The Effect of Starvation on Insulin Secretion and Glucose Metabolism in Mouse Pancreatic Islets

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1. Rates of insulin secretion, glucose utilization, lactate output, incorporation of glucose into glycogen, contents of glucose 6-phosphate, fructose 1,6-diphosphate and ATP, and maximally extractable enzyme activities of hexokinase, high- K_m glucose-phosphorylating activity ('glucokinase'), glucose 6-phosphatase and unspecific acid phosphatase were measured in isolated pancreatic islets from fed and 48-h-starved mice. 2. In the fed state insulin secretion from isolated islets was increased five- to six-fold when the extracellular glucose concentration was raised from 2.5 mM to 16.7 mM; 5 mM-caffeine potentiated this effect. The secretory response to glucose of islets from mice starved for 48 h was diminished at all glucose concentrations from 2.5 mM up to approx. 40 mM. Very high glucose concentrations (60 mM and above) restored the secretory response to that found in the fed state, suggesting that the K_m value for the overall secretory process had been increased (approx. fourfold) by starvation. Addition of 5 mM-caffeine to islets from starved mice also restored the insulin secretory response to 2.5–16.7 mM-glucose to normal values. 3. Extractable hexokinase, 'glucokinase', glucose 6-phosphatase and unspecific phosphatase activities were not changed by starvation. 4. Glucose utilization and glycolysis (measured as the rate of formation of $^3\text{H}_2\text{O}$ from $[5\text{-}^3\text{H}]\text{glucose}$ over a 2 h period) was decreased in islets from starved mice at all glucose concentrations up to approx. 55 mM. At still higher glucose concentrations up to approx. 100 mM, there was no difference between the fed and starved state, suggesting that the K_m value for the rate-limiting glucose phosphorylation had been increased (approx. twofold) by starvation. Preparation of islets omitting substrates (glucose, pyruvate, fumarate and glutamate) from the medium during collagenase treatment lowered the glucose utilization measured subsequently at 16.7 mM-glucose by 38 and 30% in islets from fed and starved mice respectively. Also the 2 h lactate output by the islets at 16.7 mM extracellular glucose was diminished by starvation. Incorporation of glucose into glycogen was extremely low, but the rate of incorporation was more than doubled by starvation. 5. After incubation for 30 min at 16.7 mM-glucose the content of glucose 6-phosphate was unchanged by starvation, that of ATP was increased and the concentration of (fructose 1,6-diphosphate plus triose phosphates) was decreased. 6. Possible mechanisms behind the correlated impairment in insulin secretion and islet glucose metabolism during starvation are discussed.

Starvation results in a decreased basal plasma insulin concentration in several species and in an impairment of glucose-stimulated insulin secretion in man and rat (Solomon *et al.*, 1968; Cahill *et al.*, 1966; Bagdade *et al.*, 1969; Malaisse *et al.*, 1967; Grey *et al.*, 1970). This decreased sensitivity of the insulin secretory mechanism to glucose has also been demonstrated *in vitro* by using rat pancreas pieces (Malaisse *et al.*, 1967) and isolated rat pancreatic islets (Buchanan *et al.*, 1969).

The mechanisms whereby these alterations in insulin secretion during starvation are produced have not been established. The substrate-site hypothesis for insulin secretion (Randle *et al.*, 1968) envisages that a metabolite of glucose, rather than glucose itself, is the trigger that starts the excitation-secretion coupling process in the β -cells. It would be

consistent with this hypothesis to predict that an impaired glucose utilization in the pancreatic islets during starvation might be at least partly responsible for the observed changes in insulin secretion. However, very little is known about the rate of glucose utilization and glucose metabolism in pancreatic islets from starved animals.

In an approach to the problem *in vivo* Matschinsky *et al.* (1971) demonstrated that insulin release provoked by glucose was completely abolished in rats starved for 5–6 days. Basal concentrations of glucose 6-phosphate, 6-phosphogluconate and fructose 1,6-diphosphate in islets were lower, but the energy potential (the concentration of ATP plus creatine phosphate) was higher during starvation than in the fed state. At 5 min after glucose injection to starved animals there was a rise in the concentrations of

glucose 6-phosphate, 6-phosphogluconate and fructose 1,6-diphosphate in the islets. Concentrations did not, however, exceed those found in fed control animals. It was concluded from this and other evidence that glucose transport and glucose phosphorylation were not impaired in islets during starvation and if glucose metabolism was in fact linked with insulin release, starvation must have uncoupled glucose metabolism from release. This conclusion can be criticized on two grounds: first, the uncoupling may be related to the fact that the insulin stores are very markedly decreased after such extended starvation periods; secondly, it cannot be deduced from the results whether the glucose phosphorylation rate was diminished or not during starvation as blood glucose concentrations after injection of glucose were different in fed and starved animals.

Grey *et al.* (1970) observed that the impairment of glucose-stimulated insulin secretion in the rat during starvation could be restored by re-feeding a high-carbohydrate diet or by intermittent injection of small amounts of glucose given intraperitoneally, and further, that actinomycin D given before re-feeding blocked the return to a normal glucose-stimulated insulin release. These experiments showed that a glucose-inducible enzyme system in the β cells might be responsible for the alteration in insulin secretion during starvation, although the evidence for induction was not completely conclusive.

