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J Clin Invest. 1977;59(4):716-722. https://doi.org/10.1172/JCI108690.

Research Article

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Factors Controlling Gastric-Glucagon Release

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ABSTRACT A system consisting of an isolated dog stomach perfused with whole blood has been designed to study gastric glucagon secretion. Under basal conditions, gastric glucagon release was 0.0–3.1 ng glucagon/100 g of stomach per min. Arginine, at an arterial plasma concentration averaging 10 mM, elicited a rapid glucagon release. This gastric glucagon release was almost completely abolished by somatostatin (100 ng/ml). The release of gastric glucagon was not affected by hyperglycemia alone but was reduced by about 40% when hyperglycemia was concomitant with an hyperinsulinemia within the physiological range. These observations support the concept that adequate concentrations of insulin are necessary in order for hyperglycemia to inhibit gastric glucagon secretion. Furthermore, it is suggested that the isolated perfused dog stomach might provide a unique tool permitting investigation of alpha-cell function in the absence of endogenously released insulin.

INTRODUCTION

As recently reported by several groups of investigators (1–3), the plasma of totally depancreatized dogs contains normal, or even increased, quantities of a material immunometrically indistinguishable from pancreatic glucagon by radioimmunoassays regarded as highly specific for this hormone. As emphasized by Sasaki et al. (4), this post-pancreatectomy immunoreactivity cannot be attributed to a cross reaction with high levels of "gut glucagon-like immunoreactivity", a group of immunometrically dissimilar polypeptides whose level is not elevated after pancreatectomy (5). However, glucagon cannot be detected in the blood of dogs that have undergone complete abdominal evisceration (6). Therefore, in the dog, the origin of extrapancreatic glucagon is likely to be an abdominal organ; other significant sources of glucagon, such as the salivary glands as reported in the rodents (7, 8), are unlikely to exist.

Sutherland and De Duve (9), almost 30 yr ago, were the first to suggest that glucagon may be present in the gastrointestinal tract. Since then, various groups (10–14) have reported that the oxyntic glandular mucosa of the canine stomach contains cells resembling pancreatic alpha(A) cells. The joint efforts of Unger's group in Dallas and Orci and his co-workers in Geneva established the presence in the gastrointestinal tract of a material biologically, physicochemically, and immunometrically identical to pancreatic glucagon (4) and also established the existence of A cells in the dog gastric fundus (4, 15).

Because of the difficulty involved in the selective investigation of gastric glucagon secretion in pancreatectomized dogs, we designed an isolated perfused dog stomach system. The aim of the present study was to determine whether such an isolated system responds to the intravascular infusion of arginine by a release of gastric glucagon as does the pancreas, and whether such a response is affected by glucose, insulin, or somatostatin, which are all factors previously demonstrated to modify profoundly the secretion of pancreatic glucagon.

METHODS

Mongrel dogs of both sexes were fasted overnight and were anesthetized with pentobarbital (30 mg/kg). Large dogs, weighing 25–40 kg, were used as blood donors for loading the perfusion apparatus. Smaller dogs (7.5–15.0 kg) were used to provide the stomach. During the surgical procedure, the stomach-donor dogs were artificially ventilated with a mixture of oxygen (95%) and CO_2 (5%).

After a large median xypho-pubic laparotomy, the stomach was completely isolated but its arterial and venous supply was carefully preserved. The procedure included splenectomy, with ligature of all the splenic branches (rami lienalis)

A preliminary note on part of this study was presented at the International Symposium on Glucagon in Dallas, Tex. on 10–12 May 1976 and published in 1976: *Metab Clin. Exp.* **25**(Suppl. 1): 1477–1479. This study was also presented in part at the 12th Annual Meeting of the European Association for the Study of Diabetes in Helsinki, Finland on 1–3 September 1976 and published in abstract form in 1976: *Diabetologia.* **12**: 405. (Abstr.)

Dr. Luyckx is an Established Investigator of the Fonds National de la Recherche Scientifique.

Received for publication 24 September 1976 and in revised form 3 December 1976.

