Measurement and Partial Characterization of Immunoreactive Glucagon in Gastrointestinal Tissues of Dogs

Soichiro Morita, M.D., Kunihiro Doi, M.D., Ph.D., Cecil C. Yip, Ph.D., and Mladen Vranic, M.D., D.Sc., Toronto, Ontario, Canada*

SUMMARY

We have reported previously that increasing amounts of immunoreactive glucagon (IRG), measured by four specific antisera, appeared in plasma of depancreatized insulin-deficient dogs. It was therefore concluded that pancreatectomy was not accompanied by glucagon deficiency in the dog, but instead excessive amounts of extrapancreatic IRG could contribute to the diabetic syndrome. In order to locate the source of extrapancreatic glucagon, tissue extracts were assayed with anti-glucagon sera 30-K and K-44, which cross-react minimally with crude gut extracts. IRG was detected in all gastrointestinal tissues and in the salivary glands, but not in extracts of liver, kidney, brain, heart atrium, and adenohypophysis. Immunologic dilution curves of extracts from all gastrointestinal tissues were parallel to those of the pure pancreatic glucagon standard, and both antisera (30-K and K-44) measured the same concentrations. The highest concentration of gastrointestinal IRG was found in the fundus and corpus of the stomach. Presence of IRG in gastrointestinal tissues of depancreatized dogs indicates that gastrointestinal cells can not only secrete but also store large amounts of IRG.

Extracts of mucosa of stomach fundus were further purified by gel filtration on Biogel P-30 columns. The immunoreactivity in the eluate was assayed by 30-K and a strongly crossreacting antibody, K-4023. One pooled fraction corresponding to marker pancreatic glucagon in its elution volume was found to contain the largest amount of IRG and the highest specific immunoreactivity (IRG/protein concentration). This fraction showed also the highest activity in a glucagon-receptor assay system. Disc gel electrophoresis in the presence of urea resolved this fraction into three immunoreactive components, one of which was identical to pancreatic glucagon in its electrophoretic mobility. It appears, therefore, that mucosa of the upper stomach in the dog contains a polypeptide similar to pancreatic glucagon.

We conclude that (a) hyperglucagonemia in the dog can result from excessive secretion of IRG not only by the pancreatic α cells but also by cells of the gastrointestinal tract; (b) the highest IRG concentration was found in fundus and corpus of the stomach and lower concentrations throughout the gastrointestinal tract; (c) the IRG component in the stomach displayed immunologic and physical properties similar to pancreatic glucagon. DIABETES *25*:1018-25, November, 1976.

We have reported previously that immunoreactive glucagon (IRG) was present in normal concentrations in serum of totally depancreatized insulin-infused dogs[1] and that insulin deprivation of depancreatized dogs induced a marked increase in serum IRG.[2] Serum IRG of depancreatized dogs was indistinguishable immunologically from pancreatic IRG by radioimmunoassay of serial dilutions of the sera of diabetic dogs using four different antisera. The observation that IRG is found in lage amounts in plasma of depancreatized dogs at a time when immunoreactive insulin (IRI) was absent provides conclusive evidence that IRG is produced not only by the pancreas but also by extrapancreatic tissues. The additional evidence was the lack of an IRI response to arginine and the absence of any remnant pancreatic tissue at autopsy.[1,2] A material cross-reacting with glucagon antibodies in plasma of depancreatized dogs was observed also by other investigators.[3,4] In insulin-treated depancreatized humans, Müller and colleagues[5] were able to detect IRG by using antibody 30-K. In contrast, using another

Presented in part at the 57th Annual Meeting of the Endocrine Society, New York, June 1975.

From the Department of Physiology and the Banting and Best Department of Medical Research, University of Toronto, Ontario, Canada.

*Currently at the Second Department of Internal Medicine, Kobe University School of Medicine, Kobe, Japan.

Address reprint requests to Mladen Vranic, M.D., D.Sc., Department of Physiology, Medical Sciences Building, University of Toronto, Ontario, Canada M5S 1A8.

