impairment in kidney function and renal IRG degradation rather than to the uremic state per se. Furthermore, it is suggested that the pathogenesis of uremic hyperglucagonemia differs in the azotemic models studied, and that the various plasma IRG fractions are handled differently by the kidney.

## METHODS

### Animals

All experiments were performed on male albino rats (ARS/Sprague-Dawley Div., The Mogul Corp., Madison, Wisc.) weighing 200–350 g. Bilateral nephrectomy, bilateral ureteral ligation, or a sham operation (renal decapsulation) was performed through a midline incision under ether anesthesia while urine autoinfusion was established under light Innovar-Vet anesthesia (0.05 ml i.m.).<sup>2</sup> After urethral ligation the urine was diverted from the bladder to a jugular vein through a subcutaneously placed silastic catheter equipped with a miniaturized one-way valve (Hakim valve, model 902-012, Cordis Laboratories, Miami, Fla.). The valve system prevented the development of hydronephrosis (valve opening pressure was 5–12 mm of H<sub>2</sub>O), while insuring unidirectional urine flow and preventing backflux of blood and clotting of the conduit. In these animals glomerular filtration rates (GFR) as well as the histologic appearance of the kidneys did not differ from those of control animals up to 3 days after urinary diversion.<sup>3</sup> Control rats for this group were lightly anesthetized with Innovar and underwent a sham operation, in which catheter placement was omitted. Unless otherwise specified food and water were withheld from uremic animals after surgery, while sham-operated rats had free access to water only until 12 h before study.

### Renal function

**GFR.** GFR was measured in urine autoinfused and sham-operated rats by the clearance of either unlabeled or methoxy [<sup>3</sup>H]inulin (New England Nuclear, Boston, Mass.). All urine autoinfused animals were studied unanesthetized. Sham-operated rats were anesthetized with Inactin (Promonta, Hamburg, W. Germany), 100 mg/kg body wt. i.p., or studied unanesthetized. Since the data obtained in these two groups of control animals did not differ, they have been pooled and are presented together.

After the i.v. injection of an appropriate inulin prime, the vesicojugular shunt was reconnected in urine autoinfusion (UA) animals, while the controls received isotonic saline (0.02 ml/min) with maintaining amounts of inulin. After an equilibration period of at least 45 min clearances were measured while the volume of urine collected was replaced intravenously. Based on measurements from pre-

liminary experiments, the inulin containing infuse (2% Ringer's lactate in water) was delivered at a rate of 0.82 ml/min in UA animals. All solutions were administered with constant infusion pumps (Model 975, Harvard Apparatus Co., Inc., Millis, Mass.).

**Renal blood flow.** Renal blood flow rates were determined 48 h after bilateral ureteral ligation or sham operation by a modification of the tracer microsphere technique of Hsu et al. (16). Animals were anesthetized with Inactin (Controls, 100 mg/kg body wt; bilateral ureteral ligation (BUL), 80 mg/kg body wt) and a carotid and femoral artery were cannulated with PE-50 tubing. A suspension of 60,000 <sup>141</sup>Ce-labeled microspheres 15  $\mu$ m in diameter (3M Co., Nuclear Division, St. Paul, Minn.) in 0.1 ml of 10% dextran was injected rapidly into the carotid artery. 10 s before the injection, withdrawal of blood was commenced at a rate of 0.68 ml/min and continued for 60 s with a continuous automatic infusion-withdrawal pump (Model 600-950, Harvard Apparatus Co.). Whole kidney and blood radioactivity were determined in a gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.) and blood flow (expressed in milliliters per minute) calculated from their ratio  $\times 0.68$ .

### Analytical methods

IRG was measured by a double-antibody radioimmunoassay and gel filtration of plasma samples (0.5–2.0 ml) was carried out as previously described (4). A specific  $\alpha$ -cell glucagon antibody (30K) obtained from Dr. Roger Unger, Dallas, Tex. was utilized. Recovery of endogenous IRG in the 65 samples studied averaged 90 $\pm$ 3%.

