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Enzymes of Glucose Metabolism in Normal Mouse Pancreatic Islets

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1. Glucose-phosphorylating and glucose 6-phosphatase activities, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, $NADP^+$-linked isocitrate dehydrogenase, 'malic' enzyme and pyruvate carboxylase were assayed in homogenates of normal mouse islets. 2. Two glucose-phosphorylating activities were detected; the major activity had K_m 0.075mM for glucose and was inhibited by glucose 6-phosphate (non-competitive with glucose) and mannoheptulose (competitive with glucose). The other (minor) activity had a high K_m for glucose (mean value 16mM) and was apparently not inhibited by glucose 6-phosphate. 3. Glucose 6-phosphatase activity was present in amounts comparable with the total glucose-phosphorylating activity, with K_m 1mM for glucose 6-phosphate. Glucose was an inhibitor and the inhibition showed mixed kinetics. No inhibition of glucose 6-phosphate hydrolysis was observed with mannose, citrate or tolbutamide. The inhibition by glucose was not reversed by mannoheptulose. 4. 6-Phosphogluconate dehydrogenase had K_m values of 2.5 and 21 μM for $NADP^+$ and 6-phosphogluconate respectively. 5. Glucose 6-phosphate dehydrogenase had K_m values of 4 and 22 μM for $NADP^+$ and glucose 6-phosphate. The K_m for glucose 6-phosphate was considerably below the intra-islet concentration of glucose 6-phosphate at physiological extracellular glucose concentrations. The enzyme had no apparent requirement for cations. Of a number of possible modifiers of glucose 6-phosphate dehydrogenase, only NADPH was inhibitory. The inhibition by NADPH was competitive with $NADP^+$ and apparently mixed with respect to glucose 6-phosphate. 6. $NADP^+$–isocitrate dehydrogenase was present but the islet homogenate contained little, if any, 'malic' enzyme. The presence of pyruvate carboxylase was also demonstrated. 7. The results obtained are discussed with reference to glucose phosphorylation and glucose 6-phosphate oxidation in the intact mouse islet, and the possible nature of the β-cell glucoreceptor mechanism.

The suggestion that glucose must be metabolized by the β-cell to elicit insulin release (Grodsky *et al.* 1963; Coore & Randle, 1964) has received support from studies on the carbohydrate metabolism of isolated pancreatic islets of the mouse, in which a correlation was found to exist between glucose metabolism and glucose-stimulated insulin release (Ashcroft, Hedeskov & Randle, 1970). In particular, an increased rate of glucose oxidation and lactate output was observed when the medium glucose concentration was raised over the range 5–15mM. The rate-limiting step for glucose utilization by mouse islets appears to be glucose phosphorylation since there is evidence that membrane transport of glucose is not rate-limiting in this tissue (Matschinsky & Ellerman, 1968, Ashcroft *et al.* 1970). The question therefore arises as to the enzymic basis for the high K_m (approx. 8mM) for glucose utilization and hence net glucose phosphorylation. Such a system may then be tentatively identified with the glucoreceptor that enables the β-cells to respond to changes in blood glucose concentration by the production of an intracellular metabolite that may trigger insulin release. We have therefore studied the properties of enzymes in homogenates of mouse islets that may be involved in glucose phosphorylation (ATP–D-glucose 6-phosphotransferase, EC 2.7.1.1 and EC 2.7.1.2; D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) to see to what extent these enzymes may account for the observed kinetics of glucose utilization by mouse islets.

There is, as yet, no firm view as to which product(s) of glucose metabolism by the β-cell may be involved in the control of insulin release. Since there is isotopic evidence for metabolism of glucose by the

pentose phosphate pathway in human and rat pancreatic islets (Field, Johnson, Herring & Weinberg, 1960; Keen, Sells & Jarrett, 1965), it has been suggested that one of the products of this pathway may be involved (Montague & Taylor, 1968*a*,*b*). This hypothesis envisages a chain of events whereby increased extracellular glucose concentrations lead via increased intra-islet glucose 6-phosphate concentrations to increased oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and thereby to insulin release. This hypothesis assumes that the intra-islet glucose 6-phosphate concentration may limit the rate of oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase. However, in tissues where the pentose phosphate pathway may furnish NADPH for fatty acid biosynthesis there is evidence that the rate of oxidation of glucose 6-phosphate by this pathway is determined by the rate of reoxidation of NADPH to $NADP^+$ rather than by the intracellular glucose 6-phosphate concentration (McLean, 1960, 1962). The influence of the [NADPH]/[$NADP^+$] ratio on the functioning of this pathway may extend to other tissues: estimates of the redox state of $NADP^+$ in rat liver cytoplasm calculated by Veech, Eggleston & Krebs (1969) have suggested [NADPH]/[$NADP^+$] ratios between 100:1 and 1000:1 depending on the nutritional state of the animal, and it has been found that NADPH is a competitive inhibitor (against $NADP^+$) of glucose 6-phosphate dehydrogenases from a variety of tissues (e.g. see Glaser & Brown, 1955; Flint & Denton, 1970). We have therefore investigated the kinetic properties of mouse islet glucose 6-phosphate dehydrogenase in order to assess the possibility of control of this reaction by intra-islet glucose 6-phosphate concentration and [NADPH]/[$NADP^+$] ratio. Some properties of 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were also determined. In addition we have measured the activities of $NADP^+$-isocitrate dehydrogenase (EC 1.1.1.42), 'malic' enzyme (EC 1.1.1.40) and pyruvate carboxylase (EC 6.4.1.1) to determine the potential capacity of the mouse islet to generate NADPH other than via the pentose phosphate pathway.

Preliminary accounts of some of these findings have been published (Ashcroft & Randle, 1968*a*; Randle & Ashcroft, 1969).

