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Metabolic Fate of Glucose in Purified Islet Cells

GLUCOSE-REGULATED ANAPLEROSIS IN β CELLS*

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Previous studies in rat islets have suggested that anaplerosis plays an important role in the regulation of pancreatic β cell function and growth. However, the relative contribution of islet β cells *versus* non-β cells to glucose-regulated anaplerosis is not known. Furthermore, the fate of glucose carbon entering the Krebs cycle of islet cells remains to be determined. The present study has examined the anaplerosis of glucose carbon in purified rat β cells using specific ^{14}C-labeled glucose tracers. Between 5 and 20 mM glucose, the oxidative production of CO_2 from [3,4-^{14}C]glucose represented close to 100% of the total glucose utilization by the cells. Anaplerosis, quantified as the difference between $^{14}CO_2$ production from [3,4-^{14}C]glucose and [6-^{14}C]glucose, was strongly influenced by glucose, particularly between 5 and 10 mM. The dose dependence of glucose-induced insulin secretion correlated with the accumulation of citrate and malate in β(INS-1) cells. All glucose carbon that was not oxidized to CO_2 was recovered from the cells after extraction in trichloroacetic acid. This indirectly indicates that lactate output is minimal in β cells. From the effect of cycloheximide upon the incorporation of ^{14}C-glucose into the acid-precipitable fraction, it could be calculated that 25% of glucose carbon entering the Krebs cycle via anaplerosis is channeled into protein synthesis. In contrast, non-β cells (approximately 80% glucagon-producing α cells) exhibited rates of glucose oxidation that were ⅓ to ⅙ those of the total glucose utilization and no detectable anaplerosis from glucose carbon. This difference between the two cell types was associated with a 7-fold higher expression of the anaplerotic enzyme pyruvate carboxylase in β cells, as well as a 4-fold lower ratio of lactate dehydrogenase to FAD-linked glycerol phosphate dehydrogenase in β cells *versus* α cells. Finally, glucose caused a dose-dependent suppression of the activity of the pentose phosphate pathway in β cells. In conclusion, rat β cells metabolize glucose essentially via aerobic glycolysis, whereas glycolysis in α cells is largely anaerobic. The results support the view that anaplerosis is an essential pathway implicated in β cell activation by glucose.

Pancreatic β cells are equipped with a sensing device that measures the levels of circulating nutrients by processes requiring cellular uptake and metabolism (Refs. 1 and 2; reviewed in Refs. 3–5). D-Glucose elicits insulin secretion only when extracellular levels exceed the basal threshold value of 3 mM (6). This feature has been largely attributed to the enzyme glucokinase, which is rate-limiting for overall glucose consumption in β cells from rat (7) and human (8) islets of Langerhans. Although targeted gene disruption in mice (9) and mutations in human diabetes (10) have strengthened the concept that glucokinase is a glucose-sensing protein, the following evidence indicates that (post)mitochondrial events are important for glucose signaling (4, 5). First, up to 80% of glucose carbon is oxidized in β cells; this is a very high fraction when compared with other cell types (11, 12). Moreover, the fraction of total glucose utilization that is further oxidized to CO_2 increases in rat β cells (13) or isolated islets (14) when glycolysis accelerates, whereas this ratio decreases in most cell types (the Crabtree effect; see Ref. 14). Second, glucose utilization in β cells does not accelerate under anaerobic conditions (the Pasteur effect), which has been explained by low lactate dehydrogenase (LDH)[1] and high mitochondrial FAD-linked glycerol-3-phosphate dehydrogenase (mGPDH) expression (12, 15). Third, the dimethyl ester of succinate, which is converted to succinate and oxidized in the Krebs cycle, is a potent "anaplerotic" secretagogue (4, 16). Fourth, islets cultured at low glucose levels (17) or in the presence of palmitate (18) exhibited parallel suppression of glucose-induced insulin release and down-regulation of pyruvate dehydrogenase (PDH). Moreover, PDH activity is acutely responsive to glucose (19). Fifth, glucose signaling in the mouse insulinoma cell line MIN6 is severely blunted by knocking out its mitochondrial genome (20).

The nature of the metabolic signals required for the activation of insulin release remains largely unknown. An increase in glucose metabolism causes a rise in islet [ATP]/[ADP] (2). This results in the closure of K_{ATP} channels (21), membrane depolarization, and influx of Ca^{2+}, which is required for exocytosis (3). Nonetheless, electrophysiological studies have suggested that other glucose-derived signals are required for exocytosis as well (22). This study explores the concept that anaplerosis is one of the K_{ATP}-independent signal-generating pathways for glucose-induced insulin release (4, 23, 24). Anaplerosis, *i.e.* filling up the Krebs cycle with intermediates that are channeled into anabolic pathways (4), requires conversion of pyruvate into oxaloacetate by pyruvate carboxylase (PC). Metabolic flux through this enzyme is quantitatively important in rat

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[1] The abbreviations used are: LDH, lactate dehydrogenase; ACC, acetyl-CoA carboxylase; mGPDH, FAD-linked glycerol-3-phosphate dehydrogenase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; TCA, trichloroacetic acid.

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islets (25) due to the abundant expression of PC (23, 24). Stimulated flow of intermediates from the Krebs cycle into synthesis of coupling factors or macromolecules is believed to sustain stimulus-secretion coupling and β cell growth. One of the hypothetical coupling factors is malonyl-CoA, which is produced by acetyl-CoA carboxylase (ACC). Glucose rapidly increases citrate content of the cells, which in turn may activate ACC (23). Chronic stimulation with glucose increases ACC mRNA abundance in INS-1 cells (26). Because ACC activity in islets is ten times higher than fatty acid synthase activity (23), malonyl-CoA accumulates rapidly in the cells (27). Thus, malonyl-CoA, the physiological inhibitor of carnitine palmitoyltransferase I (28), may act as a metabolic coupling factor in β cell signaling (4, 5, 29, 30). Anaplerosis might also be instrumental in accelerating the pyruvate/malate shuttle (24), which results in the formation of cytosolic NADPH. Finally, because glucose acutely enhances both transcription and translation in β cells, anaplerosis is expected to be required for *de novo* synthesis of metabolic precursors for regulated gene expression and growth.

