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MEASUREMENTS OF ENDOGENOUS GLUCAGON IN PLASMA AND THE INFLUENCE OF BLOOD GLUCOSE CONCENTRATION UPON ITS SECRETION*

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The development of a radio-immunoassay for glucagon capable of measuring as little as 50-millionths of a microgram was recently reported (1, 2). The high degree of specificity and sensitivity provided by this technique has made possible the following studies designed to determine if circulating endogenous glucagon can be identified in plasma, and, if so, whether its secretion is influenced by changes in blood glucose concentration. Such information might bear decisively upon the question of the hormonal status of glucagon, which, despite the classic studies of Foa and associates (3–5), has not been specifically established.

METHODS AND MATERIALS

A total of 48 mongrel dogs was employed in these studies. While the dogs were anesthetized with Nembutal (pentobarbital), a laparotomy was performed and a polyethylene or Tygon catheter inserted in a retrograde direction into the pancreaticoduodenal vein. Another catheter was placed into the portal vein distal to the pancreaticoduodenal vein and in the direction of blood flow. These two catheters were joined to a three-way stopcock, thereby permitting the normal entry of pancreaticoduodenal venous effluent into the portal vein and making possible the intermittent collection of blood samples.

Catheters were passed into each femoral vein. One was threaded into the vena cava to the approximate level of the hepatic veins and was used for sampling; the other was left in the femoral vein to be used for injections and infusions. All dogs were given heparin. Oxygen was administered by tracheal catheter in most experiments.

Specimens of pancreaticoduodenal blood were obtained by opening the three-way pancreaticoportal stopcock and allowing free flow into a test tube; vena caval blood was obtained by syringe.

Glucagon concentration was determined by means of the radio-immunoassay described in previous reports (1, 2) and glucose concentration by means of the Hoffman method (6) on the Technicon Autoanalyzer.

As described elsewhere (1, 2), the glucagon assay is based upon the ability of nonradioactive glucagon[1] to displace competitively glucagon-I^{131} from rabbit antibodies against beef-pork glucagon. Standard solutions containing from 0 to 10,000 $\mu\mu$g of nonradioactive beef-pork glucagon cause a progressive decrease in binding of glucagon-I^{131} to antibodies, as determined by paper chromatography.

Endogenous glucagon in each plasma specimen was quantitated by comparing its bound to free (B/F) lowering effect with that of the beef-pork glucagon standards used to make the standard curve. Because of the presumed immunologic dissimilarity of canine and beef-pork glucagon, concentration of the former should be expressed in "micromicrogram equivalents" of beef-pork glucagon per milliliter ($\mu\mu$g Eq per ml).

Blood samples for glucagon assay were centrifuged upon collection, and frozen until the time of assay. Undiluted plasma was employed in an assay system in which 50 or 100 $\mu\mu$g of glucagon-I^{131} and a 1:100 dilution of pooled high-titer rabbit antiserum to beef-pork glucagon were used. A separate standard curve was run with each group of determinations. The resulting B/F ratios were corrected for nonspecific migration of glucagon-I^{131} by the method of Yalow and Berson (7) on the basis of control strips in which nonimmune rabbit serum had been substituted for antiserum. These controls were run with both standards and plasma samples in every experiment.

RESULTS

Endogenous glucagon in pancreaticoduodenal venous plasma

Plasma specimens obtained from the pancreaticoduodenal vein of 48 normal fasting dogs were assayed for glucagon content. In 42 of the measurements made, the undiluted plasma sample caused a significant lowering of the B/F ratio of glucagon-I^{131}, indicating the presence in these samples of endogenous glucagon.

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[1] Kindly supplied by Dr. W. R. Kirtley, Eli Lilly Co., Indianapolis, Ind.

Glucagon concentration in normal fasting dogs ranged from 0 to 1,300 μμg Eq per ml with a mean of 543 μμg Eq per ml (Figure 1).