Accepted for publication June 7, 1976.

antiglucagon serum, Barnes and Bloom[6] did not find IRG in the plasma of their patients. Arginine was found by both groups not to stimulate IRG secretion.[5,6] Experiments with depancreatized dogs and those with depancreatized humans are not comparable, because excessive amounts of plasma IRG are found in depancreatized dogs only when the animals have been deprived of insulin for more than three days. Similarly, stimulation of extrapancreatic IRG secretion was obtained with arginine challenge only when depancreatized dogs were deprived of insulin but not when the plasma IRI was near or slightly below normal. It was suggested that serum IRG in depancreatized dogs plays a role in glucoregulation, because (a) depancreatized dogs infused with insulin had normal rate of glucose production;[7] (b) acute deprivation of insulin led to an increase in glucose production and to hyperglycemia when serum IRG was within the physiologic range[8] but not when it was suppressed by somatostatin;[9,10] (c) infusion of arginine increased serum IRG, which in turn markedly increased the rate of glucose production in depancreatized dogs deprived of insulin,[10] suggesting that the effects of extrapancreatic and pancreatic glucagon on glucose production by the liver are comparable.

Our preliminary data[2,11] as well as those of Sasaki et al.[12] showed that immunoreactive glucagon was detected in various gastrointestinal tissues of normal dogs. The highest concentration measured by specific antiglucagon antibody was found in the corpus and fundus of the stomach. The highest concentration of glucagon-like immunoreactivity measured by nonspecific antiserum was found mainly in the gastrointestinal tract distal to the stomach.[12]

The aims of the present study were (a) to measure accurately IRG concentrations in various tissues of the dog to find out whether the gastrointestinal tract and the pancreas could be the only sources of immunoreactive glucagon; and (b) to partially characterize immunoreactive glucagon in extracts of the upper stomach, where the highest IRG concentration was found.

MATERIALS AND METHODS

Extraction of Gastrointestinal Tissues

Gastrointestinal tissues were obtained from seven normal dogs fasted for 24 hours and also from two depancreatized dogs. The depancreatized dogs were initially treated with 6-12 U. of pork NPH-insulin daily for three weeks. Insulin and food were then withdrawn for a period of seven days.[2] Dogs were subjected to laparotomy under Nembutal anesthesia, and the tissues were obtained moments after the animals were sacrificed with an overdose of the barbiturate. Tissues obtained from the dogs were immediately frozen on dry ice and stored at −20° C. Mucosal scrapes were obtained from the gastrointestinal tissues after thawing.

Pancreatic tissue and the mucosal scrapes were minced, homogenized, and extracted three times with acid ethanol (95 per cent ethanol, water, concentrated HCl, V/V/V 750/250/15, 10 ml. per gram tissue). The pooled supernatant from the three extractions was brought to pH 7.5 with 2 N ammonium hydroxide and then filtered to remove the precipitates.

Acid-Ethanol Extraction of Mucosa of Gastric Fundus

The extraction procedure was essentially that described by Kenny,[13] modified by Okuno et al.[14] Mucosal scrapes from gastric fundi were minced with scissors and homogenized for one minute in ice-cold acid alcohol containing 0.01 M benzamidine (10 ml./gm. wet weight) in a Polytron homogenizer. The homogenate was stirred at 5° C. overnight, and the supernatant obtained after centrifugation (1,000 × g, 30 minutes) was adjusted to pH 7.5 with concentrated NH_4OH. The precipitates formed after several hours at 5° C. were removed by centrifugation (1,000 × g, 30 minutes). The supernatant was then precipitated with 1.7 volumes of absolute ethanol together with 2.8 volumes of ether. After standing at 5° C. overnight, the precipitates were collected by centrifugation (1,000 × g, 20 minutes) and dried under a gentle stream of nitrogen. The dry precipitates were kept at 5° C. in a dessicator over $CaCl_2$.

Gel-Filtration Column Chromatography

Columns of Bio-Gel P-30, equilibrated either to 3 M acetic acid or to 0.05 M ammonium bicarbonate, were used to separate the mucosal extract. The columns of Bio-Gel were routinely calibrated with albumin, blue-dextran, and ^{125}I-glucagon and ^{125}I-insulin before use. Gel filtration was carried out at 5° C. Details of the separation are described in the legends to the figures. In general, protein content in the column effluent was monitored at either 280 nm or 254 nm. Fractions were collected into tubes containing aliquots of concentrated solution of benzamidine so that the final concentration of benzamidine was 0.01 M. We have used Bio-Gel P-30 in 3 M acetic acid for gel filtration and found that separation characteristics were not altered after months of exposure to the acid even at room temperature.

Polyacrylamide Disc Gel Electrophoresis

Disc electrophoresis[15] was carried out in 15 per cent gel containing 4 M urea at pH 8.7. The gel was stained with coomassie blue.[16] Unstained gel was cut into 1.5-mm. sections, which were eluted with the immunoassay buffer by repeated freezing and thawing. Aliquots of the eluate were immunoassayed for glucagon.