EXPERIMENTAL

Materials and animals. Collagenase (Calbiochem, Los Angeles, Calif., U.S.A.), triethanolamine hydrochloride, $NADP^+$, NADH, ATP, ADP, AMP, 3′:5′-cyclic AMP, glucose 6-phosphate, pyruvate and auxiliary enzymes for assay purposes were from Boehringer Corp., London W.5, U.K. Sodium DL-isocitrate and L-malate were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. NADPH (type I) prepared by chemical reduction was obtained from Sigma Chemical Co. because commercial samples prepared by enzymic reduction may be contaminated with 6-phosphogluconate. Acetyl-CoA was prepared by the method of Simon & Shemin (1953). Bovine serum albumin (Fraction V) was from Armour Pharmaceuticals Ltd., Eastbourne, Sussex, U.K. Other chemicals of the purest grade available were from British Drug Houses Ltd., Poole, Dorset, U.K. [1-^{14}C]Glucose (specific radioactivity 0.2 or 2.9mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Male albino mice were fed *ad libitum* on diet 41B (Oxo Ltd., London E.C.4, U.K.) and used for preparation of islets at age 3–4 weeks after starvation overnight.

Preparation of islets. Islets were prepared by a collagenase method and harvested with a wire loop as described by Coll-Garcia & Gill (1969). The islets (100–200) were collected in 40μl of ice-cold 0.25M-sucrose–5mM-tris–0.5mM-EDTA, pH 7.0.

Islet homogenates. The islet suspension (see above) was diluted with 100–250μl of the appropriate assay buffer (see below), and islets were disrupted by sonication for 10s at position 1 on a Soniprobe (Dawe Instruments Ltd., London W.3, U.K.).

Assays of glucose-phosphorylating activity. The reaction mixture (final volume 30μl) consisted of: 100mM-triethanolamine chloride, pH 7.4, 150mM-KCl, 10mM-$MgSO_4$, [1-^{14}C]glucose (at concentrations and specific radioactivities described in the text and figures), ATP (5mM unless otherwise stated), 2mM-*N*-acetylcysteine, 1mM-EDTA and 0.1mg of bovine plasma albumin/ml. In some assays the medium also contained 2mM-phosphoenolpyruvate and 1 unit of pyruvate kinase to provide an ATP-regenerating system. Other additions to the medium are indicated in the text or figures. The reaction was started by the addition of 10μl of islet homogenate, and was carried out in small plastic tubes (6mm × 30mm) in a water bath at 37°C. The reaction was terminated by the addition of 50μl of 0.2M-HCl. The contents of the tubes were transferred quantitatively to glass-stoppered centrifuge tubes with 2 × 60μl of 0.2M-HCl, and 0.5μmol of glucose 6-phosphate (in a volume of 10μl) was added to each tube. The tubes were heated on a boiling-water bath for 15min to destroy the ATP present, and then cooled and centrifuged to bring down to the bottom of the tubes any liquid adhering to the sides. The contents of the tubes were then transferred quantitatively to Marie flasks with washes (2 × 1.25ml) of 100mM-triethanolamine buffer, pH 7.7, containing 50mM-glycylglycine, 10mM-$MgSO_4$, 0.5mM-EDTA and 1.2mM-$NADP^+$. Then 0.7 unit of glucose 6-phosphate dehydrogenase was added to each flask. This reaction (conversion of glucose 6-phosphate into 6-phosphogluconate) was complete in 15min. Then 0.07 unit of 6-phosphogluconate dehydrogenase was added, a scintillation vial containing 0.4ml of Hyamine was placed in the centre well of each flask and the flasks were sealed with serum stoppers. After 40min, 0.5ml of 0.15M-$NaHCO_3$ was injected into each flask followed by 1ml of 2M-H_2SO_4. The flasks were shaken at room temperature for 2h for absorption of $^{14}CO_2$ into Hyamine. The Hyamine was then transferred to scintillation bottles with 10ml of toluene scintillator (Synder, 1961) and radioactivity was assayed in a Nuclear–Chicago Mark I

Liquid-Scintillation Computer. Count rates were corrected to 100% counting efficiency by the channels-ratio method by using an external barium standard. Control samples lacking ATP or homogenate were taken through the entire procedure and the radioactivity assayed in these samples was subtracted from that in the experimental samples. Since the specific radioactivity of glucose was constant in kinetic experiments it was convenient to express rates of glucose phosphorylation as d.p.m. in $^{14}CO_2$ per unit time. For calculation of absolute rates of phosphorylation, the known specific radioactivity of [1-^{14}C]glucose was used to express results in terms of pmol of glucose phosphorylated with a correction for the efficiency of conversion of [1-^{14}C]glucose 6-phosphate into $^{14}CO_2$. This was less than 100% because the 6-phosphogluconate dehydrogenase reaction did not go to completion under the conditions used. The extent of conversion at this step (70–80%) was ascertained by measuring the E_{340} of a sample of reaction mixture after the glucose 6-phosphate dehydrogenase step and again after the 6-phosphogluconate dehydrogenase step. Since excess (500–1000-fold) of carrier glucose 6-phosphate was added before assay of [1-^{14}C]glucose 6-phosphate it has seemed reasonable to assume that the extent of conversion of [1-^{14}C]glucose 6-phosphate into $^{14}CO_2$ will be independent of the amount of [1-^{14}C]glucose 6-phosphate present. Aqueous samples, e.g. assay media, were assayed for radioactivity by liquid-scintillation spectrometry by using a dioxan-based scintillator (Butler, 1961).

Glucose 6-phosphatase assay. A modification of the method of Ockerman (1967) was used. A 20μl portion of islet homogenate was added to 20μl of 50mM-tris–maleate buffer, pH 6.5, containing substrate and other additions, in small plastic tubes (1cm × 6cm). The small tubes were sealed with Parafilm, placed inside larger tubes also stoppered with Parafilm and then incubated in a water bath at 37°C usually for 2h. The reaction was stopped and P_i determined by adding to each tube 1ml of Malachite Green reagent [1vol. of 4.2% $(NH_4)_6Mo_7O_{24},4H_2O$ in 4.5–5.0M-HCl and 3vol. of 0.2% Malachite Green] and 50μl of 1.5% Tween 20. The colour was developed at 37°C for 15min and then 1.5ml of 1M-HCl was added to each tube. The samples were then assayed spectrophotometrically at 666nm in 4cm-light-path cuvettes. For each assay, P_i standards were carried through the entire procedure, as were blank incubations lacking either substrate or homogenate. These controls allowed corrections to be made for any P_i present in reagents or homogenate respectively.