Effects of changes in blood glucose concentration upon endogenous glucagon concentration

1. Chronic phlorizin-induced hypoglycemia. If the hyperglycemic and glycogenolytic properties of exogenous beef-pork glucagon provide a clue as to the function of endogenous glucagon, it might be anticipated that glucagon concentration would rise when the blood glucose concentration is low. For this reason, nine dogs were made chronically hypoglycemic by means of phlorizin administration. A total dose of 10 g of 40 per cent phlorizin in propylene glycol was administered subcutaneously in five divided doses over a 4-day period.

Fasting glucagon concentration in the pancreaticoduodenal venous plasma of the hypoglycemic dogs averaged 1,976 μμg Eq per ml and ranged from 680 to 3,100 μμg Eq per ml. In a group of

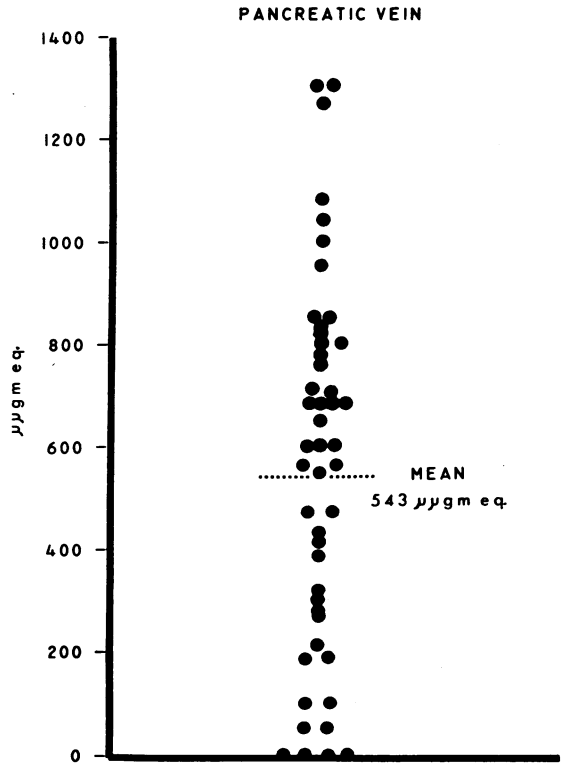

FIG. 1. PLASMA GLUCAGON CONCENTRATION IN NORMAL FASTING DOGS. The fasting level of endogenous glucagon in 48 normal dogs expressed in μμg Eq per ml of beef-pork glucagon.

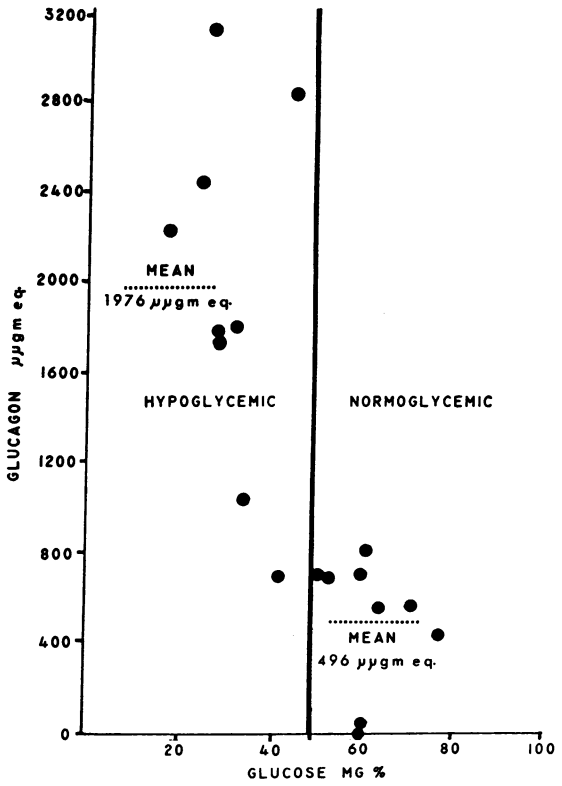

FIG. 2. RELATIONSHIP OF FASTING BLOOD SUGAR (FBS) TO PANCREATIC VENOUS GLUCAGON LEVEL IN FASTING DOGS. Endogenous pancreaticoduodenal venous plasma glucagon concentration was measured in 9 dogs made chronically hypoglycemic (FBS < 50 mg per 100 ml) by means of phlorizin administration. Nine normoglycemic control dogs (FBS > 50 mg per 100 ml) were studied simultaneously. The chronically hypoglycemic animals were found to have a considerably higher fasting glucagon level, ranging from 680 to 3,100 μμg Eq per ml, with a mean of 1,976; the controls averaged only 496 μμg Eq and ranged from 0 to 800 μμg Eq.

