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### **Research Article**

The hyperglucagonemia that occurs in vivo in animals made diabetic with alloxan or streptozotocin is not suppressed by high glucose but is suppressed by exogenous insulin. These observations together with other studies suggested that insulin-dependent glucose transport and metabolism by the alpha-cells serves as the primary mechanism controlling glucagon secretion. This hypothesis was tested in the present investigation. The possible interactions between glucose, insulin, and a mixture of 20 amino acids at physiological proportions were examined in the isolated-perfused diabetic rats. Release of insulin and glucagon were used as indicators of beta-cell and alpha-cell function. According to rigid criteria the diabetic animals entering the study were severely diabetic. It was found that in vitro: (a) basal glucagon release (measured in the absence of an alpha-cell stimulus or inhibitor) was extremely low, even lower (i.e. 10%) than the basal rates seen in controls; (b) the alpha-cells of alloxanized- and streptozotocin-treated rats responded with a biphasic glucagon release to stimulation by an amino acid mixture; (c) this alpha-cell response was reduced after both streptozotocin and alloxan; (d) glucose at 5 mM was a potent inhibitor of amino acid-induced glucagon secretion in both types of experimental diabetes; (e) in alloxan diabetes alpha-cell stimulation by amino acids can be curbed by exogenous insulin, whereas glucagon secretion by the perfused pancreas of streptozotocin diabetic rats [...]

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# Insulin and Glucose as Modulators of the Amino Acid-Induced Glucagon Release in the Isolated Pancreas of Alloxan and Streptozotocin Diabetic Rats

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**ABSTRACT** The hyperglucagonemia that occurs in vivo in animals made diabetic with alloxan or streptozotocin is not suppressed by high glucose but is suppressed by exogenous insulin. These observations together with other studies suggested that insulin-dependent glucose transport and metabolism by the  $\alpha$ -cells serves as the primary mechanism controlling glucagon secretion. This hypothesis was tested in the present investigation. The possible interactions between glucose, insulin, and a mixture of 20 amino acids at physiological proportions were examined in the isolated-perfused pancreas of normal, alloxan diabetic, and streptozotocin diabetic rats. Release of insulin and glucagon were used as indicators of  $\beta$ -cell and  $\alpha$ -cell function. According to rigid criteria the diabetic animals entering the study were severely diabetic. It was found that in vitro: (a) basal glucagon release (measured in the absence of an  $\alpha$ -cell stimulus or inhibitor) was extremely low, even lower (i.e. 10%) than the basal rates seen in controls; (b) the  $\alpha$ -cells of alloxanized- and streptozotocin-treated rats responded with a biphasic glucagon release to stimulation by an amino acid mixture; (c) this  $\alpha$ -cell response was reduced after both streptozotocin and alloxan; (d) glucose at 5 mM was a potent inhibitor of amino acid-induced glucagon secretion in both types of experimental diabetes; (e) in alloxan diabetes  $\alpha$ -cell stimulation by amino acids can be curbed by exogenous

insulin, whereas glucagon secretion by the perfused pancreas of streptozotocin diabetic rats appeared to be resistant to insulin action.

The data indicate that the modulation of glucagon secretion by glucose in vitro is independent of insulin and that other unknown factors extrinsic to the pancreatic islets are responsible for the hyperglucagonemia observed in vivo.

## INTRODUCTION

Suppression of pancreatic glucagon secretion by hyperglycemia is a characteristic feature of normal  $\alpha$ -cell function (1-3). Recent studies have suggested that in both genetic diabetes in man (2, 4) and in alloxan diabetes in animals, the  $\alpha$ -cell loses this susceptibility to inhibition by glucose (2-5). It has further been shown that insulin therapy of alloxan diabetic dogs rapidly corrects the hyperglucagonemia (5). From these observations it has been concluded that the hyperglycemic suppression of glucagon release results from enhanced glucose transport and metabolism within the  $\alpha$ -cell, and that the glucose metabolism of these cells may be an insulin-requiring process (2-5).

In contrast to the above hypothesis, Samols, Tyler, and Marks (6) have proposed an alternative mechanism which invokes direct inhibition of glucagon release by insulin and explains the various instances of hyperglucagonemia observed in vivo as resulting from either physiologic decrease of insulin release (e.g., during fasting hypoglycemia) or from pathologic impairment in hormone secretion (e.g., diabetes).

With both hypotheses it is implied that the elevated glucagon levels observed in vivo in the diabetic animal are a consequence of insulin deficiency. To test the

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above hypotheses, the interactions between insulin and glucose as modulators of amino acid-induced glucagon release was investigated in the isolated-perfused pancreas of alloxan and streptozotocin diabetic rats.

## METHODS

**Animals and perfusion system.** Male Sprague-Dawley rats weighing 300–400 g, fed ad libitum with Purina rat chow and water, were used in all experiments. Diabetes was induced with alloxan (40 mg/kg, i.v.) or streptozotocin (65–70 mg/kg, i.v.). The alloxan diabetic animals were used as pancreas donors usually 4–8 days after injecting the alloxan, while the streptozotocin diabetic animals were used 3–4 wk after treatment. The weight of the normal animals was  $333 \pm 8$  g (mean  $\pm$  SEM,  $n=20$ ), the alloxan diabetic animals  $274 \pm 6$  g ( $n=34$ ), and the streptozotocin diabetic  $222 \pm 4$  g ( $n=27$ ).

All animals were fasted 18–20 h before the study. Blood samples were obtained just before removal of the pancreas from all diabetic and control animals which had been heparinized as described (7). The samples (2 ml) were drawn by puncturing the aorta closely above the most cranial ligature [see protocol of surgical procedure as published previously (7)]. The blood was injected into tubes containing 500 kIU/ml Trasylol® (FBA Pharmaceuticals, N. Y.) and 1.25 mg EDTA/ml and was kept on ice for no longer than 30 min. The plasma was then separated by centrifugation and stored at  $-20^{\circ}\text{C}$  until assayed. The plasma sample was used to determine glucose, insulin, and glucagon and all values were corrected for dilution with Trasylol®.

The pancreas was isolated and perfused using the procedure described by Grodsky et al. (8) with minor modifications (9). The anesthetic and surgical preparation of the animals and composition of the perfusion media have been described in detail (7).

For most experimental conditions at least 10 separate perfusions were performed. The secretory stimulus was normally 10 mM of a mixture of 20 amino acids in proportions found in normal rat serum (10). In half of the experiments 5 mM glucose was present throughout the entire duration of the perfusion which lasted for 75 min. The pancreas was perfused for 15 min before exposure to the amino acid stimulus; the period of stimulation lasted 30 min. This was followed by another control period of 30 min by perfusion in the absence of the amino acids. The transitions between different conditions by switching from one circulation medium to the other were rapid and did not result in detectable changes of flow rate.

