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Glucose Metabolism in Mouse Pancreatic Islets

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1. Rates of glucose oxidation, lactate output and the intracellular concentration of glucose 6-phosphate were measured in mouse pancreatic islets incubated *in vitro*. 2. Glucose oxidation rate, measured as the formation of $^{14}CO_2$ from [U-^{14}C]glucose, was markedly dependent on extracellular glucose concentration. It was especially sensitive to glucose concentrations between 1 and 2mg/ml. Glucose oxidation was inhibited by mannoheptulose and glucosamine but not by phlorrhizin, 2-deoxyglucose or *N*-acetylglucosamine. Glucose oxidation was slightly stimulated by tolbutamide but was not significantly affected by adrenaline, diazoxide or absence of Ca^{2+} (all of which may inhibit glucose-stimulated insulin release), by arginine or glucagon (which may stimulate insulin release) or by cycloheximide (which may inhibit insulin synthesis). 3. Rates of lactate formation were dependent on the extracellular glucose concentration and were decreased by glucosamine though not by mannoheptulose; tolbutamide increased the rate of lactate output. 4. Islet glucose 6-phosphate concentration was also markedly dependent on extracellular glucose concentration and was diminished by mannoheptulose or glucosamine; tolbutamide and glucagon were without significant effect. Mannose increased islet fructose 6-phosphate concentration but had little effect on islet glucose 6-phosphate concentration. Fructose increased islet glucose 6-phosphate concentration but to a much smaller extent than did glucose. 5. [1-^{14}C]Mannose and [U-^{14}C]fructose were also oxidized by islets but less rapidly than glucose. Conversion of [1-^{14}C]mannose into [1-^{14}C]glucose 6-phosphate or [1-^{14}C]glucose could not be detected. It is concluded that metabolism of mannose is associated with poor equilibration between fructose 6-phosphate and glucose 6-phosphate. 6. These results are consistent with the idea that glucose utilization in mouse islets may be limited by the rate of glucose phosphorylation, that mannoheptulose and glucosamine may inhibit glucose phosphorylation and that effects of glucose on insulin release may be mediated through metabolism of the sugar.

Studies with rat or rabbit pancreas *in vitro* showed that the rate of release of insulin is markedly dependent on glucose concentration with a threshold concentration above 0.6mg of glucose/ml and tending to saturate at high glucose concentrations (Grodsky *et al.* 1963; Coore & Randle, 1964*a*). The more detailed measurements made with the rat pancreas by Malaisse, Malaisse-Lagae & Wright (1967) have shown that the relationship between rate of insulin release and extracellular glucose concentration may be sigmoid, with a threshold at approx. 0.9mg of glucose/ml, half maximum rates of release at approx. 1.8mg of glucose/ml and tending to a maximum rate at approx. 5mg of glucose/ml. It has been suggested, therefore, that pancreatic β-cells possess a glucoreceptor system through which alterations in extracellular glucose concentration lead to changes in the rate of insulin release (Grodsky *et al.* 1963; Coore & Randle, 1964*a*; Malaisse *et al.* 1967).

Insulin release in response to changes in extracellular glucose concentration may be visualized as involving three sequential processes. These are a glucoreceptor system, a coupling process through which the glucoreceptor system influences the release system and a release system which may involve secretory granule discharge by emiocytosis (Lacy, 1961). Two possible models for the glucoreceptor system have been considered. In the first model, glucose is envisaged as combining with the receptor molecule to form a complex which either activates the coupling system directly or which catalyses the formation of an intracellular messenger that activates the coupling system. In the second model, it is envisaged that glucose is first converted into a metabolic product which then activates the coupling system; the glucoreceptor system in this

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model is envisaged as an enzyme or enzymes involved in the metabolism of glucose. These models have been termed 'regulator-site' and 'substrate-site' respectively (Randle, Ashcroft & Gill, 1968). We would emphasize that for the hypothetical signal to be effective other conditions may have to be fulfilled: these include the correct ionic environment for the β-cell, in particular the presence of Ca^{2+}, and the availability in the β-cell of ATP and possibly 3′:5′-cyclic-AMP (Milner & Hales, 1969).

Early studies of the effects of different sugars on the rate of release of insulin *in vitro* indicated that release is stimulated only by those sugars (glucose, mannose and, to a smaller extent, fructose) which are readily metabolized by a number of mammalian tissues (Grodsky *et al.* 1963; Coore & Randle, 1964*a*). These observations provided the first evidence for the substrate-site model for the glucoreceptor system. Subsequently, it was found that D-mannoheptulose, which inhibits a number of hexokinases, rabbit liver glucokinase, and also glucose phosphorylation in rabbit liver slices (Sols & Crane, 1954; Sols, de la Fuente, Villar-Palasi & Asensio, 1958; Hernandez & Sols, 1963; Coore & Randle, 1964*b*), is a potent inhibitor of the glucose effect on insulin release (Coore, Randle, Simon, Kraicer & Shelesnyak, 1963). By contrast, phlorrhizin, which inhibits a number of glucose-transporting systems, did not modify the effect of glucose on insulin release (Coore & Randle, 1964*a*). This suggested that pancreatic β-cells may be freely permeable to glucose and that the glucoreceptor system may include enzymes controlling the rate of glucose phosphorylation. In conformity with this suggestion, Matschinsky & Ellerman (1968) have obtained evidence that the intracellular concentration of glucose in the pancreatic islets of obese-hyperglycaemic mice may closely parallel the blood glucose concentration over a wide range.

If glucose must be metabolized by the β-cell to elicit insulin release then it follows that the rate of metabolism of glucose to the postulated effector molecule may be correlated with its effects on insulin release. The effects of various agents on the release of insulin by pancreatic islets of normal mice have been described in parallel studies in this laboratory by Coll-Garcia & Gill (1969). In the present study we have measured only parameters of the metabolism of glucose and other hexoses since features of the experimental design (especially acidification of media containing islets) made it impracticable to measure insulin release accurately. In the experiments of Coll-Garcia & Gill (1969) insulin release was measured by using incubation media supplemented with pyruvate, glutamate and fumarate. These additions were made because Coore & Randle (1964*a*) had found that the insulin secretory response to glucose and other agents was enhanced thereby. These additions are, however, not essential, for insulin secretory responses in mouse islets (S. J. H. Ashcroft & J. G. Schofield, unpublished work) or in islets from other species (e.g. see Coore & Randle, 1964*a*; Malaisse *et al.* 1967; Montague & Taylor, 1968, 1969). In the present study, pyruvate, glutamate and fumarate were not added because they may complicate the interpretation of isotope data.