Our recent work (31, 32) has shown that glycolysis accelerates proportionally to extracellular glucose between 1 and 10 mM in purified β cells and non-β cells. Moreover, when differences in cellular volume are taken into account, the rate of glycolysis is similar in β cells and non-β cells (31). This similarity can be explained by the presence of glucokinase in both β cells and α cells (32) and by the fact that glucose transport is not rate-limiting for glucose metabolism (31). How can glucose, which is metabolized similarly in glycolysis, activate β cells while it inhibits α cell function? Part of the answer to this question may be found by considering anaplerosis in α cells and β cells. Indeed, it is intriguing that about 40% of glucose-derived carbon entering the citric acid cycle is carboxylated in rat islets (33). This extent of anaplerotic input is unusually high for a non-gluconeogenic (34) and non-lipogenic (23) tissue. Thus, approximately 40% of the carbons of glucose that enter the krebs cycle must leave it for a non-oxidative fate, which remains to be assessed. This consideration and the fact that the major anaplerotic enzyme PC is expressed at very high levels in rat islets (24, 35) and β(INS-1) cells (23) suggest an important function for pyruvate carboxylation in the β cell.

To further extend our understanding of the role of anaplerosis in islet tissue, we have endeavored to provide answers to the following three questions: (i) is glucose-induced anaplerosis a unique property of β cells or is it also present in islet non-β cells? (ii) Can the magnitude of glucose-induced anaplerosis in β cells be quantified? (iii) What is the fate of the glucose carbon entering the mitochondria as pyruvate but escaping the Krebs cycle? We addressed these questions by comparing glucose regulation of anaplerosis in fluorescence-activated cell sorter-purified β cells and non-β cells (approximately 80% glucagon-producing α cells; Ref. 36). The present study demonstrates that glucose metabolism distal from pyruvate differs markedly in β cells *versus* non-β cells.

EXPERIMENTAL PROCEDURES

Materials—Labeled compounds were purchased from Amersham Corp. (D-[5-^{3}H]glucose (13 Ci/mmol), D-[U-^{14}C]glucose (292 mCi/mmol), D-[1-^{14}C]glucose (54 mCi/mmol), D-[6-^{14}C]glucose (56 mCi/mmol), L-[3,5-^{3}H]tyrosine (52 Ci/mmol), $NaH^{14}CO_3$ (54 mCi/mmol), and ^3H_2O (5 mCi/ml)) or from NEN Life Science Products (D-[3,4-^{14}C]glucose (54 mCi/mmol)). Other chemicals were from Sigma or Merck (Darmstadt, Germany).

Islet Cell Preparation and Culture of INS-1 Cells—Rat islet β and non-β cells were purified from adult male Wistar rats by autofluorescence-activated cell sorting, as has been described previously (36). Purity of the β cell preparations was more than 90%, as determined by immunocytochemistry for insulin or by electron microscopical analysis. Non-β cells were composed of >80% α cells, 5–10% β cells, and 10–15% other cells. INS-1 cells (37) were cultured at 37 °C in 95% air-5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc., Paisley, United Kingdom), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. The attached cells were harvested from culture dishes (Nunc, Roskilde, Denmark) by a 5-min treatment with Mg^{2+}/Ca^{2+}-free phosphate-buffered saline, pH 7.4, containing 0.025% (w/v) trypsin (Boehringer Mannheim).

Measurement of D-Glucose Metabolism—Glucose metabolism was measured in batches of freshly isolated cells (5×10^4 β cells and 1×10^5 non-β cells) over 2-h incubations at 37 °C in 100 μl of Earle's-Hepes buffer (36) containing the indicated concentrations of glucose. Conversion of [5-^{3}H]glucose (50 μCi/ml; specific activities, 1.3–25 Ci/mol) to ^3H_2O was measured simultaneously with production of CO_2 from ^{14}C-labeled D-glucose tracers (11). Total radioactivity added to the cells was 50 μCi/ml for [1-^{14}C]glucose and [6-^{14}C]glucose, 25 μCi/ml for [U-^{14}C]glucose, and 2–5 μCi/ml for [3,4-^{14}C]glucose, resulting in specific activities of 0.1–50 Ci/mol. Cellular metabolism was stopped by the addition of 20 μl of 0.4 mol/liter citrate buffer, pH 4.9, containing 5 mM KCN, 10 μM antimycin A, and 10 μM rotenone. Hydroxyhyamine (Hewlett-Packard) was used to capture the produced $^{14}CO_2$. Tritiated water and $^{14}CO_2$ production were measured via liquid scintillation counting. Calculations of glucose oxidation took into account the CO_2 recovery (89 ± 1%), which was assessed by adding 0.05 μCi $NaH^{14}CO_3$ to separate incubation vials without cells. The contribution of the pentose phosphate pathway to total glucose utilization was calculated from the specific yields of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose oxidation as has been explained in detail in Ref. 38. The flux of glucose-related anaplerosis was quantified as the difference between [3,4-^{14}C]glucose oxidation and [6-^{14}C]glucose oxidation, which reflects the amount of glucose carbon entering the Krebs cycle without being oxidized to CO_2 (25). Cellular radioactivity accumulating during a 2-h incubation of β cells with [U-^{14}C]glucose was measured after the cells were washed four times with 0.8 ml of incubation medium, sonication in either 1 M perchloric acid or 10% TCA, and liquid scintillation counting of radioactivity in the acid-soluble and acid-precipitable fractions.

Measurement of Insulin Secretion and Citrate and Malate Accumulation in INS-1 Cells—Cells were grown in 21-cm^2 Petri dishes in regular RPMI medium at 11 mM glucose. They were then preincubated for 3 days in culture medium at 5 mM glucose because we have previously shown that such protocol allows a robust secretory response to high glucose (26). The culture medium was removed, and attached cells (8×10^6) were washed twice with phosphate-buffered saline and preincubated for 30 min at 37 °C in Krebs-Ringer bicarbonate medium (KRB) containing 10 mM Hepes (pH 7.4), 0.07% bovine serum albumin, and 4 mM glucose. Cells were then washed twice with phosphate-buffered saline and incubated for 30 min in KRB-Hepes medium containing 0.07% bovine serum albumin and different glucose concentrations. Incubation media were collected to determine insulin release (23). For measurements of citrate and malate accumulation, cells were scraped from the dishes after addition of 10% TCA. Precipitated proteins were removed by centrifugation, and the supernatants were extracted five times with ether. Samples were lyophilized and stored at −80 °C. The assays of citrate and malate are described in detail in Ref. 39.