Fluorescence measurements. In assays described below NADPH or NADH was measured by fluorescence in a purpose-built recording fluorimeter (chart width 25cm). For dehydrogenase assays the sensitivity was adjusted to yield full-scale deflexion with 2–3nmol of NADPH or NADH.

Assay of dehydrogenase activities. The activity of $NADP^+$-linked dehydrogenases was assayed at 22°C by the change in fluorescence on adding islet homogenate (equivalent to ten islets, representing 5μg dry wt. of islets) to tubes containing 2.5ml of assay medium (50mM-triethanolamine hydrochloride, 10mM-$MgCl_2$, 0.5mM-EDTA and 0.1mg of bovine plasma albumin/ml, adjusted to pH 7.5 with KOH), containing substrates and possible effectors at concentrations specified in tables, figures or text. The limit of the assay was approx. 2×10^{-6} unit of dehydrogenase activity. Difficulties were encountered in determining the effect of NADPH on glucose 6-phosphate dehydrogenase activity, since this entailed measurement of small increases in fluorescence against a large background. This difficulty was overcome by using a stopped assay for 6-phosphogluconate produced. Reaction was stopped and NADPH destroyed by adding 20μl of 5M-HCl. After neutralization and further addition of $NADP^+$, 6-phosphogluconate was assayed by the change in fluorescence on adding 0.6 unit of 6-phosphogluconate dehydrogenase. It was verified that 6-phosphogluconate was formed in amounts equivalent to NADPH under the assay conditions used.

Pyruvate carboxylase. Pyruvate carboxylase was assayed as citrate formed when islet homogenate was added to 0.5ml of assay medium containing 50mM-triethanolamine hydrochloride, 10mM-sodium pyruvate, 2.5mM-ATP, 50mM-$NaHCO_3$, 5mM-$MgCl_2$, 0.75mM-acetyl-CoA, 0.5mM-EDTA and 0.1mg of bovine plasma albumin/ml, pH 7.5. After incubation at 37°C for up to 90min the reaction was terminated by heating at 100°C for 5min, and citrate was assayed fluorimetrically by the method of Moellering & Gruber (1966). The rate of citrate formation was linear over 90min and was shown to be dependent on the presence of each of the components (islet homogenate, pyruvate, HCO_3^-, ATP and acetyl-CoA). Control experiments showed that further addition of citrate synthase was not necessary.

RESULTS

Glucose-phosphorylating activities. The rate of formation of [1-^{14}C]glucose 6-phosphate from [1-^{14}C]glucose was constant over the period of assay (30min at 37°C) and directly proportional to the amount of mouse islet homogenate used. Control incubations in which ATP was omitted showed no detectable incorporation of [1-^{14}C]glucose into [1-^{14}C]glucose 6-phosphate at any glucose concentration from 40μM to 40mM except when glucose 6-phosphate was added at high glucose concentrations. With 1mM-glucose 6-phosphate there was no detectable incorporation of radioactivity in the absence of ATP at glucose concentrations below 1mM. At higher glucose concentrations (3–20mM) the rate of incorporation in the absence of ATP was approx. 30–50% of that with ATP. The occurrence of isotope exchange would thus appear to be confined to those assays involving both high glucose concentrations and addition of unlabelled glucose 6-phosphate. The results shown in the figures and tables have been corrected for this isotope exchange.

Rates of glucose phosphorylation, v, measured at 5mM-ATPMg^{2-} were plotted against v/s, where s is glucose concentration (range 40μM–40mM), in Fig. 1. Such plots showed discontinuity, suggesting the presence of two glucose-phosphorylating activities in the homogenate, the major activity having a low K_m for glucose and a minor activity having a

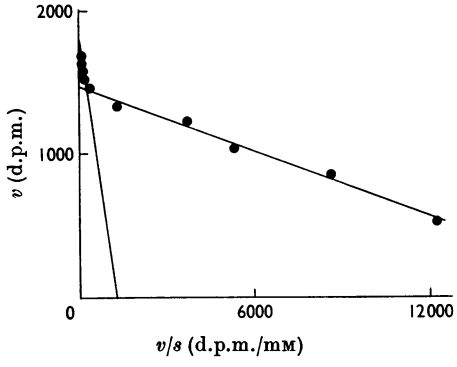

Fig. 1. Dependence of glucose-phosphorylating activity on glucose concentration. Portions of homogenate corresponding to nine islets were incubated at 37°C for 20min with [1-^{14}C]glucose (0.05–40mM; 2.4mCi/mmol) and $ATPMg^{2-}$ (5mM). The rate of formation of [1-^{14}C]-glucose 6-phosphate was measured as described in the Experimental section.

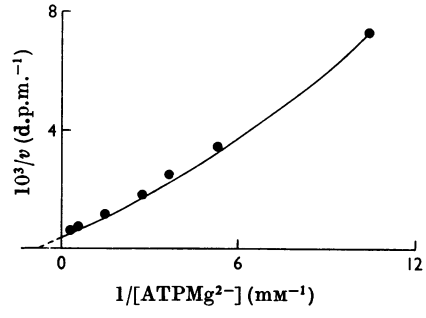

Fig. 2. Dependence of glucose-phosphorylating activity on $ATPMg^{2-}$ concentration. Portions of homogenate corresponding to nine islets were incubated at 37°C for 20min with [1-^{14}C]glucose (0.57mM; 2.8mCi/mmol) and $ATPMg^{2-}$ (0.09–3.5mM). The medium also contained the ATP-regenerating system described in the Experimental section. The [1-^{14}C]glucose 6-phosphate formed was assayed as described in the Experimental section. The results are plotted in double-reciprocal form.

high K_m for glucose. Computer analysis by a least-square method for non-linear regression showed that the data fitted an equation for two activities with different K_m values for glucose:

$$[v = V_{max.1}(1+K_{m1}/s)+V_{max.2}(1+K_{m2}/s)]$$

significantly better than the Michaelis equation for a single activity ($P<0.05$), and gave apparent K_m values for glucose of 0.075 ± 0.01 and 16 ± 6mM (means ±S.E.M.) for the two activities. Estimates of $V_{max.}$ from the intercepts on the v axis were (in pmol/islet per min at 37°C; means ±S.E.M.) 3.15 ± 0.09 for total glucose phosphorylation rate, 2.35 ± 0.2 for the low-K_m glucose-phosphorylating activity, and (by difference) 0.8 for the high-K_m glucose-phosphorylating activity. As a further control