The islets of normal mice were chosen for these studies because they contain a relatively high proportion of β-cells (approx. 80%, Brolin & Hellerström, 1967) and because they were being utilized by a number of investigators for related metabolic studies. A preliminary account of some of these findings has been published (Ashcroft & Randle, 1968*a*).

EXPERIMENTAL

Materials. Collagenase was from Calbiochem, Los Angeles, Calif., U.S.A. Other enzymes, biochemicals and triethanolamine hydrochloride were from Boehringer Corp., London W.5, U.K. D-Mannoheptulose was either a gift from Professor E. Simon (Weizmann Institute, Rehovoth, Israel) or from Pfanstiehl Laboratories, Waukegan, Ill., U.S.A. Glucagon was a gift from Eli Lilly and Co., Indianapolis, Ind., U.S.A.; adrenaline was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Bovine plasma albumin (fraction V) was from Armour Pharmaceutical Co. Ltd., Hampden Park, Eastbourne, Sussex, U.K. Tolbutamide and diazoxide were gifts from Burroughs Wellcome and Co., London N.W.1, U.K. [U-^{14}C]Glucose (2.8–3.0mCi/mmol), [1-^{14}C]mannose (27.5mCi/mmol) and [U-^{14}C]fructose (2.8mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Phlorrhizin (Hopkin and Williams, Ltd.) was recrystallized twice from hot water. D-Glucosamine and *N*-acetyl-D-glucosamine were from BDH Chemicals Ltd., Poole, Dorset, U.K. L-Lactic acid was from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

Preparation and incubation of islets. Pancreas were obtained from 3–4-week-old male albino mice, fed *ad libitum* diet 41B (Oxo Ltd., London S.E.1, U.K.) and starved overnight. Islets were separated by a collagenase method and harvested with a wire loop as described by Coll-Garcia & Gill (1969). The islets were transferred individually to each vessel in turn with repetition of the cycle until all had received the required number. For measurement of glucose oxidation batches of 10 islets were incubated in 0.15ml of bicarbonate-buffered medium (Krebs & Henseleit, 1932), gassed with O_2+CO_2 (95:5), with the additions given in the text, tables or figures. The incubation vessels were as described by Keen, Field & Pastan (1963). For measurement of islet metabolite concentrations, or of lactate formation, batches of 10 islets were incubated in 50μl of medium in glass tubes (6mm × 30mm) inserted into 15ml centrifuge tubes containing 1.2ml of 0.9% NaCl (to facilitate heat exchange) gassed with O_2+CO_2 and sealed. All incubations were made in a water bath at 37°C.

[^{14}C]*Carbon dioxide output.* After incubation, 0.5ml of methanolic Hyamine was injected into the outer vessel and islet metabolism was arrested and CO_2 liberated into Hyamine by injection of 80μl of 0.2M-HCl into the inner vessel containing the islets. After a further 2h of incubation with shaking at room temperature, radioactivity in Hyamine was assayed by liquid-scintillation spectrometry with a toluene scintillator (Synder, 1961) and a Nuclear–Chicago Mark 1 Liquid Scintillation Computer. Blank incubations without islets were carried through the whole procedure for each experimental condition investigated, and the blank radioactivity was subtracted from the experimental value. Quenching corrections were made by using the channels-ratio method with an external standard. The specific radioactivity of medium glucose and mannose was assayed by using dioxan-based scintillator for radioactivity (Butler, 1961) and spectrophotometric assays for glucose (Slein, 1963) and mannose [assayed by ADP production with hexokinase (Adam, 1963)].

Lactate output. To avoid contamination, acid-washed glassware was used throughout and handled with forceps. After incubation of islets, 70μl of 50mM-HCl was added to the inner tube and, after resealing with a rubber cap, the whole assembly (inner and outer tube) was heated at 60°C for 30min to inactivate enzymes and destroy NADH and NADPH. After cooling, 100μl was then added to 1.15ml of assay medium containing 0.4M-glycine, 0.16M-hydrazine, 0.08% EDTA (pH adjusted to 9.5 with NaOH), and 50μl of 0.5mM-NAD^+ was added. After addition of 3.6 units of lactate dehydrogenase, the assay mixture was incubated for 30min at room temperature and fluorescence was measured in a purpose-built fluorimeter (see below). Blank incubations without islets were carried through the entire procedure for all types of incubation medium. Standard L-lactate solutions [standardized by spectrophotometric assay (Hohorst, 1963)] were included in each batch of assays (300, 600 and 1200pmol in duplicate). The reproducibility of the assay as judged by agreement between 14 duplicate standards (1.2nmol of lactate) was within 5%.

Islet glucose 6-phosphate and fructose 6-phosphate concentrations. These metabolites were assayed by enzymic recycling of NADPH formed by reaction of glucose 6-phosphate with $NADP^+$ catalysed by glucose 6-phosphate dehydrogenase. The method used was essentially that of Lowry, Passonneau, Schulz & Rock (1961) and Matschinsky, Passonneau & Lowry (1968) with additional precautions to obviate interference by glucose (which may also reduce $NADP^+$). The modified procedure was as follows. After incubation, islets were packed by a 1min spin in a bench centrifuge and 48μl of incubation medium (containing most of the glucose) was removed with a calibrated fine glass capillary tube. The inadvertent removal of any islets was readily seen. Enzymes were then inactivated and NADPH destroyed by adding 20μl of 15mM-HCl and heating to 60°C for 30min (see above). After cooling at 0°C, 5μl of medium was added containing 0.5M-tris, 50μM-$NADP^+$, 2.5mg of bovine plasma albumin/ml, 5m-units of glucose 6-phosphate dehydrogenase, and dithiothreitol (0.1mg/ml) (pH8.0). After incubation for 90min at room temperature, $NADP^+$ was destroyed by the addition of 15μl of 0.3M-NaOH and heating at 75°C for 105min. After cooling, NADPH was assayed by addition of 200μl of recycling mixture containing 0.1M-tris, 5mM-2-oxoglutarate, 1mM-glucose 6-phosphate, 0.1mM-ADP, 2.5mg of bovine plasma albumin/ml, 36mM-ammonium acetate (pH8.0), 2.6 units of L-glutamate dehydrogenase and 2 units of glucose 6-phosphate dehydrogenase [separated from $(NH_4)_2SO_4$ by centrifugation and treated with charcoal to remove contaminating nucleotides]. After incubation for 40min at 37°C, the reaction was stopped by heating to 100°C for 4min and the mixture was cooled and centrifuged. A sample of supernatant was then assayed fluorimetrically for 6-phosphogluconate with 6-phosphogluconate dehydrogenase. Blanks for each type of incubation medium were prepared by incubation of samples of media without islets and by then treating them in the same way as media incubated with islets. Standard curves for glucose 6-phosphate were constructed in each type of incubation medium. These precautions were essential because the residual incubation medium frequently contained sufficient glucose to give an appreciable blank and glucose also decreased the slope of the standard glucose 6-phosphate curve. Under the conditions described the standard curves were linear from 0.5 to 20pmol of glucose 6-phosphate and the lower limit of assay was 0.5–1pmol. The reproducibility of the assay for duplicate determinations on standards was within 7% of the mean.