experiments on nine normoglycemic control animals, considered most comparable to those of the phlorizinized animals because the identical lots of glucagon-I^{131} and the same standard curves were employed, the fasting glucagon concentration averaged 496 μμg Eq per ml, with a range of 0 to 800 μμg Eq per ml (Figure 2). The higher glucagon concentration in the hypoglycemic group represents a statistically significant difference ($p < 0.01$).

2. Acute insulin-induced hypoglycemia. The effect of insulin-induced hypoglycemia upon glucagon concentration was studied in 13 fasting dogs. In 10 of the animals 0.3 to 0.78 U per kg of "glu-

TABLE I

*Acute insulin hypoglycemia and plasma glucagon concentration (μμg Eq/ml)**

Dog no.		Control period (*min*)						Time after insulin injection (*min*)											
		−90	−60	−30	−20	−5	0	+20	+30	+40	+50	+60	+70	+90	+110	+120	+130	+150	+180
1	PDG			1,300					1,440		1,400		1,480	2,900	2,500		2,400	3,200	3,200
	VCG			920		910			900		910		850	940	970		970	1,240	1,540
	BS			41					31				17	18	20		22	24	32
	Insulin (U/kg)						0.5†												
2	PDG			800		740			730		970		1,280	1,760	2,700		1,880	2,550	2,650
	VCG			420		480			500		640		670	630	660		510	600	650
	BS			61		57			46				31	28			31	51	30
	Insulin (U/kg)						0.4†												
3	PDG			50					0		400		550	680	1,220		1,500		
	VCG			0					50		200		75	310	340		400	400	
	BS			61		58			39				39		37		44		
	Insulin (U/kg)						0.3†												
4	PDG			810					695		740		750	1,030	985		1,000	1,140	1,180
	VCG												690				595		570
	BS			85					58		41		40	37	44		47	41	42
	Insulin (U/kg)						0.5†												
5	PDG			650					815		630		945	760	940		1,255	1,970	Died
	VCG			450							460			580			720		
	BS			74					38		37		36	32	46		57	91	
	Insulin (U/kg)						0.4†												
6	PDG			470		920			950		845		935	780	825		1,070	1,180	1,250
	VCG			410									470		560				350
	BS			69					54		33		35	35	41		46	58	63
	Insulin (U/kg)						0.5†												
1B	PDG			470		355			490		1,020								
	VCG			440															
	BS			57					46		35								
	Insulin (U/kg)						0.7†												
7	PDG	600	630	840		870			940			1,160		1,810		2,340			
	VCG	360				480													
	BS	52	50	52		45						22		21		18			
	Insulin (U/kg)						0.78†												
8	PDG	135		740		350			555			700		560		220		870	
	VCG	0				180			200									300	
	BS	64	59	51		47			16			13		14		15		20	
	Insulin (U/kg)						0.76†												
9	PDG	0		100		90			110			250		560		810		920	
	VCG	0				0‡								0					
	BS	61	76	84		95			59			52		49		43		45	
	Insulin (U/kg)						0.78†												
10	PDG	280	390	460		560			830			1,400		920		960		1,050	
	VCG		175			135								460					
	BS	77	58	60		53			28			18		24		22			
	Insulin (U/kg)						0.78†												
11	PDG			700	600	935		680			1,430	1,750				2,360	2,800		
	VCG			530								580				580			
	BS			51				42			26	23				14			
	Insulin							←					0.01 U/min§						→
12	PDG			950	700			770		1,030		1,340		1,670		1,640		950	
	VCG			570								560						730	
	BS			47				45		35		27		26		18		14	
	Insulin							←					0.01 U/min§						→
13	PDG			550	653			775		650		925		1,500		1,750		3,825	
	VCG			300												675			
	BS			64				50		42		37		34		27		23	
	Insulin							←					0.01 U/min§						→

* PDG = pancreaticoduodenal glucagon; VCG = vena caval glucagon; BS = blood sugar.
† Rapid injection.
‡ Hemolysis.
§ Constant infusion, U/min.

cagon-free" crystalline insulin[1] was injected rapidly by vein; in the remaining 3 dogs the insulin was infused intravenously at a rate of 0.01 U per kg per minute for a period of 150 mintes.