Radioimmunoassay of Glucagon

The filtrate obtained above from the extract was immunoassayed by the method of Unger.[17] Each filtrate was diluted serially five to 12 times with the assay diluent, and each dilution was assayed in triplicate. Two antisera that measure "pancreatic glucagon" immunoreactivity were used to determine IRG concentrations in gastrointestinal tissues: (a) 30-K (from R.H. Unger, Dallas) and (b) K-44 (from L. G. Heding, Novo Research Institute, Denmark), which showed respectively 3 per cent[12] and 10 per cent (L. G. Heding, personal communication) "cross-reactivity" with crude gut extracts. In addition, the glucagon-like immunoreactivity was also measured by another antibody K-4023 (from L. Heding, Novo Research Institute, Denmark), which cross-reacts strongly with a preparation of GLI (L. G. Heding, personal communication). In our immunoassay system, K-4023 measured five times more immunoreactivity than did antibody 30-K in plasma of 11 normal dogs (326 ± 28 pg./ml. by K-4023, as against 64 ± 7 pg./ml. by 30-K). Both antisera measured the same IRG concentration in mucosal extract of stomach fundus. On the other hand K-4023 measured three times more IRG in mucosal extract of the jejunum. ^{125}I-glucagon and highly purified standard crystalline glucagon, lot no. 9969, were also obtained from Novo Research Institute, Denmark.

RESULTS

Contents of Immunoreactive Glucagon in Pancreatic Tissue of the Normal Dogs

Figure 1 shows that the immunoassay dilution curves of the acid ethanol extracts of the splenic part of the pancreas from five normal dogs are essentially identical to the dilution curves of the highly purified crystalline pork standard. This observation demonstrates that glucagon immunoassay using antiserum 30-K does not distinguish immunologically dog pancreatic glucagon from purified pork pancreatic glucagon standard. Contents of immunoreactive glucagon (IRG) in the pancreas of five normal dogs are shown in table 1. Except for one dog, the uncinate process of the pancreas

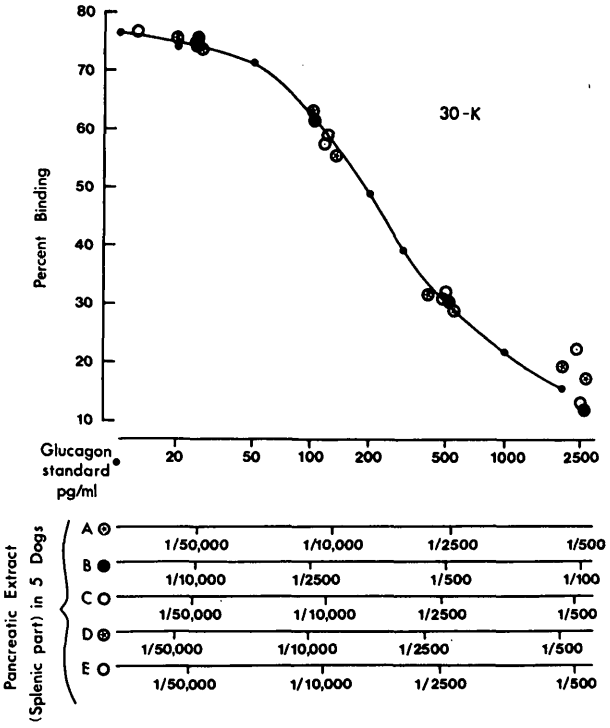

FIG. 1. Immunoassay comparison between highly purified porcine crystalline glucagon standard and acid ethanol extracts of the splenic part of the pancreas in five normal dogs (A-E). Each point represents the average of triplicate determination. All assays were carried out on the same day. Dilutions plotted in logarithmic scale were the final dilutions of the acid ethanol extract with the assay diluent.

contained very low concentrations of IRG when compared with the splenic portion.

Contents of Immunoreactive Glucagon in Dog Gastrointestinal Tissues

Figure 2 shows the immunoassay dilution curves of extracts from the stomach fundus of seven normal dogs with antiserum 30-K (figure 2a) and from six normal dogs with antiserum K-44 (figure 2b). Immunoassay dilution curves of extracts from stomach antrum, jejunum, ileum, and colon are shown in figure 3. These data clearly demonstrate that the dilution curves of the tissue extracts were not different from the standard curves obtained with purified pork pancreatic glucagon when assayed with the same antiserum.