Quantification of PC Abundance in β Cells and Non-β Cells—The expression level of PC was measured as described previously (23) by direct streptavidin blotting. Samples corresponding to 25 μg of protein from INS-1 cells, 2×10^5 flow-sorted β cells, and 6.6×10^5 non-β cells were loaded per lane on 5% SDS-polyacrylamide gels. As verified in each experiment by Ponceau red staining, the amount of total protein load per lane was similar for the three cell types.

Protein Biosynthesis—The effect of cycloheximide (40 μM) on total protein synthesis was assessed by incubating purified β cells for 2 h at 10 mM glucose in the presence of L-[3,5-^{3}H]tyrosine (250 μCi/ml; 4.8 μM). The amount of newly synthesized protein was assessed by precipitating cellular extracts in 10% TCA followed by liquid scintillation counting (40). For the incorporation of [U-^{14}C]glucose into proteins, batches of 1.5×10^5 β cells were incubated for 2 h at 37 °C in 100 μl of Earle's-Hepes buffer containing 25 μCi/ml [U-^{14}C]glucose (concentration, 10 mM; specific activity, 2.5 Ci/mol) either without or with 40 μM cycloheximide. Cells were washed four times with incubation medium to remove the excess of extracellular tracer and homogenized in 10% TCA. The amount of newly synthesized protein was assessed by liquid scintillation counting of the TCA precipitates (40) and calculated as the difference between control cells and cells incubated with cycloheximide.

Determination of LDH and mGPDH Activities in β Cells and Non-β

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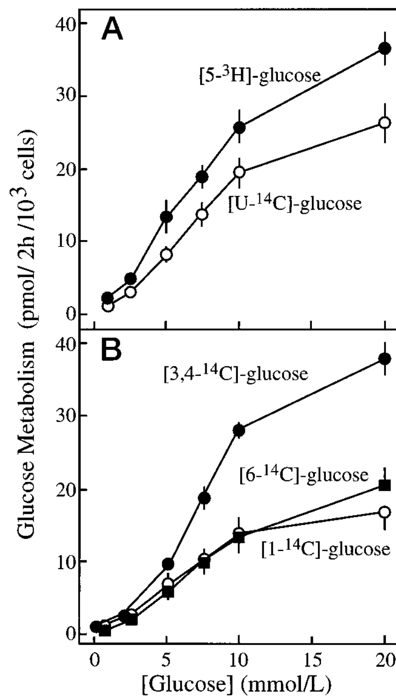

FIG. 1. **D-glucose metabolism in rat pancreatic β cells assessed by using various glucose tracers.** Results (mean values ± S.E. of 5–7 experiments) are expressed as picomoles of glucose residues utilized or oxidized per 2 h per 10^3 β cells. *A:* ●, total glucose utilization, measured as ^3H_2O production from [5-^{3}H]glucose; ○, total glucose oxidation, measured as $^{14}CO_2$ production from [U-^{14}C]glucose. *B,* glucose oxidation using different labeling positions of glucose carbon. ○, [1-^{14}C]glucose; ■, [6-^{14}C]glucose; ●, [3,4-^{14}C]glucose.

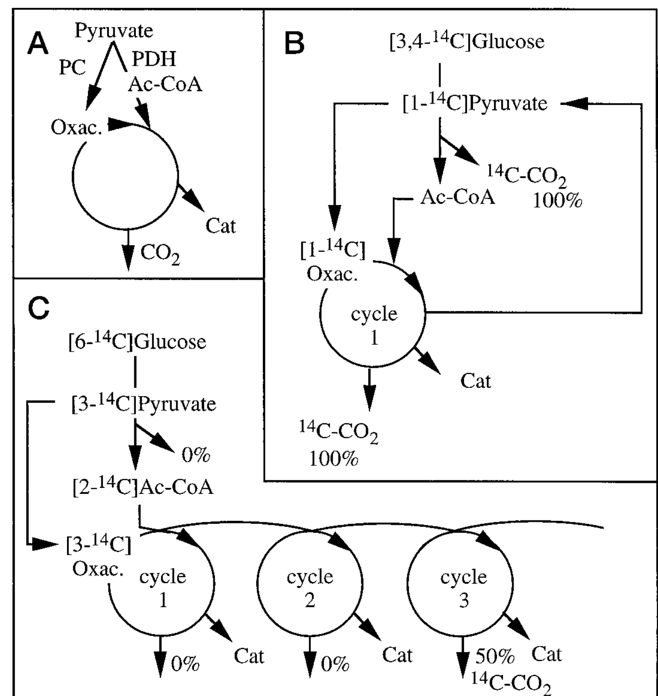

FIG. 2. **Differences in $^{14}CO_2$ production from [3,4-^{14}C]glucose and [6-^{14}C]glucose.** *A,* when anaplerosis brings extra glucose carbon into the Krebs cycle via PC, a dilution of ^{14}C label among the citric acid cycle intermediates occurs. In addition, anaplerotic input must be matched by a cataplerotic output (*Cat*) of citric acid cycle intermediates of similar magnitude. *B,* the dilution of the carbon tracer and cataplerosis are not apparent when [3,4-^{14}C]glucose is used as substrate, because (*i*) all of the [1-^{14}C]pyruvate oxidized by PDH produces $^{14}CO_2$ and unlabeled acetyl-CoA; and (*ii*) all of the labeled oxaloacetate (*Oxac*) formed from [1-^{14}C]pyruvate via the PC reaction either loses its radioactivity during the first round of the Krebs cycle or is reconverted to [1-^{14}C]pyruvate via the pyruvate/malate or pyruvate/citrate shuttles. *C,* the dilution of the radioactive tracer among the Krebs cycle intermediates following anaplerosis is important with [6-^{14}C]glucose, which produces [3-^{14}C]pyruvate, [2-^{14}C]acetyl-CoA, and [3-^{14}C]oxaloacetate. The latter two compounds must cycle at least twice before $^{14}CO_2$ is produced. For instance, in the absence of anaplerosis, the yield of $^{14}CO_2$ from [2-^{14}C]acetyl-CoA is 0% during the first and second cycles, 50% in the third cycle, and 50% of the remaining radioactivity in subsequent cycles. In the presence of anaplerosis, ^{14}C-metabolites escape the Krebs cycle during their first turn in the cycle. Thus, the calculated difference between the rates of [3,4-^{14}C]glucose and [6-^{14}C]glucose oxidation reflects the cataplerotic output of intermediates. Cataplerosis must be quantitatively identical to anaplerosis because citric acid cycle intermediates are not a sink for glucose carbon.