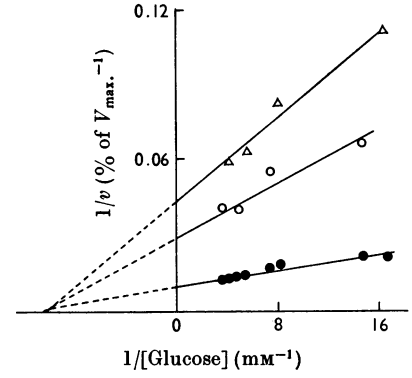

Fig. 3. Inhibition of glucose-phosphorylating activity by glucose 6-phosphate at low glucose concentrations. Portions of homogenate corresponding to nine islets were incubated for 20min at 37°C with [1-^{14}C]glucose (0.06–0.24mM; 2.9mCi/mmol), $ATPMg^{2-}$ (5mM) and glucose 6-phosphate (●, zero; ○, 0.42mM; △, 0.74mM). The [1-^{14}C]glucose 6-phosphate formed was assayed as described in the Experimental section. The results are plotted in double-reciprocal form.

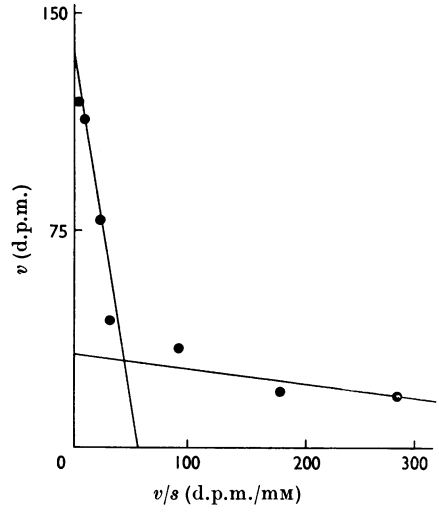

Fig. 4. Dependence of glucose-phosphorylating activity on glucose concentration in the presence of glucose 6-phosphate. Portions of homogenate corresponding to eight islets were incubated with [1-^{14}C]glucose (0.06–23mM; 0.2mCi/mmol), $ATPMg^{2-}$ (5mM) and glucose 6-phosphate (1mM). The rate of formation of [1-^{14}C]-glucose 6-phosphate was measured as described in the Experimental section.

Table 1. *Enzyme activities in homogenates of mouse pancreatic islets*

The enzymic activities of mouse islets were assayed as described in the Experimental section. One unit of activity catalyses the formation of 1 μmol of measured product/min.

Enzyme activity	Temperature (°C)	$V_{max.}$ (units $\times 10^{-6}$/islet)	$V_{max.}$ (units/g dry wt. of islet)*	K_m (mM)	Inhibitor	Type	K_i (mM)
Low-K_m glucose-phosphorylating activity	37	2.35	4.7	0.075 (glucose)	Glucose 6-phosphate	Non-competitive with glucose	0.22
				0.77 ($ATPMg^{2-}$)	Mannoheptulose	Competitive with glucose	0.25
High-K_m glucose-phosphorylating activity	37	0.80	1.6	16 (glucose)			
Glucose 6-phosphatase	37	3.50	7.0	1.0 (glucose 6-phosphate)	Glucose	Mixed	
Glucose 6-phosphate dehydrogenase	22	4.5	9.0	0.022 (glucose 6-phosphate)	NADPH	Competitive with $NADP^+$	0.019
				0.004 ($NADP^+$)		Mixed with glucose 6-phosphate	
6-Phosphogluconate dehydrogenase	22	4.6	9.2	0.021 (6-phosphogluconate)			
				0.0025 ($NADP^+$)			
$NADP^+$–isocitrate dehydrogenase	22	20.0	40.0				
'Malic' enzyme	22	0.85	1.7				
Pyruvate carboxylase	37	3.5	7.0				

* Calculated assuming a mean islet dry wt. of 0.5 μg/islet.

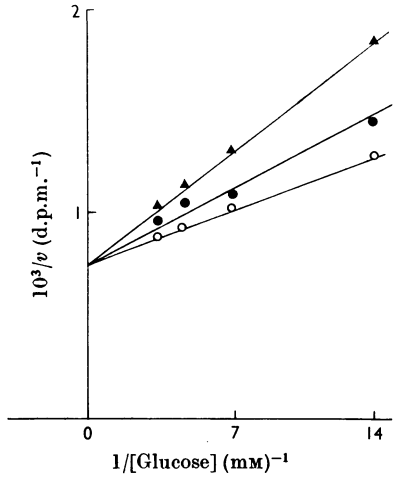

Fig. 5. Inhibition of glucose-phosphorylating activity by mannoheptulose at low glucose concentrations. Portions of homogenate corresponding to eight islets were incubated at 37°C for 20 min with [1-^{14}C]glucose (0.07–0.29 mM; 2.8 mCi/mmol), $ATPMg^{2-}$ (5 mM) and mannoheptulose (○, zero; ●, 0.095 mM; ▲, 0.19 mM). The [1-^{14}C]glucose 6-phosphate formed was assayed as described in the Experimental section and the data plotted in double-reciprocal form.