The present experiments were undertaken to see to what extent the decreased insulin secretory response to glucose during starvation was associated with a changed concentration in the islets of the glucose-receptor system postulated by Ashcroft & Randle (1970b) as involving a combination of hexokinase (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1), high- K_m glucose-phosphorylating activity (ATP-D-glucose 6-phosphotransferase, EC 2.7.1.2) and glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) and/or whether the rates at which these enzymes work in the pancreatic islets were changed during starvation. We have therefore, by incubation *in vitro* of islets isolated from fed and 48h-starved mice, measured insulin secretion as stimulated by glucose and as potentiated by caffeine and correlated these results with measurements of the maximally extractable activities of hexokinase, high- K_m glucose-phosphorylating enzyme activity and glucose 6-phosphatase, and in addition related the rate of insulin release to the rate of glucose utilization, lactate output, incorporation of glucose into glycogen and contents of glucose 6-phosphate, ATP and fructose 1,6-diphosphate. The results indicate that the decreased sensitivity of the insulin-secretory mechanism to glucose during starvation is associated with changes in the rate of glucose utilization, lactate output, incorporation of glucose into glycogen and the concentration of ATP, fructose

1,6-diphosphate and possibly cyclic AMP in the β cells, whereas the concentration of hexokinase, high- K_m glucose-phosphorylating enzyme activity and glucose 6-phosphatase in the islets is unchanged by starvation. The results suggest that 6-phosphogluconate dehydrogenase activity may be decreased during starvation.

Experimental

Materials

Luciferase from *Achromobacter fischerii*, firefly luciferase and 3,5-diaminobenzoic acid were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Other enzymes (including collagenase) and coenzymes were from Boehringer, Mannheim, Germany. Mouse insulin, ^{125}I -labelled pig insulin, anti-(pig insulin) and guinea-pig serum were gifts from Dr. J. Schlichtkrull, Novo Research Institute, Copenhagen, Denmark. Human serum albumin was from Behringswerke A.G., Marburg-Lahn, Germany. Bovine serum albumin powder was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Sodium merthiolate, Malachite Green, the sodium salt of calf thymus DNA and sodium arsenate were from British Drug Houses, Poole, Dorset, U.K. $[5\text{-}^3\text{H}]\text{Glucose}$ (2Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Aquasol was from New England Nuclear Corp., Boston, Mass., U.S.A. Dimethyl-POPOP [1,4-bis-(methyl-5-phenyloxazol-2-yl)benzene] and PPO (2,5-diphenyloxazole) were from E. Merck, Darmstadt, Germany. Myristaldehyde was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Mercaptoethanol was from Koch-Light, Colnbrook, Bucks., U.K. Other reagents used were of the purest grade obtainable.

Methods

Preparation of islets. Pancreas from male albino mice (Theillers original strain; Tuck and Son, Rayleigh, Essex, U.K.) were used. For each experiment 12–20 mice of the same age and weight (20–22g) were selected and divided into two groups. One group was fed on a standard laboratory diet *ad libitum* for 24h and one group was starved for 48h (with free access to drinking water) before being killed by decapitation. Starvation was carried out in wired metabolic cages to minimize coprophagy. Islets were separated by a collagenase method and harvested with a wire loop as described by Coll-Garcia & Gill (1969). The buffer used for the collagenase treatment was supplemented with glucose (3.3mM), and the sodium salts of glutamate (5mM), fumarate (5mM) and pyruvate (5mM).

Islet homogenates. For preparation of a homogenate 100–150 islets were collected in 40 μl of

ice-cold 0.25M-sucrose–5mM-Tris–HCl–1mM-EDTA, pH7.0. This islet suspension was diluted with 100–200 μ l of the appropriate assay buffer described below, and islets were disrupted by sonication for 20s at position 1 on a Branson Sonifier B-12.

Insulin release. Batches of five islets were incubated (with shaking) in 0.3 ml of Krebs bicarbonate medium (Krebs & Henseleit, 1932) containing human serum albumin (2mg/ml) and other additions as stated. After 2h of incubation the tubes were gently centrifuged and a sample (0.1ml) of the medium was removed, diluted with a 0.04M-sodium phosphate buffer, pH7.4, containing human serum albumin (10mg/ml) and stored at -20°C until assay by radioimmunoassay (Heding, 1966) with mouse insulin as standard.

Assays of glucose-phosphorylating activity. The activity of the glucose-phosphorylating enzymes was assayed at 37°C by the change in fluorescence on addition of 15 μ l of islet homogenate (equivalent to five to ten islets, representing 3–6 μ g dry wt. of islets) to tubes containing 600 μ l of assay medium [50mM-Tris–HCl buffer, pH7.5, 150mM-KCl, 4mM-mercaptoethanol, 1mM-EDTA, 0.05mg of bovine plasma albumin/ml, 10mM-MgCl₂, 2mM-ATP 0.7mM-(for low- K_m activity) or 70mM-(for total activity) glucose, 0.3mM-NADP⁺ and 0.03 unit of glucose 6-phosphate dehydrogenase/ml]; 1mol of glucose was assumed to be phosphorylated for each mol of NADPH formed. Since it was not known whether endogenous 6-phosphogluconate dehydrogenase in the homogenate would contribute to the NADPH formation under the experimental conditions of the above assay, another assay with added 6-phosphogluconate dehydrogenase (0.03 unit/ml of assay medium) was also carried out. The enzyme was dialysed against a 50mM-Tris–HCl buffer, pH7.5, before addition to remove sulphate, as the amount of sulphate added otherwise would inhibit the glucose 6-phosphate dehydrogenase. In this assay it was assumed that phosphorylation of 1mol of glucose gave rise to 2mol of NADPH. It was ascertained that further addition of these auxiliary enzymes did not increase the rate of the reaction.

