

Hormone Secretion and Glucose Metabolism in Islets of Langerhans of the Isolated Perfused Pancreas from Normal and Streptozotocin Diabetic Rats*

(Received for publication, April 26, 1976)

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The glucose responsiveness of α - and β -cells of normal as well as untreated and insulin-treated streptozotocin diabetic rats was tested in the extracorporeal perfusion system. Also assessed was the possible *in vitro* effect of added insulin on the glucose sensitivity of islets from untreated diabetic animals. Insulin and glucagon secretion served as functional indicators of glucose responsiveness of the two cell types. The rate of glucose entry into islet tissue was estimated, and the effect of glucose on the tissue supply of ATP and lactate and the cyclic 3':5'-AMP level of islets was measured under the above *in vitro* conditions. It was demonstrated that β -cells are more accessible to glucose than α -cells, that glucose entry into islet cells is not significantly modified by insulin and that glucose had no effect on ATP, lactate and cyclic 3':5'-AMP levels of islet tissue under any of the conditions investigated. High insulin *in vitro* elevated ATP levels of α -cell islets independent of extracellular glucose. Glucose caused insulin release from normal but not from diabetic islets and rapidly and efficiently suppressed stimulated glucagon secretion of the pancreas from normal and insulin treated diabetic rats. Glucose was less effective in inhibiting stimulated glucagon secretion by the pancreas from untreated diabetic rats whether insulin was added to the perfusion media or not. Therefore, profound differences of glucose responsiveness of α -cells fail to manifest themselves in alterations of basic parameters of glucose and energy metabolism in contrast to what had been postulated in the literature. It is, however, apparent that the glucose responsiveness of α -cells is modified by insulin by an as yet undefined mechanism.

A new concept of bihormonal etiology of diabetes mellitus has evolved during the last few years implying that both α - and β -cells of the pancreatic islets might be primarily impaired (1, 2), whereas previously the primary lesion was thought to be confined to the β -cells (3, 4). It is conjectured that both cell types have the same basic defects, *i.e.* an impairment or even inability of the endocrine cells to recognize and respond to altered glucose concentration in the blood. The reduced sensitivity to glucose manifests itself in insulin deficit and glucagon excess in plasma relative to the blood sugar level or, expressed differently, in increased glucagon to insulin ratios in the blood. The hypothesis has gained momentum as a result of the recent observation that the hypothalamic oligopeptide somatostatin reduces blood sugar in the diabetic organism (5, 6), most likely by inhibiting the excessive glucagon secretion.

In this study we continued to test this hypothesis using the

isolated perfused rat pancreas (7, 8) for assessing the secretory function of α - and β -cells *in vitro* (9, 10) and applying quantitative histochemical methods (11, 12) for studying parameters of energy and glucose metabolism in islet tissue sampled from the isolated perfused pancreas by rapid freezing techniques. Compared to previous studies from our laboratory, which were designed to investigate glucose effects on the multiphasic stimulated glucagon release, the perfusion protocols were altered here to allow assessing of the actual kinetics of glucose suppression of α -cell secretion and to provide an estimate of the initial velocity of glucose entry into islet cells. The glucose responsiveness of α - and β -cells from normal as well as insulin-treated and untreated streptozotocin diabetic animals and the possible *in vitro* effect of insulin on the glucose sensitivity of islets from untreated diabetic animals were tested in the extracorporeal perfusion system. The rate of glucose entry into islet tissue, the effects of glucose on the tissue supply of ATP and lactate and the adenosine 3':5'-monophosphate (cyclic AMP) levels of islets were measured under these conditions, and attempts were made to correlate the biochemical results with the physiological responses.

Brief exposure of islets to 10 mM glucose showed that β -cells are more accessible to glucose than α -cells, that glucose entry

* These studies were supported by United States Public Health Service Grants AM-10591, AM-17810, and NS-09667, by Clinical Research Centers Grant R-0036, and by Diabetes and Endocrinology Centers Grant AM-17094.

‡ Established Investigator of the American Diabetes Association.

§ Recipient of Research Career Development Award AM-0003.

¶ Recipient of Research Career Development Award NS-50292.

into islet cells is not significantly modified by insulin, and that glucose had no effect on ATP, lactate, and cyclic AMP levels of islet tissue under any of the conditions. Glucose caused insulin release from normal but not from diabetic islets, and rapidly and efficiently suppressed glucagon secretion of the normal and of the insulin treated diabetic pancreas. However, glucose was less effective in inhibiting glucagon secretion from untreated diabetic pancreas whether insulin was added to the perfusate or not. These functional and biochemical results are compatible with the classic view that a primary lesion of the β -cells can explain the diabetic syndrome and that it seems unnecessary to postulate an additional primary lesion in α -cells.

METHODS

Animals and Perfusion System—Male Sprague Dawley rats weighing 300 to 400 g, fed *ad libitum* with Purina rat chow and water were used in all experiments. Diabetes was induced with streptozotocin (65 to 70 mg/kg intravenously) (9). Control animals received no treatment. After diabetes had been demonstrated by blood glucose analysis (usually within 2 to 3 days following streptozotocin) all diabetic animals were treated with insulin. During the first week of treatment they received 5 units subcutaneously of NPH insulin daily. During the ensuing treatment period the daily dose was reduced to 2 units subcutaneously. This treatment allowed normal weight gain in the diabetic animals but did not normalize the blood sugar. In animals designated "untreated diabetics" insulin was withdrawn for 2 to 3 days prior to the perfusion experiment.

In contrast to the previous studies (9, 10) in this series all animals entered the experiment in the fed state in order to avoid possible complications resulting from hypoglycemia in the insulin-treated group. Blood samples were obtained just before removal of the pancreas from the heparin-treated animals. The samples were drawn by puncturing the aorta closely above the most cranial ligature. The blood was injected into tubes containing 500 Kallikrein inactivating units/ml of Trasylol (FBA Pharmaceuticals, N. Y.) and 1.25 mg of EDTA/ml and was kept on ice no longer than 30 min. The plasma was then separated by centrifugation and stored at -20° until assayed. The plasma samples were used to determine glucose, lactate, ketone bodies, glucagon, and insulin and all values were corrected for dilution with the Trasylol.

The pancreas was isolated and perfused using the procedure of Grodsky *et al.* (7) with minor modifications (8). The anesthetic and surgical preparation of the animals and composition of the perfusion media have been described in detail (8–10). Inulin was present at 0.33 mM throughout to serve as marker of the extracellular water space of islets.