TABLE I
*Perfusion Blood Characteristics**

	Beginning of perfusion		End of perfusion		Paired *t* test
Hemoglobin saturation	>98%	(15)	>98%	(15)	NS
pH	7.33±0.01	(15)	7.25±0.01	(15)	$P < 0.01$
P_{CO_2}, *mm Hg*	32.6±1.2	(15)	33.8±1.5	(15)	NS
Bicarbonate, *meq/liter*	17.9±0.3	(15)	15.7±0.5	(15)	$P < 0.01$
Lactate, *mM/liter*	3.63±0.31	(12)	4.67±0.34	(12)	$P < 0.01$

* Results are expressed as mean±SEM; the number of determinations is given in parentheses.

at the level of the long hilus but with careful preservation of the splenic and gastro-epiploic arteries; careful exclusion of all pancreatic tissue; dissection of the coeliac trunk and of the gastro-duodenal artery and vein; preservation of the right and left gastric artery and vein; and ligature and section of the hepatic pedicle.

When the stomach was completely isolated with its arterial and venous supply, 5000 U of heparin (Boots Co, Nottingham, England) was injected intravenously. The cardia was then ligated and the esophagus was sectioned. A polyethylene catheter (outer diameter 1 cm; length 15 cm) was inserted into the stomach through the pylorus, which was then ligated. The duodenum was sectioned right over the pylorus. A glass cannula of appropriate size was then inserted into the distal part of the coeliac trunk, which was tied and cut. Another glass cannula was inserted into the distal part of the portal vein to drain off all the venous blood leaving the stomach. After section of the portal vein, the stomach was completely isolated, removed from the abdominal cavity, and transferred to the perfusion machine. The duration of gastric ischemia (between the ligature of the arterial supply in situ and the beginning of the perfusion in vitro) never exceeded 4 min.

During the final stages of the isolation of the stomach, the perfusion machine was loaded with 1,100–1,200 ml of blood that was rapidly collected from both carotides of the blood donor. As previously shown necessary in this system (16), the blood was supplemented with 1,000 U/ml Trasylol (Bayer, Leverkusen, W. Germany). The perfusion system was essentially the one designed by Nizet and his co-workers for the perfusion of isolated dog kidneys (17, 18); it consists basically of a film oxygenator and a Dale-Shuster pump under external hydraulic control. The whole system was kept at 38°C by a thermostatic bath. The blood was equilibrated with a gaseous mixture containing 95% O_2 and 5% CO_2. The blood flow through the stomach was measured either by means of a side-branched graduated 25-ml pipette and temporary occlusion of the venous return, or directly by collecting the venous effluent in graduated cylinders (16). The arterial perfusion pressure in the isolated stomach was continuously measured with a mercury manometer and was adjusted if necessary by means of a Goldblatt clamp. Most perfusions were effected at a systolic pressure of 12–14 cm Hg. Stomach venous pressure was 2–4 cm H_2O. The output of gastric juice was collected in a graduated cylinder. Blood samples were simultaneously collected from the artery and the vein, and blood sampling started only after a 45–60-min equilibration period during which the blood was recirculated. Thereafter, samples were collected continuously at intervals of 10–30 s, without recirculation of the blood, before, during and after an intraarterial infusion of L-arginine monohydrochloride (Fluka AG, Buchs, Switzerland). The amino acid was diluted in saline and infused into the artery at a rate of 1 ml/min from time zero on, the concentration being adjusted to reach a final arginine concentration in the arterial plasma of about 10 mM; the arginine solution was therefore prepared extemporaneously after a preliminary determination of the flow and of the hematocrit of the blood perfusing the stomach in each particular experiment. In five experiments, arginine alone was infused. In three experiments, a somatostatin infusion was started 10 min before the arginine infusion and was continued until the end of the experiment. Linear somatostatin (Lot no 129 E 11-5 Roussel UCLAF, Paris, France), diluted in saline containing 0.25% human albumin, was infused into the artery at a rate of 0.2 ml/min, the concentration being adjusted to reach an arterial plasma somatostatin concentration of 100 ng/ml. In the last six perfusions, the blood perfusing the stomach was made hyperglycemic by the addition to the reservoir of sufficient 50% (wt/vol) glucose solution to allow a final blood glucose concentration of about 300 mg/100 ml. This addition was performed 30 min before the beginning of the arginine infusion. In three of these experiments, the blood reservoir was supplemented, 10 min before the arginine infusion, with an amount of insulin (Actrapid Novo, Novo Industries A/S, Copenhagen, Denmark; diluted in saline containing 1% crystallized bovine albumin) calculated to allow a final plasma insulin concentration of about 120 μU/ml.

