Phosphorylation of Glucose in Isolated Rat Hepatocytes

SIGMOIDAL KINETICS EXPLAINED BY THE ACTIVITY OF GLUCOKINASE ALONE

By FRANÇOISE BONTEMPS, LOUIS HUE and HENRI-GÉRY HERS

Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, U.C.L., 75.39., Avenue Hippocrate 75, B-1200 Brussels, Belgium

(Received 16 January 1978)

The conversion of glucose into glucose 6-phosphate in an extract of isolated rat hepatocytes incubated in the presence of MgATP was studied spectrophotometrically at 340 nm and also by a radiochemical procedure based on the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$. Both methods gave similar results. The glucose-saturation curve was sigmoidal and the shape of this curve was not influenced by the ionic composition of the incubation medium. The activity at 0.5 mM-glucose was only 1–2% of V_{max} , indicating a virtual absence of low- K_m hexokinase in the preparation. The radiochemical method was also used for the determination of glucose phosphorylation by intact hepatocytes. The glucose-saturation curve was also markedly sigmoidal, but the $s_{0.5}$ (substrate concentration at half-maximal velocity) and the Hill coefficient were larger than in extracts of hepatocytes. These two parameters became smaller when cells were incubated in a medium in which Na^+ ions were replaced by K^+ ions. The increased rate of phosphorylation at low glucose concentration in a K^+ medium was accompanied by an increased rate of metabolite recycling between glucose and glucose 6-phosphate and also by an increased uptake of glucose. In both media phosphorylation of glucose was inhibited co-operatively by *N*-acetylglucosamine. Calculations indicate that this inhibition would reach 100% at saturation of the inhibitor, although at lower concentrations of *N*-acetylglucosamine it was smaller than expected from the known K_i of *N*-acetylglucosamine for glucokinase. The rate of phosphorylation of glucose was proportional to the amount of glucokinase in hepatocytes from newborn rats and in conditions such as starvation and diabetes in which the total amount of glucokinase in the liver is decreased. In the same conditions, glucose 6-phosphatase activity was either normal or increased. It is concluded that the phosphorylation of glucose in isolated hepatocytes follows sigmoidal kinetics, which can be explained by the activity of glucokinase alone with no participation of low- K_m hexokinase or of glucose 6-phosphatase.

Several mechanisms for the phosphorylation of glucose by the liver are currently considered [for reviews see Walker (1966) and Weinhouse (1976)].

(1) A high- K_m glucokinase (EC 2.7.1.2) is generally assumed to play the major role in the phosphorylation of glucose. The enzyme is absent from the liver of newborn rats until day 15 after birth (Walker & Holland, 1965) and its activity is also markedly decreased by prolonged starvation and by diabetes. The residual activity observed in these two latter experimental conditions varied from 5% (Salas *et al.*, 1963; Walker & Rao, 1964; Murphy & Anderson, 1974) to higher values, up to 60% of the activity in the normal liver (DiPietro & Weinhouse, 1960; Niemeyer *et al.*, 1962; Sharma *et al.*, 1963; Walker & Rao, 1964; Migliorini, 1971). In contrast with previous reports (Salas *et al.*, 1965; Parry & Walker, 1966, 1967; Gonzalez *et al.*, 1967; Pilkis, 1972; Grossman *et al.*, 1974), more recent work indicates that the glucose-saturation curve of the enzyme is

sigmoidal (Niemeyer *et al.*, 1975; Storer & Cornish-Bowden, 1976). (2) Several low- K_m hexokinases (EC 2.7.1.1), of which the cumulative activity reaches 10–30% of that of the glucokinase measured at substrate saturation, have also been described. There is some evidence that these enzymes are present only in the non-parenchymal cells of rat or mouse liver (Sapag-Hagar *et al.*, 1969; Crisp & Pogson, 1972), but contradictory results for the rat have also been reported (Morrison, 1967; Werner *et al.*, 1972; Bonney *et al.*, 1973). (3) Glucose 6-phosphatase (EC 3.1.3.9) can also phosphorylate glucose by using pyrophosphate, several nucleoside triphosphates or carbamoyl phosphate as phosphoryl donor. Only carbamoyl phosphate is an effective substrate at neutral pH. On this basis, it has been suggested that glucose 6-phosphatase may play a role in the phosphorylation of glucose *in vivo*, particularly when glucokinase is poorly active, as in diabetes or prolonged starvation, as well as in the newborn rat liver