Five experimental protocols were used with both normal and diabetic animals: (a) stimulation with the 10 mM amino acid mixture alone; (b) stimulation with the 10 mM amino acid mixture with 5 mM glucose present; (c) stimulation with the 10 mM amino acid mixture in the presence of 1  $\mu\text{g}/\text{ml}$  insulin; (d) stimulation with the 10 mM amino acid mixture in the presence of 5 mM glucose plus 1  $\mu\text{g}/\text{ml}$  insulin; and (e) to fully assess the degree of  $\beta$ -cell damage in the alloxan and streptozotocin diabetic animals, the capacity to release insulin and glucagon was also studied with 20 mM glucose.

In all of the above experiments 0.5 ml samples were obtained at suitable intervals from the cannula in the portal vein, were cooled on ice, and after completion of the experiment frozen at  $-20^{\circ}\text{C}$  until assayed. 25 samples were collected in each experiment; of these, three were taken during the preperfusion period of 15 min (at  $t_{-8}$ ,  $t_{-2}$ , and  $t_0$ )

to obtain the basal insulin and glucagon release levels; then the medium was switched to that containing the stimulant under study and samples were collected 0.5 min after the change, then every minute for 5 min, and finally every 5 min for the remaining 25 min of stimulation. The same schedule of sampling was applied when the circulation was switched back to the control solution for studying the poststimulatory activity. The rates of insulin and glucagon release were calculated by multiplying the concentration of the respective sample by the flow rate, which was measured at frequent intervals. First phase (0–3 min), second phase (3–30 min), and total release (0–30 min) during exposure to a stimulus were obtained by planimetry and by calculating the means of the respective areas of the individual perfusions. The total release during the period of stimulation was calculated by multiplying the concentration of the respective sample by the flow rate. The net release during the period of stimulation was calculated by deducting the average basal secretion observed during the preperfusion period (7).

**Pancreatic insulin and glucagon content.** Glucagon and insulin contents were determined on acid-ethanol extracts in a representative group of whole pancreases as well as in microdissected freeze-dried islets of normal, alloxan, and streptozotocin diabetic animals. For measuring the total hormone content of the pancreas, the pancreas was exposed through a midline abdominal incision after anesthesia. Blood for the measurement of glucose, insulin, and glucagon was obtained from the inferior vena cava. The pancreas was then rapidly dissected free from its attachment to the small intestine, weighed, and frozen between blocks of dry ice. Each individual pancreas was then homogenized in 3 ml acid-ethanol (1 part concentrated HCl to 49 parts 95% ethanol). The acidic extracts were analyzed for insulin and glucagon by using multiple dilution points in both immunoassays. Microdissected freeze-dried islets were obtained from the pancreas samples by quick freezing at the termination of the 75-min perfusion and weighed as previously described (11). 10  $\mu\text{l}$  of acid ethanol was added to approximately 1  $\mu\text{g}$  of pooled islet pieces which were allowed to remain in this solution for 45 min at  $25^{\circ}\text{C}$ . The samples were then stored at  $-20^{\circ}\text{C}$  after dilution with 500  $\mu\text{l}$  of 0.2 M glycine buffer, pH 8.8, until assayed for insulin and glucagon. Each sample was analyzed using a minimum of three appropriate dilutions of the extracts for both immunoassay.

**Analytical methods.** Immunoreactive glucagon (7, 12) and insulin (13) were measured by using double antibody systems as previously described. Glucose in rat plasma was determined with a fluorometric enzymatic assay (11).

## RESULTS

**Criteria for defining diabetes as used in this study.** Diabetes was defined as a plasma glucose greater than 6 SD from the mean of the corresponding control animals observed in the 18–20-h-fasted, alloxan- and streptozotocin-treated rats immediately before pancreatic perfusion (Table I). The plasma glucose levels in the controls ranged from 3.7 to 8.2 mM, with a mean  $\pm$ SD of  $6.4 \pm 1.2$  mM ( $n=20$ ). Thus all treated rats were excluded from the study whose plasma glucose was 13.6 mM or less. The mean  $\pm$ SEM of the plasma glucose of the alloxan diabetic animals was  $34.4 \pm 2.3$  mM with a range of 14.3–63 mM ( $n=34$ ), while that of the

TABLE I  
*Plasma Glucose, Insulin, and Glucagon in Alloxan Diabetic and Normal Rats prior to Perfusion\**

	<i>n</i>	Glucose	Insulin	Glucagon
		<i>mM</i>	$\mu\text{U/ml}$	$\text{pg/ml}$
Normal	20	6.4 $\pm$ 0.3	35.0 $\pm$ 4.3	126 $\pm$ 6.0
Alloxan diabetic	34	34.4 $\pm$ 2.3	6.7 $\pm$ 1.2	671 $\pm$ 177
Streptozotocin diabetic	27	34.8 $\pm$ 1.4	5.0 $\pm$ 0.5	460 $\pm$ 51
† <i>P</i>		<0.001	<0.001	<0.001

\* All values represent the mean  $\pm$  SEM.

† Degree of significance of the difference of plasma values of alloxan and streptozotocin treated animals as compared to normals.

streptozotocin animals was 34.8 $\pm$ 1.4 with a range of 20–53 mM (*n* = 27).

In comparing the plasma glucose, insulin, and glucagon concentrations in the three groups of animals immediately before pancreatic perfusion, it was observed that the plasma insulin in the control animals was 35 $\pm$ 4.3 (mean  $\pm$ SEM) as compared to 6.7 $\pm$ 1.2  $\mu\text{U/ml}$  in the alloxan diabetic population and 5 $\pm$ 0.5 in the streptozotocin animals (Table I). Of the 34 alloxan diabetic animals, 11 had undetectable plasma insulin as compared to 6 of the 27 streptozotocin-treated animals. This is based on an insulin assay system which will accurately detect down to 2.5  $\mu\text{U/ml}$ .

Plasma glucagon was significantly elevated in both diabetic groups as compared to the controls (alloxan 671 $\pm$ 177, streptozotocin 460 $\pm$ 51, and controls 126 $\pm$ 6 pg glucagon/ml). Plasma glucagon levels ranged from 150 to 3,750 pg/ml in the alloxan diabetic animals. Of the 34 animals, 4 had markedly elevated glucagon concentrations of 2,062, 2,158, 3,530, and 3,752 pg/ml with corresponding plasma glucose concentrations of 63, 50,

60, and 62 mM. These four animals were also precoma-tose before perfusion. Despite the severity of the diabetes in these four rats, subsequent glucagon and insulin release profiles after pancreatic perfusion were indistinguishable from the less severely diabetic animals in which the plasma glucose and glucagon concentrations were 30 $\pm$ 1.4 mM and 317 $\pm$ 26 pg/ml, respectively (the mean  $\pm$ SEM being recorded). In the streptozotocin diabetic group, plasma glucagon levels ranged from 239 to 1,347 pg/ml. Similar to the alloxan diabetic animals, the highest plasma glucagons were associated with the highest plasma glucose.