In both assays the reaction was stopped after 90min of incubation by dilution with 50mM-Tris–HCl buffer, pH7.5, and the fluorescence measured immediately at 25°C on a Photovolt fluorimeter. The sensitivity was adjusted to yield full-scale deflexion with 2–3nmol of NADPH. For each assay two blanks were used, one containing the complete reaction mixture but lacking the homogenate (allowing for correction for hexokinase contamination or side activity of glucose 6-phosphate dehydrogenase) and another containing homogenate but lacking the substrates for the reaction (allowing correction for NADPH formation independent of glucose phosphorylation).

The rate of the total reaction was always more than 4–5 times the rate of the combined blank reactions. Many but not all determinations of enzyme activity were done in duplicate. Duplicated determinations never deviated from each other by more than 5–10%.

NADPH standards (checked spectrophotometrically) were run in each assay. There was no reoxidation of the NADPH formed in the assay. The rate of formation of NADPH in the two assays was linear with time over 90min and was proportional to the amount of homogenate added. The DNA content of 15 μ l of homogenate was determined in duplicate for each assay.

DNA content. DNA content of samples of the islet homogenate was assayed by the method of Kissane & Robins (1958) with calf thymus DNA as standard. The fluorimeter used was a Photovolt multiplier fluorescence meter, model 540, with a 25 μ l cuvette.

Glucose 6-phosphatase assay. A portion (0.02ml) of islet homogenate prepared as described above was incubated for 2h at 37°C with 0.01ml of 0.05M-Tris–HCl buffer, pH6.5, containing 0.045M-glucose 6-phosphate. To avoid evaporation the incubation was performed in small plastic tubes (9mm $\times$ 70mm) sealed by Parafilm and enclosed in larger tubes as described by Öckermann (1967). After incubation the P_i content was determined by a modification of the method of Itaya & Ui (1966): 1.0ml of Malachite Green reagent and 0.05ml of 1.5% Tween 20 were added directly to the incubation mixture. The colour was allowed to develop for 15min at 37°C , then 1.5ml of 1M-HCl was added and the intensity of the colour developed was measured at 660nm in 4cm micro-cuvettes. Blank incubations without homogenate and blank samples without substrate as well as the appropriate standards were included in each experiment. All determinations were in triplicate. The rate of hydrolysis of glucose 6-phosphate was linear with time for at least 3h and was proportional to the amount of homogenate used. Any unspecific phosphatase activity present in the homogenate was measured by using β -glycerol phosphate as substrate.

Incorporation of glucose into glycogen. Some 30 islets were incubated at 37°C in 15 μ l of gassed (O₂+CO₂, 95:5) Krebs bicarbonate buffer containing 3mg of [5-³H]glucose/ml (0.1mCi/mg). After 2h of incubation the reaction was stopped by addition of 10 μ l of 0.2M-HCl. The tubes were centrifuged and 23 μ l of supernatant was discarded. Then 20 μ l of 30% (w/v) KOH and 50mg of glycogen/ml were added and the tubes were put on a boiling-water bath for 1h. The tubes were cooled on ice, 2 $\times$ 25 μ l of water were added to each tube and the contents were transferred quantitatively to polypropylene tubes with bottoms consisting of dialysis membranes. The tubes were sealed and dialysed against water for 48h. After dialysis the contents were transferred to

scintillation vials containing 10ml of Aquasol and 3.5ml of water and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer. In each experiment four blank values were obtained by incubating buffer without islets.

Glucose utilization. For measurements of glucose utilization 10 islets were incubated at 37°C for 2h in 15 μ l of Krebs bicarbonate medium containing [^3H]glucose at the concentrations and specific radioactivities given in the tables and figures. Glucose utilization was determined as the formation of $^3\text{H}_2\text{O}$ from [^3H]glucose essentially as described by Ashcroft *et al.* (1972). Usually six control samples without islets were included in each assay to allow correction for $^3\text{H}_2\text{O}$ in the [^3H]glucose. In time-course studies two control samples without tissue were included for each time-interval, as blank values increased with incubation time. Blank values were less than one-quarter to one-fifth of the measured glucose utilization. The specific radioactivity of the glucose in the medium was determined by liquid-scintillation spectrometry for radioactivity and spectrophotometric assay for glucose (Slein, 1963).

Lactate output. For this 100–150 islets were incubated at 37°C in 100 μ l of Krebs bicarbonate buffer containing 3mg of glucose/ml. After 5min the whole medium was removed and 100 μ l of fresh, pre-warmed and gassed ($\text{O}_2 + \text{CO}_2$, 95:5) medium was added. The vials were further gassed for 2min and then the incubation was continued (with shaking) for 1h. The removal of and addition of fresh buffer was repeated and the islets were incubated for 1h more. Two 40 μ l samples of the removed media were immediately pipetted into 80 μ l of 0.08M-HCl and heated to 60°C for 30min and then lactate was assayed as described by Ashcroft *et al.* (1970). Tissue blanks and appropriate lactate standards made up in acidified Krebs bicarbonate buffer were included in each experiment. Lactate output was linear with time up to 2h with islets from both fed and starved mice.