Fructose 6-phosphate was assayed by the difference between glucose 6-phosphate measured in the presence and in the absence of 0.014 units of glucose phosphate isomerase (added in the first incubation).

Assay of ^{14}C in glucose 6-phosphate and glucose. In some experiments we have attempted to show formation of [^{14}C]glucose 6-phosphate and [^{14}C]glucose from [1-^{14}C]-mannose and [U-^{14}C]fructose. The procedure was as follows. After incubation, the media were acidified and CO_2 was collected in Hyamine for radioactive assay (see above). The acidified medium was transferred either directly or after ultrasonic treatment to a Marie flask containing 2ml of 100mM-triethanolamine, 50mM-glycylglycine, 10mM-$MgSO_4$, 5mM-ATP, 2mM-$NADP^+$, 0.5mM-EDTA and 0.25mM-glucose (pH7.7). Glucose was then converted quantitatively into 6-phosphogluconate by the addition of 7 units of hexokinase and 4 units of glucose 6-phosphate dehydrogenase and incubation for 10min (the extent of conversion was checked by measurement of ΔE_{340}). Incubation was then continued for a further 40min with 0.05 unit of 6-phosphogluconate dehydrogenase. The extent of conversion into ribulose 5-phosphate and HCO_3^- was then ascertained by the change in E_{340}; CO_2 was subsequently liberated by acidification, collected in Hyamine and assayed for radioactivity. For assay of radioactive glucose 6-phosphate, ATP and hexokinase were omitted and 0.5μmol of carrier glucose 6-phosphate was used in place of glucose. This method only measures radioactivity in C-1 of glucose and recovery (shown with [1-^{14}C]glucose) is 85% or better. [1-^{14}C]Mannose and [U-^{14}C]fructose do not interfere.

Fluorescence measurements. In the fluorimetric assays described above for NADH and NADPH the fluorimeter used was built in the laboratory and measurements were recorded on a chart 25cm wide. The sensitivity was adjusted according to the amount to be assayed such that full scale deflection was given by 0.5 to 5nmol of NADH or NADPH. The exciting light of wavelength 340nm was delivered at right angles to the front face of the cuvette

and the emitted light above 420nm taken at an angle of 45°. The cuvette chamber was jacketed at 22°C.

Islet volumes. The mean diameter of 80 mouse islets measured with a micrometer eye-piece fitted to the dissecting microscope was 157μm. Assuming a spherical shape this corresponds to a mean islet volume of 2.15nl. The weight of 40 mouse islets heated to 105°C for 18h was 20μg which gives a mean islet dry weight of 0.5μg. This corresponds to an islet water volume of 2nl if it is assumed that dry solids account for 20% of the wet weight of islets. (The latter could not be measured with any accuracy.) The two methods both suggest that the mean islet water volume is of the order of 2nl per islet.

Calculation and expression of results. Rates and concentrations were measured on individual batches of 10 islets and are expressed per 10 islets. The extent to which individual batches of islets vary in weight is not known but it is presumably similar to the variation in mean islet volume which was found with different batches of 10 islets to be of the order of 10%. The concentrations of glucose 6-phosphate and fructose 6-phosphate have also been calculated per unit volume of islet water assuming the latter to be 2nl per islet. The actual concentration in intracellular water will be greater because this estimate of islet water volume includes extracellular water. For purposes of comparison with other tissues, rates of glucose oxidation and lactate formation have also been given per g dry wt. of islets (mean islet dry weight of 0.5μg). Rates of $^{14}CO_2$ formation from radioactive glucose, mannose, or fructose have been calculated either as ng-atoms of sugar carbon or pmol of sugar by using the known specific radioactivity of these sugars.

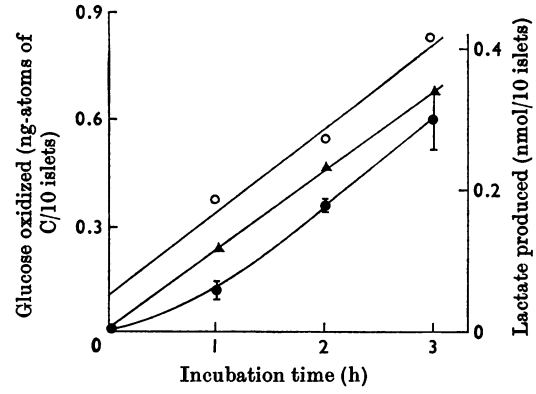

Fig. 1. Time-course of glucose oxidation and lactate output by mouse pancreatic islets. For measurement of glucose oxidation three batches of 10 islets were incubated in medium (0.15ml) containing [U-^{14}C]glucose (●, 0.2mg/ml; 1.58mCi/mmol; ▲, 3.3mg/ml, 3mCi/mmol) at 37°C for the time shown. The output of $^{14}CO_2$ was assayed and the rate of glucose oxidation calculated as described in the Experimental section. For measurement of lactate output two batches of 10 islets were incubated in medium (50μl) containing 3mg of glucose/ml at 37°C for the time shown. ●, Glucose oxidation; ▲, 0.1 × glucose oxidation; ○, lactate output. Each result is given as the mean or as the mean ± S.E.M.

RESULTS

Glucose oxidation

The term glucose oxidation is used here to refer to the conversion of glucose carbon into carbon dioxide calculated from the incorporation of ^{14}C from [U-^{14}C]glucose into carbon dioxide and the specific radioactivity of medium glucose under conditions of incubation given in the Figures and Tables. The true rate of glucose oxidation may exceed this estimate because of the presence in the islets of pools of unlabelled metabolites of glucose at the beginning of incubation, and the possibility of some delay in temperature equilibration of the incubation medium. Technical difficulties have not permitted measurement of the incorporation of ^{14}C into metabolites of glucose in the islet and, because of this, true rates of glucose oxidation could not be calculated. Studies of the time course of glucose oxidation shown in Fig. 1 give an estimate of this difference. At a concentration of 3.3mg of glucose/ml of medium identical rates of glucose oxidation were observed in the first, second or third hours of incubation. However, with 0.2mg of glucose/ml of medium the rate of glucose oxidation in the first hour (0.12ng-atom of glucose carbon/10 islets) was only one-half of that seen in the second and in the third hour (0.24ng-atom of glucose carbon/10 islets). A fixed incubation time of 2h was used in all subsequent experiments and the calculated rate of glucose oxidation at low extracellular glucose concentrations may thus be some 30% lower than the true rate. The magnitude of changes in the calculated rate of glucose oxidation which are described below are large in relation to this possible source of error.