Additional information demonstrating that alloxan and streptozotocin treatment eliminated practically all of the  $\beta$ -cells was obtained by measuring the insulin and glucagon contents of the whole pancreas (Table II) in a separate group of animals treated in the same manner. Plasma concentrations of glucose, insulin, and glucagon in the alloxan and streptozotocin diabetic animals were similar and all animals were diabetic as defined above (compare plasma data in Table I with that in Table II). Pancreatic insulin content of the normal animals was 758 $\pm$ 53 mU/g wet weight (*n* = 5) as compared to 13.4 $\pm$ 3.4 mU/g wet weight (*n* = 3) in the alloxan diabetic animals (i.e. 1.4% of controls) and 44.0 $\pm$ 8 mU/g wet weight (*n* = 5) in the streptozotocin diabetic group (i.e., 5% of controls) (*P* < 0.001 for both diabetic groups as compared to normals) (Table II). In contrast to insulin, glucagon content was similar in all three groups: 5.0 $\pm$ 1.1 in normals, 6.8 $\pm$ 1.1 in alloxan diabetics, and 5.8 $\pm$ 0.6  $\mu\text{g/g}$  wet weight in streptozotocin diabetics (all values exhibiting *P* values > 0.05 as compared to normals) (Table II).

Further evidence that we were dealing with practically pure  $\alpha$ -cell islets in the diabetic animals can be seen in Table III in which the insulin and glucagon content of microdissected islets from selected perfusion experiments were analyzed. Total insulin content in the islets of the diabetic animals was 5–6% of the normal. More con-

TABLE II  
*Plasma Glucose, Insulin, and Glucagon and Pancreatic Insulin and Glucagon in Normal, Alloxan, and Streptozotocin Animals\**

	<i>n</i>	Plasma			Pancreas	
		Glucose	Insulin	Glucagon	Insulin	Glucagon
		<i>mM</i>	$\mu\text{U/ml}$	$\text{pg/ml}$	<i>mU/g wet wt</i>	$\mu\text{g/g wet wt}$
Normal	5	7.2 $\pm$ 0.2	30.7 $\pm$ 7.2	191 $\pm$ 9	758 $\pm$ 53	5.0 $\pm$ 1.1
Alloxan	3	26.1 $\pm$ 0.9†	5.8 $\pm$ 1†	377 $\pm$ 65†	13.4 $\pm$ 3.4†	6.8 $\pm$ 1.1
Streptozotocin	5	23.1 $\pm$ 1.8†	10.3 $\pm$ 2†	404 $\pm$ 30†	44.0 $\pm$ 8.0†	5.8 $\pm$ 0.6

\* All values represent the mean  $\pm$  SEM.

† All parameters representing alloxan and streptozotocin diabetic animals significantly different from normal at *P*  $\leq$  0.01.

vincingly, the ratios of 3.7 and 4.0 of the total glucagon content in the alloxan and streptozotocin islets, respectively, as compared to the glucagon content in the normal islets, is good evidence that the tissue being analyzed in the diabetic pancreases was practically pure  $\alpha$ -cell islets since it has been previously demonstrated morphologically that the  $\alpha$ -cell mass of the rat pancreas represents approximately 25% of the total islet (14, 15).

*Glucagon and insulin release from the perfused pancreas of normal and diabetic animals with 10 mM amino acid mixture with and without 5 mM glucose.* The results of perfusion studies with normal and diabetic pancreases using 10 mM amino acid mixture as the stimulus for secretion both with and without 5 mM glucose present are illustrated in Fig. 1. The typical biphasic amino acid-induced glucagon release pattern, as previously described (7, 16), was observed in the normal as well as in the alloxan and streptozotocin diabetic pancreases. In the normal pancreas (Fig. 1 A), 5 mM glucose infused from the start of the perfusion (starting at  $t_{-15}$ ) significantly decreased the prestimulatory release of

TABLE III  
*Insulin and Glucagon Content of Freeze-Dried Islets Dissected from the Perfused Pancreas\**

	n	Insulin mU/ $\mu$ g dry tissue	Glucagon ng/ $\mu$ g dry tissue	Relative islet gluca- gon levels†
Normals	4	1.43 $\pm$ 0.07	4.3 $\pm$ 0.8	1.0
Alloxan	11	0.07 $\pm$ 0.02	15.8 $\pm$ 1.6	3.7
Streptozotocin	6	0.09 $\pm$ 0.01	17.3 $\pm$ 0.7	4.0

\* Perfusions of all pancreases were performed as shown in protocol 2 (Table I) which contained 5 mM glucose throughout the 75-min perfusion. The pancreas was rapidly frozen in liquid nitrogen after termination of perfusion. Freeze-dried microdissected islet samples were then obtained as described previously (11).

† Ratio of glucagon in islets of diabetics to glucagon in normal islets.