From 10 to 24 separate perfusions were performed for different experimental conditions. The secretory stimulus was a 10 mM concentration of a mixture of 19 amino acids in proportions found in rat serum (Fig. 1, protocols 1 through 3). As compared to previous studies (9, 10) cystine was left out of the amino acid mixture. The amino acid mixture was present throughout the entire perfusion which lasted 23 min in protocols 1 and 2 and 35 min in protocol 3. After perfusion with the

amino acid mixture for 20 min, 10 mM glucose was superimposed in half the experiments in each group, either for 3 min, as in protocols 1 and 2, or for 15 min, as in protocol 3. In one set of experiments, 1 μ g/ml of insulin was included in the perfusion medium throughout (protocol 2). Protocol 1 was used with the pancreas from controls, treated diabetics, and untreated diabetics. Protocols 2 and 3 were used with untreated diabetics only.

In all perfusion experiments 0.5-ml samples of perfusate were obtained at suitable intervals from the cannula in the portal vein, cooled on ice, and, after completing the experiment, frozen at -20° until assayed. In protocols 1 and 2, usually nine samples were collected, and in protocol 3 the number of samples was increased to 14. The rates of insulin and glucagon release were calculated by multiplying the concentration of the respective sample by the flow rate, (5 to 6 ml/min) which was measured at frequent intervals.

Sampling of Pancreas and Tissue Preparation for Quantitative Histochemistry—The pancreas was sampled at the termination of the perfusion at 23 min (Fig. 1, protocols 1 and 2) using quick freezing as described previously (12, 13).

Microtome sections of 30 μ m thickness were cut in a cryostat at -20° and then freeze-dried as described in previous publications (12, 13). Islets were dissected freehand without prior staining and weighed using a quartz fiber fishpole balance (14). To obtain samples for lactate measurements, gloves were worn during dissection and the materials that come in contact with the tissue during the process of dissecting and weighing were thoroughly cleaned with alcohol and acetone.

Inulin, Glucose, Lactate, ATP, and Cyclic AMP in Islet Tissue—Oil well methods in combination with enzymatic cycling and fluorometry were used for measuring inulin, glucose, lactate, and ATP in islet tissue. Methods for inulin (15), glucose, and ATP (11, 12) have been described previously.

The inulin method was modified in that the tissue sample was heated in 0.1 N NaOH. This pretreatment destroyed free glucose and enzymes that would interfere in the enzymatic assay for fructose. Subsequently, fructose is liberated from the inulin by hydrolysis in weak acid. The actual procedure for inulin was as follows. The samples, placed in the oil well rack, were suspended in 0.07 μ l of 0.1 N NaOH, covered with oil, and heated for 25 min in an oven set at 105° . After cooling to 25° 0.05 μ l of 0.2 N HCl was added and the heating step was repeated (25 min in an oven set at 105°) in order to hydrolyze inulin. After cooling, 1.5 μ l of the reagent for measuring fructose was added. The composition of the reagent was described in the original procedures for measuring fructose (12, 15). The racks were incubated for 45 min and the reaction was terminated by adding 0.32 μ l of 1 N NaOH. A heating step followed (20 min at 75°). TPNH resulting from fructose was then measured by enzymatic cycling as described previously. The enzymes were adjusted to allow amplification of 3000 times. Inulin standards ranged from 0.3 to 1.6 pmol (Fig. 2).

The lactate assay was a micro modification of a procedure published previously (16, 17). Islet samples were suspended in 0.07 μ l of 0.05 N HCl under oil and were heated for 20 min at 75° . After cooling and neutralizing with 0.017 μ l of 2-amino-2-methyl-propyl alcohol base 0.125 μ l of the following reagent was added: 0.1 M 2-amino-2-methyl-

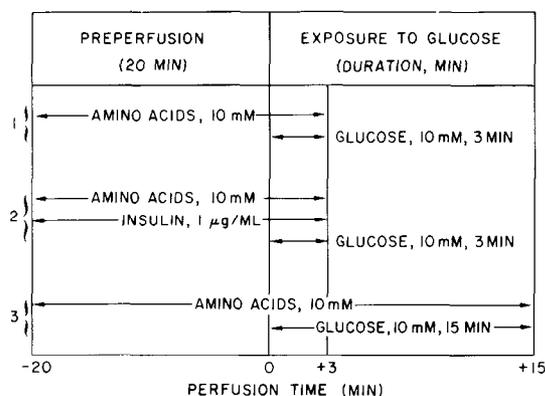


FIG. 1. Experimental protocols for perfusion studies.

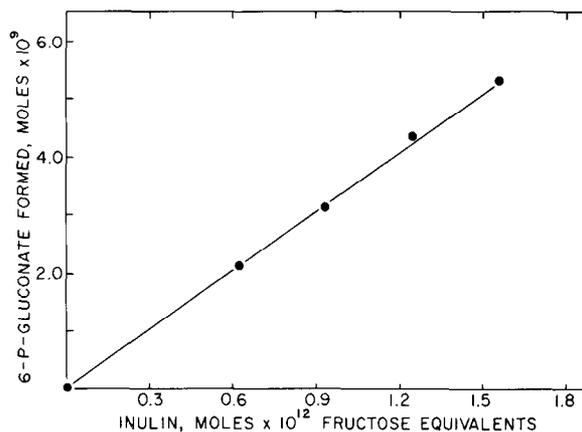


FIG. 2. Yield of product in the oil well assay for inulin. The ordinate records the amount of 6-P-gluconate derived from amounts of inulin shown on the abscissa. The amplification in the cycling step was approximately 3000-fold. Each point is the mean of duplicates.

propyl alcohol buffer, pH 9.9; DPN, 650 μ M; sodium glutamate, 5 mM; lactic acid dehydrogenase (beef heart), 75 μ g/ml; and glutamate-pyruvate transaminase, 75 μ g/ml. The oil well rack was then incubated for 45 min at 22° and the reaction was terminated with alkali (1.08 μ l of 0.125 N NaOH) and heating (20 min at 75°). The DPNH formed was measured by enzymatic cycling according to the procedure of Kato *et al.* (18). This reaction was performed in 50 μ l in glass tubes (10 \times 75 mm). The amplification needed for this application was 4000-fold. Lactate standards ranging from 5×10^{-13} to 3×10^{-12} mol were carried through the entire process. Great care was taken to avoid contamination of tissue and materials with lactate and therefore gloves had to be worn while carrying out this enzymatic assay.

For measurements of cyclic AMP, 5 to 7 islet or exocrine tissue samples were dissected, weighed, and pooled in glass tubes (6 \times 50 mm). The pooled samples weighed between 0.3 and 1.0 μ g. Forty microliters of 10% trichloroacetic acid were added to the tubes which were allowed to sit at room temperature for 15 to 30 min. The tubes were gently mixed and 35 μ l of the extract were removed and placed into clean tubes. (A dissecting microscope was used during this transfer to prevent inadvertent transfer of tissue samples.) The trichloroacetic acid extracts were then evaporated to dryness in a vacuum centrifuge. This procedure completely removed all trichloroacetic acid. The dried residue was dissolved in 50 μ l of H₂O and then acetylated and assayed for cyclic AMP as previously described (19). Measurements were made in triplicate. Treatment of tissue extracts with beef heart phosphodiesterase destroyed all immunoreactive material indicating that the measured substance was indeed cyclic AMP.