Blood glucose concentration was determined by the method of Hoffman (19) adapated to the Technicon AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y.). Plasma α-amino-nitrogen was determined by colorimetry (20). For hormone assays, 0.4 ml of a solution containing Trasylol, 5,000 U/ml, and Na_2EDTA, 12 mg/ml, was added to 3.6 ml of blood. The mixture was immediately centrifuged at 4°C and the separated plasma was stored at −20°C. Plasma glucagon was determined in duplicate assays by a classical radioimmunoassay procedure (21), using porcine ^{131}I-labeled glucagon as tracer, 30K antiserum (provided by Dr. R. H. Unger, Dallas, Tex.) and dextran-charcoal separation of free and antibody-bound hormone. Gastric-glucagon production by the stomach was calculated by multiplying the veno-arterious difference in plasma glucagon concentration by the plasma flow (the latter being derived from the blood flow and the hematocrit). Plasma insulin was determined as previously described (22). At the end of the experiment, the stomach was carefully dissected and weighed. In some preliminary experiments it was fixed for routine histologic examination.

RESULTS

Perfusion conditions and characteristics of the isolated perfused dog stomach. As shown in Table I,

TABLE II
Characteristics of the Isolated Perfused Dog Stomach

	Mean±SEM	*n*	Range
Body weight of the stomach donor dog, *kg*	11±0.4	20	7.5–15.0
Weight of stomach, *g*	130±8	20	70–200
Weight of stomach as percent of body weight	1.18±0.05	20	0.87–1.55
Stomach blood flow, *ml/100 g/min*	62.6±3.8	20	42–100
Perfusion blood hematocrit	47.5±0.8	20	38–52
Stomach plasma flow, *ml/100 g/min*	32.9±2.2	20	22–55
Basal gastric juice production, *ml/100 g/h*	15.2±2.8	14	2.7–35.0
Gastric juice pH	4.33±0.62	12	1.8–7.0

the hemoglobin O_2 saturation of the perfusing blood remained over 98% throughout the experiment. At the end of the perfusion, a slight metabolic acidosis, partly due to a moderate accumulation of lactate, was recorded. Plasma-glucagon concentration in the perfusing blood, before the introduction of the stomach in the circuit, averaged 105±14 pg/ml (n = 31; range 32–390 pg/ml) and the mean basal concentration of plasma insulin was 6.9±0.9 μU/ml (n = 19; range 0.0–12.6 μU/ml). The characteristics of the isolated perfused stomach are given in Table II. In all experiments, the stomach produced significant amounts of gastric juice during the equilibration period before arginine infusion. In most instances, the gastric juice was acidic; the total HCl output ranged from 0 to 0.9 meq/h. At the end of the perfusion the appearance of the stomach was normal on both its serous and mucous sides; however, a few histological controls demonstrated the presence of a moderate edema involving all layers.