ATP assay. For this six islets were incubated for 30min in 0.05ml of gassed ($\text{O}_2 + \text{CO}_2$, 95:5) Krebs

bicarbonate buffer containing 0.42 or 3mg of glucose/ml. Then 0.025 ml of 1M- HClO_4 was added and the tubes were sonicated for 20s at position 1 on a Branson Sonifier B-12. Then 2ml of an arsenate-phosphate buffer, pH7.4 (0.1M- NaH_2AsO_4 with 40mM- MgSO_4 –0.01M- KH_2PO_4 with 4mM- MgSO_4 – H_2O , 2:1:3, by vol) was placed in a polypropylene tube (11mm $\times$ 50mm) that had been soaked with water overnight and washed with double-distilled water. A portion (20 μ l) of the homogenate was added, the tube was placed in an ordinary scintillation vial, and the ATP content was determined in triplicate by the luciferase assay described by Stanley & Williams (1969). A set of ATP standards made up in 0.01M-potassium phosphate buffer were included in each experiment: 1–30 μ l of a 1 μ M-ATP solution was added to the polypropylene tubes together with 20 μ l of a Krebs bicarbonate- HClO_4 solution of the same composition as the acidified incubation medium. The extra amount of phosphate buffer added with the standards did not affect the luciferase assay. A Packard Tri-Carb liquid-scintillation spectrometer, model 2002, operated at room temperature was used. The photomultipliers were switched out-of-coincidence. The amplification was 100% and the discriminators were set between 10 and infinity.

Glucose 6-phosphate and (fructose 1,6-diphosphate plus triose phosphates). Either 10 (for glucose 6-phosphate) or 40 (for fructose 1,6-diphosphate+triose phosphates) islets were incubated at 37°C in 28 μ l of gassed ($\text{O}_2 + \text{CO}_2$, 95:5) Krebs bicarbonate buffer containing 0.5 or 3mg of glucose/ml. After $\frac{1}{2}$ h the reaction was stopped by addition of HCl and the islet contents of glucose 6-phosphate and (fructose 1,6-diphosphate+triose phosphates) were measured by a luciferase method as described in detail by Ashcroft *et al.* (1973a).

Results

Table 1 shows that 48h starvation caused a decrease in the average mouse weight from 21.4 ± 0.09 to 16.9 ± 0.13 g, i.e. approx. 20%. The DNA content

Table 1. *Effect of starvation on the weight of the mice and the DNA content of the islets*

Male mice of similar weight and age were chosen and either fed on a standard laboratory diet for 24h or starved for 48h. The DNA content of the isolated islets was determined as described in the Experimental section. Results are given as means $\pm$ S.E.M. with the numbers of determinations in parentheses.

	Day of selection	Fed for 24h	Starved for 48h
Weight (g/mouse)	21.4 ± 0.09 (50)	22.2 ± 0.08 (50)	16.9 ± 0.13 (50)*
DNA (ng/ten islets)		148 ± 5 (22)	163 ± 6 (22)†

* $P < 0.001$ versus fed control.

† Not significant versus fed control.

Fig. 1. Insulin secretory responses of islets isolated from fed and 48 h starved mice to glucose (0.5–12 mg/ml)

Batches of five islets isolated from either fed (●) or 48h-starved (▲) mice were incubated for 2h in Krebs bicarbonate buffer containing albumin and various concentrations of glucose. Insulin secretory responses were determined as described in the text. Each result is given as the mean±S.E.M. with the number of batches of islets in parentheses. The difference in insulin-secretory response between the fed and starved state was statistically significant at all glucose concentrations tested ($P<0.001$) except for 10.5mg of glucose/ml, where no difference could be demonstrated ($P>0.7$).

of islets from fed mice was not different from that of islets from 48h-starved mice.

Insulin release

The rate of insulin release from islets of fed mice was increased five- to six-fold when the extracellular glucose concentration was raised from 2.5mM to 16.7mM; 5mM-caffeine potentiated this effect to 15-fold. Fig. 1 shows that the insulin secretory response of islets from starved mice was diminished at all glucose concentrations tested from 2.5mM up to approx. 40mM. However, very high concentrations of glucose (above 60mM) restored the secretory response to normal values. This shows that the V_{max} value for the overall secretory response is unchanged by starvation whereas the K_m value for the same process is increased approx. three- to four-fold. It is unlikely that the effect of very high glucose concentrations on insulin release has been mediated by hyperosmolarity effects, as Lernmark (1971) has shown that hyperosmolarity up to an excess of 100mM has no effect on insulin secretion from isolated islets from obese hyperglycaemic mice.

The rates of glucose-stimulated insulin release as potentiated by 5mM-caffeine were not impaired by starvation. This holds equally well for 2.5mM- and 16.7mM-glucose in the medium (Table 2).

Glucose phosphorylation

Table 3 gives maximally extractable activities of glucose-phosphorylating enzyme activities expressed both per islet and per 100ng of DNA. Two different assays were applied. In the presence of exogenous 6-phosphogluconate dehydrogenase in the assay, total glucose-phosphorylating activity, hexokinase and high- K_m glucose-phosphorylating activity were not affected by starvation. When the assay was carried out without exogenous 6-phosphogluconate

Table 2. Effect of starvation on glucose- and caffeine-stimulated insulin release

Batches of five islets from fed or 48h-starved mice were incubated for 2h at 37°C in 600μl of medium containing glucose and caffeine at the indicated concentrations. The released insulin was determined by radioimmunoassay as described in the Experimental section. Results are given as means±S.E.M. with the numbers of determinations in parentheses.