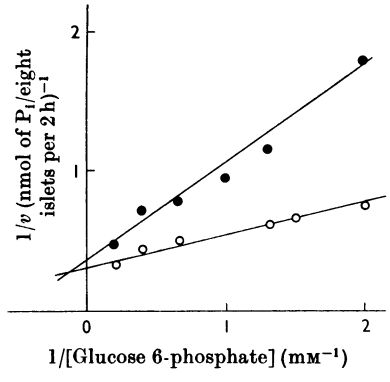

Fig. 6. Inhibition of glucose 6-phosphatase activity by glucose. Glucose 6-phosphatase activity was measured as described in the Experimental section at glucose 6-phosphate concentrations from 0.5 to 5 mM in the presence (○) and absence (●) of 12.5 mM-glucose.

similar assays were made with crystalline yeast hexokinase diluted to give equivalent phosphorylating activity. Plots of v against v/s were linear with no evidence of discontinuity, and the expected values for $V_{max.}$ and K_m (glucose) were obtained. The effect of $ATPMg^{2-}$ on reaction velocity at 0.57 mM-glucose is shown in Fig. 2. Reciprocal plots showed evidence of curvature in each of a number of different experiments. This curvature is unlikely to be due to breakdown of ATP at low $ATPMg^{2-}$ concentrations by islet phosphatase activity because it was still seen when an ATP-regenerating system was used. The apparent K_m for $ATPMg^{2-}$ at 0.6 mM-glucose was 0.77 ± 0.12 mM (Table 1).

The effects of glucose 6-phosphate on the rate of glucose phosphorylation are shown in Figs. 3 and 4. At low glucose concentrations (60–260 μM) glucose 6-phosphate was a non-competitive inhibitor against glucose (K_i 0.22 mM). With the full range of glucose concentrations (20 μM–16 mM) interpretation of effects of glucose 6-phosphate was complicated by isotope exchange at glucose concentrations above 1 mM (see above). At the lower glucose concentrations (below 1 mM) glucose 6-phosphate decreased $V_{max.}$ in complete accordance with the non-competitive inhibition of the low-K_m activity mentioned above (calculated from the intercept on the v axis in Fig. 4). The high-K_m glucose-phosphorylating activity was apparently not inhibited by glucose 6-phosphate, so that the discontinuity noted in Fig. 1 was enhanced by glucose 6-phosphate (Fig. 4). An apparent K_m 4 mM for glucose was observed for the high-K_m activity in the presence of 1 mM-glucose 6-phosphate.

Reciprocal plots showing the effect of mannoheptulose on the rate of glucose phosphorylation at low glucose concentrations (70–300 μM) are given in Fig. 5. Mannoheptulose was a competitive inhibitor against glucose (K_i 0.25 mM). The preponderance of low-K_m glucose-phosphorylating activity made it difficult to determine whether or not mannoheptulose inhibited the high-K_m activity.

Glucose 6-phosphatase activity. The rate of release of P_i from glucose 6-phosphate at 37°C was constant for at least 2 h and proportional to the amount of homogenate used. Reciprocal plots of glucose 6-phosphate concentration and reaction velocity were linear (Fig. 6) and the K_m for glucose 6-phosphate was 1.0 ± 0.14 mM. The action of several compounds on the rate of hydrolysis of glucose 6-phosphate was tested. Glucose (10 and 20 mM) was inhibitory, whereas mannose (10 and 20 mM), mannoheptulose (14.3 mM), citrate (5 mM) and tolbutamide (0.2 and 1 mg/ml) had no detectable effect on the rate of hydrolysis of 5 mM-glucose 6-phosphate. The inhibition by glucose (10 mM) was not reversed by mannoheptulose (14.3 mM). The kinetics of the inhibition by glucose were analysed by a computer programme (England, 1968) and the inhibition by glucose was found to be mixed (Fig. 6). The data could be fitted to the equation:

$$v = V_{max.}/[(1 + i/K_1)K_m/s + 1 + i/K_2]$$

where K_1 and K_2 are constants for the competitive

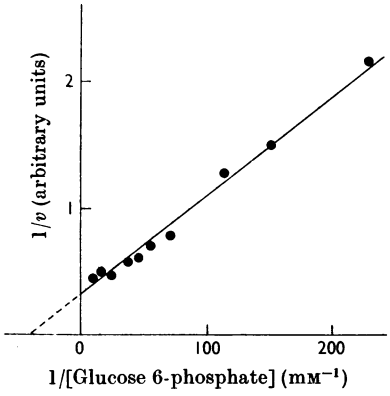

Fig. 7. Dependence of glucose 6-phosphate dehydrogenase activity on glucose 6-phosphate concentration. Portions of homogenate corresponding to nine islets were incubated at 22°C for 90min with glucose 6-phosphate (4–88 μM) and NADP$^+$ (0.2mM). The rates of formation of NADPH were measured as described in the Experimental section. The results are plotted in double-reciprocal form.

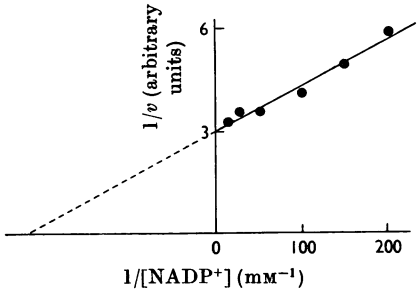

Fig. 8. Dependence of glucose 6-phosphate dehydrogenase activity on NADP$^+$ concentration. Portions of homogenate corresponding to seven islets were incubated at 22°C for 90min with glucose 6-phosphate (0.35mM) and NADP$^+$ (5–82 μM). The rates of formation of NADPH were measured as described in the Experimental section. The results are plotted in double-reciprocal form.

and non-competitive elements of the inhibition respectively and the other symbols have their usual connotation. K_1 and K_2 were found to be 8.9 and 38mM respectively.

The total glucose 6-phosphatase activity in mouse islet homogenates was 3.5 ± 0.5pmol/islet per min at 37°C (Table 1).

Glucose 6-phosphate dehydrogenase. The formation of NADPH from glucose 6-phosphate and NADP$^+$ with mouse islet homogenate proceeded linearly for at least 90min, and the rate was directly proportional to the amount of homogenate used.

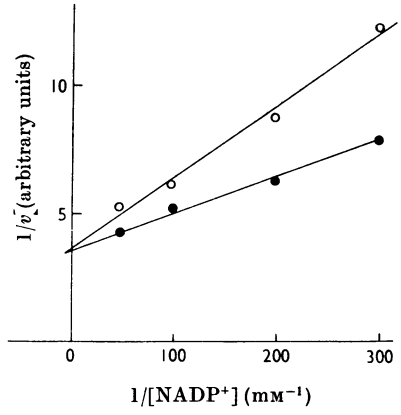

Fig. 9. Inhibition of glucose 6-phosphate dehydrogenase activity by NADPH at various NADP$^+$ concentrations. Portions of homogenate corresponding to nine islets were incubated at 22°C for 90min with glucose 6-phosphate (0.4mM) and NADP$^+$ (3.3–20 μM) in the absence of NADPH (●) and in the presence of 42 μM-NADPH (○). Rates of formation of 6-phosphogluconate were measured as described in the Experimental section. The results are plotted in double-reciprocal form.