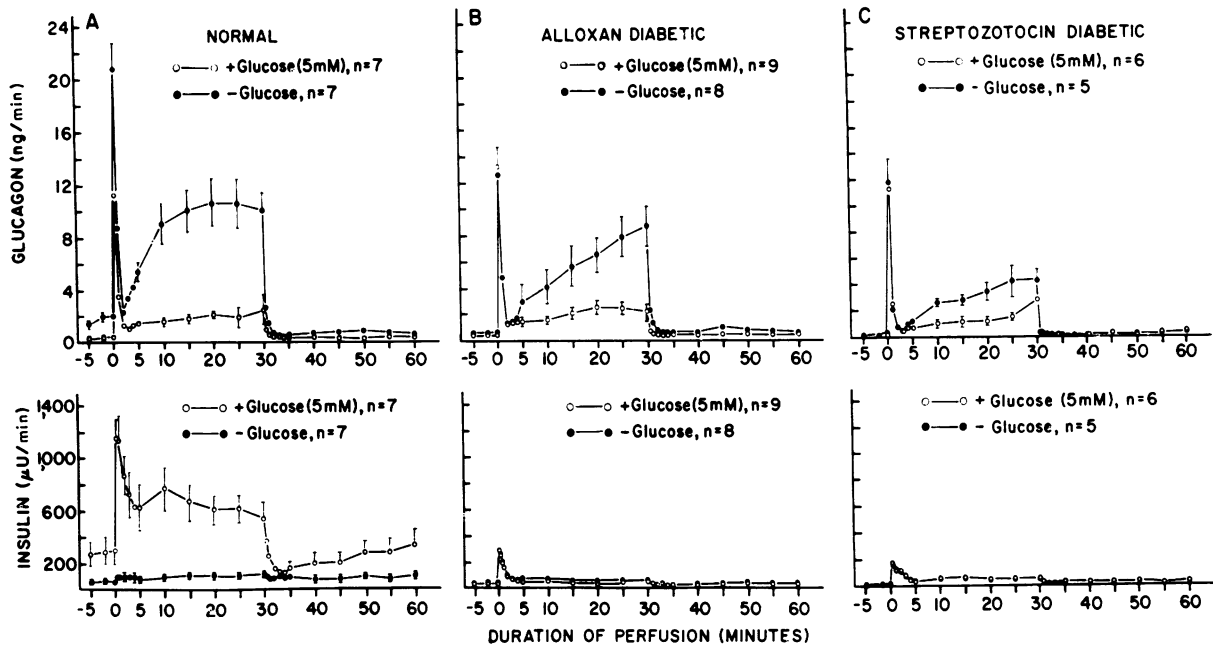


FIGURE 1 Glucose modulation of amino acid-induced glucagon and insulin release from the isolated perfused pancreas of normal and diabetic rats. The amino acid stimulus (10 mM) was applied between  $t_0$  and  $t_{30}$  and glucose (5 mM), when included, was present throughout the entire perfusion experiments ( $t_{-15}$  to  $t_{60}$ ). The means $\pm$ 1 SEM of the indicated number of experiments are recorded. Closed circles represent the release profiles in the absence of glucose and open circles represent the data obtained in the presence of 5 mM glucose. In the case of the insulin release profiles in diabetics the results obtained in the absence and presence of glucose are shown without SE's and because of the similarity of results, many of the points (solid circles) are omitted in order to preserve the clarity of the figure. The results from controls, alloxan diabetic, and streptozotocin-treated animals are depicted in panels A, B, and C, respectively.

TABLE IV  
*Comparison of the Action of a 10 mM Amino Acid Mixture on the Two Phases of  
Glucagon and Insulin Release in Normal and Diabetic Pancreases*

	n	First phase,* $t_0-t_3$		Second phase,* $t_3-t_{30}$		Total,* $t_0-t_{30}$	
		Glucagon	Insulin	Glucagon	Insulin	Glucagon	Insulin
		ng/3 min	mU/3 min	ng/27 min	mU/27 min	ng/30 min	mU/30 min
Normal	7	17.8±2.2	0.12±0.1	194±35.0	1.22±0.9	211.8±36.0	1.34±0.9
Alloxan diabetic	8	11.4±1.9‡	0.34±0.1	135±32.5	0.63±0.3	146.4±33.0	0.97±0.4
Streptozotocin diabetic	5	8.7±1.0§	0.29±0.1	76.7±15.0	0.48±0.4	85.4±14.0§	0.77±0.5

\* The values represent the mean ± SEM of the integrated secretion rates of insulin and glucagon release above base line as obtained by planimetry of the areas under the curves extending over the indicated time periods.

‡,§,|| Significantly different from control rates: ‡,  $P < 0.05$ ; §,  $P < 0.02$ ; ||,  $P < 0.01$ .

glucagon ( $t_{-5}$  to  $t_0$ ) as compared to the results during preperfusion in the absence of glucose. In contrast to the normal pancreas and despite the finding that plasma glucagon levels were markedly elevated in vivo, the basal prestimulatory glucagon release in both the alloxan and streptozotocin diabetic pancreas was significantly decreased when perfused without glucose and no further inhibition of glucagon release occurred when 5 mM glucose was included in the perfusion fluid (Fig. 1 B and C, also see Fig. 5).

Stimulation with 10 mM amino acid mixture without glucose present resulted in qualitatively similar glucagon release profiles in controls and the two forms of

diabetes. However, similar to the low basal secretion rates seen in vitro in diabetes, the stimulated glucagon release ( $t_0$  to  $t_{30}$ ) was blunted for both alloxan and streptozotocin diabetic pancreases as compared to the normal (Fig. 1). The difference in total release above basal secretion in the alloxan diabetic pancreases, although lower than in controls, did not attain statistical significance, while the streptozotocin diabetic pancreases showed markedly decreased total glucagon release as compared to the controls ( $85.4 \pm 14$  vs.  $211.8 \pm 36$  ng/30 min,  $P < 0.02$ ) (Table IV). In both diabetic prototypes the first phase of release ( $t_0$  to  $t_3$ ) was significantly depressed (Fig. 1, Table IV).

TABLE V  
*Comparison of the Action of 5 mM Glucose on the Two Phases of Glucagon and Insulin Release  
Caused by a 10 mM Amino Acid Mixture in Normal and Diabetic Pancreases*

	n	First phase,* $t_0-t_3$		Second phase,* $t_3-t_{30}$		Total,* $t_0-t_{30}$	
		Glucagon	Insulin	Glucagon	Insulin	Glucagon	Insulin
		ng/3 min	mU/3 min	ng/27 min	mU/27 min	ng/30 min	mU/30 min
Normal							
AA‡ (10 mM)	7	17.8±2.2	0.12±0.1	194±35.0	1.22±0.9	211.8±36.0	1.4±0.9
AA (10 mM) + G (5 mM)	7	10.0±1.6	2.27±0.4	38.0±7.4	9.90±1.8	48.0±8.8	12.8±2.0
§P		<0.05	<0.001	<0.001	<0.001	<0.001	<0.001
Alloxan diabetic							
AA (10 mM)	8	11.4±1.9	0.34±0.1	135±32.5	0.63±0.3	146.4±33.0	0.7±0.4
AA (10 mM) + G (5 mM)	9	12.0±1.5	0.29±0.1	42.0± 9.5	0.42±0.2	54.0±9.5	0.71±0.3
§P		NS	NS	<0.05	NS	<0.01	NS
Streptozotocin diabetic							
AA (10 mM)	8	8.7±1.0	0.29±0.1	76.7±15.0	0.48±0.4	85.4±14.0	0.77±0.5
AA (10 mM) + G (5 mM)	6	8.4±2.0	0.37±0.1	27.9±9.4	1.1±0.4	36.3±11.2	1.47±0.5
§P		NS	NS	<0.02	NS	<0.02	NS

\* The values represent the mean ± SEM of the integrated secretion rates of insulin and glucagon release above base line obtained by planimetry of the areas under the curves extending over the indicated time periods.

‡ Abbreviations: AA, amino acid mixture; G, glucose.

§ Degree of significance between perfusions with and without glucose.

|| Significantly different ( $P \leq 0.05$ ) from control perfusions with 10 mM amino acid mixture.

Insulin release with the 10 mM amino acid stimulus was negligible and did not differ significantly between the three groups. The pancreases of both diabetic prototypes demonstrated a small transient response, but again the integrated release above basal secretion rates did not differ from that of the normal perfused pancreas in the absence of glucose (Fig. 1, Tables IV and V).