Contents of IRG in the tissue extracts were then determined from the dilution curves with the use of 5-12 different dilutions of the extracts. Results are shown in table 2. The highest content of IRG was found in the corpus and fundus of the stomach, and much lower contents were found in stomach antrum, duodenum, jejunum, ileum, and colon. Results obtained with either antiserum 30-K or K-44 were quantitatively very similar in all time extracts tested. In one

TABLE 1

Concentration (ng./gm. wet weight) of immunoreactive glucagon in pancreatic tissue of five normal dogs with assayed 30-K antiserum

	A	B	C	D	E
Splenic portion	17,573	3,066	14,485	13,698	11,769
Uncinate process	8,931	70	78	10	60

normal dog, small amounts of IRG were detected in extracts of mucosa of the esophagus (5 ng./gm. wet weight), sigmoid colon (12 ng./gm.), rectum (27 ng./gm.), and salivary glands (3 ng./gm.). IRG was not detected in extracts of the liver, kidney, brain, heart atrium, and adenohypophysis. IRG in the gastrointestinal tissues was also determined in two dogs four weeks after pancreatectomy and seven days after insulin and food withdrawal. As shown in table 3, significant amounts of IRG were found in the gastrointestinal tissues. Immunoassay dilution curves of extracts of these tissues were also indistinguishable from the pancreatic glucagon-standard curve.

Partial Characterization of Glucagon Immunoreactivity in Dog Stomach Extracts

Since the stomach fundus and corpus had the highest content of IRG, the nature of this IRG was further characterized by gel-filtration chromatography and

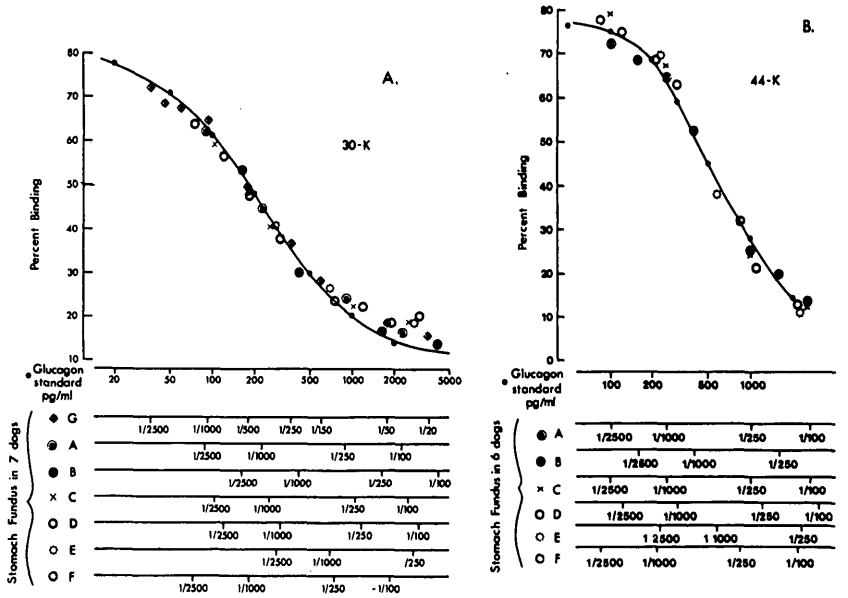

FIGURE 2

Immunoassay comparison between highly purified porcine crystalline glucagon standard and acid ethanol extracts of the stomach fundus and corpus in normal dogs (A-G). Each point represents the average of triplicate determination. The assays were carried out with use of two different antisera, 30-K (A) and K-44 (B). Dilutions plotted in logarithmic scale were the final dilutions of the acid ethanol extract.