Reciprocal plots of glucose 6-phosphate concentration and reaction velocity (Fig. 7) gave K_m 22 ± 2 μM (mean ± S.E.M.) for glucose 6-phosphate. The K_m for glucose 6-phosphate was apparently independent of the initial NADP$^+$ concentration over the range 7–200 μM. The K_m for NADP$^+$ from reciprocal plots was 4 ± 0.1 μM at initial glucose 6-phosphate concentrations between 300 and 400 μM (Fig. 8).

Glucose 6-phosphate dehydrogenase activity in mouse islet homogenates was inhibited by NADPH. At a fixed glucose 6-phosphate concentration of 330 μM and varied NADP$^+$ concentrations (3.3–20 μM) inhibition was strictly competitive with an apparent K_i 19 μM (Fig. 9). With fixed concentrations of NADPH (41 μM) and NADP$^+$ (6.6 μM) and varied glucose 6-phosphate concentrations (25–170 μM) inhibition was apparently of the mixed type (Fig. 10). The apparent K_m for glucose 6-phosphate was increased to 50 μM and $V_{max.}$ was decreased by 40%. Essentially comparable changes were seen with [NADPH]/[NADP$^+$] ratios 25:12 or 18:6.6. At glucose 6-phosphate concentrations near to its K_m and in the absence of added NADPH no modification of reaction velocity was detected with 10mM-glucose, 14.8mM-mannoheptulose, 1mM-glucose 1-phosphate, 1mM-fructose 6-phosphate or 1mM-fructose 1,6-diphosphate, 4mM-ATPMg^{2-}, 1mM-3′:5′-cyclic AMP, 2mM-P_i or 1mg of tolbutamide/ml. There was no detectable requirement for Na^+, K^+, Ca^{2+} or Mg^{2+} (see Table 2).

The total activity of mouse islet glucose 6-phosphate dehydrogenase measured from the intercepts of reciprocal plots with saturating concentrations of one or other substrate was 4.5pmol/min per islet at 22°C, corresponding to approx. 9 units/g dry wt. of islets (Table 1). This is comparable with the value obtained in islets of obese–hyperglycaemic mice by Matschinsky, Kauffman & Ellerman (1968).

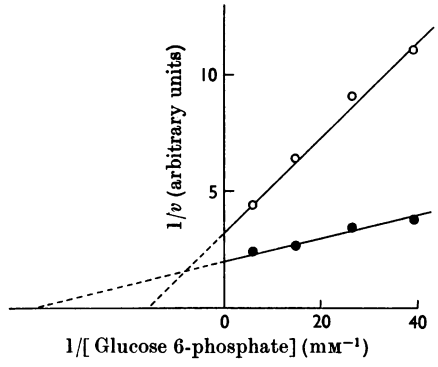

Fig. 10. Inhibition of glucose 6-phosphate dehydrogenase activity by NADPH at various glucose 6-phosphate concentrations. Portions of homogenate corresponding to nine islets were incubated at 22°C for 90min with glucose 6-phosphate (0.025–0.17mM) and $NADP^+$ (6.6μM) in the absence of NADPH (●) and in the presence of 41μM-NADPH (○). Rates of formation of 6-phosphogluconate were measured as described in the Experimental section. The results are plotted in double-reciprocal form.

6-Phosphogluconate dehydrogenase. The total activity of 6-phosphogluconate dehydrogenase in mouse islet homogenates was 4.6pmol/min per islet at 22°C, corresponding to approx. 9.2 units/g dry wt. of islets (Table 1). The K_m values were 21μM for 6-phosphogluconate (with 400μM-$NADP^+$) and 2.5μM for $NADP^+$ (with 320μM-6-phosphogluconate). The total activity observed is of the same order as that reported for islets of obese–hyperglycaemic mice by Matschinsky *et al.* (1968).

$NADP^+$–isocitrate dehydrogenase, pyruvate carboxylase and 'malic' enzyme. Other reactions that may generate NADPH in tissues include $NADP^+$–isocitrate dehydrogenase, and 'malic' enzyme [$NADP^+$–malate dehydrogenase (decarboxylating)] in conjunction with pyruvate carboxylase and NAD^+–malate dehydrogenase. The activity of $NADP^+$–isocitrate dehydrogenase (measured at 22°C with 30μM-$NADP^+$, 5mM-DL-isocitrate and 5mM-magnesium chloride) was 20pmol/min per islet, corresponding to approx. 40 units/g dry wt. of islets (Table 1). Pyruvate carboxylase was detected at an activity of 3.5pmol/min per islet or 7 units/g dry wt. of islets (measured at 37°C with 10mM-pyruvate, 50mM-bicarbonate, 2.5mM-ATP and 0.75mM-acetyl-CoA) (Table 1). Islet homogenates showed malate-dependent reduction of $NADP^+$ equivalent to 'malic' enzyme activity of

Table 2. *Possible modifiers of glucose 6-phosphate dehydrogenase in homogenates of mouse pancreatic islets*

The substances shown below were tested for possible effects on islet glucose 6-phosphate dehydrogenase assayed as described in the Experimental section. Under the conditions shown in the Table glucose 6-phosphate dehydrogenase activity was not affected by any of the substances given.