Glucose concn. (mM)	Caffeine concn. (mM)	Insulin release (μunits/2h per five islets)	
		Fed	Starved for 48 h
3	5	175 ± 28 (27)	176 ± 23 (18)
16.7	5	2597 ± 298 (27)	2750 ± 317 (25)

Table 3. *Effect of starvation on the activity of hexokinase, glucokinase and glucose 6-phosphatase*

Glucose-phosphorylating enzyme activity was measured at 37°C by the change in fluorescence on addition of islet homogenate to a reaction mixture as described in the Experimental section. The high- K_m glucose-phosphorylating enzyme activity was calculated as the difference between the low- K_m and the total glucose-phosphorylating activity. The glucose 6-phosphatase activity was determined from the amount of P_i liberated from glucose 6-phosphate during incubation, and unspecific phosphatase activity was calculated from the amount of P_i liberated from β -glycerophosphate during incubation as described in the Experimental section. Results are given as means $\pm$ S.E.M. with the numbers of determinations in parentheses. Glucose-phosphorylating activity is expressed as pmol of glucose phosphorylated/min. Glucose 6-phosphatase and unspecific phosphatase activities are expressed as pmol of P_i liberated/min.

	6-Phospho- gluconate dehydrogenase present in the assay	Fed		Starved for 48 h	
		(pmol/min per islet)	(pmol/min per 100 ng of DNA)	(pmol/min per islet)	(pmol/min per 100 ng of DNA)
Total glucose-phosphorylating activity	—	3.23 $\pm$ 0.22 (19)	21.7 $\pm$ 1.0 (18)	2.94 $\pm$ 0.16 (19)	18.4 $\pm$ 0.9 (18)*
	+	2.37 $\pm$ 0.26 (10)	16.3 $\pm$ 0.8 (10)	2.09 $\pm$ 0.20 (10)	14.0 $\pm$ 1.2 (10)
Low- K_m glucose-phosphorylating activity (hexokinase)	—	1.54 $\pm$ 0.09 (19)	10.5 $\pm$ 0.5 (18)	1.34 $\pm$ 0.08 (19)	8.4 $\pm$ 0.5 (18)**
	+	1.13 $\pm$ 0.13 (10)	7.7 $\pm$ 0.4 (10)	1.01 $\pm$ 0.09 (10)	6.7 $\pm$ 0.5 (10)
High- K_m glucose-phosphorylating activity (glucokinase)	—	1.69 $\pm$ 0.14 (19)	11.2 $\pm$ 0.7 (18)	1.60 $\pm$ 0.11 (19)	10.0 $\pm$ 0.7 (18)
	+	1.24 $\pm$ 0.14 (10)	8.6 $\pm$ 0.5 (10)	1.08 $\pm$ 0.11 (10)	7.3 $\pm$ 0.7 (10)
Glucose 6-phosphatase	—	3.1 $\pm$ 0.3 (4)	32.7 $\pm$ 1.2 (3)	3.5 $\pm$ 0.4 (4)	29.9 $\pm$ 1.0 (3)
Unspecific phosphatase	—		7.5 $\pm$ 0.2 (3)		9.6 $\pm$ 0.6 (3)

* $P < 0.025$ versus fed control.

** $P < 0.005$ versus fed control.

Table 4. *Effect of starvation on uptake of [5- 3 H]glucose, lactate output and incorporation of [5- 3 H]glucose into glycogen*

For measurements of glucose uptake batches of ten islets from fed or 48 h-starved mice were incubated for 2 h in 0.015 ml of medium containing 2.5 mM-[5- 3 H]glucose (sp. radioactivity 25–30 mCi/mmol) or 16.7 mM-[5- 3 H]glucose (sp. radioactivity 3–4 mCi/mmol). The glucose uptake was calculated from the amount of 3 H $_2$ O formed during incubation. For measurement of incorporation into glycogen 25 islets were incubated in 0.015 ml of medium containing 16.7 mM-[5- 3 H]glucose (sp. radioactivity 0.1 mCi/mg) for 2 h. After addition of carrier, glycogen was isolated and radioactivity determined as described in the Experimental section. For measurement of lactate output 100–150 islets were incubated for 2 h in 0.1 ml of medium containing 16.7 mM-glucose, and lactate in the medium was determined as described in the Experimental section. The results are given as means $\pm$ S.E.M. with the numbers of determinations in parentheses.

Glucose concn. (mM)	Fed		Starved for 48 h	
	... 2.5	16.7	2.5	16.7
Glucose uptake (pmol/2h per ten islets)	209 $\pm$ 20 (9)	1196 $\pm$ 52 (15)	136 $\pm$ 10* (9)	814 $\pm$ 52** (15)
Incorporation of glucose into glycogen (pmol/2h per ten islets)		2.4 $\pm$ 0.3 (11)		5.6 $\pm$ 1.4*** (11)
Lactate output (pmol/2h per ten islets)		317 $\pm$ 48 (6)		180 $\pm$ 35*** (6)

* $P < 0.01$ versus fed control.

** $P < 0.001$ versus fed control.

*** $P < 0.05$ versus fed control.

dehydrogenase all the values were higher, which means that endogenous 6-phosphogluconate dehydrogenase had contributed to some extent to the measured NADP reduction. In this case a significant decrease in 'hexokinase' and 'total glucose-phosphorylating activity' (expressed per 100 ng of DNA) was noted (the activities were contaminated with endogenous 6-phosphogluconate dehydrogenase). This finding suggests that 6-phosphogluconate dehydrogenase may be suppressed in islets from

starved mice. The values found for total glucose-phosphorylating activity are very close to those given by Ashcroft & Randle (1970a), who used a radioactive assay, but we found that 50% of the total activity was high- K_m glucose-phosphorylating activity as opposed to their 25%. The reason for this discrepancy is not clear but may be related to the different assays applied, as Matschinsky & Ellerman (1968), using a fluorimetric assay, also found approx. 50% in islets from obese hyperglycaemic mice.