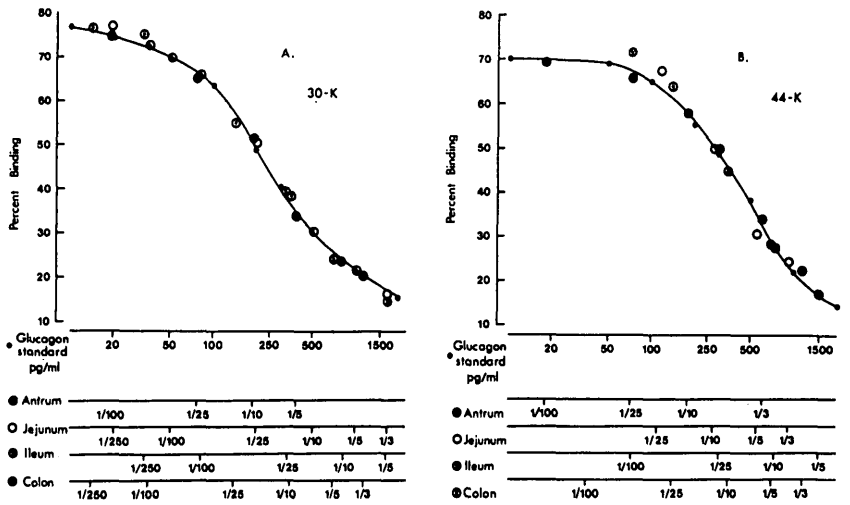

FIGURE 3

Immunoassay comparison between highly purified porcine crystalline glucagon standard and acid ethanol extracts of the gastrointestinal tissue in one normal dog with use of antiserum 30-K (A) and antiserum K-44 (B).

TABLE 2

Concentration (ng./gm. wet weight) of immunoreactive glucagon in gastrointestinal mucosa of normal dogs*

Dog		A	B	C	D	E	F	G	Mean ± S.E.M.
Stomach fundus and corpus	30-K	1,723	2,695	1,988	1,746	2,967	2,178	1,180	2,216.2 ± 209
	K-44	1,910	2,629	1,897	1,709	2,458	2,491		2,182.3 ± 158
Stomach antrum	30-K	22	10					7	13.3 ± 4.5
	K-44	21	12						
Duodenum	30-K						29	8	
	K-44						22		
Jejunum	30-K	37	192	145			193	42	121.9 ± 34.8
	K-44	20	251	153			190		153.3 ± 48.9
Ileum	30-K	69	61	82			72	71	71.3 ± 3.38
	K-44	62	53	71			104		72.3 ± 11.2
Colon	30-K	34	37	28				38	34.2 ± 2.2
	K-44	34	32	22					29.6 ± 3.6

*Concentrations of immunoreactive glucagon were determined with two antisera in the assay system: 30-K and K-44.

disc gel electrophoresis. Routinely the acid ethanol extract of mucosal scrapes of the stomach fundus of five dogs (see Methods and Materials) was fractionated on Bio-Gel P-30 in 3 M acetic acid. Figure 4 shows a typical separation of the extract. In this case, six pooled fractions were obtained. Immunoassay of the pooled fractions showed that fraction VI was most active and contained the highest specific immunoreactivity (IRG/A 280 nm.). Both antisera 30-K and K-4023 measured quantitatively the same amount of IRG in this fraction. As determined on the calibration of this column, fraction VI contained polypeptides similar to pancreatic glucagon in molecular weight. Although not determined in this case, a separate experiment under similar conditions showed that the column eluate after fraction VI contained little or no IRG. In a preliminary study the six pooled fractions were assayed also for their reactivity in a receptor system[18] using rat liver plasma membrane. Fraction VI was the most active and its dilution curve paralleled that of standard pancreatic glucagon. Fraction VI was then chromatographed on Bio-Gel P-30 in 0.05 M ammonium bicarbonate, and only one immunoreactive peak with elution volume similar to that of ^{125}I-glucagon was obtained when assayed with either antiserum 30-K or K-4023 (figure 5). Immunoassay dilution curve of this peak paralleled that of standard pancreatic glucagon (figure 6). Polyacrylamide disc gel electrophoresis of this immunoreactive peak resolved it into three distinct immunoreactive components (figure 7). One of these was similar to pancreatic glucagon in its electrophoretic mobility. The faster-moving component corresponded to desamido-glucagon in its mobility. The slower-moving component, barely entering the gel, was more reactive with antiserum K-4023 than with

TABLE 3

Glucagon concentrations (ng./gm. wet weight) in the gastrointestinal mucosa of two depancreatized dogs kept without food and without insulin for one week

	Px Dog I		Px Dog II	
	30-K	K-44	30-K	K-44
Stomach fundus and corpus	1,037	747	691	610
Duodenum	17		11	
Jejunum	47	39	34	26
Ileum	218	188	208	131