Assay conditions			
[Glucose 6-phosphate] (μM)	[NADP] (μM)	Buffer	Substance tested
27	200	50mM-Triethanolamine hydrochloride adjusted to pH7.5 with KOH, 0.5mM-Na_2EDTA, 10mM-$MgCl_2$, 0.1mg of albumin/ml	Glucose (10mM) Mannoheptulose (3mg/ml) Glucose 1-phosphate (1mM) Fructose 6-phosphate (1mM) Fructose 1,6-diphosphate (1mM) $ATPMg^{2-}$ (4mM) 3′:5′-cyclic AMP (1mM) P_i (2mM) Tolbutamide (1mg/ml)
27	200	As above but $MgCl_2$ omitted	ATP^{4-} (4mM)
200	50	100mM-Tris base adjusted to pH7.5 with HCl, 0.1mg of albumin/ml	K^+ (100mM) Na^+ (50mM) Ca^{2+} (5mM) Mg^{2+} (10mM)

0.8pmol/min per islet or 1.6 units/g dry wt. of islets (measured at 22°C with 30μM-$NADP^+$, 1mM-L-malate and 3mM-manganese chloride) (Table 1). Since NAD^+–malate dehydrogenase may show some activity with $NADP^+$, the small activity that we have detected may not be due to 'malic' enzyme.

DISCUSSION

The proportion of β-cells in normal mouse islets is reported to be about 80% (Brolin & Hellerström, 1967). It has seemed reasonable, for purposes of discussion, to assume that the enzyme activities that we have studied in islet homogenates are present in β-cells, but the possible contribution of other cell types needs to be borne in mind.

Glucose-phosphorylating activities. It has been found in the present study that the major glucose-phosphorylating activity in normal mouse islet has a low K_m for glucose and is inhibited non-competitively by glucose 6-phosphate and competitively by mannoheptulose (against glucose). These results are similar to those obtained in an earlier study in which the method of assay employed anion-exchange chromatography to separate [6-^{3}H]glucose 6-phosphate formed from [6-^{3}H]-glucose (Ashcroft & Randle, 1968*b*). The present results have also indicated the presence of a small proportion of the total glucose-phosphorylating activity with a high K_m for glucose. This high-K_m activity was not detected in the earlier study although the method of assay (see above) was capable of detecting glucokinase in extracts of mouse liver. The probable reason for this is the high precision and sensitivity of assay required to detect the relatively small proportion of high-K_m activity in mouse islet extracts. The major limiting factor in this type of assay is the background radioactivity in control samples. In this respect the method used in the present experiments is superior, partly because of the greater radiochemical purity of [1-^{14}C]glucose as compared with [6-^{3}H]-glucose, and partly because the assay of [1-^{14}C]-glucose 6-phosphate avoids the use of anion-exchange resins. The radioactivity method that we have developed for these experiments has one definite advantage over fluorimetric and spectrophotometric methods, in that it permits investigation of the effects of added glucose 6-phosphate (see below). The relative, and absolute, amounts of the two activities, and the K_m values for glucose, are of the same order as those obtained by Matschinsky & Ellerman (1968), using extracts of freeze-dried islets from obese–hyperglycaemic mice, and a fluorimetric assay of glucose 6-phosphate formation. Evidence for inhibition of glucose-phosphorylating activity in extracts of rat islets by mannoheptulose has also been given by Malaisse, Lea & Malaisse-Lagae (1968), employing a spectrophotometric assay for glucose 6-phosphate formation.

Investigation of the high-K_m activity has been made difficult by the presence of a relative excess of the low-K_m activity and by the occurrence of isotope exchange when unlabelled glucose 6-phosphate is added at high glucose concentrations. The possibility, for example, that the two activities may be due to a single enzyme showing a change in mechanism at high glucose concentrations has not been excluded. In the present study, the apparent existence of two glucose-phosphorylating activities was only readily demonstrable when assays were conducted in the presence of glucose 6-phosphate. The basis for this experiment was the observation that in liver the low-K_m enzyme (hexokinase) is inhibited non-competitively by glucose 6-phosphate, whereas the high-K_m enzyme (glucokinase) is relatively insensitive. The results obtained here suggest that islet glucose-phosphorylating activities may be similar in these respects, but it would be premature to assign the name 'glucokinase' to the high-K_m activity without separation and purification of the two apparent activities. The problems of separation and purification of an enzyme from the small amounts of islet tissue obtainable (less than 1mg) are, however, formidable.

Glucose 6-phosphatase activity. The results described here show that homogenates of normal mouse islets contain enzymic activity catalysing the liberation of P_i from glucose 6-phosphate. Several of the observations suggest that this activity may be important in regulating the concentration of glucose 6-phosphate in islets. First, the amount of activity was similar in magnitude to the total glucose-phosphorylating activity. Secondly, the K_m for glucose 6-phosphate was approx. 1mM whereas islet glucose 6-phosphate concentrations were 0.15 and 0.8mM at extracellular glucose concentrations of 0.8 and 3mg/ml respectively (Ashcroft *et al.* 1970). Finally, the finding that the glucose 6-phosphatase activity was inhibited by glucose at physiological concentrations (for example, at 1mM-glucose 6-phosphate, 10mM-glucose produced a 50% inhibition of glucose 6-phosphatase) seemed consistent with a regulatory role for this enzyme in the control of glucose metabolism in the islet. On the other hand, the failure to demonstrate significant formation of [^{14}C]glucose by islets incubated in the presence of [^{14}C]mannose (Ashcroft & Randle, 1968*a*) might argue against appreciable glucose 6-phosphatase activity in the intact islet. However, it has also been found that equilibration between fructose 6-phosphate and glucose 6-phosphate may be impaired in mouse islets during mannose metabolism (Ashcroft *et al.* 1970). In view of this finding, our failure to demonstrate conversion of

mannose into glucose is not necessarily inconsistent with activity of glucose 6-phosphatase in the intact islet.

The kinetic behaviour of the glucose 6-phosphatase is in broad agreement with the results of Täljedal (1969) on glucose 6-phosphatase in homogenates of islets from obese-hyperglycaemic mice. In both studies glucose was found to be a mixed inhibitor with a major competitive element. Mannose, however, did not inhibit the hydrolysis of glucose 6-phosphate.

The lack of effect of tolbutamide on islet glucose 6-phosphatase does not support the suggestion of Lazarus (1959) that sulphonylureas may stimulate insulin release by raising the β-cell concentration of glucose 6-phosphate through an inhibition of glucose 6-phosphatase. Citrate, which has been shown to stimulate insulin release and to raise the glucose 6-phosphate concentration in rat islets (Montague & Taylor, 1969), is reported to inhibit liver glucose 6-phosphatase (Nordlie & Lygre, 1966). However, no effect of citrate on mouse islet glucose 6-phosphatase could be demonstrated, and it seems more likely that effects of citrate on islet glucose 6-phosphate concentration may be due to inhibition of phosphofructokinase (Matschinsky, Rutherford & Ellerman, 1968).