Unlabeled inulin was measured by an AutoAnalyzer method (17) (Technicon Instruments Corp., Tarrytown, N. Y.), while methoxy [<sup>3</sup>H]inulin concentration in blood and urine was determined using a liquid scintillation counter (Packard Instrument Co., Inc.). Urea was determined by a modification of the Berthelot method (18).

### Statistical methods

Results are presented as mean $\pm$ SEM. The statistical significance of differences between group means was assessed by the Student's *t* test, *P* values less than 0.05 being considered significant.

## RESULTS

**IRG levels and renal function in uremic rats.** 24 h after surgery IRG levels were significantly elevated (*P* < 0.001) in bilaterally nephrectomized (Nx) animals (575 $\pm$ 95 pg/ml, *n* = 8) and rats with both ureters ligated (492 $\pm$ 54 pg/ml, *n* = 12), when compared to sham-operated controls (180 $\pm$ 26 pg/ml, *n* = 12) and UA animals (208 $\pm$ 35 pg/ml, *n* = 8). Blood urea nitrogen (BUN) levels were similar in all three experimental groups (Nx, 168 $\pm$ 8 mg/100 ml; BUL, 171 $\pm$ 10 mg/100 ml; and UA, 208 $\pm$ 21 mg/100 ml), and were significantly higher than control values (25 $\pm$ 3 mg/100 ml, *P* < 0.001). In UA animals, despite marked azotemia, glomerular filtration (8.57 $\pm$ 0.61 ml/min per kg body wt) was similar to that of controls (8.27 $\pm$ 0.31 ml/min per kg body wt), as were the levels of plasma IRG (Fig. 1).

<sup>2</sup> Innovar-Vet Injection (Pitman-Moore, Inc., Div. Johnson & Johnson, Washington Crossing, N. J.) contains 0.4 mg fentanyl citrate and 20 mg droperidol/ml.

<sup>3</sup> Emmanouel, D. S., M. D. Lindheimer, and A. I. Katz. Uremia in rats with normal kidneys: A model for the study of renal function in a uremic environment. In preparation.

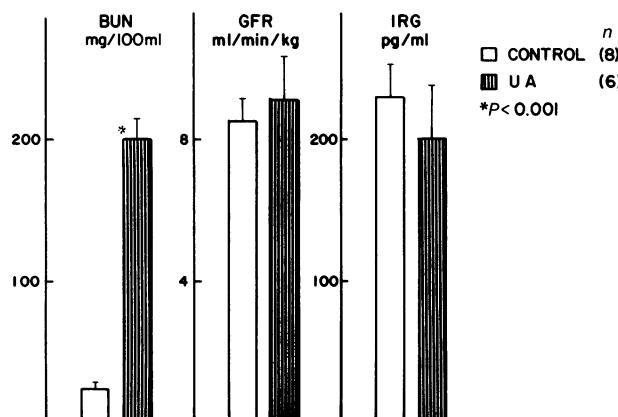


FIGURE 1 GFR, BUN, and plasma IRG in sham-operated (C) and urine autoinfused (UA) rats 24 h after operation. GFR and IRG are similar, despite the highly significant difference in the levels of BUN between the two groups.

48 h after nephrectomy or ureteral ligation the levels of circulating IRG ( $N_x$ ,  $606 \pm 65$  pg/ml,  $n = 14$ ; BUL,  $486 \pm 109$  pg/ml,  $n = 11$ ) remained essentially unchanged when compared to the concentrations observed 24 h previously despite progression of azotemia (BUN,  $298 \pm 15$  mg/100 ml in  $N_x$  and  $287 \pm 25$  mg/100 ml in BUL rats).

Renal blood flow of animals which had both ureters ligated 48 h previously ( $59.4 \pm 5.3$  ml/min per kg body wt,  $n = 6$ ), was similar to that of sham-operated rats ( $57.7 \pm 6.7$  ml/min per kg body wt,  $n = 8$ ).