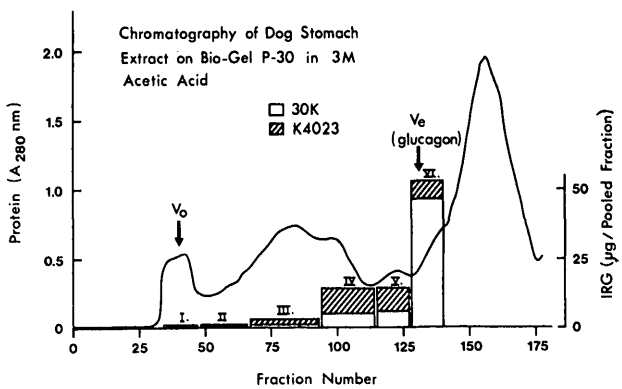

FIG. 4. Gel filtration of acid ethanol extract of mucosal scrapes from the stomach fundus of five normal dogs. The ether-alcohol precipitates obtained from the acid ethanol extract were dissolved in 15 ml. of 3-M acetic acid. The material applied to a column (5 cm. x 100 cm.) of Bio-Gel P-30 (100-200 mesh) was equilibrated to 3-M acetic acid. The column was eluted with 3-M acetic acid at a flow rate of about 18 ml./hr. Fractions of 8 ml. were collected. Fractions were pooled as indicated and assayed for IRG with antiserum 30-K and K-4023. The elution volume (Ve) of glucagon in this column under similar conditions had been determined by using ^{125}I-glucagon. Vo indicates the void volume of the column.

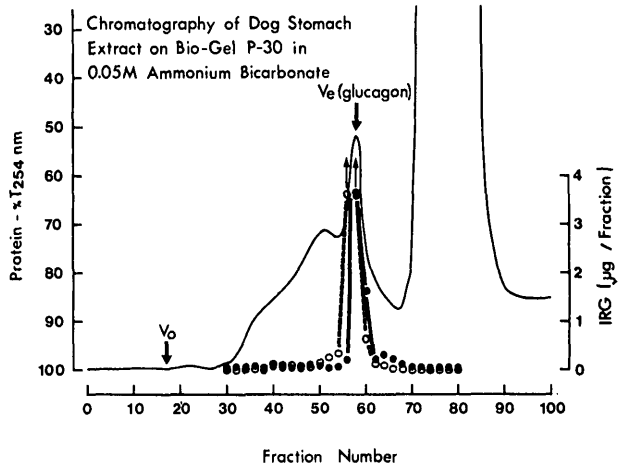

FIG. 5. Gel filtration of pooled fraction VI obtained as shown in figure 4. Fraction VI was reduced in volume by ultrafiltration at 5° C. and was then lyophilized. The dry material was dissolved in 4 ml. of 0.05-M NH_4HCO_3 and chromatographed on a column (2.5 cm. x 100 cm.) of Bio-Gel P-30 (100-200 mesh) equilibrated to 0.05-M NH_4HCO_3. The column was eluted with the same buffer at a rate of 15 ml./hr. Fractions of 7.5 ml. were collected and assayed for IRG with antiserum 30-K (o—o) or K-4023 (o---o). Ve of glucagon as indicated was determined with ^{125}I-glucagon.

30-K and most likely represents degradation products of the component identified as being similar to pancreatic glucagon.

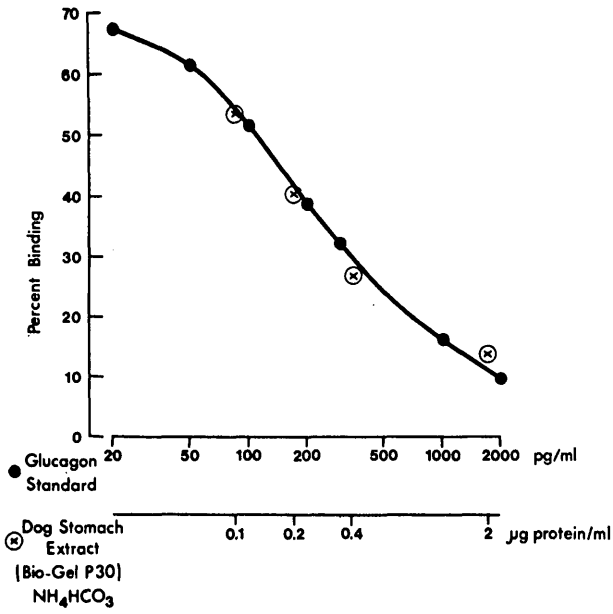

FIG. 6. Immunoassay comparison between porcine crystalline glucagon standard and the partially purified immunoreactive material obtained as shown in figure 5. The fractions containing the immunoreactive material were pooled. Dilutions of the material giving the final protein concentrations as indicated were assayed for IRG in triplicates with antiserum 30-K.