Other Analytical Methods—Immunoreactive glucagon and insulin were measured by using double antibody systems as previously described (10, 20, 21). Glucose, lactate, and ketone bodies in plasma were measured by enzymatic assays, either fluorometrically (22) or spectrophotometrically (23).

RESULTS

Criteria for Defining Diabetes in This Study (Table I)—Three lines of evidence show that the animals entering the study were severely diabetic. First, only those animals were used which showed fasting blood sugars over 14 mM, 2 to 3 days after streptozotocin treatment and before starting the insulin treatment (data not shown); second, there was no (or minimal) glucose-induced insulin release demonstrable from the isolated perfused pancreas of diabetic animals; and third, the method of inducing diabetes used here has been amply validated in preceding studies (9, 17). Data given in Table I also demonstrate that the method of inducing diabetes was highly effective and that treatment with insulin improved the diabetic

conditions. The body weights and the serum levels of ketones, insulin, and glucagon indicate the influence of insulin treatment. It is noteworthy that the glucagon levels of fed animals were about twice the levels found in a previous study following an overnight fast. The average plasma glucose of fed unstressed control animals was 7.4 ± 0.2 mM ($n = 24$). At the time of killing the blood sugar of controls had risen to 19.3 ± 0.63 mM ($n = 14$). The high blood sugar levels are explained by the fed state at the time of surgery and by the fact that the blood sampling was performed after a stressful evisceration of the animal in preparation for the pancreas perfusion. The fed state and the surgical intervention most likely also explain the high serum lactate in normals and diabetics alike.

Glucagon and Insulin Release from Perfused Pancreas with 10 mM Amino Acid with and without 10 mM Glucose (Figs. 3 and 4)—Perfusion of normal pancreas with a physiological mixture of 19 amino acids, at a concentration of 10 mM, leads to high glucagon release (Fig. 3). Since no samples were taken during the early period, only the second phase of release is seen (compare Refs. 9 and 10). The rate of release is almost twice that observed in previous studies (9, 10). This may be due to the fed state of the animals or due to the change of the perfusion protocol. Consistent with published data is the complete absence of a β -cell response to the amino acid mixture alone (10) (Fig. 4). Addition of 10 mM glucose to the amino acid stimulus resulted in suppression of glucagon release and stimulation of insulin release in the normal pancreas (Figs. 3 and 4). The β -cells responded more rapidly to the glucose pulse than the α -cells, as indicated by the fact that the rate of insulin release rose significantly within 30 s after switching to glucose, whereas glucagon suppression did not become significant until 1 min after the switch (compare Figs. 3 and 4, control perfusion). About 1.5 min elapsed before glucagon release was suppressed by 50%. The endocrine response observed here to 10 mM glucose consisted of the entire first phase of insulin release and almost complete cessation of glucagon release.

With the pancreas from untreated diabetic rats there was a greatly reduced α -cell response to the amino acid stimulus (approximately one-third of the control rate) consistent with the results of a previous study (9) (Fig. 3). Glucose suppression of α -cell function was delayed and was clearly seen only when

TABLE I

Body weights and plasma levels of glucose, lactate, β -hydroxybutyrate, insulin, and glucagon of normal as well as treated and untreated diabetic animals prior to perfusion

Condition (<i>n</i>)	Animal weight	Glucose	Lactate	β -Hydroxybutyrate	Insulin	Glucagon
	<i>g</i>	<i>mmol/liter plasma</i>	<i>mmol/liter plasma</i>	<i>mmol/liter plasma</i>	<i>ng/ml plasma</i>	<i>ng/ml plasma</i>
A. Controls (14)	383 ± 13^a	19.3 ± 0.6^b	5.6 ± 0.40	0.19 ± 0.01	1.94 ± 0.30	0.304 ± 0.015
B. Untreated diabetics						
Experiment 1 (14)	286 ± 9	33.9 ± 1.4	4.8 ± 0.65	6.10 ± 0.60	0.06 ± 0.01	0.772 ± 0.123
Experiment 2 (14)	295 ± 13	42.9 ± 1.0	6.2 ± 0.45	4.00 ± 0.75	0.14 ± 0.03	0.708 ± 0.175
Experiment 3 (16)	324 ± 10	39.6 ± 1.0	5.7 ± 0.40	6.80 ± 0.60	0.10 ± 0.02	0.527 ± 0.076
C. Experiments 1 to 3 (44)	304 ± 5	38.6 ± 0.6	5.5 ± 0.22	5.80 ± 0.21	0.09 ± 0.01	0.659 ± 0.047
D. Treated diabetics (20)	319 ± 4	38.3 ± 2.0	7.3 ± 0.40	1.36 ± 0.31	2.80 ± 1.20	0.424 ± 0.030
Statistical treatment of data						
A versus C	<0.001	<0.001	N.S. ^c	<0.001	<0.001	<0.01
A versus D	<0.001	<0.001	<0.001	<0.001	N.S.	<0.01
C versus D	N.S.	N.S.	<0.001	<0.001	<0.001	<0.02

^a All values represent the means \pm S.E.

^b The corresponding plasma glucose values in the fed unstressed animals were 7.4 ± 0.2 , 34.8 ± 1.3 , and 27.6 ± 1.1 mM in controls, untreated, and treated diabetics, respectively.

^c N.S., not significant.

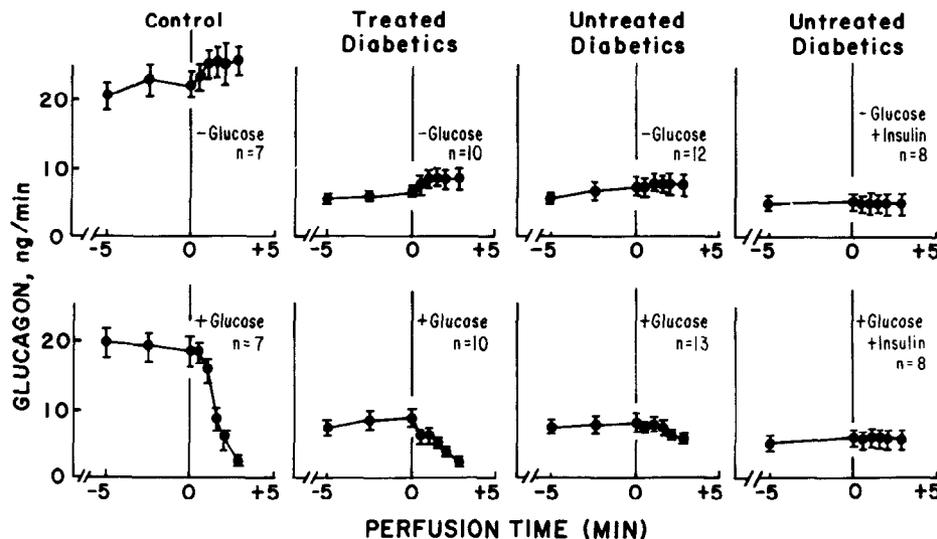


FIG. 3. Glucose suppression of amino acid-induced glucagon secretion in the isolated perfused pancreas from normal and diabetic rats. The concentration of the amino acid mixture was 10 mM and 10 mM glucose was present from t_0 to t_3 . Porcine insulin was added as indicated at 1 μ g/ml, present during the entire duration of the perfusion. For further information see Fig. 1. Means \pm S.E. of indicated numbers of perfusion experiments are recorded.