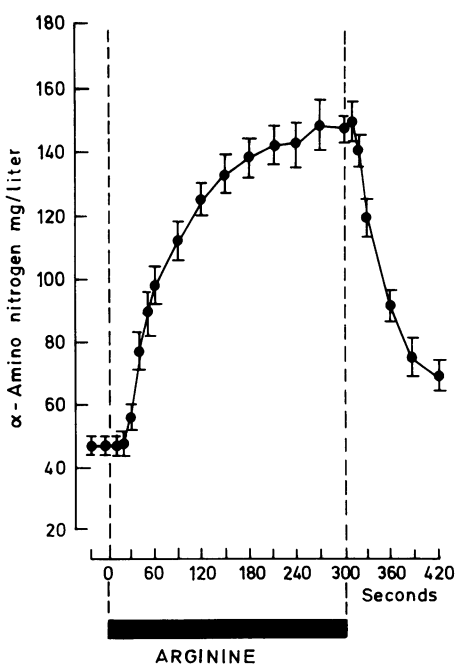

FIGURE 1 Time course of the changes in α-amino-nitrogen in the gastric venous plasma in response to the intra-arterial infusion of arginine. Results are expressed as mean ± SEM (n = 17).

Gastric glucagon release under basal conditions. Immediately before arginine infusion, blood glucose and plasma α-amino-nitrogen were within the normal range: 72.7±4.0 mg/100 ml (n = 20) and 4.65±0.34 mg/100 ml (n = 17), respectively. Basal glucagon release averaged 7.0±3.0 ng/100 g stomach per 5 min (n = 7; range 0.0–15.5 ng/100 g per 5 min). In a single experiment, saline was infused at a rate of 1 ml/min into the artery for 10 min; under these conditions basal glucagon release averaged 4.5±0.5 ng/100 g per 5 min.

Gastric glucagon release in response to arginine infusion. As indicated under Methods arginine was infused at a rate calculated to reach an arterial plasma concentration of about 10 mM. Assuming that infused arginine is distributed solely in the plasma,[1] retrospective calculations on the basis of the actual plasma flows recorded in every experiment indicated that, in the 17 perfusion experiments reported here, the mean concentration of arginine in the arterial plasma was in fact 9.9 ± 0.3 mM. As indicated in Fig. 1, plasma α-aminonitrogen concentrations in the venous effluent rose progressively, reached a plateau during the 3rd and the 5th min of the perfusion, and then declined abruptly after cessation of the infusion. By paired comparison, the first statistically significant rise in the venous-plasma concentration of α-amino-nitrogen was obtained within 20–30 s after the beginning of the intraarterial infusion of arginine (9.6 ±1.9 mg/100 ml; $P < 0.01$). Comparison with dye infusion indicated that this delay corresponded almost entirely to the circulatory time in the system. As shown in Fig. 2, arginine infusion resulted in rapid rise in glucagon output. Glucagon output began to increase 30–40 s after the beginning of the intra-arterial arginine infusion, reached a peak value at about 60 s and then declined progressively. A transient off-response was sometimes observed immediately after the cessation of the infusion. The total amount

[1] It is known that amino acids are transported into erythrocytes and that, at the equilibrium, the amino acid concentration in whole blood is about the same as in plasma (48). We have considered that such a diffusion might probably be neglected in our experimental conditions where infused arginine reached the stomach capillary bed in about 10 s.

of glucagon released during the arginine infusion averaged 50.1±10.4 ng/100 g per 5 min (Table III), that is, 6–10 times more than the basal release.

Effect of somatostatin on the arginine-induced gastric glucagon release. As shown in Table III, 100 ng/ml somatostatin almost completely inhibited gastric glucagon release which, under these conditions, averaged 8.8±2.4 ng/100 g per 5 min, a value similar to the release obtained in basal conditions and which represents 17.6% of the release obtained with arginine alone.

Effect of glucose alone or glucose plus insulin on arginine-induced gastric glucagon release. As shown in Table IV, the arginine-induced gastric glucagon release was unaffected by hyperglycemia of the perfusing blood. When such hyperglycemia was concomitant with an hyperinsulinemia of 115±20 μU/ml, arginine-induced gastric glucagon release was reduced by about 40%. As shown in Fig. 3, no major difference in the kinetic of the release was observed under either condition.