**Plasma IRG patterns in uremic rats.** Representative plasma IRG elution profiles and the contribution of the different IRG components to the total immunoreactivity measured in the four groups of rats are shown in Figs. 2 and 3, respectively. The IRG elution profile in the rat is similar to that of man (4, 6) and comprises three peaks (A, B, and C). Peak A eluted in the void volume, coinciding with the  $\gamma$ -globulin marker (mol wt  $>40,000$ ). This peak was not always present in plasma of control or urine autoinfused animals. Peak C corresponded to the 3,500-mol wt pancreatic glucagon marker. This fraction was almost entirely responsible for the hyperglucagonemia observed in animals with bilateral ureteral ligation and comprised  $82.6 \pm 4.1\%$  of their total circulating IRG. Peak B was found only in nephrectomized animals in which it accounted for  $36.8 \pm 6.6\%$  of the total plasma IRG. This peak, which was present in every sample obtained as early as 2 h after renal ablation, eluted just beyond the proinsulin marker, corresponding to a mol wt of approximately 9,000. Peak C accounted for  $57.6 \pm 5.0\%$  of the circulating IRG in nephrectomized rats.

The elution profiles of plasma from control or urine autoinfused animals were similar, and the cir-

culating IRG in these groups consisted mainly of the 3,500-mol wt fraction ( $88.2 \pm 5.9\%$  and  $89.0 \pm 3.3\%$  of total IRG, respectively).

**Evolution of the alterations in IRG composition in nephrectomized rats.** The material in Peak B became detectable within 60–90 min after renal ablation (Fig. 4), and its concentration continued to increase with time, reaching peak levels at 24 h. Peak C increased much more rapidly and appeared to level off 2–4 h after nephrectomy. Peak A did not change in a

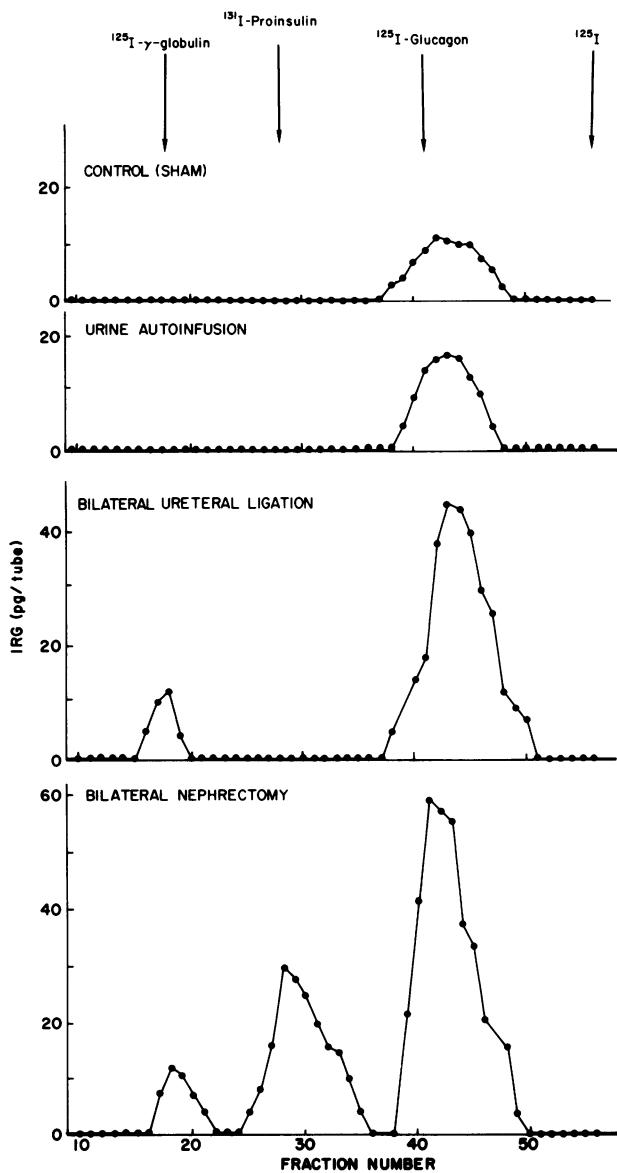


FIGURE 2 Representative elution profiles of circulating IRG 24 h after surgery in a sham-operated animal, a urine autoinfused rat, an animal with bilateral ureteral ligation, and a nephrectomized rat. The 9,000-mol wt fraction was found only in  $N_x$  rats.

consistent manner, although its concentration tended to correlate with the levels of total IRG. Fig. 5 illustrates representative plasma IRG elution profiles before, and at various intervals after nephrectomy. Differences in the respective metabolic clearance rates could account for these observations (14), but the data at hand do not allow the exclusion of other possibilities, such as differences in the rate of secretion.