Hydrolysis of glucose 6-phosphate

Glucose 6-phosphatase activity (see Table 3) was present in amounts comparable with the total glucose-phosphorylating activity, confirming the results of Ashcroft & Randle (1970a) and Matschinsky & Ellerman (1968). The glucose 6-phosphatase activity, expressed per islet or per 100ng of DNA, was not increased by starvation. The unspecific phosphatase activity towards β -glycerophosphate was approximately one-quarter of the glucose 6-phosphatase activity and was not changed by starvation either (Table 3).

Incorporation of glucose into glycogen

The incorporation of glucose into glycogen (see Table 4) was exceedingly low, less than 1% of the glucose utilization. Starvation caused approximately a doubling of this incorporation when islets were incubated at a high glucose concentration.

Glucose utilization and lactate output

Glucose utilization was measured as the rate of formation of $^3\text{H}_2\text{O}$ from $[5\text{-}^3\text{H}]\text{glucose}$ and represents the combined rates of flow of glucose carbon through the glycolytic pathway and the pentose cycle pathway, but it does not include the flow of glucose carbon into glycogen. As the rate of glycogen synthesis is very low and as the flow of glucose through the pentose cycle pathway in islets also is low (Ashcroft *et al.*, 1972), the values given almost exclusively represent glucose uptake and glycolysis.

The glucose utilization at 2.5mM- and 16.7mM-glucose was linear with time for at least 2h in both fed and starved animals (Fig. 2).

Fig. 3 shows that starvation caused a decrease in the combined flow of glucose carbon through the glycolytic pathway and the pentose cycle pathway when this combined flow was measured over a 2h period at glucose concentrations from 2.5mM up to approx. 55mM. Thus, starvation must have caused a decrease in glycolytic flux in any case and possibly also a decrease in pentose-cycle activity at the mentioned glucose concentrations. However, at very high glucose concentrations (70–100mM) there was no difference in glucose utilization between the fed and starved state, suggesting that the K_m value for glucose utilization and thereby for the rate-limiting glucose phosphorylation (Hellman *et al.*, 1971) had been increased by starvation, whereas the V_{max} value for glucose utilization was unchanged. This compares very well with our results on the maximally extractable amount of hexokinase, glucokinase and glucose 6-phosphatase in the islets under these conditions.

Fig. 2. Time-course of glucose uptake by pancreatic islets from fed and 48h-starved mice

For measurement of glucose uptake batches of islets, isolated from fed and 48h-starved mice, were incubated in Krebs bicarbonate buffer (0.015M) containing $[5\text{-}^3\text{H}]\text{glucose}$ at 37°C for the time shown. The amount of $^3\text{H}_2\text{O}$ formed was measured and the glucose uptake calculated as described in the Experimental section. Each result is given as the mean $\pm$ S.E.M. with the number of batches of islets in parentheses. ●, Islets from fed mice, glucose 3mg/ml, 3.7mCi/mmol; △, islets from 48h-starved mice, glucose 3mg/ml, 3.7mCi/mmol; ○, islets from fed mice, glucose 0.4mg/ml, 25mCi/mmol.

The effect of very high glucose concentrations on glucose utilization in the starved state is not an effect of hyperosmolarity as further addition of urea to different glucose concentrations did not significantly change glucose utilization.

Thus in five paired experiments with islets from starved mice, the glucose utilization at 38mM-glucose was 1507 ± 83 pmol/2h per ten islets (means $\pm$ S.E.M.). At 38mM-glucose with added 37mM-urea the value was 1609 ± 186 .

It could be argued that lipolysis in the islets from starved mice might have decreased the specific radioactivity of the triose phosphate pool compared with the specific radioactivity of extracellular glucose from which utilization rates are calculated and that this would give erroneously low glucose utilization results in the starved state. However, we have shown (see Table 5) that the combined pool of triose phosphate and fructose 1,6-diphosphate is diminished during starvation and further, measurements of lactate outputs clearly demonstrated that glycolysis is impaired in the islets during starvation. Lactate output was linear with time for up to 2h (results not shown), and Table 4 shows that at 16.7mM-glucose lactate output was decreased by 43% by starvation.

Fig. 3. Glucose utilization of islets from fed and 48h-starved mice as a function of the glucose concentrations in the medium

Batches of ten islets, isolated from fed (●) or 48h-starved (▲) mice, were incubated in Krebs bicarbonate buffer (0.015ml) containing $[5\text{-}^3\text{H}]\text{glucose}$ at various concentrations for 2h at 37°C . The amount of $^3\text{H}_2\text{O}$ formed was measured and the glucose uptake calculated as described in the Experimental section. Each result is the mean $\pm$ s.e.m. with the number of batches of islets given in parentheses. The difference in glucose utilization between islets from fed and starved mice was statistically significant at all glucose concentrations tested ($P < 0.01$) except for the two highest glucose concentrations (14mg/ml and 17.5mg/ml), where no difference could be demonstrated ($P > 0.05$ and $P > 0.8$ respectively).