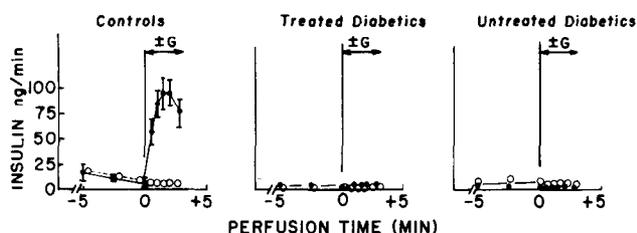


FIG. 4. Glucose-induced insulin release in the isolated perfused pancreas of normal and diabetic rats. The amino acid mixture (10 mM) was present throughout in all experiments. In about half of the experiments 10 mM glucose was added from t_0 to t_3 . In most instances only the mean values are given. Standard errors are recorded at essential parts of the release profiles. There were from 7 to 12 experiments in each group (as to experimental design see Fig. 1; regarding the corresponding data, compare Tables II to IV and Fig. 3).

the glucose infusion was extended to 15 min (Fig. 5). About 3 min were required to result in 50% suppression of glucagon release. There was no insulin secretion either with amino acids alone or with the amino acid/glucose combination. These data agree with our previous result, showing effective glucose suppression of stimulated glucagon secretion in the diabetic pancreas *in vitro* in the virtual absence of insulin (9) and agree also with data by Frankel *et al.* (24) obtained with the pancreas from diabetic Chinese hamsters as well as with data by Weir who used the acutely diabetic rat pancreas (25). *In vivo* treatment of diabetic animals with insulin improved the glucose sensitivity of the α -cells in the *in vitro* setting (Fig. 3). The suppressive action of glucose on α -cells manifested itself more rapidly than in the pancreas from untreated animals. It is noteworthy, however, that the total release of glucagon in response to the amino acid stimulus was not altered as compared to the pancreas of untreated animals. Further, insulin treatment *in vivo* did not improve β -cell function, as indicated by the complete lack of insulin release *in vitro* (Fig. 4). Insulin at 1 μ g/ml added to the perfusate throughout the entire duration of the experiment failed to improve glucose suppressibility. In fact in the set of experiments there was no glucose suppression demonstrable during the brief period

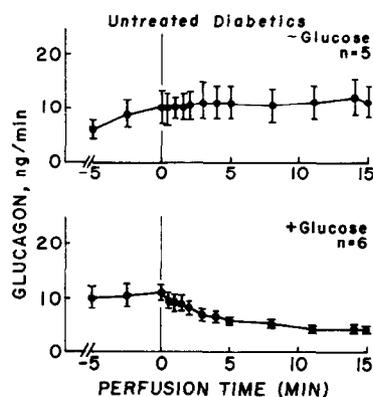


FIG. 5. Glucose suppression of amino acid-induced glucagon release in the isolated perfused pancreas of untreated diabetic rats. The amino acid mixture was present throughout at 10 mM. Glucose (10 mM) was superimposed from t_0 to t_{15} . Means \pm S.E. of indicated numbers of experiments are recorded. Insulin was virtually absent during the preperfusion period and glucose failed to elicit any response from t_0 to t_{15} (similar to results shown in Fig. 4, but not recorded here).

tested (Fig. 3). The data indicate however, that high levels of insulin alone reduce amino acid-stimulated glucagon release, confirming results of earlier studies with the pancreas from alloxan-treated rats (9). The average rates of glucagon release prior to adding glucose (t_{15} to t_0) were 7.1 ± 0.5 and 7.1 ± 0.4 ng/min in the untreated and treated diabetics alike. Addition of insulin *in vitro* (1 μ g/ml) decreased the stimulated glucagon release to 5.3 ± 0.5 ng/min (-25% , $p < 0.05$).

Glucose Penetration into Islets of Normal and Diabetic Pancreas (Tables II and III)—Following 3 min of perfusion of the normal pancreas with 10 mM glucose, the glucose content of normal islets had risen from 4.4 ± 0.9 to 34.3 ± 1.7 mmol/kg of dry tissue (Table II). Accumulation of glucose in α -cell islets from untreated and insulin treated diabetics is similarly swift, showing an increase of 21.3 and 22.1 mmol/kg of dry tissue, respectively, within 3 min. This increase is approximately two-thirds of the total glucose accumulation seen in normal mixed islets. Perfusion with 1 μ g/ml of insulin did not change the glucose accumulation in islets of untreated diabetics *in*

TABLE II

Effect of insulin on glucose and inulin levels of islets from isolated perfused pancreas

The results for each pancreas are shown individually. These individual values represent the means \pm S.E. from usually six islet samples. Also given are the means \pm S.E. for each group. Perfusions were performed in the presence and absence of 10 mM glucose in the perfusion medium. The exposure to glucose following a preperfusion of 20 min lasted for 3 min, after which period the pancreas was sampled by quick freezing.