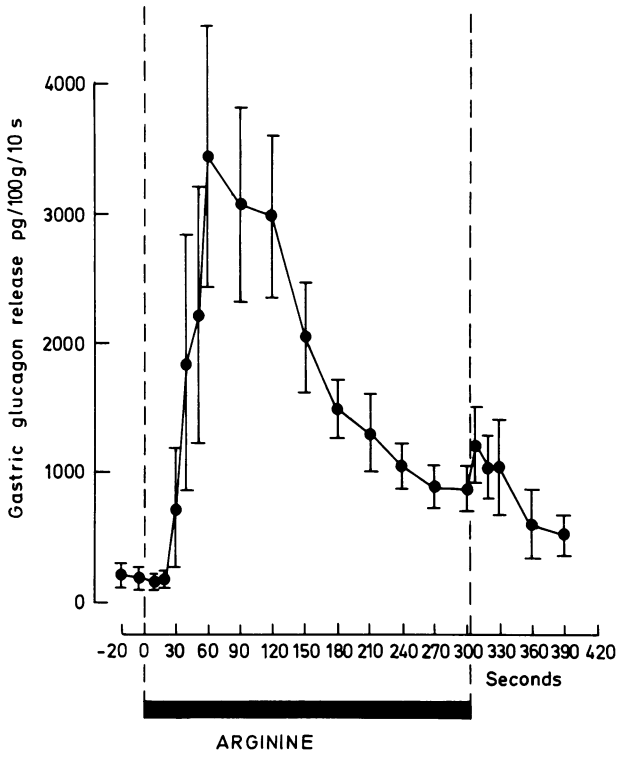

FIGURE 2 Gastric glucagon release in response to intra-arterial arginine infusion. Results are expressed as mean ± SEM (n = 5).

DISCUSSION

The recent identification of "true A cells" (4, 15, 23) as well as the presence of glucagon (23–25) in the canine stomach made necessary a systematic analysis of the factors controlling the release of gastric glucagon. Since the pancreas still remains the major organ synthesizing and releasing glucagon, studies in vivo would necessitate the use of pancreatectomized animals. Even in such animals, changes in peripheral plasma glucagon levels may not solely reflect changes in gastric glucagon secretion since other parts of the gastrointestinal tract may contain and release small amounts of glucagon and since salivary glands have also recently been reported to contain glucagon. It must be said, however, that the amount of glucagon reported in the dog salivary glands are extremely low as compared with other animal species such as rodents (7, 8), a finding which is indirectly confirmed by the absence of circulating glucagon in abdominally eviscerated

TABLE III
Gastric Glucagon Release in Response to a 5-Min Arginine Infusion

	Calculated arginine concentration	Peak α-amino nitrogen in venous blood	Glucagon release
	mM	*mg/liter*	*ng/100 g/5 min*
Arginine alone	12.0	207	38.1
	7.0	202	83.5
	10.9	142	64.6
	9.9	121	28.1
	10.5	158	36.2
Mean±SEM	9.9±0.9	166±17	50.1±10.4
Arginine + somatostatin (*100 ng/ml*)	10.5	137	4.7
	9.2	146	13.1
	9.2	121	8.7
Mean±SEM	9.6±0.4	135±7 (NS vs. arginine)	8.8±2.4 ($P < 0.01$ vs. arginine)

TABLE IV
*Gastric Glucagon Release in Response to a 5-Min Arginine Infusion**

	Perfusing blood enriched with		
	Glucose alone	Glucose + insulin	Comparison
Calculated arginine concentration, *mM*	10.0±0.5	9.6±1	NS
Peak α-amino nitrogen in venous plasma, *mg/liter*	153±16	150±12	NS
Blood glucose, *mg/100 ml*	312±19	319±13	NS
Plasma insulin, *μU/ml*	7±2	115±20	$P < 0.01$
Glucagon release, *ng/100 g/5 min*	55.9±5.1	34.7±3.5	$P < 0.01$

* Results are given as mean±SEM; three perfusions were performed in each experimental condition.

dogs (6). Preliminary studies have been reported on the release of canine gastric glucagon in anesthetized animals with appropriate cannulation of the gastric veins (26). A precise study of the mechanisms controlling gastric glucagon release would involve the use of an isolated perfused organ, a method which has already provided invaluable information on the control of pancreatic glucagon release (27, 28).