The results are recorded in Table I and shown graphically in Figure 3. In all but 4 of the 11 animals (Dogs 4–6 and 8) significant elevation in pancreaticoduodenal venous plasma glucagon

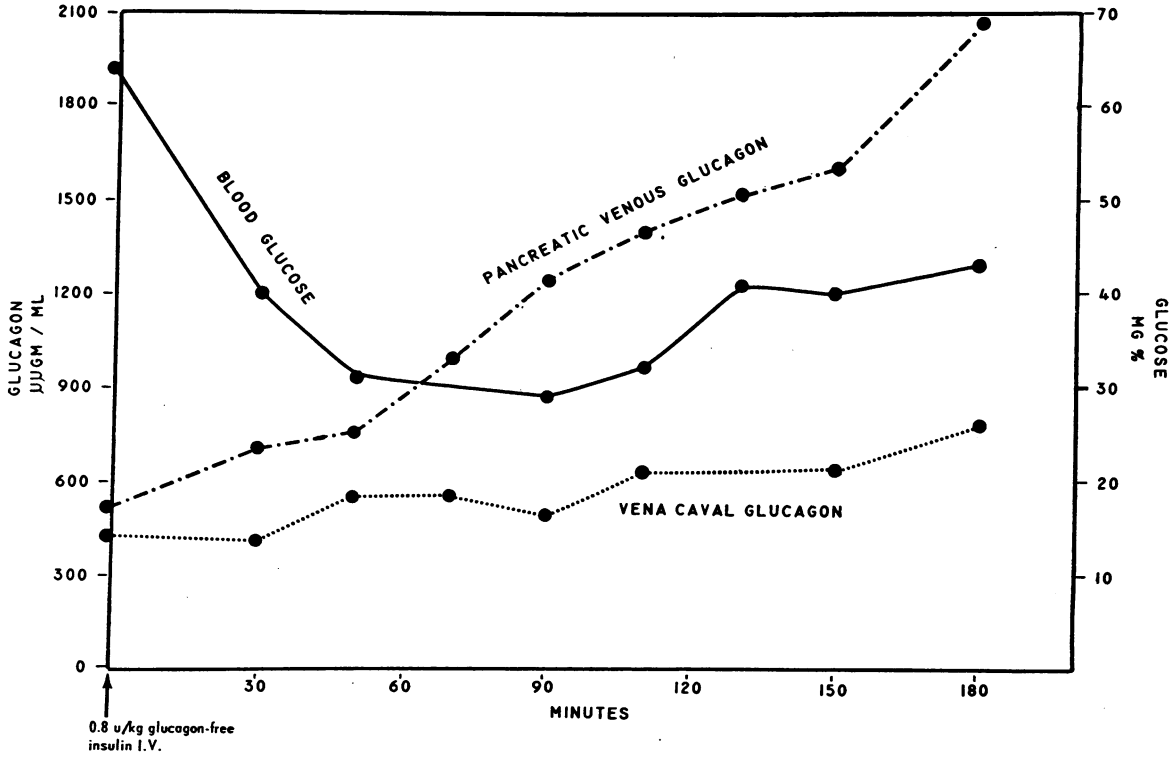

FIG. 3. PLASMA GLUCAGON CONCENTRATION DURING ACUTE INSULIN HYPOGLYCEMIA IN 10 DOGS. The effect of acute hypoglycemia induced by the rapid intravenous injection of "glucagon-free" insulin in doses of 0.3 to 0.78 U per kg upon mean glucagon concentration of 10 dogs is shown above. Because of variation in the timing of specimens and in the duration of the individual experiments, some of the points on the mean curves represent less than 10 observations (Table I). Dog 1B was omitted because of the paucity of data. The mean level of glucagon in the pancreaticoduodenal venous plasma is seen to rise gradually during the first hour after insulin injection, and to reach significantly elevated values during the second hour of hypoglycemia. Mean glucagon concentration in the vena caval plasma rose only slightly.