Conditions	Glucose		Inulin ^a	
	No glucose	Glucose, 10 mM	No glucose	Glucose, 10 mM
	mmol/kg dry tissue		mmol/kg dry tissue	
Controls				
1	4.4 \pm 0.8	28.5 \pm 3.7	6.8 \pm 0.8	11.8 \pm 1.2
2	6.3 \pm 0.7	34.8 \pm 3.1	9.0 \pm 0.9	11.7 \pm 0.6
3	3.5 \pm 0.3	33.6 \pm 1.9	11.0 \pm 1.0	5.3 \pm 0.8
4	0.7 \pm 0.2	37.5 \pm 2.7	8.2 \pm 0.7	9.6 \pm 1.4
5	7.3 \pm 0.6	40.6 \pm 3.5	9.3 \pm 0.9	14.2 \pm 2.1
6	3.3 \pm 1.6	33.3 \pm 1.2	7.2 \pm 0.8	9.5 \pm 1.1
7	5.0 \pm 0.9	37.1 \pm 1.4	13.5 \pm 0.8	8.8 \pm 0.8
Means \pm S.E.	4.4 \pm 0.9	34.3 \pm 1.7	9.2 \pm 1.0	10.1 \pm 1.3
Untreated diabetics				
1	1.2 \pm 0.6	21.1 \pm 1.5		13.7 \pm 1.6
2	4.3 \pm 0.8	17.8 \pm 1.6	13.5 \pm 2.5	8.0 \pm 0.4
3	3.3 \pm 0.4	37.6 \pm 6.2	11.6 \pm 1.3	17.1 \pm 2.4
4	4.8 \pm 1.1	26.6 \pm 2.0	7.9 \pm 0.8	13.9 \pm 2.6
5	4.6 \pm 2.5	26.4 \pm 1.7	8.7 \pm 1.5	10.7 \pm 1.1
6	6.0 \pm 1.5	19.1 \pm 1.1	6.9 \pm 0.6	7.4 \pm 0.9
7	12.8 \pm 1.5	33.9 \pm 1.5	9.1 \pm 2.2	16.4 \pm 2.3
8	9.2 \pm 1.7	37.5 \pm 3.2	6.6 \pm 0.7	18.8 \pm 2.8
9	9.2 \pm 1.7	39.8 \pm 4.0	11.1 \pm 1.8	9.9 \pm 1.5
10	5.9 \pm 0.2	27.0 \pm 5.1	10.3 \pm 1.9	9.8 \pm 1.2
11	9.7 \pm 1.8	19.3 \pm 0.4	9.7 \pm 1.8	10.3 \pm 2.3
Means \pm S.E.	6.5 \pm 1.0	27.8 \pm 2.0	9.5 \pm 0.5	12.1 \pm 0.9
Treated diabetics				
1	8.9 \pm 1.1	17.7 \pm 2.6		12.3 \pm 1.1
2	6.9 \pm 0.7	29.3 \pm 1.4	8.3 \pm 1.1	
3	8.8 \pm 0.4	32.6 \pm 4.0	11.4 \pm 4.2	10.4 \pm 2.3
4	12.7 \pm 1.6	19.0 \pm 2.4	11.3 \pm 0.5	
5	5.6 \pm 0.9	36.0 \pm 3.9	8.6 \pm 1.4	8.6 \pm 1.5
6	7.0 \pm 2.5	25.6 \pm 1.1	5.9 \pm 1.6	10.9 \pm 1.7
7	5.4 \pm 0.9	28.3 \pm 4.9	13.6 \pm 2.0	10.5 \pm 2.2
8	6.0 \pm 1.2	32.3 \pm 3.8	5.6 \pm 1.1	
9	10.3 \pm 1.4		10.4 \pm 1.8	
10	10.9 \pm 2.1			
Means \pm S.E.	8.2 \pm 0.7	30.3 \pm 2.3	9.5 \pm 0.9	10.6 \pm 0.6
Untreated diabetics (insulin in vitro, 1 μg/ml)				
1	9.2 \pm 1.5	28.3 \pm 2.9	9.0 \pm 1.3	5.6 \pm 0.4
2	7.6 \pm 0.4	47.1 \pm 3.7	7.5 \pm 1.6	9.3 \pm 0.8
3	10.8 \pm 1.1	19.7 \pm 1.9	11.3 \pm 1.9	7.0 \pm 0.5
4	7.8 \pm 0.3	30.5 \pm 1.1	11.1 \pm 1.1	6.4 \pm 0.6
5	8.3 \pm 1.0	36.4 \pm 3.1	10.3 \pm 0.9	6.0 \pm 2.8
6	13.6 \pm 1.4	20.6 \pm 2.0	10.6 \pm 1.5	9.8 \pm 1.0
7	9.7 \pm 1.4	25.9 \pm 1.9	9.2 \pm 1.2	7.5 \pm 1.2
8	8.3 \pm 0.5	45.8 \pm 5.3	8.0 \pm 1.2	8.7 \pm 1.3
Means \pm S.E.	9.4 \pm 0.7	31.8 \pm 3.3	9.6 \pm 0.4	7.5 \pm 0.5

^a Inulin levels are expressed in terms of hexose equivalents (30 eq/mol of inulin).

vitro, the increase being similarly 22.4 mmol/kg of dry tissue during the 3 min. Tissue glucose had nearly equilibrated with the perfusate glucose within the short period chosen here, as indicated by results of a few measurements performed on islets sampled after 15 min of exposure to 10 mM glucose (protocol 3). In islets from two normal animals the absolute glucose levels were 31.2 \pm 3.2 and 34.8 \pm 3.3 mmol/kg of dry tissue and from two untreated diabetics they were 26.9 \pm 1.9 and 21.5 \pm 2.1 mmol/kg of dry tissue, all within the range found after brief (3 min) exposure to glucose (compare with Tables II and III).

Islet tissue dissected from the pancreas following perfusion in the absence of extracellular glucose contained substantial amounts of glucose, confirming the results of a previous study from this laboratory (17). The levels found here ranged from 4.4 mM in islets of controls to 9.4 mM in islets from untreated diabetics receiving insulin treatment *in vitro*. That these levels are about twice as high as were previously reported (17) may be due to the nutritional state and the shorter duration of perfusion (fed *versus* fasted and 23 *versus* 75 min). Because of the specificity of the assay it is reasonable to assume that glucose is being measured. It is less likely that fructose release from inulin or glucose released from dextran can explain the data since this tissue glucose blank lacks proportionality to the inulin levels of the islets (compare the results in controls with the results of islets following *in vitro* treatment with high insulin) (Table II). Further, it was found that neither inulin nor dextran carried through the assay procedures resulted in TPNH formation as measured here. It is also unlikely that this glucose pool is derived from glycogen since dilute acid fails to hydrolyze the α -glucosidic bond (11, 12).

In order to allow some insight into the tissue distribution of glucose, the inulin spaces of islets were determined. When the pancreas was perfused with 0.33 mM inulin and in the absence of glucose, the extracellular space was remarkably constant no matter what the condition, *i.e.* 0.87, 0.91, 0.91, and 0.90 liters of water/kg of dry tissue in controls, untreated diabetics, treated diabetics, and untreated diabetics plus insulin *in vitro*, respectively. The extracellular space measured here is about 1.5 times larger than found *in vivo*. The most plausible explanation is that in the perfusion situation this compartment is expanded as a result of the artificial circulatory conditions. Surprisingly, it was found that in the presence of 10 mM glucose in the perfusate the inulin spaces of diabetic islets was reduced following pharmacological doses of insulin *in vitro* (from 1.11 to 0.67 liters/kg of dry tissue, *i.e.* -38%, Table III). There is currently no obvious biochemical correlate that might explain the phenomenon.