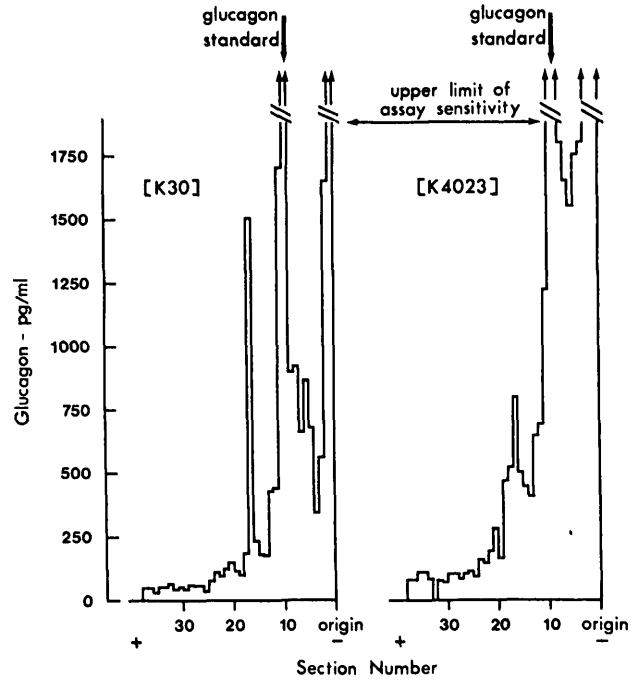

FIG. 7. Polyacrylamide disc gel electrophoresis of an aliquot of the partially purified immunoreactive material obtained as shown in figure 5. Electrophoresis was carried out in 15 per cent gel containing 4-M urea at pH 8.7. Standard porcine crystalline glucagon was electrophoresed in parallel in a separate gel tube and stained. The unstained gel containing the sample was cut into 1.5-mm. sections. Each section was eluted with 0.1 ml. of the immunoassay diluent. The eluate from each section was assayed for IRG with antiserum 30-K and K-4023 as indicated.

DISCUSSION

In this study, considerable amounts of immunoreactive glucagon were found in various tissues of the gastrointestinal tract of the dog when assayed with antiserum 30-K or K-44. Both antisera measured quantitatively similar amounts of IRG in these tissues. The highest concentration was found in the fundus and corpus of the stomach. The distribution of immunoreactive glucagon in the gastrointestinal tract is thus similar to the distribution pattern of glycogenolytic activity of gut tissues observed in the early work of Sutherland and De Duve.[19] The observation that near normal concentration of IRG was found in gastrointestinal tissues of depancreatized dogs that had secreted a normal amount of IRG during the period of insulin treatment and excessive amounts during insulin deprivation suggests that the gut is not only capable of secreting but also of storing IRG.

Since IRG was not detected in other tissues of the dog, the gastrointestinal tissues are likely the source of extrapancreatic glucagon in blood of the depancreatized dogs.

A-cells indistinguishable from pancreatic glucagon-producing A-cells have been identified in the stomach mucosa. In the gastrointestinal tract distal to the stomach a different type of A-cells is found, and it is thought that they are operative in secretion of glucagon-like substances.[12] Earlier histologic work suggesting that A-cells similar to pancreatic A-cells are present in the gut has been reviewed previously.[2] Histologic evidence for the presence of true A-cells in mucosa of nongastric tissues is not available. Since excessive secretion of gastrointestinal glucagon occurs in diabetic dogs, it may be that excessive glucagon secretion in human diabetics does not necessarily reflect an altered function of pancreatic A-cells but may originate at least in part from excessive secretion by gastrointestinal A-cells.[2]

Sasaki et al.[12] have shown that in the dog, the antiserum 78J, which strongly cross-reacts with glucagon-like materials, and antiserum 30-K detected essentially the same concentration of IRG in extracts of stomach fundus, but antiserum 78J detected a much higher concentration of IRG in extracts of duodenum, jejunum, and ileum. This indicates that stomach fundus contains essentially only immunoreactive glucagon, while other gastrointestinal tissues contain large amounts of glucagon-like substances.