The surgical procedure described here for the isolation of the stomach can be carried out within 60–75 min by a surgical team of two. The use of a perfusion machine, utilizing whole blood as a perfusion fluid and originally designed for the perfusion of isolated dog kidneys (16–18), permitted us to study the function of this isolated dog stomach. Controls indicated perfect oxygenation of the blood throughout the experiment. At the end of the perfusion, a moderate metabolic acidosis, partly due to the accumulation of laatate, was recorded. This is probably due to lactate production by both the erythrocytes and the stomach since erythrocytes, but also digestive mucosa, are mainly known to exhibit significant anaerobic glycolysis (29). Indications of the satisfactory behavior of the isolated perfused stomach include persistence of strong gastric contractions throughout the experiment, significant production of an acidic juice, and a normal macroscopic and microscopic appearance of the organ at the end of the perfusion, apart from a moderate edema noticed on the histologic samples.

Under basal conditions, gastric glucagon release was absent or minimal, a finding which is in agreement with previous data obtained in the anesthetized dog (26). The release of gastric glucagon increased markedly and rapidly after a short arginine infusion which reached a 10-mM concentration in the arterial plasma; this is indeed a strong stimulus of pancreatic glucagon secretion both in vivo (review in 30) and in vitro (31, 32). Therefore, the increased plasma glucagon observed after arginine infusion in insulin-deprived depancreatized dogs (3, 26, 33) is likely to be, at least in part, of gastric origin. The stimulation of the release of gastric glucagon occurs within seconds after the arrival of arginine at the A cells. The kinetics of the release have been studied only during a 5-min arginine infusion; this is essentially due to our decision to avoid recirculation of blood during the infusion, and therefore the relatively limited amount of blood obtainable from a single donor dog for loading the perfusion machine and the relatively high blood flow through the isolated stomach limit the duration of the experiment. The possibility of a two-phase release of glucagon, one rapid and one more sustained, as described for the pancreas (31, 32), has not been investigated in the present experiments.

Somatostatin, which is a potent inhibitor of glucagon secretion (34), almost completely blocked the arginine-induced gastric glucagon release. This finding

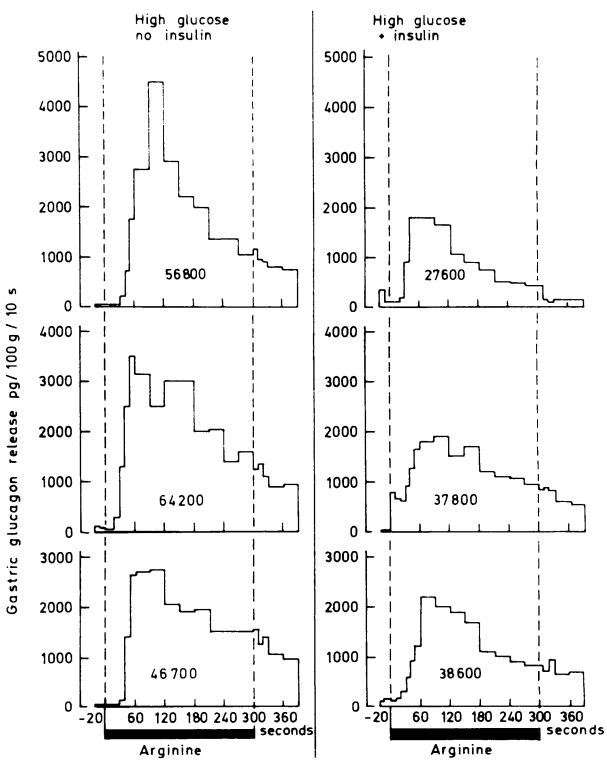

FIGURE 3 Gastric glucagon release in response to intra-arterial arginine infusion. On the left panel, the perfusing blood was enriched with glucose alone (mean blood glucose concentration: 312±19 mg/100 ml); no exogenous insulin was added (mean plasma insulin concentration: 7±2 μU/ml). On the right panel, the perfusing blood was simultaneously enriched with glucose (319±13 mg/100 ml) and insulin (115 ±20 μU/ml of plasma). The figures in the histograms corresponded to the total amount of glucagon (in picograms) released during the 5-min arginine infusion.

is in agreement with the previously reported decrease in plasma glucagon after somatostatin infusion in the depancreatized dog (5, 33).