Using these data on inulin distribution it can be calculated that the intracellular glucose space of controls is about two-thirds of an assumed total water space of 3 liters/kg dry tissue (17), whereas the intracellular glucose space of the islets from untreated diabetics contributes about one-third of the total water space. Brief insulin treatment *in vitro* with 1 μ g/ml of insulin increased the intracellular glucose space from 1.0 to 1.5 liter/kg (Table III). This change does not attain statistical significance, however. The level of free intracellular glucose is, within experimental error, the same in the islets from all three diabetic groups, with the lowest levels at 5 and the highest level of 6.6 mM. Similarly, in a previous study (17) insulin, when given *in vivo* or *in vitro* had no effect on the intracellular glucose level.

Lactate Levels of Islet Tissue (Table IV)—The average lactate content of islet tissue was similar in all experimental

TABLE III

Effect of insulin on tissue distribution of glucose and inulin in pancreatic islets of perfused pancreas

The values of this table are derived from the results represented in Table II. Free glucose represents the difference between the glucose values found in the presence and absence of 10 mM glucose in the perfusate, calculated for each individual pancreas. The means \pm S.E. of the differences are given. Inulin represents the means \pm S.E. of the values obtained in the presence of 10 mM glucose (fructose equivalents divided by 30). The total glucose space was obtained by dividing the individual values representing the free glucose levels by 10.5 (*i.e.* by the concentration (mM) of glucose in the perfusate corrected for

solids). Here also means \pm S.E. are recorded. The inulin spaces represent the quotient of inulin tissue values in terms of mM and of 0.35 mM, the concentration of inulin in the perfusate water. The intracellular (I.C.) glucose space represents the difference between total glucose space and inulin (or extracellular) space. The total glucose space of normal islets is of comparable magnitude as the assumed total water space (about 3 liters/kg of dry tissue). Also given is a statistical treatment of the data obtained with conditions A through D.

Condition	Free glucose	Inulin	Total glucose space	Inulin space	I.C. glucose space
	mmol/kg dry tissue		liters/kg dry tissue		
A. Controls	30.7 \pm 1.5	0.31 \pm 0.03	3.15 \pm 0.14	0.93 \pm 0.10	2.21 \pm 0.17
B. Untreated diabetics	21.4 \pm 2.3	0.37 \pm 0.03	2.23 \pm 0.17	1.11 \pm 0.10	1.00 \pm 0.11
C. Treated diabetics	20.0 \pm 3.0	0.32 \pm 0.02	1.92 \pm 0.27	0.98 \pm 0.06	1.10 \pm 0.35
D. Untreated diabetics + insulin	22.1 \pm 4.3	0.23 \pm 0.02	2.11 \pm 0.41	0.67 \pm 0.06	1.48 \pm 0.39
Statistical treatment of data					
A versus B	<0.01	N.S. ^a	<0.01	N.S.	<0.01
A versus C	<0.01	N.S.	<0.01	N.S.	<0.01
A versus D	N.S.	<0.02	<0.05	<0.02	N.S.
B versus C	N.S.	N.S.	N.S.	N.S.	N.S.
B versus D	N.S.	<0.01	N.S.	<0.01	N.S.
C versus D	N.S.	<0.01	N.S.	<0.01	N.S.

^a N.S., not significant.

groups, whether glucose was present in the perfusate or not (Table IV). The values ranged from about 6.5 mM in the islets of control pancreas perfused without glucose to 9.7 mM in islets of the pancreas of untreated diabetics perfused without glucose but containing high insulin (1 μ g/ml). There was substantial scatter from animal to animal. These high values are consistent with the extremely high serum lactates observed at the time of killing. Further, expressed on a wet weight basis, the values are within the range of tissue lactate concentrations measured in many other tissues *i.e.* between 1.5 to 2.5 mM. It would seem from this that under certain experimental circumstances lactate is a poor indicator of glycolysis (26).

ATP Content of Normal and Diabetic Islets (Table IV)—Consistent with previous results of ATP measurements in islets of normal (13, 17) and diabetic pancreas (17) it was found that for short term perfusions as used here (23 min) the energy supply was in the physiological range. These results in the perfused pancreas differ from the results obtained with isolated islets obtained either by freehand dissection or by collagenase digestion (27). In the latter cases it was found that ATP levels had dropped substantially in the course of islet isolation and that high glucose was needed to normalize ATP concentration. This high degree of ATP preservation is certainly a distinguishing feature of the perfused pancreas system. Exposure to 10 mM glucose from 3 min in addition to the amino acid mixture has no significant effect on ATP levels under any circumstances (Table IV). It cannot be overlooked that with insulin treatment *in vivo* and *in vitro* the ATP values of islets from diabetic animals are significantly higher, whether glucose is added to the perfusate or not. The ATP increment is most pronounced after perfusion with high insulin for 23 min (+22%, $p \leq 0.01$). A similar increase of ATP in α -cell islets due to insulin has been observed previously in the isolated perfused pancreas (17), the change being +24% but lacking statistical significance. These data indicate that insulin is capable of influencing the phosphate potential of α -cells.

Levels of Cyclic AMP in Islet Tissue of Perfused Pancreas (Table V)—The recent development of a highly sensitive radioimmunoassay (19, 28) allowed us to measure cyclic AMP in islet tissue and the corresponding exocrine samples. These measurements revealed a striking histochemical heterogeneity of the cyclic AMP system of pancreatic tissue: the cyclic AMP levels of islets were 5 to 7 times higher than the levels of exocrine tissue. The difference is so pronounced that for the pancreas the cyclic nucleotide can be considered an islet tissue marker, again attesting to the reliability of the dissection procedure used here. The levels found in the perfusion setting are similar to the levels reported previously with isolated perfused pancreatic islets (29). In the quoted study, cyclic AMP levels between 1.5 to 4 pmol/100 islets were observed. Because the average dry weight of islets is 1.3 μ g (30), this is equivalent to 11.5 to 30 pmol/mg of dry weight. Perfusion with glucose for 3 min did not alter the cyclic AMP level under any circumstances. Since only one time point was analyzed here no statement can be made regarding whether or not the cyclic AMP system is involved in glucose potentiation or glucose suppression of amino acid-induced insulin and glucagon release, respectively. However, the data presented clearly demonstrate the feasibility of studying dynamics of the cyclic AMP system of pancreatic islets in the isolated perfused pancreas, thus avoiding possible complications that might arise from using isolated islets obtained by the collagenase procedure.

DISCUSSION

Reduced Glucose Suppressibility of α -Cells in Diabetes—In order to help elucidate how glucagon secretion from α -cells is regulated physiologically and how this regulation might be impaired in diabetes, studies with the isolated perfused pancreas system have been performed by several investigators using the pancreas from diabetic animals (9, 24, 25). Even though the forms of diabetes differed markedly, it was shown in

TABLE IV

Effect of glucose and insulin on levels of ATP and lactate in islets of perfused pancreas

Results of experiments in the absence and presence of 10 mM glucose are given. The results of individual perfusion experiments are recorded. Each value represents the means \pm S.E. from six islet samples. Also given are the means \pm S.E. of the different experimental groups. The numbers of experiments are indicated.