When 5 mM glucose was present in the perfusate, marked and significant suppression of amino acid-stimulated glucagon release occurred in the normal pancreas. As previously demonstrated (7), both phases of glucagon release were suppressed and insulin secretion was markedly stimulated (Fig. 1 A, Table V). Similar to glucagon release from the normal pancreas, 5 mM glucose significantly inhibited the total amino acid-induced glucagon secretion in both the alloxan and streptozotocin diabetic pancreases. But unlike the situation in the normal pancreas, glucose was incapable of inhibiting the first phase of release. Only the second phase of release was significantly inhibited in both diabetic prototypes (Fig. 1 B and C, and Table V).

Inhibition of second-phase glucagon secretion in the diabetic pancreases occurred in the virtual absence of insulin release. Total insulin release was similar in both alloxan and streptozotocin diabetic pancreases, with and without glucose in the perfusion media, and was of the same magnitude as that observed in the normal pancreas when perfusion was performed with the 10 mM amino acid mixture in the absence of glucose (Fig. 1 A, B, C, Table V). These data thus demonstrate that unlike the *in vivo* situation in the diabetic animal in which even very high levels of glucose are ineffective in inhibiting glucagon release (2-5), glucose alone in physiological concentrations and in the absence of significant insulin secretion from the isolated perfused pancreas is a potent inhibitor of stimulated glucagon secretion.

**Effect of exogenous insulin on glucagon release in the perfused diabetic pancreas.** Perfusions were performed to evaluate the effect of exogenous insulin on amino acid-stimulated glucagon release from the diabetic pancreas. Insulin in a concentration of 1  $\mu$ g/ml infused from the beginning and continued throughout the duration of the perfusion had no effect on amino acid-induced glucagon secretion of the normal pancreas in the presence or absence of 5 mM glucose (Fig. 2, Table VI).

In contrast to the results obtained with the normal pancreas, perfusion of the alloxan diabetic pancreases (Fig. 3, Table VI) with insulin resulted in significant suppression of total amino acid-stimulated glucagon release  $146.4 \pm 33$  ng/30 min, in the absence of 5 mM glucose vs.  $64.4 \pm 15$  ng/30 min in the presence of insulin,  $P < 0.05$  (Fig. 3, Table VI). Addition of 5 mM glucose, together with the insulin throughout the duration of the

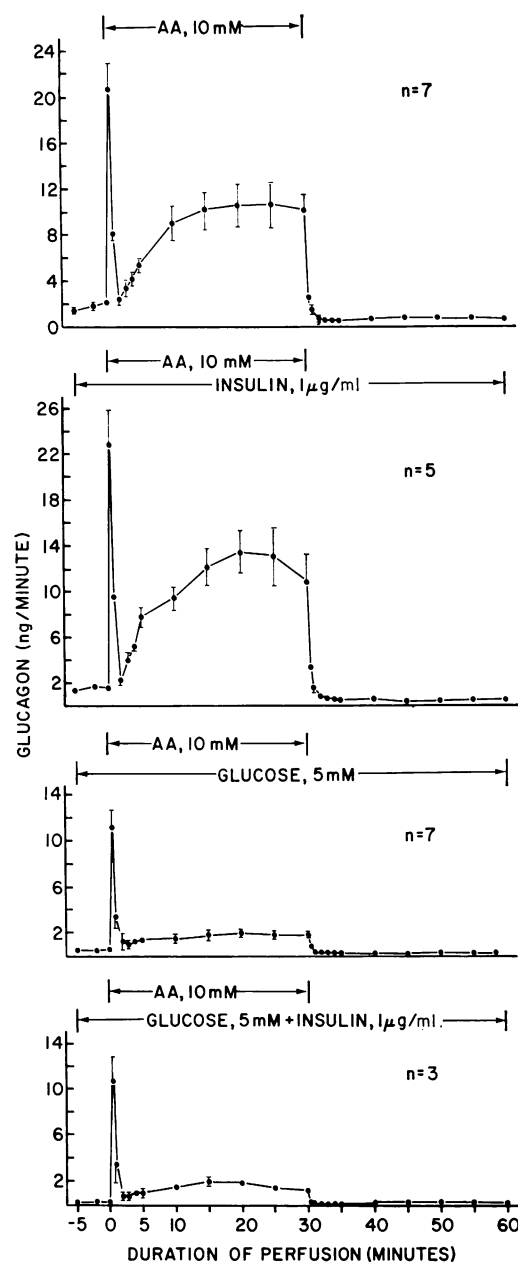


FIGURE 2 Interactions of glucose, insulin, and the combination of insulin plus glucose in modulating amino acid-induced glucagon release from the normal pancreas. The means  $\pm$  SEM of the indicated number of experiments are recorded. The amino acid stimulus was applied between  $t_0$  and  $t_{30}$  and glucose (5 mM), and insulin (1  $\mu$ g/ml) either alone or in combination, when included were present throughout the entire perfusion experiment ( $t_{-15}$  to  $t_{60}$ ).

perfusion, had no additional significant inhibitory effect on amino acid-stimulated glucagon release (Fig. 3, Table VI).

In the presence of insulin, the total amino acid-stimulated glucagon release above basal secretion rates in

TABLE VI  
*Effect of Insulin Replacement on the Two Phases of Amino Acid-Stimulated  
Glucagon Release in Normal and Diabetic Pancreases*

	n	Glucagon release*		
		First phase, <i>t<sub>0</sub>-t<sub>1</sub></i>	Second phase, <i>t<sub>0</sub>-t<sub>30</sub></i>	Total, <i>t<sub>0</sub>-t<sub>30</sub></i>
		ng/3 min	ng/27 min	ng/30 min
Normal				
AA‡ (10 mM)	7	17.8±2.2	194±35.0	211.8±36.0
AA (10 mM) + 1 µg/ml Ins	5	21.0±3.0	233±40.0	254.0±43.0
§P		NS	NS	NS
AA (10 mM) + G (5 mM)	7	10.0±1.6	38.0±7.4	48.0±8.8
AA (10 mM) + G (5 mM) + 1 µg/ml Ins	3	9.3±2.4	38.3±6.1	47.6±6.4
§P		NS	NS	NS
Alloxan diabetic				
AA (10 mM)	8	11.4±1.9	135±32.5	146.4±33.0
AA (10 mM) + 1 µg/ml Ins	8	9.2±1.5	55.2±14.9	64.4±15.0
§P		NS	<0.05	<0.05
AA (10 mM) + G (5 mM)	9	12.0±1.5	42.0±9.5	54.0±9.5
AA (10 mM) + G (5 mM) + 1 µg/ml Ins	5	9.3±2.5	35.0±22	44.3±25
§P		NS	NS	NS
Streptozotocin diabetic				
AA (10 mM)	5	8.7±1.0	76.7±15.0	85.4±14.0
AA (10 mM) + 1 µg/ml Ins	6	12.8 ± 1.4	61.2±15	74.0±16.0
§P		NS	NS	NS
AA (10 mM) + G (5 mM)	6	8.4±2.0	27.9±9.4	36.3±11.2
AA (10 mM) + G (5 mM) + 1 µg/ml Ins	6	10.3±2.0	9.7±2.8	20.0±3.0
§P		NS	NS	NS

\* The values represent the mean ± SEM of the integrated secretion rates of glucagon release above base line obtained by planimetry of the areas under the curves extending over the indicated time periods.