If the islets were prepared without substrates (glucose, pyruvate, fumarate and glutamate) in the medium during collagenase treatment, the subsequently measured glucose utilization at 16.7mm-glucose was decreased by 38% ($P < 0.001$) and 30% ($P < 0.025$) in islets from fed and starved mice respectively.

Islet contents of glucose 6-phosphate, (fructose 1,6-diphosphate+triose phosphates) and ATP

In previous work on islets isolated from mice starved overnight it was shown that glucose 6-phosphate attains a steady-state concentration after 10–15min of incubation at high glucose (Ashcroft *et al.*, 1970). The intermediates, glucose 6-phosphate, (fructose 1,6-diphosphate+triose phosphates) and ATP have accordingly been measured after 30min of incubation.

Table 5 shows that the content of glucose 6-phosphate in the islets after 30min of incubation at 16.7mm-glucose was not significantly changed by starvation, whereas there was a 30% decrease in glucose 6-phosphate concentration when islets from starved mice were incubated at 2.5mm-glucose. The contents of glucose 6-phosphate are very close to those found with a completely different method of enzymic recycling (Ashcroft *et al.*, 1970). The content of (fructose 1,6-diphosphate+triose phosphates) when islets were incubated at a high glucose concentration was significantly decreased in islets from starved animals. After 30min of incubation at high glucose concentration there was a significantly higher ATP content in islets from the starved mice.

Table 5. Effect of starvation on the steady-state concentration of glucose 6-phosphate, ATP and (fructose-1,6-diphosphate+triose phosphates)

Batches of 5–40 islets from fed or 48h-starved mice were incubated for $\frac{1}{2}$ h at 37°C in medium containing glucose at the indicated concentration. The amount of glucose 6-phosphate, ATP and (fructose 1,6-diphosphate+triose phosphates) was determined as described in the Experimental section. The results are given as means $\pm$ s.e.m. with the number of batches of islets shown in parentheses.

Glucose concn. (mm) ...	Fed		Starved for 48h	
	2.5	16.7	2.5	16.7
Glucose 6-phosphate (pmol/ten islets)	3.6 ± 0.4 (15)	11.3 ± 0.4 (30)	$2.8 \pm 0.2^*$ (19)	10.5 ± 0.6 (30)
ATP (pmol/islet)	6.8 ± 0.4 (26)	7.3 ± 0.3 (37)	$7.5 \pm 0.4^\dagger$ (21)	$8.9 \pm 0.3^{**}$ (31)
(Fructose 1,6-diphosphate + triose phosphates) (pmol/ten islets)		0.44 ± 0.04 (24)		$0.30 \pm 0.04^{***}$ (14)

* $P < 0.05$ versus fed control.

** $P < 0.001$ versus fed control.

*** $P < 0.02$ versus fed control.

† Not significant versus fed control.

Only a slight increase in ATP concentration in the islets was noticed when the glucose concentration was raised from 2.5mM to 16.7mM and only in the starved state was the difference statistically significant.

Discussion

It has been assumed in the present work that the parameters measured were expressions of β -cell activity, as the proportion of β cells in normal mouse islets is about 80% (Brolin & Hellerström, 1967), but the possible contribution of other cell types must be borne in mind. Measurements of DNA content showed that the number of cells in islets isolated from starved mice was not different from that of islets from fed mice. Whether the proportion of β cells changes with nutritional state is not known.

Insulin secretion during starvation

The well-known glucose intolerance during prolonged starvation can partly be accounted for by a decreased sensitivity of the tissues to insulin (Feldman & Lebowitz, 1970) and partly by a decreased sensitivity of the insulin-secretory mechanism to glucose, as shown for rat pancreas pieces by Malaisse *et al.* (1967) and confirmed in the present work, for isolated mouse islets. The present results indicate that very high extracellular glucose concentrations can restore the insulin-secretory response to the normal value. The shift of the dose-response curve to the right suggests that the maximal secretory capacity is unchanged by starvation but that the K_m value (glucose) for the overall secretory mechanism has been increased (approx. three- to four-fold).

The glucose-stimulated insulin secretion in the presence of 5mM-caffeine was, however, not impaired by starvation. This agrees with the results of Grey *et al.* (1970) for aminophyllin-induced insulin release in the rat *in vivo* and seems to indicate that the defect in the insulin release mechanism does not involve more distal parts of the secretory mechanism or a markedly decreased pancreatic insulin content. It is noteworthy that the described pattern of insulin release during starvation resembles that found in the pre-diabetic state (Cerasi & Luft, 1969) and in foetal and neonatal pancreatic tissue (Lambert *et al.*, 1970). The fact that caffeine can restore the defective insulin secretion to normal values could suggest that the concentration of cyclic AMP may be below a critical value in islets from starved mice. It is very likely that this is the case, since Howell *et al.* (1973) have reported that adenylate cyclase activity and the activity of cyclic AMP-dependent protein kinase, which may reflect cyclic AMP concentrations in the islets, are both significantly decreased during starvation. The effect of starvation on insulin secretion and islet adenylate cyclase activity is unlikely to be mediated via the

gastrointestinal hormones secretin and pancreozymin, since refeeding with casein and amino acids does not restore the glucose-stimulated insulin release to normal values (Turner & Young, 1973). Increased concentrations of adrenalin during starvation (Feldman & Lebowitz, 1970) could also be thought of as a possible cause of the diminished secretory response to glucose, as adrenalin is a known inhibitor of insulin secretion (Coore & Randle, 1964). According to Voyles *et al.* (1973) adrenalin seems, however, to have an inhibitory mechanism different from that caused by starvation.