Effect of glucose concentration. These results are shown in Fig. 2. The rate of glucose oxidation by mouse islets was found to depend markedly on the extracellular glucose concentration. The most striking feature of these results is the sigmoid or biphasic shape of the curve relating glucose oxidation rate to glucose concentration, which showed evidence of a threshold at approximately 1mg of glucose/ml. Below this concentration there was little increase in the rate of glucose oxidation as the glucose concentration was raised. Between concentrations of 1 and 1.8mg of glucose/ml increasing the glucose concentration led to a marked increase in the rate of glucose oxidation. At concentrations above 1.8 mg of glucose/ml the rate of oxidation tended towards a maximum of 1.32ng-atoms of glucose carbon/h per 10 islets (or 0.26mg-atoms of glucose carbon/h per g dry wt. of islet). Least squares analysis showed that an equation for a sigmoid curve gives a significantly better fit ($P<0.01$) for the relationship between glucose oxidation and extracellular

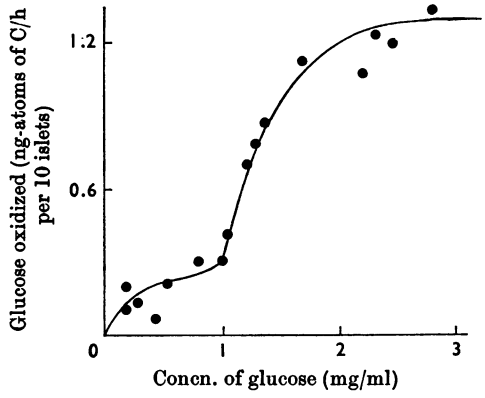

Fig. 2. Effect of glucose concentration on the rate of glucose oxidation by mouse pancreatic islets. Batches of 10 islets were incubated for 2h at 37°C in medium (0.15ml) containing [U-^{14}C]glucose (0.2–2.6mCi/mmol) at the given concentrations. The output of $^{14}CO_2$ was assayed and the rate of glucose oxidation calculated as described in the Experimental section. Each point is the mean of duplicate incubations.

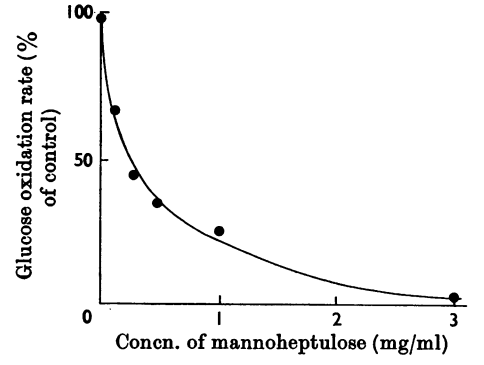

Fig. 3. Effect of various concentrations of D-mannoheptulose on the rate of glucose oxidation by mouse pancreatic islets. [U-^{14}C]Glucose concentration was 1.77mg/ml (2.9mCi/mmol). Other conditions were as given in the legend to Fig. 2.

glucose concentration, than either a linear or a quadratic equation. The concentration of glucose that gives a half maximum rate of glucose oxidation was approximately 1.4mg/ml (8mM).

Effect of mannoheptulose, phlorrhizin and some glucose derivatives. These results are shown in Fig. 3 and Table 1. Mannoheptulose was a potent inhibitor of glucose oxidation. At a glucose concentration of 1.77mg/ml, 50% inhibition was produced by 0.2mg of mannoheptulose/ml and apparently total suppression of glucose oxidation was frequently observed with appropriate relative concentrations. Phlorrhizin, on the other hand, at a concentration of 1.4mg/ml had no detectable effect on the rate of glucose oxidation at glucose concentrations of 0.55 or 2.6mg/ml. In experiments with D-glucosamine, *N*-acetyl-D-glucosamine and D-2-deoxyglucose, concentrations of glucose were chosen above the threshold value but below the value which gave near maximum oxidation rates so that inhibitory or stimulatory effects might be detected. D-Glucosamine inhibited glucose oxidation by about 50% (7mM-glucose; 18.5mM-glucosamine). At the concentrations used, *N*-acetyl glucosamine and 2-deoxyglucose had no detectable effect on glucose oxidation.

Effect of some modifiers of insulin release or insulin biosynthesis

These results are shown in Table 2. Adrenaline (1μM) or diazoxide (0.12mg/ml), which block the glucose effect on insulin release (Coll-Garcia & Gill, 1969; Milner & Hales, 1969) had no significant effect on the rate of glucose oxidation by mouse islets. The presence of Ca^{2+} has been shown to be essential for glucose stimulation of insulin release by rabbit and rat pancreas (Milner & Hales, 1969; Grodsky & Bennett, 1966). However, the increase in glucose oxidation rate by mouse islets at high glucose concentrations was not dependent on extracellular Ca^{2+}. Thus in the absence of Ca^{2+} the rate of oxidation of glucose was increased some sevenfold on raising the medium glucose concentration from 0.52 to 2.89mg/ml and both the magnitude of the change and the absolute rates of oxidation were comparable to those observed in the presence of Ca^{2+} at the same glucose concentrations (Fig. 2).

Glucagon (5μg/ml) and tolbutamide (0.2mg/ml) stimulate insulin release by mouse islets (Coll-Garcia & Gill, 1969) and arginine stimulates insulin release by rat islets (Malaisse & Malaisse-Lagae, 1968). Tolbutamide stimulated glucose oxidation moderately whereas arginine and glucagon had no significant effect. Cycloheximide at a concentration (1mM) that totally inhibits pancreatic insulin synthesis *in vitro* (Steiner, Cunningham, Spigelman & Aten, 1967) had no significant effect on the glucose-oxidation rate.

Lactate formation

The time-course of lactate formation is shown in Fig. 1. If it is assumed that some lactate is present at the start of incubation (equivalent to a rate of conversion into lactate of 2μmol of glucose equivalent/h per g dry wt. of islets) then the rate of lactate formation is essentially constant during 3h of incubation. This assumption may be reasonable since the collection of islets in incubation vessels

Table 1. *Effects of* D-*mannoheptulose, phlorrhizin,* D-2-*deoxyglucose,* D-*glucosamine and* N-*acetyl*-D-*glucosamine on glucose oxidation by mouse pancreatic islets*

Batches of 10 islets were incubated for 2h at 37°C in medium (0.15ml) containing [U-^{14}C]glucose at the specific radioactivity and concentration shown, with other additions as given. The output of $^{14}CO_2$ was assayed and the rate of glucose oxidation calculated as described in the Experimental section. The results are either the means of duplicate incubations or given as means±S.E.M. with the numbers of observations in parentheses.