Cells—Measurement of LDH activity in purified β and non-β cells was done as described by Sekine *et al.* (12) with minor modifications. Pellets from 3×10^5 cells were suspended in 10 mM Tris-HCl buffer, pH 7.0, containing 2 mM EDTA, 1 mM DTT and lysed by three cycles of freeze-thawing. Enzymatic activity was determined in the supernatant fraction after centrifugation (2 min at $10^4 \times g$) at 4 °C in 20 mM Hepes buffer, pH 7.2, in the presence of 0.05% (w/v) bovine serum albumin (fraction V; Sigma), 20 μM NADH, and 2 mM pyruvate (monosodium salt, freshly prepared solution); the consumption of NADH was measured in a Hitachi F-2000 fluorometer (Hitachi, Tokyo, Japan) at 340/460 nm. Determination of mGPDH (EC 1.1.99.5) was performed as described by MacDonald (15) using batches of 2×10^5 β cells and non-β cells.

RESULTS

The Use of Different Glucose Tracers to Measure Glucose Metabolism in Purified β Cells—Glucose utilization in β cells, as assessed from the production of ^3H_2O from [5-^{3}H]glucose, was markedly dependent on the extracellular glucose concentration (Fig. 1*A*). Confirming our previous observations in flow-sorted β cell subsets (11), the fraction of total glucose utilization that was further oxidized to CO_2 was high using [U-^{14}C]-labeled glucose as substrate: 69% at 1 mM glucose, 75% at 5 mM glucose, and 82% at 10 mM glucose. Because data obtained with this tracer reflect mean oxidation rates of all six carbon atoms of glucose, no information can be retrieved about oxidative pathways that are preferentially used by the cells. We therefore incubated β cells with glucose tracers that were labeled at specific carbon atoms. Oxidation of [6-^{14}C]glucose to $^{14}CO_2$, reflecting Krebs cycle activity at the level of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, increased 30 times when medium glucose was raised from 1 to 20 mM (Fig. 1*B*). However, the specific yield of $^{14}CO_2$ from [6-^{14}C]glucose was much lower than that of [U-^{14}C]glucose. To explain this difference, we considered two possibilities. First, oxidative pathways in addition to the Krebs cycle, in particular the pentose phosphate pathway, contribute to total glucose oxidation in β cells. Second, high anaplerotic input into the cycle provides a possible explanation for this phenomenon. Thus, with respect to glucose labeled in position 6, anaplerosis allows dilution of the [^{14}C] tracer among Krebs cycle intermediates at a carbon position that does not yield $^{14}CO_2$ during the first and second round in the cycle. In addition, some labeled intermediates escape the cycle (cataplerosis; Fig. 2*A*) during the first two rounds. By contrast, uniformly labeled glucose yields labeled CO_2 during the first two cycle rounds.

To examine the first possibility, we compared [1-^{14}C]glucose and [6-^{14}C]glucose oxidation. Whereas both [1-^{14}C]glucose and [6-^{14}C]glucose enter the citric acid cycle as [2-^{14}C]acetyl-CoA, only [1-^{14}C]glucose produces $^{14}CO_2$ during the oxidative part of the pentose phosphate pathway (38). Fig. 1*B* shows that the specific yield of $^{14}CO_2$ obtained after incubating the cells with [1-^{14}C]glucose and [6-^{14}C]glucose was almost the same over the whole tested range of glucose concentrations. This indicates that the oxidative flux through the pentose phosphate pathway is very low and is detectable only when cells were exposed to 5

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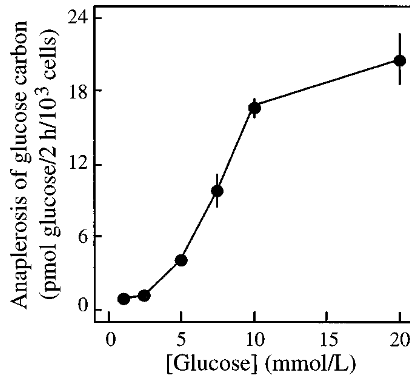

FIG. 3. **Difference between [3,4-^{14}C]glucose oxidation and [6-^{14}C]glucose oxidation in β cells.** This difference reflects the fraction of pyruvate (de)carboxylation that escapes from further oxidation in the citric acid cycle (see Fig. 2). Results (mean values ± S.E.; n = 5) are from the same experiments as in Fig. 1.

mM substrate or less (range between 0.3 and 0.6 pmol of glucose equivalents/2 h/10^3 cells). The contribution of the pentose phosphate pathway to overall glucose utilization declined with rising glucose levels: 18 ± 4%, 10 ± 3%, and 6 ± 5% at 1, 2.5, and 5 mM glucose, respectively, and below detection limit at 7.5 mM glucose or higher. Low contribution of the pentose phosphate pathway to overall glucose utilization has been described in rodent islets (25, 41), but acute suppression of this pathway by elevated glucose has not, to our knowledge, been reported before.

A consequence of a low contribution of the pentose phosphate pathway to overall glucose utilization is that [3,4-^{14}C]glucose will be converted to [1-^{14}C]pyruvate (Fig. 2*B*). [1-^{14}C]Pyruvate enters the mitochondria, where it is either decarboxylated by PDH, yielding $^{14}CO_2$, or carboxylated by PC, yielding [1-^{14}C]oxaloacetate (Fig. 2*B*). This intermediate enters the Krebs cycle, where it produces $^{14}CO_2$ during the first cycle or exits from the cycle to reconvert to [1-^{14}C]pyruvate via the citrate-pyruvate or pyruvate-malate shuttles (Fig. 2*B*). Therefore, even in the presence of anaplerosis, almost all of the label in [3,4-^{14}C]glucose that is converted to [1-^{14}C]pyruvate is recovered in the form of $^{14}CO_2$ (Fig. 2*B*).