‡ Abbreviations: AA, amino acid mixture; G, glucose; Ins, insulin.

§ Degree of significance of the difference between perfusions with and without insulin.

the streptozotocin diabetic pancreases was decreased. However, the magnitude of suppression by insulin under both conditions with or without glucose did not achieve a level of statistical significance (Fig. 4, Table VI).

*Effect of 20 mM glucose on insulin and glucagon release in normal and diabetic pancreases.* To further test the severity of diabetes induced by alloxan and streptozotocin, perfusion studies with 20 mM glucose as the agonist were performed (Fig. 5). It has previously been demonstrated that this concentration of glucose results in maximal insulin release in the isolated perfused pancreas of the normal rat (17). In the normal pancreas 20 mM glucose resulted in a marked increase in insulin secretion. The total release corrected for baseline secretion during the 30-min stimulation was  $23.5 \pm 2.9$  mU ( $n=16$ ). The release profile differed in three major aspects from those observed using the 10 mM amino acid mixture as agonist; (a) insulin release was nearly monophasic, (b) after removal of the stimulus the

fall towards the basal secretion rate was delayed, and (c) during the poststimulatory phase without glucose in the perfusion, insulin release remained significantly greater than in the preperfusion period (Fig. 5 A). These observations are in agreement with previous studies in which high glucose was used as the stimulant (18).

In comparison to the normal pancreas, insulin secretion from pancreases of both the alloxan and streptozotocin diabetic animals was negligible (total stimulated insulin release was  $2.4 \pm 1.0$  mU/30 min with alloxan and  $2.5 \pm 0.6$  mU/30 min with streptozotocin) (Fig. 5 A).

Glucagon secretion with 20 mM glucose in the normal pancreas was suppressed from a basal level of approximately 2 ng/min to 0.25 ng/min. When glucose was removed from the perfusate after 30 min, glucagon release slowly increased to preperfusion levels (Fig. 5 B). In contrast to the normal pancreas, basal glucagon release



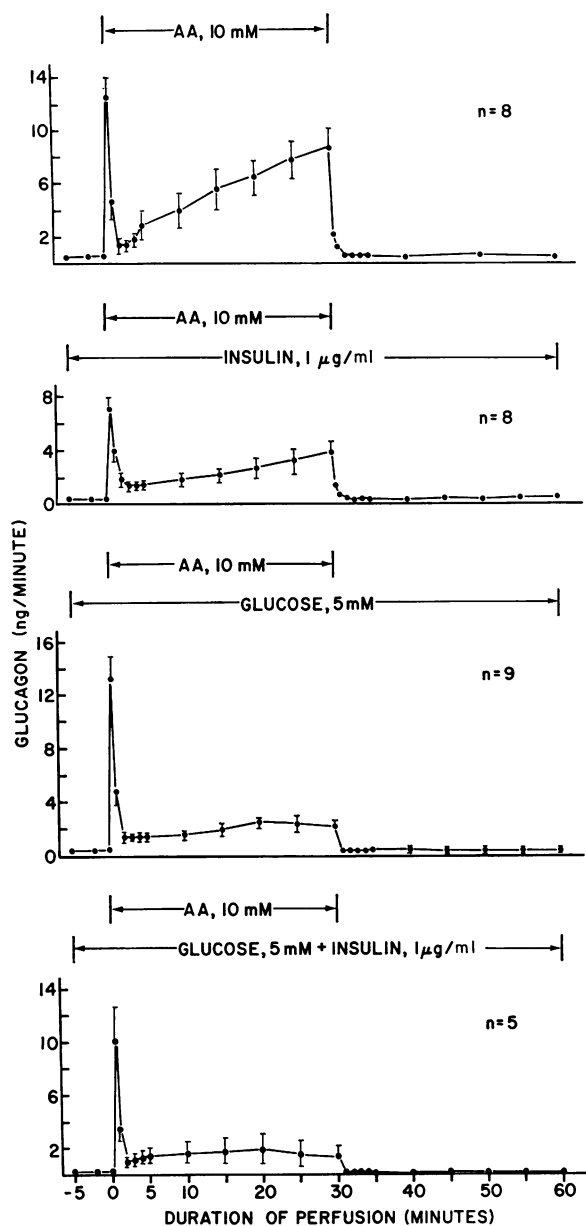


FIGURE 3 Interactions of glucose, insulin, and the combination of insulin plus glucose in modulating amino acid-induced glucagon release from the alloxan diabetic pancreas. The experimental design is the same as that described in the legend to Fig. 2.

( $t_s$  to  $t_0$ ) in both the alloxan and streptozotocin diabetic pancreases was low, as observed in corresponding experiments described above. These basal rates for both diabetic prototypes were approximately 10% of the basal secretion rate of the normal and of the same magnitude as that attained in the normal after suppression with 20 mM glucose. During perfusion of the diabetic pancreases with 20 mM glucose there was no further suppression of

the basal rate. Similarly after removal of glucose from the perfusate, no significant change in release occurred during the poststimulatory phase (Fig. 5 B).

## DISCUSSION

The possible impact of the present study rests almost entirely on the characteristics of the diabetic animals