Condition	ATP		Lactate	
	No glucose	Glucose, 10 mM	No glucose	Glucose, 10 mM
	<i>mmol/kg dry tissue</i>			
A. Controls				
1	10.2 \pm 0.3	13.0 \pm 0.4	7.9 \pm 0.9	6.9 (2)
2	8.1 \pm 0.4	7.1 \pm 0.7	4.9 \pm 0.7	7.7 \pm 1.0
3	12.0 \pm 0.4	11.7 \pm 0.7	8.6 \pm 1.1	5.3 \pm 0.5
4	13.3 \pm 0.4	10.3 \pm 0.6	6.8 \pm 1.4	7.5 \pm 1.2
5	11.3 \pm 0.7	11.9 \pm 0.5	6.5 \pm 1.4	11.9 \pm 1.7
6	11.3 \pm 0.5	13.5 \pm 0.5	5.6 \pm 0.7	3.7 \pm 0.4
7	9.8 \pm 0.5	11.7 \pm 0.6	5.0 \pm 0.4	4.7 \pm 0.8
Means \pm S.E.	10.8 \pm 0.7	11.3 \pm 0.9	6.5 \pm 0.5	6.8 \pm 1.2
B. Untreated diabetics				
1	10.0 \pm 0.6	10.5 \pm 0.4	7.9 \pm 0.5	17.8 \pm 1.4
2	11.3 \pm 0.3	13.7 \pm 0.4	14.2 \pm 1.4	6.5 \pm 1.3
3	11.2 \pm 0.4	11.5 \pm 0.8	8.9 \pm 1.1	6.2 \pm 0.8
4	12.6 \pm 0.3	12.0 \pm 0.3	5.0 \pm 1.6	11.0 \pm 1.2
5	11.9 \pm 0.1	12.1 \pm 0.3	6.6 \pm 0.5	4.4 \pm 0.8
6	12.8 \pm 0.2	11.6 \pm 0.7	6.1 \pm 0.4	6.2 \pm 1.0
7	9.4 \pm 0.7	14.9 \pm 0.3	10.4 \pm 1.0	4.3 \pm 0.6
Means \pm S.E.	11.3 \pm 0.5	12.4 \pm 0.6	8.4 \pm 1.3	8.1 \pm 1.9
C. Treated diabetics				
1	12.7 \pm 0.5	14.0 \pm 0.2	4.5 \pm 0.8	3.9 \pm 0.4
2	13.3 \pm 0.8	13.1 \pm 0.9	11.0 \pm 1.4	5.9 \pm 0.5
3	14.0 \pm 0.6	13.4 \pm 0.3	14.8 \pm 1.7	6.8 \pm 1.1
4	10.7 \pm 0.5	8.6 \pm 0.7	11.3 \pm 0.8	8.3 \pm 1.4
5	13.2 \pm 0.6	13.4 \pm 0.6	4.2 \pm 0.7	7.4 \pm 2.1
6	15.8 \pm 0.4	15.0 \pm 0.3	6.4 \pm 0.8	8.3 \pm 0.9
7	11.2 \pm 0.3	10.2 \pm 0.3	11.6 \pm 0.8	10.7 \pm 0.7
8	14.5 \pm 0.4	14.5 \pm 0.3	4.4 \pm 0.3	11.4 \pm 0.8
9	12.7 \pm 0.3	13.3 \pm 0.5	4.2 \pm 0.4	3.9 \pm 0.2
10	12.7 \pm 0.3		8.0 \pm 0.4	
Means \pm S.E.	13.1 \pm 0.5	12.9 \pm 0.7	8.0 \pm 1.1	7.4 \pm 0.8
D. Untreated diabetics plus insulin <i>in vitro</i>				
1	13.0 \pm 0.5	15.7 \pm 0.9	6.1 \pm 0.5	8.7 \pm 1.1
2	13.6 \pm 0.1	13.8 \pm 0.4	15.2 \pm 1.8	6.5 \pm 0.9
3	14.0 \pm 0.4	14.3 \pm 0.4	10.5 \pm 1.1	5.5 \pm 0.6
4	14.1 \pm 0.5	13.7 \pm 0.3	8.6 \pm 1.4	8.7 \pm 1.0
5	14.7 \pm 0.1	14.9 \pm 0.3	8.5 \pm 1.0	7.3 \pm 1.4
6	15.1 \pm 0.1	13.4 \pm 0.1	14.2 \pm 0.8	5.4 \pm 0.8
7	13.7 \pm 0.2	16.6 \pm 0.4	6.1 \pm 0.9	8.9 \pm 0.6
8	14.2 \pm 0.4	15.3 \pm 0.3	8.4 \pm 1.0	9.0 \pm 0.7
Means \pm S.E.	14.1 \pm 0.3	14.7 \pm 0.4	9.7 \pm 1.1	7.5 \pm 0.5
Statistical treatment of data				
A versus B	N.S. ^a	N.S.	N.S.	N.S.
A versus C	<0.01	N.S.	N.S.	N.S.
A versus D	<0.01	<0.01	<0.05	N.S.
B versus C	<0.02	N.S.	N.S.	N.S.
C versus D	N.S.	<0.05	N.S.	N.S.

^a N.S., not significant.

TABLE V

Levels of cyclic 3':5'-AMP in islets of Langerhans and acinar tissue of isolated perfused pancreas of normal and diabetic rats

The data represent the means \pm S.E. of an indicated number of experiments. In each experiment 5 to 7 islets were pooled for preparing the acid extract (see "Methods"). The numbers of experiments entering the analyses are given in parentheses.