The production of $^{14}CO_2$ from [3,4-^{14}C]glucose rose 25-fold upon increasing glucose from 1 to 20 mM (Fig. 1*B*). The rate of [3,4-^{14}C]glucose oxidation was higher than [U-^{14}C]glucose oxidation. Second, in the range of 5–20 mM glucose, almost all glucose that was utilized was further oxidized as can be seen when glucose usage is compared with [3,4-^{14}C]glucose oxidation (Fig. 1, *A* and *B*). Third, the rate of [3,4-^{14}C]glucose oxidation was about 2-fold higher than oxidation of [1-^{14}C]glucose or [6-^{14}C]glucose. As discussed above, anaplerosis may explain why the production of $^{14}CO_2$ from [6-^{14}C]glucose is relatively low in comparison to that observed with [3,4-^{14}C]glucose. Thus, glycolytic conversion of [6-^{14}C]glucose to [3-^{14}C]pyruvate results in the production of [2-^{14}C]acetyl-CoA, which requires two complete cycles before any $^{14}CO_2$ is produced (Fig. 2*C*). When no anaplerosis occurs, the specific activity of all Krebs cycle intermediates rises, until the input of radioactivity as [2-^{14}C]acetyl-CoA equals that of $^{14}CO_2$-production. In that case, no difference with $^{14}CO_2$-production from [3,4-^{14}C]glucose is detected. However, when anaplerosis occurs, extra glucose carbon entering the cycle as [3-^{14}C]oxaloacetate will be diverted into anabolic pathways (cataplerosis; Fig. 2). In that case, radioactivity entering both as [2-^{14}C]acetyl-CoA and [3-^{14}C]oxaloacetate has ample time to escape oxidation. Therefore, the difference between $^{14}CO_2$ production from [3,4-^{14}C]glucose and [6-^{14}C]glucose is an index of the anaplerotic flux of glucose carbon into the Krebs cycle. As indicated in Fig. 3, differences between [3,4-^{14}C] glucose oxidation and [6-^{14}C]glucose oxidation exhibited a sigmoidal substrate concentration dependence with half-maximal effect at 7.5 mM glucose. The quantitative importance of this difference and the glucose dependence between 5 and 10 mM are both apparent (Fig. 3).

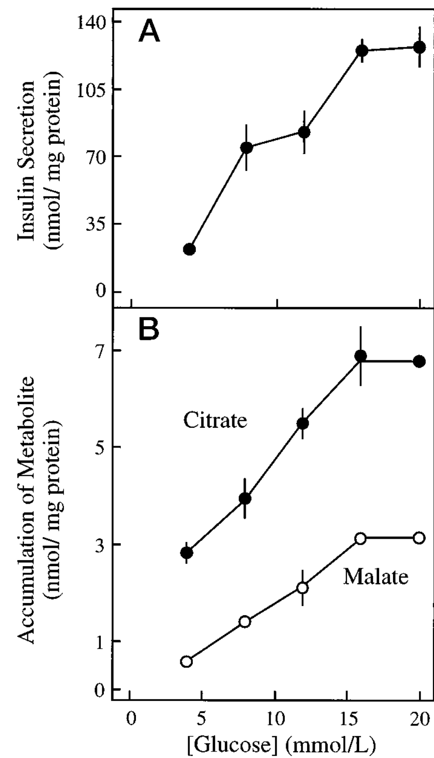

FIG. 4. **Insulin release and accumulation of citrate and malate in INS-1 cells.** Results represent mean values ± S.E. of four experiments. *A*, insulin secretion; *B*, cellular accumulation of citrate (●) and malate (○). Cells (8 × 10^6) were incubated for 30 min at the indicated concentrations of glucose.

In summary, two conclusions emerge from this series of experiments. First, the ratio of glucose oxidation to glucose utilization (in the range of 5–20 mM glucose) is close to 1 in β cells, when the production of ^3H_2O from [5-^{3}H]glucose is compared with [3,4-^{14}C]glucose oxidation. Second, an important difference exists between the degree of oxidation of carbons 3 and 4 of glucose *versus* that of carbons 1 and 6. This difference can be explained by a high anaplerotic input of glucose carbon into the citric acid cycle.

Glucose-induced Accumulation of Citrate and Malate in INS-1 Cells—Fully compatible with the view that pyruvate carboxylation is high in β cells are the measurements of the INS-1 cell content of citrate and malate (Fig. 4). INS-1 cells were used for these experiments because the low amount of available material obtained precluded the same measurements in flow-sorted islet β cells. It should be emphasized that the rates of glucose utilization and oxidation of INS-1 cells are similar to fluorescence-activated cell sorter-purified β cells (data not shown; see also Ref. 12). Fig. 4 shows that glucose caused a dose-dependent rise in the cellular contents of citrate and malate, which closely correlated to the dose dependence of glucose-induced insulin release. Half-maximal and maximal effects of glucose on insulin secretion and the accumulation of both metabolites were observed at 10 and 16 mM glucose, respectively.

Comparison of D-Glucose Utilization and Oxidation in Purified β Cells and Non-β Cells—We next assessed the cell specificity of the metabolic organization in β cells by comparing

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TABLE I
Glucose metabolism in purified rat β cells and non-β cells

Different glucose tracers were used to assess glucose metabolism. ^3H_2O production from [5-^{3}H]glucose reflects glucose utilization; $^{14}CO_2$ production from [3,4-^{14}C]glucose and [6-^{14}C]glucose reflects oxidation at the level of PDH and the Krebs cycle, respectively. To facilitate comparison between β cells and non-β cells, metabolic flux was expressed per liter of cellular space, which was determined previously (31) for purified β cells (0.62 ± 0.05 nl/10^3 cells) and non-β cells (0.27 ± 0.03 nl/10^3 cells). Data represent mean values ± S.E. of n experiments. Significance of differences was calculated using the unpaired Student's t test.

Metabolic assay	Metabolic flux			
	β cells (n = 9)	Non-β cells (n = 3)	β cells	Non-β cells
	mmol/liter × 2 h		*% of glucose utilization*	
[5-^{3}H]glucose utilization				
1 mM glucose	4.3 ± 0.6	7.4 ± 0.2[a]		
10 mM glucose	50 ± 6	48 ± 9		
[3,4-^{14}C]glucose oxidation				
1 mM glucose	2.4 ± 0.2[b]	1.1 ± 0.2[c]	56	15
10 mM glucose	45 ± 2[b]	7.4 ± 2[d]	90	15
[6-^{14}C]glucose oxidation				
1 mM glucose	1.1 ± 0.1	1.5 ± 0.4	26	20
10 mM glucose	24 ± 3	11 ± 2[a]	48	23

[a] Difference between β cells and non-β cells: $P < 0.05$.
[b] Difference between [3,4-^{14}C]glucose oxidation and [6-^{14}C]glucose oxidation in β cells: $P < 0.001$.
[c] Difference between β cells and non-β cells: $P < 0.01$.
[d] Difference between β cells and non-β cells: $P < 0.001$.