(for reviews, see Nordlie, 1971, 1974). Although this hypothesis has been considered with favour by several experts in the field (Friedmann *et al.*, 1967; McCraw *et al.*, 1967; Ryman & Whelan, 1971; Weinhouse, 1976), it seems that it can be rejected for several theoretical and practical reasons, which have been detailed elsewhere (Hers, 1976). One is that the utilization of glucose by the liver is strongly inhibited by *N*-acetylglucosamine and glucosamine (Spiro, 1958), which are potent inhibitors of glucokinase (Parry & Walker, 1966), although not of glucose 6-phosphatase (Lygre & Nordlie, 1969).

In the present work, the phosphorylation of glucose was measured by the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$ in the presence of phosphohexose isomerase (EC 5.3.1.9), as proposed by Katz & Rognstad (1975). This method was applied both to extracts of isolated hepatocytes incubated in the presence of MgATP and to suspensions of intact cells. In the latter case, the phosphorylation of glucose was investigated in relation to the ionic composition of the medium, the presence of an inhibitor of glucokinase and the abundance of glucokinase, hexokinase and glucose 6-phosphatase in the hepatocytes. Part of this work has been published in a preliminary form (Bontemps *et al.*, 1977).

Materials and Methods

Chemicals

Glucose, alloxan monohydrate, dithiothreitol, NADP⁺, glucose oxidase, peroxidase, collagenase (type I) and 6-phosphogluconate dehydrogenase were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glucose 6-phosphate dehydrogenase and ATP were from Boehringer G.m.b.H. (Mannheim, Germany), *N*-acetylglucosamine was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.), sodium pentobarbital from Serva (Heidelberg, Germany), Sephadex G-25 (medium grade) from Pharmacia Fine Chemicals (Uppsala, Sweden), and Dowex AG1 (200–400 mesh, X8) from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Other biochemical reagents were from Merck (Darmstadt, Germany) and were of analytical grade.

$[2\text{-}^3\text{H}]\text{Glucose}$ and $[\text{U}\text{-}^{14}\text{C}]\text{glucose}$ were from The Radiochemical Centre (Amersham, Bucks., U.K.). $[2\text{-}^3\text{H}]\text{Glucose}$ was separated from a contaminant that represented up to 10% of the initial radioactivity by paper chromatography in ethyl acetate/pyridine/water (12:5:4, by vol.).

Animals

Newborn or adult (250–300g) Wistar rats were used. The newborns were left with their mother; the

adults were kept on a standard commercial diet, or deprived of food during 12 or 48h, but given 0.15M-NaCl *ad libitum*. Diabetic animals were used 3 days after the injection of alloxan (60mg/kg body wt, given intravenously); their urine contained at least 0.25mg of glucose/ml.

Isolation and manipulation of hepatocytes

Hepatocytes from adult rats were isolated as previously described (Hue *et al.*, 1975), except that the cells were suspended and incubated in a Krebs–Henseleit (1932) bicarbonate buffer (Na⁺ medium). Hepatocytes from newborn rats were obtained in a similar manner, except that no cannula was put into the vena cava and that livers were perfused in an open circuit; the hepatocytes obtained from two or three of these livers perfused simultaneously were pooled and used in a single experiment. A sample (2ml) containing about 100mg of cells was shaken (120 strokes/min) in stoppered 20ml vials at 37°C. The gas phase was O₂/CO₂ (19:1). The substrates were added after 20min of incubation, which was then continued for 30 or 60min. When the effect of K⁺ was studied, the hepatocytes were incubated in the bicarbonate buffer in which Na⁺ ions were replaced by K⁺ ions, and vice versa (K⁺ medium).