Glucose metabolism in pancreatic islets during starvation

The amount of hexokinase, high- K_m glucose-phosphorylating activity and glucose 6-phosphatase was not changed by starvation. This finding compares well with the results of Lauris & Cahill (1966) on the corresponding mouse liver enzymes, which, in contrast with the rat liver enzymes, are not affected by starvation either. Matschinsky *et al.* (1971) also reported that starvation did not change hexokinase activity in rat islets. These findings are in keeping with the observation that the V_{max} value for glucose utilization in the islets was unchanged by starvation. However, the shift to the right of the dose-response curve for glucose utilization during starvation suggests that K_m (glucose) for the rate-determining step in glucose utilization, which in view of the rapid permeation of glucose into islets (Hellman *et al.*, 1971) is likely to be phosphorylation of glucose, has been increased (approx. twofold). The mechanism for this change in K_m value for glucose phosphorylation must necessarily remain a matter for speculation. The diminished glucose utilization and flow of glucose carbon through the Embden-Meyerhof pathway found at 16.7mM extracellular glucose was associated with slightly decreased concentrations of glucose 6-phosphate (although not statistically significant), increased concentrations of ATP and decreased concentrations of (fructose 1,6-diphosphate + triose phosphates). These changes in metabolite concentrations do not prove, but are at least not incompatible with, the hypothesis that glucose phosphorylation at this glucose concentration is inhibited in islets during starvation.

Lack of hexose diphosphatase in the β cells seems to preclude increased gluconeogenesis as an explanation of the observed decrease in glucose utilization (see Brolin *et al.*, 1968).

The data on glucose-phosphorylating enzyme activity suggested that 6-phosphogluconate dehydrogenase activity in the islets may be decreased during starvation. This may not influence the flow through the pentose cycle pathway, as 6-phosphogluconate dehydrogenase is present in a large excess and the

flow seems to be regulated by the availability of NADP⁺ (Ashcroft & Randle, 1970a). If the loss in activity is drastic, however, one could expect a decrease in NADPH production, and this might have some bearing on the impaired glucose-stimulated insulin release, as it is known that NADPH may react with the insulin granule membrane to facilitate insulin release (Watkins & Cooperstein, 1970). Direct measurements of the flow of glucose carbon through the pentose cycle pathway during starvation are needed to clarify this point.

Relation between insulin secretion and glucose metabolism and possible glucoreceptor mechanisms

Irrespective of how the glucose utilization at physiological glucose concentrations has been adjusted to a lower value during starvation we have here a new correlation between islet glucose metabolism and insulin release which adds to the remarkable degree of correlation between the metabolic effects of glucose, mannose, mannoheptulose and glucosamine and their effects on insulin release described by Ashcroft *et al.* (1970). The results are therefore consistent with the idea that glucose must be metabolized in the islets to elicit insulin release. This hypothesis was also supported by some recent experiments carried out in this laboratory which showed that the concentration of hexose phosphates was significantly increased within 2–5 min after a sudden exposure to a glucose pulse in both mouse and rat islets *in vitro* (Ashcroft *et al.*, 1973a), which means that changes in metabolite concentrations may have paralleled or preceded the established rapid change in the rate of insulin release under these conditions.

The energy potential in the islets does not seem to regulate insulin secretion, as the decreased insulin response to glucose after starvation was associated with an increased ATP concentration. Further, the increased secretion rate after tolbutamide is accompanied by a decrease in ATP concentration (Ashcroft *et al.*, 1973b).

If the glucose-phosphorylating enzymes indeed are the β -cell glucoreceptor then the amount of the receptor is unchanged by starvation but its sensitivity is decreased insofar as the signal arising from stimuli of equal strength (and created via glucose metabolism) is diminished compared with the fed state. This decreased signal and response can be reversed by glucose both *in vivo* (Grey *et al.*, 1970) and *in vitro*. Thus both the decreased glucose utilization and the decreased insulin release were restored to normal values at very high extracellular glucose concentrations, as shown in the present paper. Perhaps the same phenomenon can explain the observation that addition of substrates (and among these glucose) to the medium during the collagenase

treatment augmented the subsequently measured glucose utilization.

It is unlikely that primary changes in insulin release rate (in this case in some way caused by starvation) or a primarily decreased concentration of cyclic AMP in the islets has changed the glucose metabolism, as it has been shown that a number of modifiers of insulin release and insulin synthesis such as alteration of the concentration of Ca²⁺, addition of glucagon, caffeine, adrenalin, arginine and cycloheximide, have no effect on glucose oxidation or O₂ uptake (Ashcroft *et al.*, 1970; Hedekov *et al.*, 1972).

However, it has been shown by Howell *et al.* (1973) that either glucose itself or (more likely) a metabolite of glucose in long-term incubations (over 1 h) can change adenylate cyclase activity of the islets and thereby presumably concentrations of cyclic AMP.

The most likely explanation of the effect of starvation on insulin secretion is therefore that the prolonged hypoglycaemia of starvation in itself (or a changed concentration of a glucose metabolite in the islets) has decreased the sensitivity of the substrate-site glucoreceptor *in situ* by increasing the K_m value for glucose phosphorylation and utilization, and this results in an impaired insulin response to glucose, an impairment which is further accentuated at physiological glucose concentrations by a decreased concentration of cyclic AMP also caused by the impaired glucose metabolism. This would also explain the observation that the K_m value for the overall secretory process was increased approx. fourfold whereas the K_m value for the glucose utilization was increased only twofold.

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