glucose oxidation and utilization in islet β cells and non-β cells (approximately 80% α cells; Ref. 36). When normalized for the differences in cellular volume, glycolytic activity at 10 mM glucose was the same in β cells and non-β cells (Table I). This similarity has been described before (31) and was explained by the presence of glucokinase in both β cell and non-β cell preparations (32). In contrast to the comparability in rates of total glucose utilization, three important differences between β cells and non-β cells were noted at the level of mitochondrial glucose metabolism. First, rates of glucose oxidation were different in the two cell types, particularly at high glucose levels (Table I). For [3,4-^{14}C]glucose oxidation, this difference was already noted at 1 mM substrate; increasing the glucose level to 10 mM accelerated [3,4-^{14}C]glucose oxidation 20-fold in β cells as compared with only 7-fold in non-β cells. Using [6-^{14}C]glucose as a substrate, no differences between β cells and non-β cells were noted at 1 mM, but oxidation was again more accelerated at 10 mM glucose in β cells than in non-β cells. Second, the ratios of glucose oxidation to total glucose utilization were high in β cells and increased with rising substrate concentrations, whereas in non-β cells these ratios were much lower and were independent of the substrate concentration. The difference was the largest for the ratio of [3,4-^{14}C]glucose oxidation to total glucose utilization, which was up to 6 times higher in β cells than in non-β cells. Third, rates of [3,4-^{14}C]glucose oxidation exceeded those of [6-^{14}C]glucose oxidation in β cells approximately by a factor of 2, whereas in non-β cells, no differences were observed. This indicates that pyruvate carboxylation in non-β cells was below the detection limit of our experimental system.

Together, these data indicate that glucose metabolism in islet α and β cells is similar at the level of glycolysis but diverges markedly beyond pyruvate formation. In β cells, aerobic glycolysis is followed almost exclusively by pyruvate channeling to the mitochondrion. A major extent of the pyruvate carbons enter the citric acid cycle by anaplerosis in β cells. By contrast, glycolysis in non-β cells is mostly anaerobic, and anaplerosis with glucose-derived carbons is extremely low in these cells.

Expression of Lactate Dehydrogenase, FAD-linked Glycerol-3-Phosphate Dehydrogenase, and Pyruvate Carboxylase in Purified β Cells and Non-β Cells—Low LDH activity and high expression of mGPDH in flow-sorted rat β cells has been described before (12) and was considered important for the strictly aerobic mode of glycolysis in these cells. Because flow-sorted non-β cells exhibit a much more anaerobic glycolysis than β-cells (Table I) and because mGPDH has not yet been determined in these cells, we felt that the data of Sekine *et al.* (12) needed confirmation, with the extension of measuring mGPDH activity in non-β cells. As Table II shows, LDH activity was approximately 2-fold higher in non-β cells than in β cells. Furthermore, mGPDH activity was 2-fold higher in β cells. Consequently, the LDH/mGPDH activity ratio was 4 times more elevated in non-β cells as compared with β cells ($p < 0.01$), favoring anaerobic glycolysis in non-β cells and and aerobic glycolysis in β cells.

PC is the major anaplerotic enzyme in mammalian cells (4) and is very abundant in islet tissue, accounting for up to 0.4% of total islet protein (35). However, the distribution of PC among different islet cells is not known. The results in Fig. 5 indicate that PC is expressed at a high level in both INS-1 cells and purified rat β cells, whereas on the other hand, the enzyme is not abundant in flow-sorted rat non-β cells. Densitometric analysis of the results from three independent cell preparations in each group indicated that PC abundance is about 7-fold higher in β cells than in non-β cells. Taking into account that the average volume of β cells is 2.5 times that of non-β cells and that 3-fold more non-β cells were loaded on the polyacrylamide gels, it can be estimated that the β cells contain, on the average, about 20 times (7 × 3) more PC than non-β cells. Knowing that non-β cells are contaminated to the extent of 5% by β cells, the difference between the expression level of PC between α cells and β cells is likely much larger. Thus, the data in Fig. 5 are in accordance with the observed high anaplerosis of glucose-derived carbon in β cells only.

Protein Synthesis from Glucose Carbon—To investigate the metabolic fate of glucose carbon that is utilized during glycolysis but escapes mitochondrial oxidation to CO_2, purified β cells were labeled for 2 h with [U-^{14}C]glucose, washed, and homogenized in either 1 M perchloric acid or 10% TCA. The effect of glucose upon the accumulation of ^{14}C-labeled molecules was more pronounced in the acid precipitate than in the acid-soluble fraction: mean values ± S.E. at 1 and 10 mM glucose from 3 experiments were 0.5 ± 0.1 and 4.2 ± 0.4 pmol glucose equivalents/10^3 cells, respectively, for the perchloric acid supernatants (8-fold difference) and 0.1 ± 0.02 and 5.1 ± 0.6 pmol glucose equivalents/10^3 cells for the perchloric acid pellet (50-fold difference). Total accumulation of ^{14}C-labeled molecules (pmol glucose equivalents) in the cellular extracts (TCA supernatant plus pellet) was the same as the difference between glucose utilization and CO_2 production (Table III).

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TABLE II
Lactate dehydrogenase and FAD-linked glycerol-3-phosphate dehydrogenase activities in purified rat β cells and non-β cells

LDH and mGPDH activities were measured as described under "Experimental Procedures." To facilitate comparison between β cells and non-β cells, enzymatic activities were expressed per liter of cellular space (see legend of Table I). Data represent mean values ± S.E. of three different experiments.

Enzyme activity	β cells	Non-β cells
LDH (mmol of NADH consumed × liter^{-1} × min^{-1})	4.6 ± 0.5	8.6 ± 1.5
mGPDH (mmol of dye reduced × liter^{-1} × min^{-1})	9.3 ± 0.6	4.0 ± 0.4[a]
LDH/mGPDH ratio	0.5 ± 0.1	2.1 ± 0.4[a]

[a] Significance of differences between β cells and non-β cells was calculated by the unpaired Student's *t* test. $P < 0.01$.

This indirectly confirms the concept (12) that very little lactate is produced and exported by these cells. Because D-glucose has a profound stimulatory effect on protein synthesis in pure β cells, even in the absence of exogenous amino acids (11, 40), a fraction of the anaplerotic/cataplerotic carbon flux from [^{14}C]glucose into the TCA precipitable material may represent newly synthesized proteins. To estimate the size of this anabolic pathway, purified β cells were incubated at 10 mM glucose with cycloheximide, which inhibited 95% of total protein synthesis (Table III). Glucose utilization and oxidation remained unchanged in the presence of cycloheximide. However, the ^{14}C radioactivity recovered from the TCA pellet was reduced by about 50% of the control value in the presence of the protein synthesis inhibitor ($p < 0.01$) (Table III). Thus, glucose-derived carbon entering the citric acid cycle via anaplerosis is channeled into glucose-stimulated synthesis of proteins. This anabolic pathway accounts for up to 50% of the acid-precipitable ^{14}C radioactivity accumulating in β cells and 25% of the glucose carbon entering the Krebs cycle via anaplerosis.