concentration appeared within 120 minutes of insulin administration, during which time venous glucose concentration had reached its nadir. In general, the pattern in these experiments appears to be one of gradual rather than of prompt elevation in glucagon concentration, reaching statistically significant proportions ($p < 0.01$) during the second and third hours of severe hypoglycemia. This rise is quite obviously not the result of any injected exogenous glucagon contaminating the "glucagon-free" insulin,[2] since it appeared long after the injection.

In most of the 13 experiments, the glucagon concentration in the vena caval plasma failed to reflect the rising glucagon concentration noted in the pancreaticoduodenal venous plasma. Only in a few animals with extreme pancreaticoduodenal hyperglucagonemia were late elevations of vena caval plasma glucagon concentration encountered.

Five dogs were given infusions of normal saline without insulin. No significant changes in glucagon concentration were observed (Table II).

Effect of intense hyperglycemia upon induced hyperglucagonemia

The foregoing experiments were modified for the purpose of determining whether the hyperglucagonemia noted in association with profound hypoglycemia could be influenced by the sudden induction of intense hyperglycemia. Three normal dogs were given a rapid injection of glucagon-free insulin in a dose of 0.8 U per kg. At 1 hour after the injection, at which point the glucose concen-

[2] According to Dr. W. R. Kirtley, Indianapolis, Ind., the "glucagon-free" insulin which he kindly furnished contained approximately 0.01 per cent glucagon.

TABLE II

*Glucagon concentration during infusion of normal saline (μμg Eq/ml)**

Dog no.		0	15†	20	30	50	60	70	90	100	110	130	140	150	180
1C	PDG	680	760		660	630		920	800		830	850		920	
	VCG	620							580					710	
	BS	53				66		70	74		86	77		60	
2C	PDG	1,040	850		1,120	1,100		1,100			1,400	1,540			830
	VCG	570						400							200
	BS	45			48	47		52			46	46			43
3C	PDG	750		900			450			510			440		890
	VCG														
	BS‡	90		88			90			88			86		92
4C	PDG	195		210			70			0			160		180
	VCG														
	BS‡	120		126			120			110			98		90
5C	PDG	190		0			105			150			235		370
	VCG														
	BS‡	103		95			100			110			110		173

* See Table I for abbreviations.
† Minutes after infusion.
‡ Arterial blood.

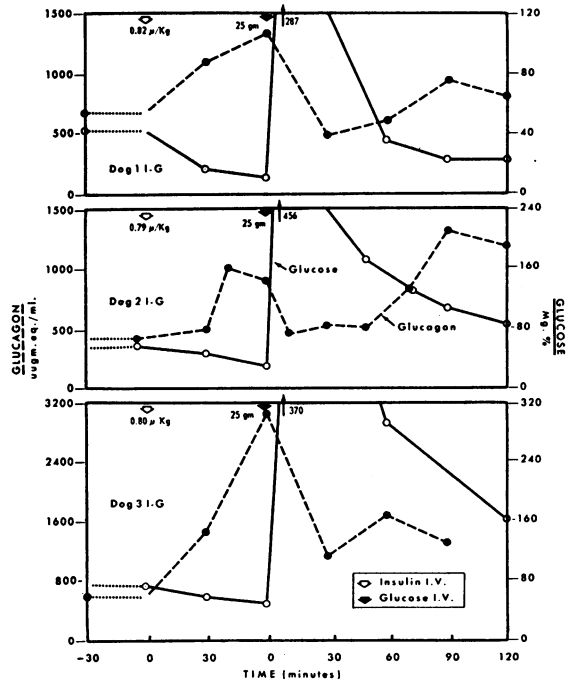

FIG. 4. EFFECT OF HYPERGLYCEMIA ON INSULIN-INDUCED HYPERGLUCAGONEMIA. Three dogs were given a rapid intravenous injection of 25 g of glucose during a period of hypoglycemia and hyperglucagonemia induced by insulin. In each case, the intense hyperglycemia was associated with an abrupt fall in pancreaticoduodenal venous plasma glucagon concentration. In Dogs 1 I-G and 2 I-G, this suppression of hyperglucagonemia persisted until the glucose concentration reached or approached the normal range, whereupon a rise was once again encountered.