## DISCUSSION

The role of the kidney in the degradation of peptide hormones including that of glucagon has been appreciated for many years (19), and therefore the finding of elevated plasma levels of IRG in uremic subjects and animals with experimental renal failure was not surprising (1-7, 15, 20). Sherwin et al. (5) observed that the metabolic clearance rate of the exogenous hormone was greatly prolonged in uremic patients, while the calculated basal systemic delivery rate (an index of endogenous glucagon secretion) remained unchanged. Similarly, Lefebvre and Luyckx (15) demonstrated that acute renal pedicle ligation in dogs led to a prompt and substantial elevation in circulating IRG, while the simultaneously measured pan-

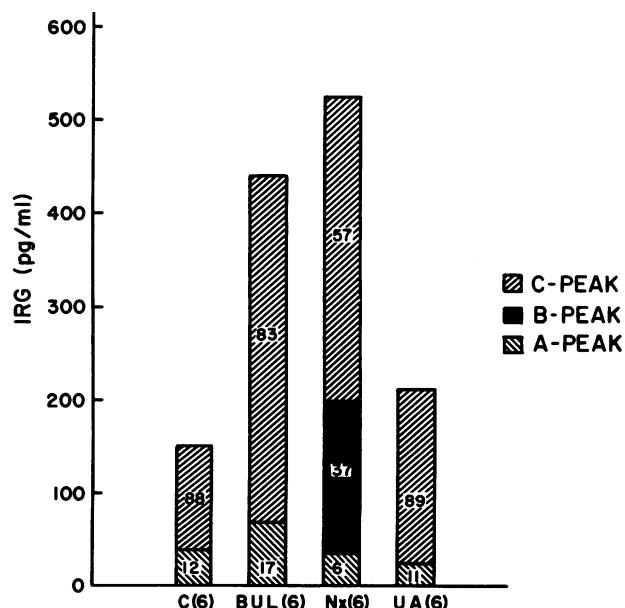


FIGURE 3 Contribution of the different IRG components to the total immunoreactivity in sham operated (C), ureteral ligated (BUL), nephrectomized (Nx), and urine autoinfused rats (UA) 24 h after operation. The height of the bars illustrates each component's contribution to the total IRG (expressed in picograms per milliliter), while the number inserts indicate the relative contribution in percent. IRG composition differs substantially in animals made uremic by different methods.

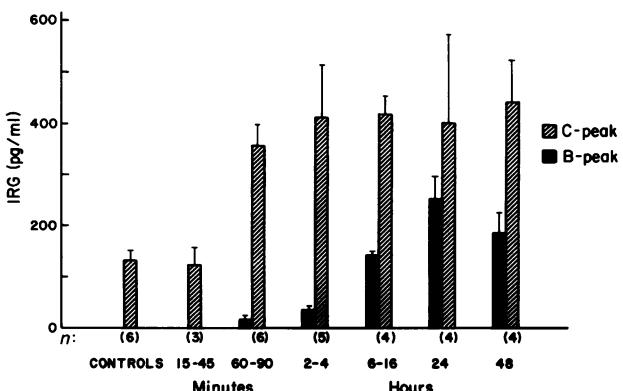


FIGURE 4 Evolution of the alterations in the pattern of circulating IRG in rats studied at various time intervals after nephrectomy. Note the different rates of rise of the 9,000- (Peak B) and 3,500- (Peak C) mol wt fractions. Peak A levels did not change with time after nephrectomy.

creatic glucagon secretion rate remained unaffected. However, in both studies it was assumed that the plasma glucagon immunoreactivity comprised a single species, and that its metabolic clearance was similar to that of endogenous glucagon circulating in the non-uremic state (5). It has been recently reported, though, that the hyperglucagonemia observed in patients with renal insufficiency is accompanied by significant and characteristic changes in the composition of circulating glucagon immunoreactivity (4, 6). For these reasons, we studied the pathogenesis of hyperglucagonemia in uremic animals by measuring total plasma IRG, and examining in addition the fractions which contribute to its immunoreactivity. Uremia was induced in rats by three different methods, and in each experimental group total IRG concentration and its composition were studied at comparable levels of BUN.