Glucose has long been recognized to be an inhibitor of glucagon release (review in 30). The mechanism by which it exerts this effect is still largely unknown. Several observations suggest that insulin might play a major role in the glucose-induced glucagon suppression (30) and it has been suggested that the action of insulin in this respect would be to permit glucose to enter the A cell (35–37). An experimental approach to this question with the pancreas or isolated islets is particularly difficult; even after streptozotocin administration, one is never sure that some release of endogenous insulin does not occur (38), causing appreciable intra-islet concentrations of insulin. The isolated perfused dog stomach might provide a unique tool permitting investigation of A-cell function in the absence of endogenously released insulin; there is no evidence of the presence of beta cells in the stomach (39) and, as we have seen, the amounts of insulin in the perfusion blood obtained from overnight fasted dogs were always low. Furthermore, determinations of insulin levels in the effluent blood, at the time of maximal arginine-induced glucagon release, never showed any rise. In these conditions, it is extremely interesting that hyperglycemia alone did not reduce the arginine-induced gastric glucagon release, but did so only when it was accompanied by a rise in plasma insulin within the physiological range. The 40% reduction in the arginine-induced release of glucagon in the presence of glucose (300 mg/100 ml) and insulin (115 μU/ml) confirms the important role of insulin in permitting glucose to inhibit glucagon secretion. This observation supports the idea of an exquisite sensitivity of the gastric A cell to glucose (26) providing adequate concentration of insulin is present, and probably explains the rapid fall in plasma glucagon after administration of insulin to the diabetic pancreatectomized dog (5).

Even if no doubt persists concerning the presence of functioning A cells in the canine stomach, the presence of such cells in man is still not certain (40). Several authors reported the persistence of circulating glucagon in pancreatectomized man (41–43) while others did not (44, 45). With Vranic et al. (46), we suggest that the presence of circulating insulin, even at relatively low concentration, might favor the suppressive effect of glucose on extra-pancreatic A cells, as demonstrated in the present study. Therefore, we think that the concept of the "pancreatectomized man as a model of diabetes without glucagon" (44) must be accepted only if experimental data are provided of the total insulin lack of these patients at the time of study. Prolonged insulin deprivation and appropriate assays of circulating insulin (47) showing that insulin lack is indeed complete are prerequisites to the demonstration that there is no extrapancreatic source of glucagon in man.

Note added in proof. Since submission of this manuscript, Munoz-Barragan et al. (49) reported evidence that in normal conscious dogs, the gastric fundus is not a major source of gastric glucagon, a finding in agreement with the low release of gastric glucagon under basal conditions reported above. In contrast, Blazquez et al. (50) reported that (*a*) in the insulin-deprived depancreatized dog, the stomach is a major source of glucagon; (*b*) gastric glucagon secretion is stimulated by intravenous and intragastric arginine administration; (*c*) it is not influenced by intravenous or intragastric glucose administration; and (*d*) glucagon release is suppressed by physiological levels of insulin.

ACKNOWLEDGMENTS

We thank A. S. Beedle, Ph.D. for his help in the preparation of this manuscript. We are indebted to Prof. A. Nizet for providing laboratory facilities and to Dr. Wald (Bayer, Brussels) for the generous gift of Trasylol. We acknowledge with thanks the skillful technical assistance of Mr. and Mrs. H. Thoumsin, A. Rombeaux, C. Cartenstadt, C. Borremans and R. Lallemand. We are indebted also to E. Vaessen-Petit for her secretarial assistance.

This work was supported by the Fonds National de la Recherche Scientifique and the Fonds de la Recherche Scientifique Médicale of Belgium.

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