tration was at a mean of 35 mg per 100 ml and the glucagon concentration was rising (averaging 1,800 μμg Eq per ml), 25 g of glucose in 50 per cent solution was rapidly infused, raising the blood glucose concentration to a mean of 405 mg per 100 ml. This extreme change in blood glucose concentration was associated with a decline in pancreaticoduodenal glucagon concentration from 1,800 to a mean of 692 μμg Eq per ml within 20 to 30 minutes. Suppression of hyperglucagonemia persisted throughout the period of hyperglycemia, but was followed, in two instances, by a return to high values as the blood glucose level re-entered a normal range. These results are shown in Figure 4.

Two phlorizinized animals were subjected to similar study in order to determine if the hyperglucagonemia associated with chronic hypoglycemia could be suppressed by induction of intense hyperglycemia. After 3 baseline samples had been obtained, 25 g of glucose, as the 50 per cent solution, was rapidly infused by vein, causing an abrupt though only moderate rise in blood glucose concentration. As shown in Figure 5, this rise was accompanied by a significant decline in glucagon concentration which remained suppressed until glucose concentration had declined to a normal range, whereupon a return to high levels was observed.

Analysis of trans-hepatic glucagon concentration gradients

The pancreatic origin of glucagon should, on the basis of dilution alone, assure a higher concentration of glucagon in the pancreatic venous effluent than in the post-hepatic venous blood. Furthermore, the avidity with which glucagon is bound to liver tissue during its initial trans-hepatic passage (8, 9) would further contribute to a substantial gradient across this organ. If the material measured by the immunoassay was, in fact, endogenous canine glucagon, its concentration would be greatest proximal to the liver. In the course of 48 experiments of various types, 106 simultaneously obtained specimens of vena caval and pancreaticoduodenal plasma were compared to determine whether such a gradient was consistently encountered. In Figure 6, plasma glucagon concentration in the pancreaticoduodenal vein was plotted against that in the simultaneously obtained vena caval plasma. In 94 of 106 paired specimens, the points were above the median line, indicating a higher concentration in the pancreaticoduodenal venous plasma. Most of the exceptions to this were at extremely low concentrations and of doubtful significance.

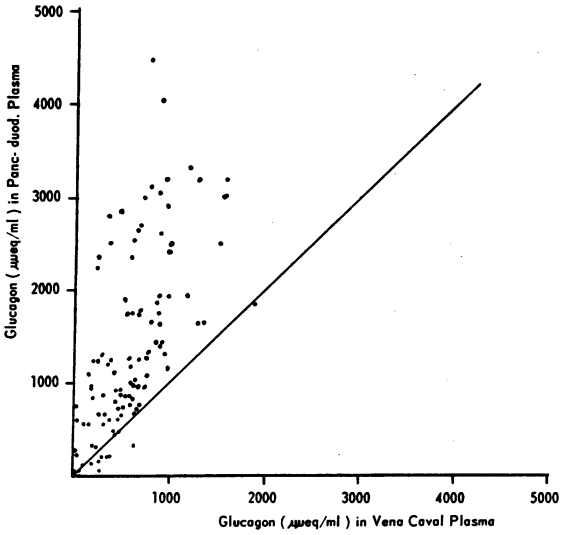

FIG. 6. COMPARISON OF GLUCAGON LEVELS IN PANCREATICODUODENAL AND VENA CAVAL PLASMA. To demonstrate the frequency of a trans-hepatic glucagon gradient, glucagon concentration in pancreaticoduodenal venous plasma was plotted against vena caval plasma glucagon concentration in 108 simultaneous samples obtained during these studies. Ninety-four of the points fall above the median line, indicating the higher concentration of glucagon in the pancreaticoduodenal plasma.