Glucose in medium (mg/ml)	Glucose in medium (mCi/mmol)	Other additions	Glucose oxidation rate (ng-atoms of glucose carbon oxidized/h per 10 islets)
0.55	2.6	None	0.21
		Mannoheptulose (3mg/ml)	0
1.5	0.44	None	1.1
		Mannoheptulose (6mg/ml)	0
2.4	0.27	None	1.2
		Mannoheptulose (6mg/ml)	0
0.26	2.36	None	0.13
		Phlorrhizin (1.4mg/ml)	0.10
2.07	0.30	None	1.1
		Phlorrhizin (1.4mg/ml)	1.0
2.60	0.49	None	1.2
		Phlorrhizin (1.4mg/ml)	1.2
1.30	2.9	None	0.79±0.13 (4)
		D-Glucosamine (4mg/ml)	0.37±0.02 (4)*
1.47	2.9	None	0.98±0.07 (4)
		N-Acetyl-D-glucosamine (15mg/ml)	0.99±0.07 (4)
1.62	2.8	None	1.5 ±0.07 (4)
		D-2-Deoxyglucose (6mg/ml)	1.4 ±0.22 (4)

* $P<0.05$.

Table 2. *Effect of adrenaline, glucagon, tolbutamide, diazoxide,* L-*arginine, and* Ca^{2+} *on rates of glucose oxidation by mouse pancreatic islets*

The conditions of incubation are as given at the head of Table 1. The specific radioactivity of glucose was 2.8mCi/mmol. The number of batches of islets incubated was 3 or 4 in each case and results are given as means±S.E.M.

Glucose in medium (mg/ml)	Glucose in medium (mCi/mmol)	Other additions	Glucose oxidation rate (ng-atoms of glucose carbon oxidized/h per 10 islets)
1.34	2.8	None	0.94±0.11
		Adrenaline (1μM)	0.81±0.15
		Glucagon (5μg/ml)	0.72±0.17
1.64	2.9	None	1.51±0.14
		Diazoxide (0.12mg/ml)	1.48±0.27
0.80	2.9	None	0.48±0.02
		Tolbutamide (0.2mg/ml)	0.64±0.05*
1.30	2.9	None	1.0 ±0.12
		Arginine (10mM)	0.96±0.08
0.52		None	0.22‡
0.52	2.8	No Ca^{2+}; 0.2mM-EDTA	0.24±0.04
2.89		None	1.32‡
2.89	2.8	No Ca^{2+}; 0.2mM-EDTA	1.69±0.12†
1.51	2.9	None	1.13±0.12
		Cycloheximide (1mM)	1.17±0.13

* $P<0.05$.
† $P<0.01$ for difference from 'No Ca^{2+}' with 0.52mg of glucose/ml.
‡ These values were read off from Fig. 2.

Table 3. *Effect of glucose concentration,* D-*mannoheptulose,* D-*glucosamine, and tolbutamide on output of lactate by mouse pancreatic islets*

Batches of 9–20 islets were incubated for 3 h at 37°C in medium (50 μl) containing glucose and other additions as shown. The output of lactate was measured and calculated as described in the Experimental section. Results are given as means ± S.E.M. with the numbers of batches of islets shown in parentheses.

Glucose in medium (mg/ml)	Other additions	Lactate output rate (nmol of lactate/h per 10 islets)
Zero	None	<0.05(3)
0.6	None	0.09±0.018(8)
1.3	None	0.14±0.015(3)
1.8	None	0.19±0.008(4)
2.5	None	0.21±0.020(2)
3.0	None	0.29±0.026(11)
Zero	Mannoheptulose (3 mg/ml)	<0.05(3)
1.8	Mannoheptulose (6 mg/ml)	0.16±0.011(4)
3.0	Mannoheptulose (3 mg/ml)	0.32±0.12 (5)
3.0	Mannoheptulose (6 mg/ml)	0.22±0.045(3)
1.3	Glucosamine (4 mg/ml)	0.09±0.002(3)*
0.6	Tolbutamide (0.2 mg/ml)	0.18±0.017(6)†

* $P<0.05$.
† $P<0.02$.

takes approximately 45 min and lactate may accumulate during this time. The amount involved was too small for reliable assay. In subsequent experiments incubations were made for 3 h.

The results are given in Table 3. The mean rate of lactate formation at 3 mg of glucose/ml was 29 μmol of glucose equivalent/h per g dry wt. of islets. Increasing medium-glucose concentrations led to an increase in lactate formation (shown to be statistically significant with $P<0.01$ as between glucose concentrations of 0.6 mg/ml and either 2.5 or 3 mg/ml). D-Glucosamine (4 mg/ml) significantly inhibited lactate production with 1.3 mg of glucose/ml and tolbutamide (0.2 mg/ml) significantly enhanced lactate formation with 0.6 mg of glucose/ml. Mannoheptulose (3 or 6 mg/ml) with glucose (1.8 or 3 mg/ml) failed to inhibit lactate production significantly in contradistinction to the marked inhibitory effects of similar concentrations of mannoheptulose on rates of glucose oxidation (Table 1) and the concentration of glucose 6-phosphate (Table 4). Similarly, mannoheptulose had no detectable effects on lactate formation measured in the absence of glucose.

Islet glucose 6-phosphate concentrations

In initial experiments the concentration of glucose 6-phosphate was measured in batches of mouse islets incubated for 15, 30 or 45 min in medium containing 1.4 mg of glucose/ml. The mean concentrations of glucose 6-phosphate (in pmol/10 islets) were 7.2 (at 15 min), 5.4 (at 30 min) and 5.4 (at 45 min) in duplicate incubations. In subsequent experiments a fixed incubation period of 30 min was used.

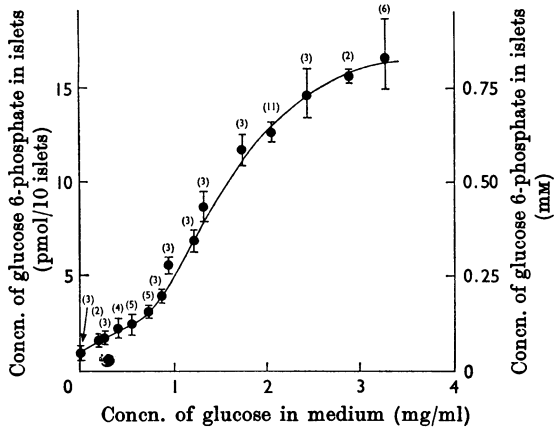

Fig. 4. Effect of glucose concentration on islet glucose 6-phosphate concentration. Batches of 10 islets were incubated at 37°C for 30 min in medium containing glucose at the given concentrations. For methods and calculations see the Experimental section. Results are given as the S.E.M. and the number of batches of islets at each glucose concentration is shown in parentheses.