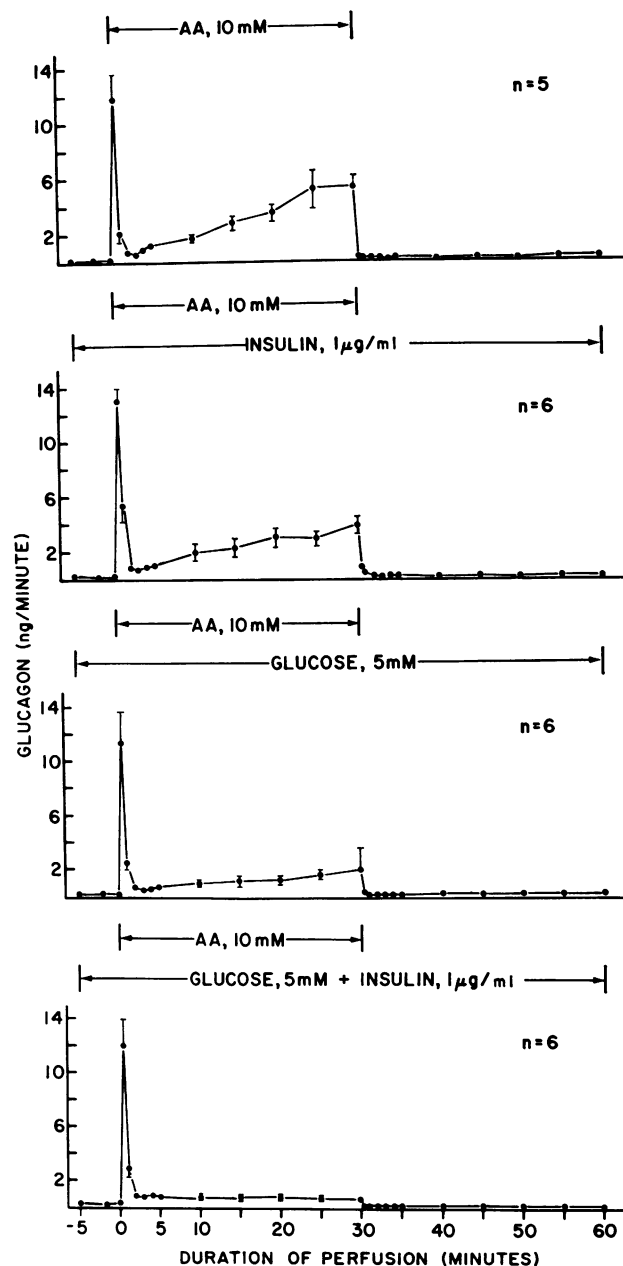


FIGURE 4 Interactions of glucose, insulin, and the combination of insulin plus glucose in modulating amino acid-induced glucagon release from the streptozotocin diabetic pancreas. The experimental design is the same as that described in the legend to Fig. 2.

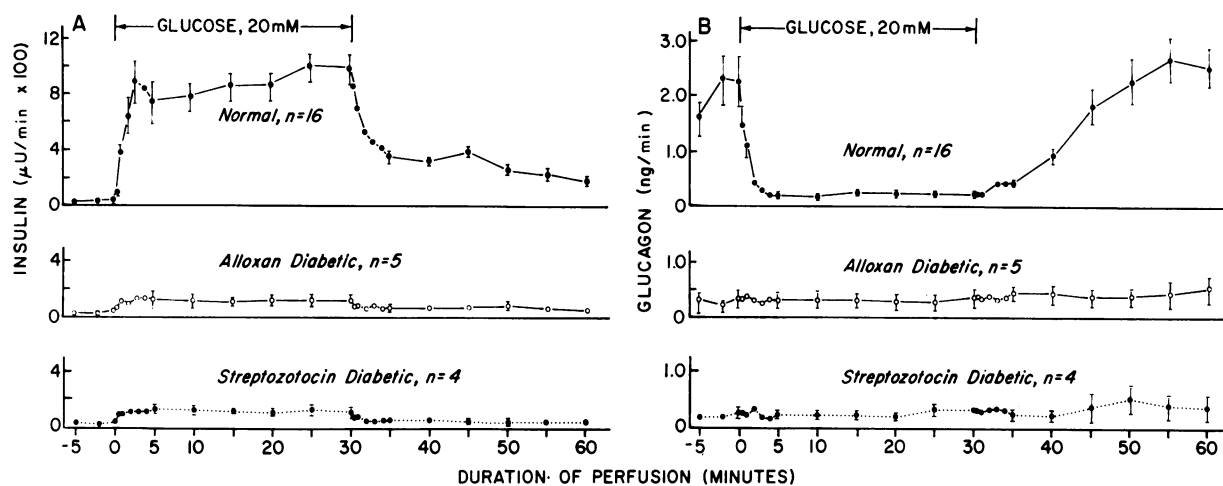


FIGURE 5 The effect of 20 mM glucose on insulin and glucagon release from the isolated perfused rat pancreas of normal, alloxan, and streptozotocin diabetic rats. In all experiments the perfusate consisted of the standard buffer without glucose from  $t_{-15}$  to  $t_0$ . 20 mM glucose was added at  $t_0$  and continued through  $t_{30}$ , at which time the perfusate was switched back to the buffer without glucose ( $t_{30}$  to  $t_{60}$ ). The insulin responses are recorded in panel A and the glucagon responses in panel B for normal, alloxan, and streptozotocin diabetic pancreases, respectively. The mean  $\pm$  SEM, and number of experiments performed are indicated.

used as pancreas donors for the perfusion studies. The criteria adopted here for defining the diabetic state have therefore been very stringent.

In the present study it was discovered that in vitro, the pancreatic  $\alpha$ -cells devoid of adjacent  $\beta$ -cells and in the presence of insignificant amounts of insulin, show very low basal activity in the absence of an exogenous stimulus, as was observed in the  $\alpha$ -cell of normal islets. Qualitatively, the diabetic  $\alpha$ -cell responds in a normal fashion to physiological stimulation by amino acids and to glucose inhibition of amino acid-stimulated glucagon release. This result seems paradoxical, at first glance, in view of the marked hyperglucagonemia and the apparently well-established refractoriness to high glucose of diabetic  $\alpha$ -cells in vivo. It is also surprising that in the absence of glucose the rate of basal glucagon release in the isolated pancreas from diabetics is even lower (i.e., about 10%) than the limited base-line secretion in the controls. This decreased activity is certainly not due to depletion of the glucagon reserves of the diabetic  $\alpha$ -cells as the results of direct pancreatic analyses demonstrate. This low base-line secretion is in accord with the decreased in vitro responsiveness to amino acid stimulus of the  $\alpha$ -cells of alloxanized animals ( $-30\%$ ) and (more pronounced) of streptozotocin-treated ( $-60\%$ ) animals.

In spite of the general similarities between amino acid-stimulated glucagon release and the inhibition of this release by glucose in the perfused pancreas of the normal and the diabetic animal, it must not be overlooked that in the diabetics 5 mM glucose inhibited only

the second phase without affecting the first phase of release. It should be noted that in both forms of diabetes the amino acid-induced first-phase release was already reduced and of the same magnitude as seen in perfusions of the normal pancreas when stimulated with 10 mM amino acids together with 5 mM glucose. Again the question arises whether the first-phase response is absolutely or relatively insensitive to glucose suppression and, provided there is only a relative decrease of glucose suppressibility, it would seem important to establish in more detail the dose dependency of glucose suppression of stimulated glucagon secretion from the diabetic pancreas in vitro.