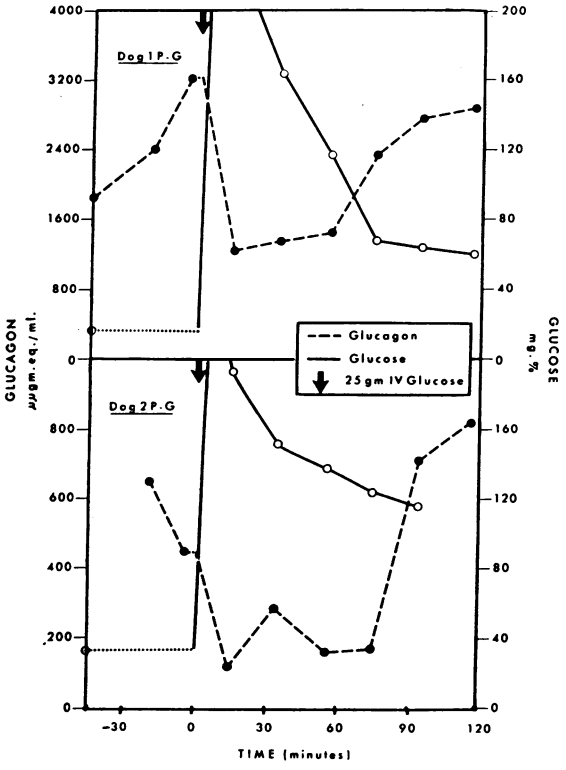

FIG. 5. EFFECT OF HYPERGLYCEMIA ON PHLORIZIN-INDUCED HYPERGLUCAGONEMIA. Hyperglycemia was induced in 2 chronically hypoglycemic phlorizinized dogs by the rapid intravenous injection of 25 g of glucose. A drop in pancreaticoduodenal venous glucagon concentration took place in both experiments. Suppression of the glucagon level persisted until the blood glucose level approached normal, whereupon a sharp rebound was observed in each case.

These findings offer additional support to the contention that the assayed substance is glucagon. The observed gradient appeared to increase with the pancreaticoduodenal glucagon concentration, indicating that peripheral venous glucagon concentration is a poor mirror of glucagon secretion.

Effect of passage through cellulose column on endogenous glucagon concentration

Experiments patterned after those employed by Yalow and Berson in their study of endogenous insulin (7) were conducted to determine whether endogenous canine glucagon, like glucagon-I^{131}, is adsorbed by cellulose. Beef-pork glucagon-I^{131} was added in negligible traces to the plasma of a

phlorizinized dog. Aliquots of this mixture were measured for radioactivity and assayed for endogenous canine glucagon. The remaining 10 ml of the mixture, containing a total of 16,800 $\mu\mu$g Eq of dog glucagon and 90 $\mu\mu$g of glucagon-I^{131}, was passed through a powdered cellulose column. Both endogenous canine glucagon and the added beef-pork glucagon-I^{131} were readily adsorbed by the cellulose. Concentrations of both components present in the eluate were so low as to be quantitatively unreliable, being in the vicinity of 4 and 20 per cent, respectively, of the original concentrations. The results demonstrate that, at the concentrations studied, endogenous canine glucagon shares with glucagon-I^{131} a high affinity for the cellulose.

DISCUSSION

The notion that glucagon might be a second pancreatic hormone was first suggested by its co-discoverers, Kimball and Murlin, in 1923 (10). The failure to identify a clinical syndrome clearly attributable to either glucagon deficiency or excess, and the lack of a method sufficiently specific and sensitive to measure endogenous glucagon in circulation have stimulated a considerable amount of indirect physiologic experimentation designed to prove that glucagon is a hormone. This work has been thoroughly considered in several recent reviews of the subject (5, 11, 12). Foremost of these efforts were the cross-circulation experiments of Foa and co-workers (3, 4) in which the pancreaticoduodenal venous effluent of insulin-hypoglycemic dogs was found to cause hyperglycemia in recipient dogs. However, specific proof that glucagon, rather than amylase, serotonin, or other glycogenolytic substances, was the cause of the hyperglycemic response was still lacking.