Fig. 4 shows the relationship between extracellular glucose concentration and islet glucose 6-phosphate concentration. The relationship is characterized by a relatively small rise in glucose 6-phosphate as the glucose concentration is increased from 0 to 1 mg/ml (increase in glucose 6-phosphate approx. 3.5 pmol/10 islets) and a relatively greater rise between 1 and 2 mg of glucose/ml (increase in glucose 6-phosphate approx. 8.5 pmol/10 islets). Above 2 mg of glucose/ml the rise in glucose 6-phosphate was again relatively small and its concentration tended towards a maximum. Least squares analysis showed that an equation for a sigmoid curve gives a significantly better fit for the relationship between glucose 6-phosphate concentration and glucose concentration than either a linear or a quadratic equation ($P<0.01$). The approximate concentrations of glucose 6-phosphate in islet water were 50 μM in the absence of extracellular glucose; 0.2 mM at 1 mg of glucose/ml and 0.7 mM at 2 mg of glucose/ml. These are minimum estimates of the intracellular concentration since the volume of islet water used in the calculation (2 nl/islet) was the total islet water volume. Mannoheptulose (2.5 mg/ml) with 2 mg of glucose/ml lowered the glucose 6-phosphate concentration

Table 4. *Effect of* D-*mannoheptulose,* D-*glucosamine,* D-2-*deoxyglucose, tolbutamide and glucagon on islet glucose* 6-*phosphate concentration*

Batches of 10 islets were incubated for 30min at 37°C in medium (50μl) containing glucose and other additions as shown. For methods and calculations see the Experimental section. Results are given as means± S.E.M. with the numbers of batches of islets shown in parentheses.

Glucose in medium (mg/ml)	Other additions	Islet glucose 6-phosphate (pmol/10 islets)	Islet glucose 6-phosphate (mM)
2.0	None	12.7±0.7 (3)	0.63
	Mannoheptulose (2.5mg/ml)	3.6±0.1 (3)†	0.18
1.3	None	7.8±1.5 (3)	0.39
	Glucosamine (4mg/ml)	3.7±0.4 (3)*	0.18
1.6	None	9.0±0.1 (3)	0.45
	2-Deoxyglucose (6mg/ml)	‡21.9±0.8 (3)†	1.09
0.6	None	2.9±0.1 (3)	0.14
	Tolbutamide (0.2mg/ml)	2.9±0.9 (3)	0.14
0.6	None	3.3±0.4 (3)	0.16
	Glucagon (5μg/ml)	3.6±0.5 (3)	0.18

* $P<0.05$.
† $P<0.001$.
‡ It is not known what part of this estimate represents 2-deoxyglucose 6-phosphate.

to the value observed with 0.8mg of glucose/ml. Glucosamine (4mg/ml) with 1.3mg of glucose/ml also lowered the glucose 6-phosphate concentration. Control experiments showed that neither of these compounds nor glucosamine 6-phosphate interferes with the assay of glucose 6-phosphate by glucose 6-phosphate dehydrogenase. 2-Deoxyglucose (6mg/ml) with 1.6mg of glucose/ml produced a large apparent increase in glucose 6-phosphate but it was found that 2-deoxyglucose 6-phosphate may be partly measured in the glucose 6-phosphate assay. Therefore it is not known whether the apparent increase is due to glucose 6-phosphate or to 2-deoxyglucose 6-phosphate in the islets. No effect of tolbutamide (0.2mg/ml) or of glucagon (5μg/ml) on islet glucose 6-phosphate was detected. These results are shown in Table 4.

Metabolism of mannose and fructose

[1-^{14}C]Mannose was metabolized to $^{14}CO_2$ by mouse islets. The rate of oxidation increased 26-fold as the extracellular concentration was increased from 0.8mM to 29mM. The rate of oxidation of mannose as measured with [1-^{14}C]mannose was less than that of an equimolar concentration of glucose as measured with [U-^{14}C]glucose. Since glucose 6-phosphatase activity has been detected in mouse islets (Ashcroft & Randle, 1968*b*; Taljedhal, 1969) we have attempted to detect the formation of [1-^{14}C]glucose from [1-^{14}C]mannose, but no such formation could be detected. The sensitivity and reproducibility were such that the formation of approximately 30pmol of glucose at the lowest mannose concentration and 330pmol of glucose at the highest mannose concentration could easily have been detected. [U-^{14}C]Fructose was also oxidized by mouse islets and the rate of oxidation was less than that of equivalent concentrations of mannose or glucose. No formation of [U-^{14}C]glucose from [U-^{14}C]fructose was detected. Control incubations and radioactivity analyses with media containing [1-^{14}C]mannose suggested a possible 0.1% contamination with [1-^{14}C]glucose. In view of the low extent of this contamination and the fact that the apparent rate of oxidation was dependent on total mannose concentration rather than on the amount of radioactive mannose added, it seems reasonable to conclude that the $^{14}CO_2$ was formed from mannose and not from contaminating glucose, i.e. that mannose is oxidized. The degree of contamination of the [U-^{14}C]fructose was appreciably higher and the possibility that some of the $^{14}CO_2$ may have been formed from contaminating glucose has not been excluded. These results are shown in Table 5.

Also shown in Table 5 is the radioactivity in glucose 6-phosphate after incubation with [1-^{14}C]-mannose; no significant radioactivity was detected. This led us to determine the concentration of glucose 6-phosphate in the islets after incubation in media containing mannose. These results are shown in Table 6. Increasing the mannose concentration over the range of 0.25–5mg/ml had only a small effect on islet glucose 6-phosphate concentration whereas the fructose 6-phosphate concentration was increased substantially. These results, in conjunction with the radioactive data, suggest that phosphorylation and oxidation of mannose is associated with poor equilibration between fructose 6-phosphate and glucose 6-phosphate by glucose phosphate isomerase. However, the marked rise in fructose 6-phosphate concentration might

Table 5. *Metabolism of mannose and fructose by mouse pancreatic islets*

Medium (150 μl) containing [1-^{14}C]mannose or [U-^{14}C]fructose in amounts and radioactivities shown was incubated for 2 h at 37°C with and without the numbers of islets shown. For details of analytical methods see the Experimental section.