Enzyme activities and glucose phosphorylation in the intact islet. In the intact mouse islet, rates of glucose oxidation show a sigmoid relationship to extracellular glucose concentration with a threshold at approx. 5mM-glucose, K_m approx. 8mM for glucose, and inhibition by mannoheptulose (Ashcroft *et al.* 1970). The amounts and kinetic properties of glucose-phosphorylating and glucose 6-phosphatase activities determined in the present study go some way towards accounting for these characteristics of glucose oxidation. Thus the presence of glucose-phosphorylating activity with an apparently high K_m for glucose, and glucose 6-phosphatase activity inhibited by physiological concentrations of glucose may provide a basis for the high K_m for glucose uptake. The inhibition by mannoheptulose of glucose oxidation and the concomitant fall in intra-islet glucose 6-phosphate concentration may be attributed in part to the inhibition of islet glucose-phosphorylating activity described here. However, the kinetics of inhibition by mannoheptulose of glucose-phosphorylating activity at low glucose concentrations do not entirely account for the profound inhibition of glucose oxidation in the intact islet. It may not be possible to obtain meaningful results on the effects of mannoheptulose on the high-K_m glucose-phosphorylating activity without separation and purification.

From the quantitative point of view, the net rate of glucose phosphorylation at given glucose and glucose 6-phosphate concentrations can be predicted from the results presented here for islet glucose-phosphorylating and glucose 6-phosphatase activities and compared with the observed rates of glucose uptake in the intact islet. The islet glucose 6-phosphate concentration at 17mM-glucose is 0.8mM and this may correspond to an intracellular concentration of approx. 1mM (assuming that the extracellular water is 20% of the total water in islets). At these concentrations of glucose and glucose 6-phosphate, the net rate of glucose phosphorylation (predicted from the results given in Fig. 4 for glucose-phosphorylating activity and from the kinetic constants for glucose 6-phosphatase activity given in Table 1) is 0.7μmol/min per g dry wt. of islets. This is in fair agreement with the observed value of 1.1μmol/min per g dry wt. of islets for the combined rates of glucose oxidation and lactate output in the intact islet at an extracellular glucose concentration of 17mM (Ashcroft *et al.* 1970). At lower glucose concentrations, however, the correlation between observed and predicted values is less good, and the properties of the glucose phosphorylating and glucose 6-phosphatase activities do not as yet account satisfactorily for the sigmoid relationship between extracellular glucose concentration and the rate of glucose oxidation.

Studies on the glucose metabolism of intact islets have shown no major inconsistency with the view that glucose phosphorylation in the β-cell may be involved in the glucoreceptor mechanism (Ashcroft *et al.* 1970), and the present studies have suggested the presence of three enzyme activities that could contribute to a glucoreceptor mechanism based on the rate of net glucose phosphorylation. The kinetic properties of these activities are not incompatible with such a mechanism, but do not as yet provide a satisfactory quantitative basis for a glucoreceptor model.

Control of glucose 6-phosphate dehydrogenase. The activities of mouse islet glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Table 1) indicate that the potential capacity of mouse islets to oxidize glucose 6-phosphate is high. Indeed the values found here are considerably in excess of the observed maximum rate of glucose oxidation in this tissue (Ashcroft *et al.* 1970). Clearly the oxidation of glucose by this pathway must be subject to control in the intact islet. In the present studies we have considered the possible importance of intra-islet glucose 6-phosphate concentrations and the [NADPH]/[$NADP^+$] ratio as regulators of glucose 6-phosphate dehydrogenase activity. The results obtained suggest that the cytosol [NADPH]/[$NADP^+$] ratio may be an important factor in controlling the rate of this reaction as is proposed for tissues where NADPH is utilized for fatty acid biosynthesis (McLean, 1962). NADPH was found to be a competitive

inhibitor (against $NADP^+$) of mouse islet glucose 6-phosphate dehydrogenase; the islet [NADPH]/[$NADP^+$] ratio is not known, but if it is in the range 100–1000 as calculated for rat liver cytosol (Veech *et al.* 1969) then it could have an important regulatory role on glucose 6-phosphate dehydrogenase activity.

On the other hand, the K_m values of glucose 6-phosphate dehydrogenase for glucose 6-phosphate (20–50 μM) were considerably lower than the islet glucose 6-phosphate concentrations (200–800 μM) measured over the physiological range of glucose concentrations (5–17 mM) (Ashcroft *et al.* 1970). If these values obtain in the islet, then the rate of oxidation of glucose 6-phosphate by the dehydrogenase may not be materially influenced by the islet glucose 6-phosphate concentration over this range of glucose concentrations. The possibility that the K_m for glucose 6-phosphate could be modified in the intact islet by some intracellular metabolite was considered but a number of possible modifiers were found to be without effect under the conditions used (Table 2). However, there was evidence suggesting that the K_m for glucose 6-phosphate could be influenced by the [NADPH]/[$NADP^+$] ratio. To what extent this effect could be of importance in the intact islet is difficult to assess, partly because of lack of knowledge of the islet [NADPH]/[$NADP^+$] ratio and partly because we have been unable to obtain an adequate amount of islet homogenate in any one experiment for a sufficiently detailed kinetic analysis of the effects of [NADPH]/[$NADP^+$] ratio on the K_m for glucose 6-phosphate.

Since a possible role of the pentose phosphate pathway is the provision of NADPH for biosynthetic or release processes it was of interest to establish whether other routes for NADPH generation were available in islet tissue. Substantial activity of $NADP^+$–isocitrate dehydrogenase was found (Table 1) and therefore the islet [NADPH]/[$NADP^+$] ratio may not be solely dependent on the dehydrogenases of the pentose phosphate pathway. The quantitative importance of glucose metabolism via the pentose phosphate pathway in normal mammalian islets has yet to be established.

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