Our results demonstrate that plasma glucagon immunoreactivity is heterogeneous in the rat as it is in humans (4, 6), and comprises three fractions: a 3,500-mol wt component (Peak C), and two larger fractions (Peaks B and A) with mol wt of approximately 9,000 and in excess of 40,000 daltons, respectively. While the material in Peak C corresponds to the "true" pancreatic glucagon marker and appears to be identical with the native hormone, the nature of the higher mol wt fractions of plasma glucagon is not well defined at present. They do not appear to represent simple aggregates of the 3,500-mol wt material or noncovalent binding of this glucagon species to other plasma proteins. Exposure of these fractions to trypsin demonstrated that Peak A is resistant to the action of the enzyme, while the material in Peak B can be converted to immunoreactive fragments of smaller mol wt, some of which are similar in size to

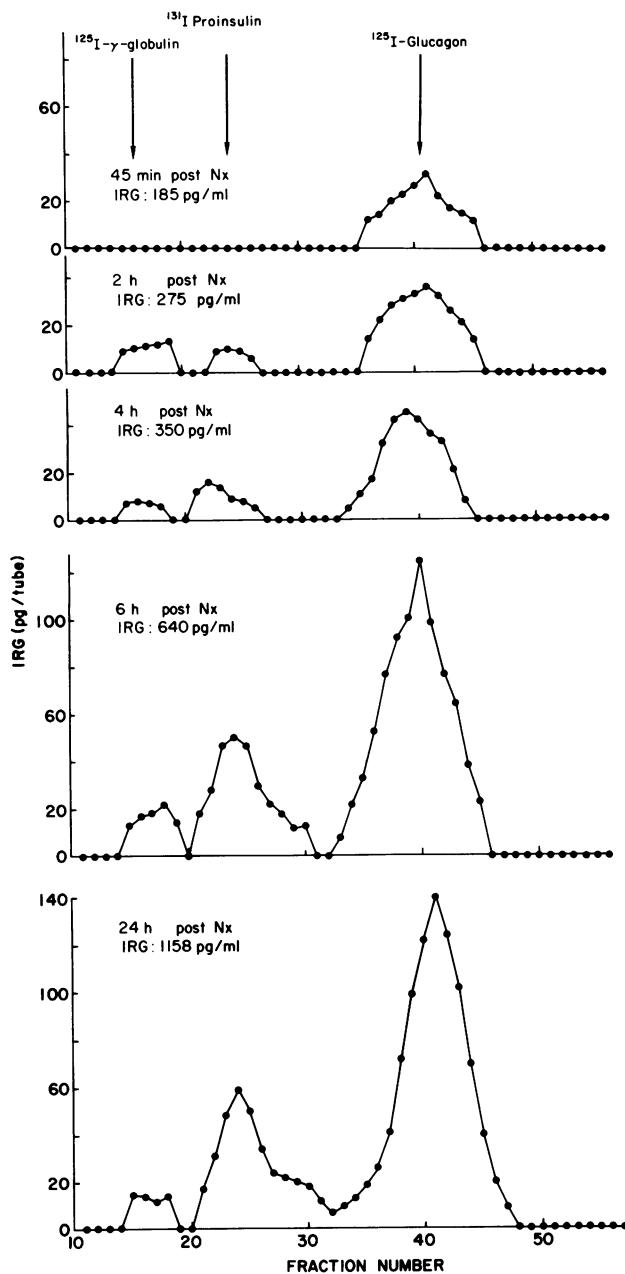


FIGURE 5 Representative plasma IRG elution profiles before and at various intervals after nephrectomy. Note the progressive increment in Peak C, and in particular Peak B, with time elapsed since renal ablation.

the native hormone (6). The latter observation lends support to the possibility that the 9,000-mol wt component might be a glucagon precursor.