Medium	No. of islets	Mannose or fructose oxidized (pmol/h)		nCi in C-1	
		Total	Per 10 islets	Glucose	Glucose 6-phosphate
Mannose (0.12 μmol and 0.89 μCi/150 μl)	100	38	3.8	1.0	—
	0	0	0	1.0	—
Mannose (0.88 μmol and 2.4 μCi/150 μl)	70	280	40	0.7	0.08
	0	0	0	0.7	0.08
Mannose (4.35 μmol and 1.3 μCi/150 μl)	107	1050	98	1.5	—
	0	0	0	1.5	—
Fructose (1.86 μmol and 0.52 μCi/150 μl)	50	145	29	6	—
	0	0	0	7	—

Table 6. *Effect of mannose and fructose on islet glucose 6-phosphate and fructose-6-phosphate concentrations*

Batches of 10 islets were incubated for 30 min at 37°C in medium (50 μl) containing mannose or fructose at the concentrations shown. For methods and calculations see the Experimental section. Results are given as means ± S.E.M. with the numbers of batches of islets shown in parentheses.

Medium	Concn. in islets (pmol/10 islets)		
	Glucose 6-phosphate	Glucose 6-phosphate plus fructose 6-phosphate	Fructose 6-phosphate by difference
Mannose (0.25 mg/ml)	1.27 ± 0.2 (3)	2.14 ± 0.26 (9)	0.87
Mannose (2.5 mg/ml)	2.0 ± 0.13 (6)*		
Mannose (4.0 mg/ml)	2.55 ± 0.66 (3)		
Mannose (5.0 mg/ml)	1.32 ± 0.11 (3)	4.27 ± 0.39 (9)‡	2.95
Fructose (0.25 mg/ml)	1.50 ± 0.35 (3)		
Fructose (5.0 mg/ml)	3.43 ± 0.40 (3)†		

* $P < 0.05$ (versus mannose, 0.25 mg/ml).
† $P < 0.02$ (versus fructose, 0.25 mg/ml).
‡ $P < 0.001$ (versus mannose, 0.25 mg/ml).

suggest that fructose 6-phosphate does equilibrate readily with mannose 6-phosphate. By contrast, fructose produced a more marked rise in glucose 6-phosphate concentration, although this was substantially less than that seen at high glucose concentrations.

DISCUSSION

Parameters of mouse islet metabolism; rates of oxidation of glucose, mannose and fructose and output of lactate; concentrations of glucose 6-phosphate. The calculated rates of glucose oxidation by normal mouse islets in the present investigations were 44 μmol of glucose/h per g dry wt. of islets at glucose concentrations above 2 mg/ml (maximum rate) and 8 μmol of glucose/h per g dry wt. of islets at 0.8 mg of glucose/ml (threshold concentration). The rates for [1-^{14}C]mannose were 20 μmol of mannose/h per g dry wt. of islets at 5 mg/ml and 8 μmol of mannose/h per g dry wt. of islets at 1 mg/ml; the rate for [U-^{14}C]fructose was 6 μmol of fructose/h per g dry wt. of islets at 2.2 mg of fructose/ml. The only other published values are those of Jarrett & Keen (1968*a*) for rat islets prepared by microdissection after ligation of the pancreatic ducts. If it is assumed that efficiency of counting was 60% in the experiments of Jarrett & Keen (1968*a*) and that rat islets are of similar weight to mouse islets, then they obtained mean rates (in μmol/h per g dry wt. of islets) of 51 at 2 mg of glucose/ml; 16 at 2 mg of mannose/ml; and 10 at 2 mg of fructose/ml (with [U-^{14}C]glucose, mannose or fructose). The two sets of values may thus be of the same absolute and relative order. Hellerström (1967) has given values for the oxygen consumption of islets, prepared by microdissection from pancreas of obese–hyperglycaemic mice, incubated in Cartesian divers in Krebs–Ringer phosphate buffer. The oxygen consumptions (in μmol of O_2/h per g

dry wt. of islet) were 340 at 3mg of glucose/ml, 271 at 3mg of mannose/ml, 249 at 3mg of fructose/ml and 226 in the absence of exogenous substrate or in the presence of 0.5mg of glucose/ml. If these values are applicable to normal mouse islets then at 3mg of glucose/ml, oxidation of glucose may account for some 80% of the islet oxygen consumption.

The production of lactate in the present study (μmol of glucose equivalent/h per g dry wt. of islets) was approx. 29 at 3mg of glucose/ml and 10 at 0.6mg of glucose/ml. There are no other published values for islet lactate output. Our observations indicate that the rate of lactate output was approx. 50% of the rate of glucose oxidation. This is of the same order as that seen with muscle preparations *in vitro* but much higher than for preparations of rat epididymal adipose tissue *in vitro*.

The concentration of glucose 6-phosphate in mouse islets (in μmol/ml of islet water) was 0.15 at 0.8mg of glucose/ml and 0.75 at 2.9mg of glucose/ml. Montague & Taylor (1969) measured glucose 6-phosphate concentrations in rat islets that were prepared and incubated under comparable conditions and (assuming similar islet wt. and water content) obtained values (in μmol/ml of islet water) of 0.4 at 0.8mg of glucose/ml and 0.7 at 2.7mg of glucose/ml. These values are thus of the same absolute order but the increase observed when the extracellular glucose concentration was raised from 0.8 to 2.7mg/ml was somewhat smaller in the rat islets. This could represent a species variation or possibly interference by extracellular glucose under the conditions of assay employed. Montague & Taylor (1969) failed to observe the sigmoid curve that we found but our experience suggests that this may have been due to the inclusion of insufficient points at glucose concentrations below 1mg/ml. Matschinsky & Ellerman (1968) measured glucose 6-phosphate in islets of obese–hyperglycaemic mice *in vivo*. The values (in μmol/ml of islet water) were 0.05 at 0.8mg of glucose/ml of blood, 0.1 at 2.7mg of glucose/ml of blood, with a sharp increase above 4mg of glucose/ml of blood to reach 0.8 at approx. 5mg of glucose/ml of blood. The values are thus of the same absolute order but the marked rise in glucose 6-phosphate concentration occurred at a much higher extracellular glucose concentration. This difference could be a feature of the obese–hyperglycaemic syndrome or reflect differences *in vivo* or possibly differences in the speed at which islet metabolism was arrested.

It has not proved practicable to measure mouse islet glucose uptake or mouse islet glycogen synthesis. If lactate production and glucose oxidation together account for a substantial proportion of the glucose taken up, then rates of glucose uptake were of the order of 70μmol of glucose/h per g dry wt. of islets at 3mg of glucose/ml; and 14μmol of glucose/h per g dry wt. of islets at 0.6mg of glucose/ml.