The second significant finding is the marked insulin suppressibility of the  $\alpha$ -cells from alloxanized animals which is in clear contrast to the normal  $\alpha$ -cells which are absolutely refractory to insulin. From the present data it might seem that the  $\alpha$ -cells of streptozotocin-induced diabetic animals are as resistant to insulin as the normal  $\alpha$ -cells. This difference between the two forms of diabetes may be real and a result of the duration of diabetes (3–4 wk vs. 4–8 days) or may be merely apparent due to the fact that a possible inhibition by insulin is more difficult to demonstrate when stimulated release is already markedly decreased (i.e., only one-half the rate seen with alloxan diabetic animals). Clearly, more studies are needed before it can be accepted that a fundamental difference exists between the two forms of diabetes regarding insulin suppressibility of  $\alpha$ -cell function.

These data clearly indicate that in the in vitro per-

fusion system, insulin is not necessary for glucose suppression of amino acid-mediated glucagon secretion, and further, that the  $\alpha$ -cells in vitro are not hyperresponsive to amino acid challenge as is observed in vivo (1-3). Taken together these observations suggest that other factors in the in vivo diabetic animal are responsible for the observed hyperglucagonemia.

Recent investigations have demonstrated the important role of the autonomic nervous system and more specifically of catecholamines in modulating glucagon secretion (19-23). In the perfused dog pancreas, glucagon secretion is stimulated by low concentrations of epinephrine which in turn is not inhibited by glucose (21). Vagal nerve stimulation is also associated with augmented glucagon secretion (19). Gerich, Karam, and Forsham (22) and Gerich, Langlois, Noacco, Schneider, and Forsham (23) have clearly demonstrated in man that the pancreatic  $\alpha$ -cell possesses both  $\alpha$ - and  $\beta$ -adrenergic receptors. Christensen and Denmark have shown a close correlation between the degree of diabetic control and plasma catecholamines: Patients with poorly controlled diabetes mellitus demonstrate a rise in norepinephrine which paralleled the degree of metabolic derangement. High epinephrine values were observed in patients with severe ketoacidosis (24). In the perfused pancreas the potential effects of the autonomic nervous system as well as circulating epinephrine are removed. The striking difference in glucagon release observed in vivo and in vitro may in part be related to secondary effects of insulin insufficiency resulting in increased sympathetic nervous system activity and/or increased secretion of epinephrine from the adrenal medulla. The decrease of glucagon release in vitro in both diabetic prototypes in the basal and stimulated state may simply reflect a relative refractoriness of the basic release mechanism after the removal of chronic neuroendocrine stimulation.

Other recent studies have demonstrated the important role of the kidney in the metabolism of glucagon (25, 26). Therefore, in states of prerenal azotemia, occurring in diabetic ketoacidosis, decreased renal perfusion and hence a decreased rate of glucagon degradation could also account for the hyperglucagonemia observed in vivo.

The interesting observations made by two independent groups (27, 28), that marked hyperglucagonemia develops in totally depancreatized dogs when these animals are taken off exogenous insulin and allowed to develop ketoacidosis, also may provide a partial explanation for the high glucagon concentrations observed in vivo. These studies suggest that other sites, such as the gastrointestinal tract, may be a source of "pancreatic" glucagon and that a polypeptide which is immunologically very similar to pancreatic glucagon, which is se-

creted in large amounts in the insulin-insufficient state, is reacting with antibodies previously claimed to be highly specific for pancreatic glucagon (12, 27, 28).

We are not aware of other reported studies utilizing the pancreatic perfusion system in diabetic rats. In vitro investigations utilizing isolated islets prepared by the collagenase procedure or employing incubated pancreas pieces from normal and streptozotocin diabetic rats have yielded variable results (29-31). Glucagon secretion from incubated-isolated islets of normal rats showed only minimal and inconsistent suppression by glucose and the glucose molecule was not capable of suppressing glucagon secretion by pancreatic pieces (29-31). In contrast to the normal islets, both low glucose (30 mg/100 ml) and high glucose (300 mg/100 ml) resulted in one study in paradoxical stimulation of glucagon release by isolated islets and pancreas pieces of streptozotocin-treated animals (30), whereas Howell, Edwards, and Whitfield were unable to demonstrate abnormal glucagon release patterns while investigating isolated islets from streptozotocin-treated guinea pigs (32). The variability of results obtained in the above studies may be related to differences in species, degree of diabetes, and/or to damage of the  $\alpha$ -cells due to the collagenase treatment (30). But most importantly, all the cited studies neglect the possibility that the  $\alpha$ -cells are in a state of rest in the absence of a stimulus and tacitly assume that the  $\alpha$ -cells are spontaneously active.

In contrast to the alloxan and streptozotocin diabetic rat, the studies by Frankel et al. (33), utilizing the pancreatic perfusion technique in the genetically diabetic Chinese hamster, have demonstrated delayed suppression by glucose (300 mg/100 ml) of basal glucagon secretion, since 10-15 min exposure to glucose resulted in suppression to the degree observed in the normal. Basal glucagon secretion from these pancreases was the same as that of the normal hamster. In the above study and a second preliminary report, these investigators have demonstrated further differences from the alloxan and streptozotocin diabetic models including: a relative rather than absolute deficiency of insulin, increased concentrations of pancreatic glucagon in the diabetic animals as compared to controls, and hyperresponsiveness of glucagon release after challenge with arginine (33, 34).

The present study or data available in the literature are not sufficient to clarify the biochemical mechanisms underlying the glucose modulation of glucagon release from either the normal or diabetic pancreases. Recent observations from our laboratory made with islet tissue dissected from freeze-dried sections of the pancreases used in the present study, demonstrate that in the alloxan diabetic pancreas perfused with 5 mM glucose, the level of glucose within the  $\alpha$ -cell was virtually the same

as that observed in the normal islet (15 vs. 18 mmol/kg dry tissue). ATP levels were also alike (i.e., 12 mmol/kg dry tissue). Insulin had no effect in increasing glucose content within the islets. When the pancreas was perfused in the absence of glucose, ATP concentration in the  $\alpha$ -cell islet was the same as with glucose present but two- to threefold higher than that found in the intact islet. In the  $\alpha$ -cell islet from the streptozotocin diabetic rat, glucose content was definitely decreased compared to controls (12 vs. 18 mmol/kg dry tissue) but insulin was unable to elevate the glucose concentration of the  $\alpha$ -cell islets. Similar to the  $\alpha$ -cell from alloxan diabetics, ATP in the  $\alpha$ -cell islets of streptozotocin diabetics was maintained at a level which was, two- to threefold higher than that found in the normal islet, when the perfusate lacked glucose (35).

The hormone release data presented above together with the biochemical data just outlined and to be published in detail indicate that glucose uptake and metabolism, as well as the energy potential of the  $\alpha$ -cell, might be insulin independent and that neither glucose metabolism nor ATP availability play a primary role in the mechanisms responsible for glucose modulation of glucagon secretion.

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