Our present observation that immunologic dilution curves not only of the stomach but also of other gastrointestinal tissues were indistinguishable from dilution curves of the pancreatic glucagon standard indicates that immunoreactive glucagon is present not only in the stomach but also in other gastrointestinal tissues. It is not likely that concentrations of IRG measured in these tissues merely reflect cross-reactivity with GLI, because one would expect that a dilution curve of an antigen, such as GLI, cross-reacting with an antibody highly specific for pancreatic glucagon, would be different from those of the specific antigen standard (i.e., the purified pancreatic glucagon). It is possible, therefore, that when cross-reactivity of an antiserum specific for pancreatic glucagon is assessed by using crude gut extracts, the IRG measurements, at least in part, are not due to cross-reactivity but to measurement of immunoreactive glucagon that might be present in such extracts.

The IRG present in the dog gastric tissue has been further established in this study to be similar to pancreatic glucagon because of the following observations: (a) Its molecular weight was similar to pancreatic glucagon as determined by gel filtration in acid and alkaline medium; (b) it was immunologically identical to pancreatic glucagon as illustrated by the parallelism between its dilution curve and the standard curve obtained with purified pancreatic glucagon; (c) its electrophoretic mobility in the presence of urea was identical to that of pancreatic glucagon. Sasaki et al.[12] have also found an immunoreactive component in extracts of porcine duodenum and have concluded that it was indistinguishable from pancreatic glucagon. These workers also detected the presence of an immunoreactive material smaller than pancreatic glucagon in gel filtration on columns equilibrated with ammonium bicarbonate. In the course of this study, we have detected also a small component at variable concentrations in the extracts of the dog gastric tissue, but only under conditions when the degradation of glucagon was not minimized. The use of benzamidine as a nonspecific protease inhibitor, the handling of the extracts and fractions in the cold, and the storage of fraction in freeze-dry form have apparently minimized the breakdown of the stomach IRG.

It has been demonstrated recently that gel filtration resolved the plasma of normal dogs into four immunoreactive glucagon fractions and that plasma of depancreatized dogs gave similar results.[20] These observations suggest that plasma glucagon in normal and depancreatized dogs is similar. Such heterogeneity, however, was not observed in the present study on the extracts of dog stomach mucosa. This difference could be due to different extraction procedures and/or to the possibility that the storage and secreted forms of glucagon might not be the same. Furthermore, the heterogeneity observed in the plasma might have been the result of degradation and aggregation in the plasma.

We have provided evidence for the similarities between gastrointestinal and pancreatic glucagon. However, the control of secretion of pancreatic is different from that of the secretion of gastrointestinal glucagon: (a) strenuous exercise increases plasma IRG in normal but not in depancreatized dogs;[8] (b) arginine increases plasma IRG in depancreatized dogs only when they were insulin-deprived.[7,10] On the other hand, insulin and somatostatin decreased plasma IRG in both normal and depancreatized dogs.[9,10] Thus, it appears that under normal conditions the interplay between pancreatic glucagon and insulin has a regulatory function during exercise or after protein meals. Under abnormal conditions, when gastrointestinal secretion of glucagon is not restrained by insulin, the gastrointestinal A-cells would secrete excessive amounts of glucagon. It is conceivable that during the phylogenetic development the pancreatic A-cells had acquired an increased sensitivity to the signals provided by various

physiologic challenges.[8] It may be that deficiencies of the control of glucagon secretion in some diabetic patients are due to the fact that in these patients, in contrast to normal subjects, relatively more IRG is secreted from the gastrointestinal tract than from the pancreas. Therefore, the regulation of secretion of glucagon in these patients becomes abnormal. A more complete knowledge about structure, synthesis, and control of secretion of gastrointestinal glucagon may thus lead to a better understanding and, therefore, more specific management of some aspects of diabetes.

ACKNOWLEDGMENTS

We are grateful to Dr. Lavinia Lickley for performing the pancreatectomies and to Dr. A. K. Tung for performing the receptor assay of glucagon. Mrs. Nada Kovacevic, P. Eng, and Mrs. J. Wilkins are thanked for their excellent assistance. The help of Dr. E. Marliss and Dr. G. Steiner in setting up the glucagon immunoassay is gratefully acknowledged.

The work was supported in part by Medical Research Council of Canada (grants MT-2196 and MT-3034), E. C. Stevens Postdoctoral Fellowship of the University of Toronto (to Dr. Morita) and Connaught Laboratories, Ltd., Toronto. The gift of Hoechst Pharmaceuticals, Ltd., Montreal (Festal tablets) is also gratefully acknowledged.

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