Islet glucose metabolism and insulin release. It was found in the present study that rates of glucose oxidation and lactate formation and the concentration of glucose 6-phosphate in mouse islets are markedly elevated by increasing extracellular glucose concentrations, which were shown in parallel experiments to elicit insulin release (Coll-Garcia & Gill, 1969). Moreover, the curves relating extracellular glucose concentration to glucose oxidation rate or glucose 6-phosphate concentration in mouse islets are very similar to the curve relating extracellular glucose concentration to the rate of insulin release in rat pancreas (Malaisse *et al.* 1967). Comparable detailed information on the rate of release of insulin by mouse islets as a function of glucose concentration is not available but results published by Coll-Garcia & Gill (1969) and by Montague & Taylor (1969) indicate that mouse and rat islets may show similar insulin-secretory responses at a number of different extracellular glucose concentrations. Hence there would appear to be a positive correlation between the effects of extracellular glucose concentration on the rates of glucose oxidation and lactate formation and the concentration of glucose 6-phosphate, and the rate of insulin release. This correlation could also be extended to the effects of other sugars and sugar derivatives. Glucose oxidation was inhibited markedly by mannoheptulose and by glucosamine, which inhibit insulin release (Coll-Garcia & Gill, 1969; Coore & Randle, 1964*a*) whereas neither process was affected by 2-deoxyglucose, *N*-acetylglucosamine and phlorrhizin. Mannoheptulose and glucosamine also suppressed the increase in islet glucose 6-phosphate that was induced by an increase in extracellular glucose concentration. Glucosamine, but not mannoheptulose, also diminished lactate formation at a high extracellular glucose concentration. Jarrett & Keen (1968*b*) have also reported inhibition of glucose oxidation in mouse islets by mannoheptulose but not by 2-deoxyglucose.

Coore & Randle (1964*a*) suggested that islet glucose uptake may be limited by the rate of glucose phosphorylation and that mannoheptulose and glucosamine may block glucose effects on insulin release by inhibiting phosphorylation of the sugar. The finding that islet glucose concentrations are very similar to extracellular glucose concentrations (Matschinsky & Ellerman, 1968) and that phlorrhizin does not influence the rate of glucose oxidation in mouse islets is consistent with the idea of phosphorylation as the rate-limiting step in glucose utilization. If glucose oxidation and lactate formation account for a substantial proportion of islet glucose uptake, then our measurements of

these parameters and of islet glucose 6-phosphate concentration may indicate that the rate of glucose phosphorylation is markedly dependent on extracellular glucose concentration over the physiological range, and that mannoheptulose and glucosamine may inhibit glucose phosphorylation in mouse islets. Some caution is necessary in interpreting effects of mannoheptulose because, although it inhibited glucose oxidation and lowered glucose 6-phosphate concentration markedly, it did not appear to lower lactate output. Moreover, the kinetics of inhibition of mouse islet hexokinase by mannoheptulose (Ashcroft & Randle, 1970) do not completely account for the profound inhibition of glucose oxidation by this sugar. There is the possibility, therefore, that mannoheptulose may have other effects on islet glucose metabolism.

The correlation between the rates of oxidation and rates of release of insulin may also be extended to mannose and fructose. These sugars are oxidized at a lower rate than glucose and they are less effective in initiating insulin release by mouse islets (S. J. H. Ashcroft & J. G. Schofield, unpublished work) or rabbit or rat islets (Coore & Randle, 1964*a*; Grodsky *et al.* 1963).

The results may, therefore, be consistent with the view that glucose must be metabolized by the β-cell in order to elicit insulin release. An alternative interpretation would be that changes in glucose utilization are secondary to changes in the rate of release or synthesis of insulin that may involve ATP utilization. This latter interpretation, however, seems less likely in view of the observations that omission of Ca^{2+}, or addition of adrenaline or diazoxide which can abolish the effect of glucose on insulin release (Grodsky & Bennett, 1966; Coore & Randle, 1964*a*; Howell & Taylor, 1966) did not affect glucose oxidation. Moreover, glucagon, which stimulates insulin release from mouse islets (Coll-Garcia & Gill, 1969) was also without significant effect on glucose oxidation, as was arginine, another stimulator of insulin release (Malaisse & Malaisse-Lagae, 1968). Further, increasing extracellular glucose concentrations were accompanied by raised intracellular glucose 6-phosphate concentrations, whereas an effect of insulin release on glucose uptake, mediated through ATP utilization, might be expected to be associated with a fall in glucose 6-phosphate concentration as a consequence of phosphofructokinase activation. As regards insulin synthesis, the lack of effect of the protein-synthesis inhibitor, cycloheximide, on islet glucose oxidation suggests that the latter process is not controlled by the rate of insulin synthesis.

It should be emphasized that the 'substrate-site' model does not require inhibition of glucose metabolism by all compounds that inhibit insulin release. Insulin release is envisaged as a multi-stage process and modification of a step beyond the glucoreceptor system (i.e. the coupling or release processes) could affect insulin release without necessarily affecting glucose metabolism. Thus the lack of effect of adrenaline, diazoxide and absence of Ca^{2+} on the rate of glucose oxidation could be interpreted as an action exerted at some later stage in the release process. Conversely, glucagon and arginine may stimulate insulin release in a manner independent of glucose metabolism, by an effect exerted at a later stage in the release process.

Metabolism of mannose and fructose. The differences in the relative rates of oxidation of fructose, mannose and glucose may reflect the characteristics of the enzyme(s) catalysing phosphorylation of these sugars. An elevated fructose concentration produced a rise in islet glucose 6-phosphate concentration, although the concentration attained was considerably less than that seen with a similar glucose concentration. However, mannose at concentrations up to 5mg/ml did not markedly affect islet glucose 6-phosphate concentrations, although the islet fructose 6-phosphate concentration was substantially increased. Further, there was no detectable incorporation of label from [1-^{14}C]mannose into islet glucose 6-phosphate. Mannose oxidation by islets may thus proceed with poor equilibration between fructose 6-phosphate and glucose 6-phosphate.

Identity of the metabolite signal. These results are consistent with the idea that enzymes controlling the rate of glucose phosphorylation may constitute the glucoreceptor and that some product(s) of glucose metabolism may mediate glucose effects on release of insulin. The nature of the product(s) that may trigger the release process is not yet established. The present results suggest that changes in glucose 6-phosphate concentration could mediate effects of extracellular glucose concentration. This could account for the inhibitory effects of mannoheptulose and glucosamine but not for the stimulatory effects of mannose or of xylitol (Montague & Taylor, 1968) on insulin release. Glucose 6-phosphate concentration may not, therefore, be an obligatory signal for effects of these compounds. The closely related metabolites fructose 6-phosphate (present study) or 6-phosphogluconate (Montague & Taylor, 1969) are possible alternatives to glucose 6-phosphate, though the effect of mannose on 6-phosphogluconate concentration and of xylitol on fructose 6-phosphate concentration are not known. It would appear that this particular problem is more likely to be resolved through studies of the coupling and release processes.

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