The radioimmunologic assay for glucagon has provided a highly sensitive technique which, in addition, appears to be specific (2). The definite and, at times, marked competitive inhibition which most specimens of pancreaticoduodenal venous plasma were noted to exert upon binding of glucagon-I^{131} to antibody indicates the presence therein of endogenous canine glucagon, or of some unknown but immunologically indistinguishable substance. The results of certain of the foregoing experiments provide additional evidence tending to identify the inhibitory material as glucagon.

First, the concentration of this material was consistently higher in the pancreatic venous effluent than in the post-hepatic venous plasma. Second, like glucagon-I^{131}, this material was readily adsorbed by a cellulose column. Finally, its concentration was noted to vary with changes in blood glucose concentration. For these reasons, and because of the previously demonstrated specificity of the assay, the material measured is considered to be endogenous canine glucagon.

A rise in the concentration of endogenous glucagon in the pancreaticoduodenal venous plasma was shown to accompany both acute and chronic hypoglycemia, and could be suppressed by the rapid induction of intense hyperglycemia. These relationships are in precisely the direction predictable from the hyperglycemic, glycogenolytic action of exogenous glucagon and from the earlier work of Foa, Weinstein and Smith (3). These data provide specific evidence that glucagon is secreted into plasma at rates which vary with changes in blood glucose concentration and should dispel any remaining doubt as to its status as a hormone concerned with blood glucose regulation.

However, the role of glucagon secretion in normal physiology and its relationship to blood glucose concentration in the normal organism have not necessarily been defined by these studies, since extreme and unphysiologic alterations of blood glucose concentration were induced so as to provoke easily measurable variations in glucagon concentration. Hypoglycemia is a highly unphysiologic state which never occurs normally, but it may represent an intense exaggeration of the physiologic situation of postabsorptive carbohydrate deprivation, in which hypoglycemia is prevented by increased hepatic glucose production. Glucagon is admirably suited to play a major role in the maintenance of hepatic output. The demonstrations of hypersecretion of glucagon in response to an exaggerated need for glucose production may, therefore, be interpreted as favoring the concept, frequently advanced in the past (5, 13, 14), that glucagon functions normally as the mobilizer of hepatic glucose in the postabsorptive state. In such a role, glucagon would be serving primarily the vital glucose-dependent, insulin-independent cells of the central nervous system by maintaining a steady flow of glucose to the periphery. The

islet cells would then constitute a bi-hormonal organ of glucose distribution to tissues.

SUMMARY AND CONCLUSIONS

The development of a highly sensitive and specific radio-immunoassay for glucagon made possible these studies designed to identify circulating endogenous glucagon in plasma and to determine whether glucagon secretion is influenced by alteration in blood glucose concentration.

Plasma obtained from the pancreaticoduodenal vein of 48 normal fasting dogs was found to contain a mean of 543 $\mu\mu$g Eq per ml of glucagon, with a range of 0 to 1,300 $\mu\mu$g Eq per ml. In 9 dogs made chronically hypoglycemic by means of phlorizin administration, an average of 1,976 $\mu\mu$g Eq per ml was present, with a range of 680 to 3,100 $\mu\mu$g Eq per ml. Of 13 dogs made acutely hypoglycemic by means of administration of "glucagon-free" insulin, a gradual rise in glucagon concentration was noted in most, with significant elevations appearing during the second and third hours of severe hypoglycemia. In both the chronically and acutely hypoglycemic animals, the induction of intense hypoglycemia by means of the rapid intravenous injection of 25 g of glucose was followed by an abrupt suppression of hyperglucagonemia to baseline values; this suppression lasted until the glucose level returned toward normal, whereupon a brisk rise in glucagon concentration often appeared.

These results provide the first specific identification of endogenous glucagon in plasma and demonstrate the influence of blood glucose concentration upon its secretion. They provide strong support for the view that glucagon is a hormone with a role in blood glucose regulation.

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