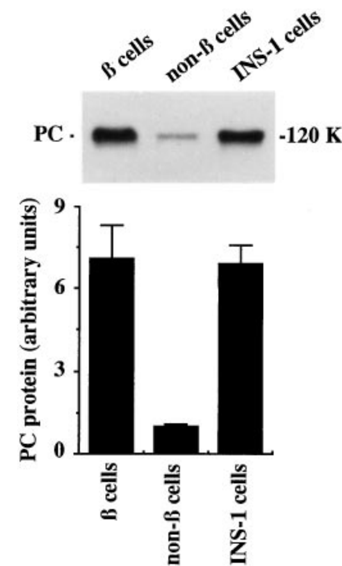

FIG. 5. **Pyruvate carboxylase protein abundance in rat pancreatic β cells, non-β cells, and INS-1 cells.** *Top panel,* representative blot of cellular protein extracted from from β cells, non-β cells, and INS-1 cells. PC abundance was assessed via the peroxidase-streptavidin technique (24). *Bottom panel,* mean results ± S.E. of densitometric scanning of 4–7 experiments.

TABLE III
Effect of cycloheximide on protein synthesis, glucose utilization, and [U-^{14}C]glucose metabolism in β cells

Measurements of protein synthesis, glucose utilization, glucose oxidation, and ^{14}C recovery were performed after 2 h incubation of purified rat β cells at 10 mM glucose, as described in "Experimental Procedures." Data represent mean values ± S.E. of three (protein synthesis) or five (metabolic flux) individual experiments.

Assay	Control	Cycloheximide (40 μM)
Total protein synthesis (cpm/cell × 2 h)		
	44 ± 2	2.4 ± 0.1[a]
Metabolic flux (pmol glucose/2 h × 10^3 cells)		
Glucose utilization	33.9 ± 2.3	34.7 ± 2.0
Glucose oxidation	24.1 ± 1.4	22.2 ± 1.7
^{14}C in TCA supernatant	5.2 ± 0.6	7.0 ± 0.9
^{14}C in TCA pellet	5.9 ± 0.7	3.3 ± 0.4[a]

[a] Significance of differences between control cells and cycloheximide-treated cells was calculated with the unpaired Student's *t* test. $P < 0.01$.

DISCUSSION

Glucose Metabolism Is Aerobic in Rat β Cells and Mainly Anaerobic in Non-β Cells—Our results show that glucose metabolism in rat β cells is essentially aerobic, supporting the idea (12) that lactate output is minimal in these cells. Studies using isolated islets (25, 42) reported lower ratios of glucose oxidation over glucose utilization (between 20 and 30% in most studies). We considered the possibility of oxidative glucose metabolism being an artifact of isolated β cells unlikely. First, isolated β cells are responsive to acute glucose stimulation, both in terms of glucose-regulated proinsulin biosynthesis (11) and glucose-induced insulin release (40). Second, glucose metabolism and glucose sensing are very well correlated in these cells (11). Third, whereas glucagon, somatostatin and other agents that alter cellular cyclic AMP levels markedly influence the amplitude of glucose-induced insulin secretion from isolated β cells (43), these islet hormones do not affect the rate of glucose oxidation in purified β cells (44). Fourth, oxidative glucose metabolism is not a feature of freshly isolated β cells because it is present in β cells maintained for 10 days in culture (45).

What may provide an explanation for the much lower oxidative fraction of glucose metabolism in whole islets? Anaerobic glycolysis is a likely factor, because lactate output from whole islets is much higher (2 pmol/min/islet; Ref. 42) than in purified β cells (12). Islet non-β cells (endocrine or non-endocrine) with elevated rates of anaerobic glycolysis may be responsible for this difference. On the basis of the present study, we believe that isolated islet non-β cells contribute little to lactate output from whole islets. Indeed, they represent 20% of the total islet mass and exhibit rates of glucose utilization that are very similar to those of β cells (31), whereas their oxidative capacity is indeed lower and their LDH activity is 2-fold higher than that of β cells (Table II). Contaminating cells in islets with very high rates of anaerobic glycolysis (*e.g.* exocrine cells) may be considered. Alternatively, high rates of lactate output may also originate from β cells in the centers of isolated islets that are prone to oxygen depletion and necrosis (46). Taken together, the large differences in the ratio of glucose oxidation to glucose utilization between whole islets on the one hand and flow-sorted β cells or INS-1 cells on the other hand stress the importance of the choice of the experimental model in metabolic studies related to glucose sensing.

Glucose-regulated Anaplerosis Is β Cell Specific—Anaplerosis ensures the maintenance of the pool of Krebs cycle intermediates when these are simultaneously used for biosynthetic purposes, such as synthesis of proteins, lipids, or heme (47). When fueled by glucose metabolism, anaplerosis is initiated by PC, which catalyzes the formation oxaloacetate from pyruvate. Glucose has been reported to induce the expression of the PC gene in rat islets (17). The islet abundance of PC is only equaled by gluconeogenic tissues such as the liver and kidney (23, 24). MacDonald (24) provided evidence for a new function

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of islet PC in β cell signaling, *i.e.* the rapid formation of oxaloacetate serving as a substrate for the pyruvate-malate shuttle, which provides cytosolic NADPH, a putative coupling factor. The present study provides support for this hypothesis. Thus, high PC expression and glucose-regulated anaplerosis have been observed in β cells but not in non-β cells. In addition, the dose dependence of malate accumulation in INS cells correlated closely with that of insulin release.