Plasma from uremic animals exhibits substantial differences in IRG composition depending on the mode of induction of uremia (Figs. 2 and 3). Despite the marked elevation of BUN, the levels and composi-

tion of IRG remained unchanged in urine auto-infused animals, indicating that the disordered chemical environment of uremia is not sufficient by itself to account for the hyperglucagonemia of renal insufficiency. In contrast, animals rendered uremic by either bilateral nephrectomy or bilateral ureteral ligation exhibited highly significant elevations in total plasma IRG levels. Despite the similar BUN levels in these two groups, the elution profile of BUL and Nx rat plasma differed substantially (Figs. 2 and 3). In animals with ligated ureters the IRG increment was almost entirely due to elevation of the 3,500-mol wt fraction (Peak C), whereas in nephrectomized rats more than one-third of the total IRG eluted in the 9,000-mol wt region (Peak B).<sup>4</sup> This observation suggests a different mechanism for the increments in total IRG in the two groups of animals, and in addition provides evidence that the various IRG fractions are handled differently by the kidney.

Glucagon is readily metabolized by renal tissue, as evidenced by the high extraction ratios and relatively low urinary clearance rates of the hormone (1, 21, 22). Labeled glucagon is absorbed from the lumen by proximal tubular cells (19), which contain specific glucagon degrading enzymes in both their brush border and cytosol (23, 24). Glomerular filtration followed by tubular reabsorption and/or uptake from the peritubular capillary network are the mechanisms responsible for the renal extraction of many other low mol wt proteins. While lysozyme handling by the rat kidney occurs mainly by the former route (25), a sizeable fraction of the renal extraction of a number of other biologically important peptides also involves peritubular uptake. This mechanism appears to contribute importantly to the renal catabolism of insulin, proinsulin, C-peptide (26), gastrin (27-29), secretin (30), cholecystokinin (31),  $\beta_2$ -microglobulin (32), immunoglobulin light chains (33), and probably synthetic 1-34 bovine parathyroid hormone (34).

Bastl et al. observed that glucagon extraction by the normal rat kidney exceeded significantly its filtered load, and concluded that IRG degradation by the renal tubule involved both glomerular filtration and peritubular uptake (22). Studies by Lefebvre et al. in dogs indicated that renal glucagon extraction could be accounted for entirely by glomerular filtration and tubular reabsorption, but the possibility

<sup>4</sup> IRG elution profile of nephrectomized rats resembled closely that of uremic patients on regular hemodialysis (4, 6), while no sample from BUL animals contained detectable amounts of the 9,000-mol wt material. Although species difference could account for this observation, it seems more likely that the patients studied had negligible renal blood flow rates as suggested by their extremely low creatinine clearance (less than 5 ml/min), and were therefore functionally anephric.

of direct uptake from peritubular capillaries could not be ruled out (21). Our results, while not strictly comparable since we considered the individual IRG components in addition to its total concentration, also indicate that renal glucagon handling involves both glomerular filtration and peritubular uptake. Since bilateral ureteral ligation leads to marked reduction or complete cessation of glomerular filtration while renal blood flow is maintained, it is apparent that the 3,500-mol wt component increases when GFR is reduced, while the 9,000-mol wt fraction does not accumulate even when GFR is very low or possibly absent, provided that renal tissue remains adequately perfused. These findings suggest that the 9,000-mol wt material may be handled by peritubular uptake, while the 3,500-mol wt fraction is handled primarily by glomerular filtration and tubular reabsorption.

#### ACKNOWLEDGMENTS

The authors are grateful to Ms. V. Armstrong and Ms. G. Sims for preparation of the manuscript.

This work was supported by grants from the National Institutes of Health (AM 19250, AM 13601, AM 13941, and AM 17046—Diabetes-Endocrinology Center), the Chicago Heart Association, and the Bertha and Henry Brownstein Foundation.

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