Condition	Islets	Acinar tissue
	<i>pmol/mg dry tissue</i>	
No glucose		
Controls	23.5 \pm 8.8 (7)	3.3 \pm 0.4 (8) ^a
Untreated Diabetics	31.9 \pm 6.4 (6)	4.9 \pm 1.2 (7) ^a
Treated Diabetics	23.2 \pm 3.6 (10)	4.0 \pm 0.4 (9) ^a
Glucose, 10 mM		
Controls	23.5 \pm 4.0 (8)	3.6 \pm 0.5 (7) ^a
Untreated Diabetics	30.8 \pm 6.9 (7)	5.2 \pm 0.8 (8) ^a
Treated Diabetics	28.6 \pm 9.1 (8)	4.4 \pm 0.5 (8) ^a

^a Significantly different from the corresponding islet values, $p < 0.01$.

all instances that in one form or another glucose suppressibility of stimulated glucagon release was impaired. In our series with chronic streptozotocin diabetic animals, reduced sensitivity of α -cells to glucose was more readily apparent with the protocols used in the present study, where the actual kinetics of glucose suppression of amino acid-stimulated glucagon secretion were investigated. Glucagon suppression *in vitro* was delayed in diabetes and insulin treatment *in vivo* improved the response, increasing the initial velocity of glucose suppression of glucagon release. In the preceding study of this series (9) preperfusion of the pancreas even with very low levels of glucose (5 mM) inhibited amino acid-stimulated glucagon release efficiently. It was noticed, however, that the first phase of release was not suppressed by glucose in the diabetic pancreas. Furthermore, whereas in the normal pancreas glucose lowered stimulated glucagon release to or below the base-line established in the preperfusion period, this was not observed with the diabetic pancreas and even 20 mM glucose did not affect basal glucagon secretion. Finally, the relative potencies of glucose as an inhibitor and of amino acids as stimulators were altered disproportionately in the pancreas from streptozotocin and alloxan diabetic animals, indicating that glucose becomes a relatively less effective inhibitor in diabetes. Altogether, data collected with the isolated perfused pancreas support the concept that glucose suppression of α -cells is impaired in diabetes.

There is, however, one major difficulty. The α -cells of chronically diabetic animals, insulin-treated or not, show very low basal release *in vitro* (about 1/10 of the rate seen in controls) and reach only 30 to 50% of the response seen in normals when 10 mM of an amino acid mixture is used as stimulant (see also Refs. 9 and 10). This weak response seems paradoxical in view of the well established hyperglucagonemia seen *in vivo* in these diabetic animals. It was postulated (9) that the hyperglucagonemia *in vivo* might be due to other powerful α -cell stimulants operative in the diabetic in the *in vivo* setting (e.g. catecholamines, enteric peptides, etc.) or to less effective degradation of the hormone, or both. It is also possible that the recently discovered somatostatin system of the pancreas (31, 32) functions less constrained *in vitro* than it might *in vivo*. The paradoxically low response *in vitro* is not seen in the pancreas of acutely diabetic animals (25) nor in the pancreas of the genetically diabetic chinese hamster (24). Considering all

the recognized and unknown factors involved in the release and degradation of glucagon *in vivo*, we now feel that reduced secretory response of the α -cells *in vitro* observed in the present system under very special circumstances is not incompatible with the hyperglucagonemia seen *in vivo*.

The results of the present study demonstrate again that glucose suppression of α -cells can occur independent of a concomitant release of insulin from the β -cell but the data also indicate that insulin treatment of the animal *in vivo* improves glucose suppressibility *in vitro*. Recent studies from this laboratory (33) with the isolated perfused pancreas made acutely diabetic by infusion of alloxan *in vitro* are consistent with this interpretation since glucose suppression of glucagon release was unimpaired even though insulin release had been completely blocked by alloxan.

Glucose Metabolism of α -Cell Islets of Treated and Untreated Diabetic Rats—The islets of Langerhans are comprised of different cell types (β -, α_1 -, and α_2 -cells, neuronal elements, and stroma cells, to mention just major components) (34). This compromises our ability to interpret biochemical measurements in islet tissue. In islet samples dissected here from the normal pancreas, β -cells contribute most of the tissue, *e.g.* 75%. They are considered here as β -cell islets. In islets from streptozotocin-treated rats, β -cells have been virtually eliminated (9, 17, 35) but the relative contribution of α_1 - and α_2 -cells, and non-endocrine cells is not established. However, it seems to be a fair approximation to consider these islets as α -cell islets since this cell type contributes probably as much as 75% of the remaining tissue mass.

It has been postulated that the glucose metabolism of α -cells is impaired in diabetes, that this leads to a fuel shortage of the cells (*e.g.* an ATP deficit), and that, as a result of this, enhanced glucagon secretion occurs (36, 37). Consistent with the biochemical results previously reported (17), no obvious impairment of energy metabolism was seen in this investigation. There was no difference in the ATP levels of glucose suppressible and nonsuppressible α -cell islets. In fact, high insulin perfused *in vitro* caused a significant elevation of ATP (+25%), without enhancing glucose sensitivity of the α -cells. It is unlikely therefore that α -cell ATP content is the primary factor involved in glucose regulation of glucagon secretion. Another line of evidence pointing against the hypothesis that the glucose effect on α -cells is mediated through ATP is our previous observation that the maintenance of α -cell ATP is largely glucose-independent (17).

The data presented here suggest that neither the process of glucose transport itself nor the intracellular glucose level are the primary factors determining glucose responsiveness of the α -cells. Net uptake (15 mmol/kg of tissue \times 3 min) and intracellular glucose concentration (6.6 mM) were highest in pancreas from untreated animals exposed to insulin *in vitro*, in which case there was no glucose suppressibility detectable within the period tested (3 min). Detailed time and concentration dependency studies of possible functional and metabolic effects of insulin treatment administered *in vivo* or *in vitro* in the perfusion system are obviously needed.

It was concluded in two preceding reports that glucose uptake and metabolism as well as the energy potential of the α -cell might be entirely insulin-independent (9, 17). Because of the present data this latter view needs to be modified. The capacity of high insulin *in vitro* to increase the ATP content of α -cell islets, albeit only slightly, suggests that insulin might modify the intermediary metabolism of α -cell islets. Since a

rise of ATP was also seen in the absence of extracellular glucose but with 10 mmol of extracellular amino acids present, the insulin effect may have been due to increased amino acid uptake or utilization, or both.

The question whether insulin might enhance glucose uptake of α -cell islets is not clearly answered by the present data. A small increase of the glucose space was apparent following insulin treatment *in vitro* but the change was not significant statistically. Improved methods for measuring the extracellular and total water spaces and for quantitating the initial velocity of sugar entry need to be developed for α -cell islets of the isolated perfused pancreas, to reach a definite answer.

As it now stands a simple causal relationship between improvement of intermediary and energy metabolism by insulin and the enhanced glucose responsiveness of the pancreas following insulin treatment *in vivo* is far from being established. The fact that insulin action on α -cells can manifest itself in elevation of ATP levels even in the absence of extracellular glucose without a demonstrable enhancement of glucose suppression of glucagon release illustrates the complexity of this unsettled issue. Possible effects of insulin on intermediary metabolism and energy yielding processes in general and on mechanisms strictly related to the cell membrane (*e.g.* the adenyl cyclase system (29) and postulated fuel receptors (10)) must be considered.

Despite the uncertainties surrounding the regulation of glucose suppression of α -cells in the normal and diabetic states, it seems to us that the diabetic syndrome can be fully explained by a primary lesion to the β -cells. Disturbances of the glucagon system are most likely secondary and are ameliorated by treatment with insulin.

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J. Biol. Chem. 1976, 251:6053-6061.

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