Measurements in purified β cells indicate that anaplerosis is quantitatively important and exquisitely regulated by glucose. In agreement with this observation is the fact that in cultured islets, approximately 60% of pyruvate entering the mitochondria is directly oxidized via PDH, whereas the remaining 40% is carboxylated by PC (33). The relative pyruvate use via PC and PDH could not be calculated in the present study because we have not measured the yields of $^{14}CO_2$ from [1,4-^{14}C] *versus* [2,3-^{14}C]succinate, [1-^{14}C] *versus* [2-^{14}C]acetate and [2-^{14}C] *versus* [6-^{14}C]glucose (33). Instead, the magnitude of anaplerosis was estimated by a radiometric method using [6-^{14}C]glucose and [3,4-^{14}C]glucose. The estimated rates of $^{14}CO_2$ production from glucose were highest when [3,4-^{14}C]glucose was used as a tracer, reaching 90–100% of total glucose consumption when glucose levels exceeded 5 mM (Fig. 1 and Table I). On the other hand, $^{14}CO_2$ production from [6-^{14}C]glucose was much lower. As is explained in detail in Fig. 2 and under "Results," anaplerosis can account for such difference. Because the estimated value of this flux is markedly regulated by glucose (Fig. 3), it can be postulated that the anaplerotic flux is correlated and perhaps even responsible for the glucose-dependent synthesis of metabolic coupling factor(s). Indeed, the concentration dependence of this flux is clearly sigmoidal and exhibits the most pronounced glucose dependence between 5 and 10 mM substrate, the range at which insulin secretion is physiologically regulated.

Interestingly, using the same radiometric technique, no anaplerotic flux of glucose carbon was detectable in purified islet non-β cells (Table I). This observation is consistent with the much lower PC protein abundance in these cells and contrasts with the similarity in total glucose utilization (31) and glucokinase expression (32) between β and non-β cells. These data are also consistent with the observation that glucose increases NADPH autofluorescence in rat β cells but not in rat non-β cells, a phenomenon that has been the basis for autofluorescence-activated separation of islet cells (36). However, the possibility cannot be discounted that glucose-induced anaplerosis and high expression of PC are present in somatostatin-secreting δ cells, which are stimulated by glucose and which constitute a small (about 10%) fraction in the sorted non-β cell preparations (36).

What Is the Fate of Glucose Carbon Entering the Krebs Cycle via Anaplerosis?—Despite the fact that it is a difficult task when studying islet cells, the present paper has attempted to delineate some pathways into which anaplerosis of glucose carbon is channeled. For this purpose the cells were labeled with [U-^{14}C]glucose and acid extracted. Whereas glucose increased the radioactivity in both the acid-soluble and acid-insoluble pools, the effect on the acid-insoluble pool was much larger. This difference may be explained as follows. The acid-soluble pool might represent a whole range of metabolites whose specific activities and concentrations rise as a consequence of accelerated glucose metabolism. In contrast, the increase in radioactivity in the acid-insoluble fraction might reflect not only the specific activities of the precursors but also the activation of biosynthetic processes by glucose. The best studied glucose-regulated biosynthesis in islet cells is that of insulin and other islet cell proteins (11, 40, 48). The results with cycloheximide indicate that glucose carbon is channeled into *de novo* synthesis of amino acids required for glucose-stimulated protein synthesis in β cells: cycloheximide prevented incorporation of 2.6 pmol/10^3 cells/2 h of glucose equivalents into TCA-precipitable material, which corresponds to 45% of radioactivity accumulating in the acid-insoluble pool. Freshly isolated β cells synthesize 67 fmol of preproinsulin/10^3 cells/2 h at 10 mM glucose (40), requiring 7.5 pmol of aminoacyl tRNA. Using [^{3}H]tyrosine and [^{3}H]histidine as markers, it was further estimated that at least the same amount of amino acid is required by purified β cells to synthesize non-insulin proteins (40), bringing the total cellular need to 15 pmol of amino acids/2 h/10^3 cells. Thus, it appears that glucose carbon contributes significantly to the pool of nonessential amino acids required for protein synthesis.

The remaining 55% of glucose carbon accumulating in the acid-insoluble fraction of the cell extracts may represent labeling of other macromolecules, such as aminoacyl tRNAs, mRNAs, lipids, and glycogen. Because chronic exposure of rat β cells to 10 mM glucose leads to modest accumulation of glycogen in the cells (45), the amount accumulating during 2 h of incubation may be small compared with overall glucose utilization. Similarly, previous studies (23, 49), as well as lipid extractions of β cells labeled with [U-^{14}C]glucose, indicate that glucose conversion into lipids represents a small fraction (5% or less) of total glucose utilization (data not shown). Furthermore, because accelerated glycolysis and glycerol phosphate production are likely to enhance esterification of unlabeled fatty acids to [U-^{14}C]glycerol phosphate (49), most of the radioactivity in the lipid extracts may be due to the glycerol part of the molecules (49) and not related to anaplerosis. With respect to the concept proposing that an "anaplerotic malonyl-CoA" pathway and lipid esterification are implicated in the short term or long term control of insulin release (4, 5, 23, 30), the present study has added one supporting piece of evidence: both the anaplerotic flux of glucose-derived carbon and the accumulation of cellular citrate, the carbon precursor of malonyl-CoA and allosteric activator of ACC (50), correlate with insulin secretion.

Pentose Phoshate Pathway in β Cells—The contribution of the pentose phosphate pathway to total glucose utilization was low, and it decreased with rising glucose levels. Previous studies (25, 41) have already pointed out the low activity of this pathway in islets. This study extends these observations by showing that low pentose phosphate pathway activity is a metabolic feature of β cells. Furthermore, the present data suggest that acceleration of glucose metabolism acutely suppresses flux through this pathway. The suppression can be explained by the following chain of events: glucose-accelerated metabolism increases anaplerosis (Ref. 25 and this study), accelerating the pyruvate/malate shuttle (24) and resulting in an increased cytosolic [NADPH]/[NADP$^+$] ratio (36, 48); this condition is likely to inhibit glucose-6-phosphate dehydrogenase, the flux-generating enzyme of the pentose phosphate pathway, as has been described in most cells (47). Thus, the dose dependence of the glucose carbon flux through the pentose pathway does not correlate with insulin release, indicating that pentose phosphate pathway intermediates are not directly implicated in the short term control of insulin secretion.

Conclusions—The organization of glucose metabolism distal from glycolysis is different in pancreatic β cells *versus* non-β cells. Glycolysis is aerobic in β cells and an important part of glucose carbon is diverted into anabolic pathways, in particular the *de novo* synthesis of amino acids and proteins. Anaplerosis is regulated by glucose in β cells only, and this correlates with the high expression level of PC in this cell type. In contrast, glycolysis is mainly anaerobic in α cells, and the low expression

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of PC and high LDH content explain why glucose-dependent anaplerosis is undetectable in these cells.

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Metabolic Fate of Glucose in Purified Islet Cells: GLUCOSE-REGULATED ANAPLEROSIS IN β CELLS

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