An extract of isolated hepatocytes was prepared in the cold (0–4°C) as follows. The cells were harvested by centrifugation at 3000 rev./min for 2min and the pellet was homogenized in 3 vol. of a solution containing 120mM-KCl, 1mM-dithiothreitol and 20mM-glycylglycine, pH 7.4, by 30 strokes in a conical sintered-glass tissue grinder (Kontes Glass Co., Vineland, NY, U.S.A.). The homogenate was centrifuged at 12000 *g* for 10min and the supernatant was filtered through a Sephadex G-25 column (1.7 cm×25cm) previously equilibrated with the homogenization medium. This extract was used for the kinetic investigation of the phosphorylation of glucose in the presence of MgATP. For the radiochemical method the extract was used as such; for the spectrophotometric assay, it was further diluted with 4 vol. of the homogenization medium. A similar procedure was used to prepare an extract of total liver previously homogenized with a Potter–Elvehjem apparatus. The removal of microsomal fraction by centrifugation of the extract at 100000 *g* for 30min did not change the assayable glucokinase activity.

To measure the total content of glucokinase and hexokinase of the hepatocytes, a more rapid procedure for the preparation of the cell extract was used. Samples (0.5ml) of the cell suspension were frozen in a cooling mixture (solid CO₂ in acetone). They were thawed on the same day and centrifuged for 1min at 3000 rev./min just before the assay. A 50μl portion of the supernatant was used for the spectrophotometric measurement of glucokinase at

100mM-glucose and of hexokinase at 0.5mM-glucose (see below).

Measurement of the phosphorylation of glucose

When the phosphorylation of glucose was measured in an extract of hepatocytes, the incubation medium contained 50mM-glycylglycine, pH 7.4, 100mM-KCl, 2.5mM-dithiothreitol, 5mM-ATP, 5mM-magnesium acetate and various concentrations of glucose; the reaction was carried out at 22–25°C. The formation of glucose 6-phosphate was measured either spectrophotometrically at 340nm or by a radiochemical method.

In the spectrophotometrical procedure, the incubation mixture contained also 0.5mM-NADP⁺ and 0.7 unit of glucose 6-phosphate dehydrogenase (where 1 unit of activity is the amount of enzyme required to convert 1 μ mol of substrate/min) and 50 μ l of the cell extract in a total volume of 1ml. Under these conditions, 2 μ mol of NADPH was produced/ μ mol of glucose 6-phosphate; it was checked that the rate of reaction was not influenced by adding an excess of purified 6-phosphogluconate dehydrogenase. Activities were corrected for the A_{340} changes observed in the absence of ATP. In the radiochemical procedure, 0.5ml of the incubation mixture containing 0.25 μ Ci of [2-³H]glucose and 0.1ml of the cell extract were incubated for 15min. The reaction was stopped by the addition of equimolar amounts of ZnSO₄ and Ba(OH)₂ (Weichselbaum & Somogyi, 1941) and the mixture was vigorously shaken. After centrifugation at 3000 rev./min, the supernatant was passed through a column (0.5cm $\times$ 4cm) of Dowex AG1 (borate form; 200–400 mesh; X8) that retained the labelled glucose (Hammerstedt, 1973). The eluate was used for the measurement of ³H₂O formed by the action of hexose phosphate isomerase present in the extract (Rose & O'Connell, 1961). Removal of ³H from glucose was calculated by multiplying the amount of glucose by the proportion of added radioactivity that was released into water.

This radiochemical method was also applied to isolated hepatocytes (Clark *et al.*, 1973; Katz *et al.*, 1975).

All results are expressed per g of protein present in the homogenate; 1g of packed cells contains 220mg of protein (Hue *et al.*, 1975) and 1g of fresh liver contains 233 $\pm$ 11 (S.E.M., $n=5$) mg of protein.

Other analytical methods

Glucose 6-phosphate was measured enzymically (Hohorst, 1963) in a neutralized perchlorate extract of hepatocytes. Glucose was measured by the glucose oxidase method (Huggett & Nixon, 1957) in an

extract of cells deproteinized with equimolar amounts of ZnSO₄ and Ba(OH)₂ as described by Weichselbaum & Somogyi (1941). The radioactivity of [2-³H,U-¹⁴C]glucose was measured after deionization and desiccation of the sample to remove labelled ions and ³H₂O (Hue & Hers, 1972). Glucose 6-phosphatase activity was measured as described by Hers (1964). Radioactivity was measured with a liquid-scintillation spectrometer by using the scintillation mixture of Patterson & Greene (1965).

The protein concentration was measured by the procedure of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Phosphorylation of glucose by an extract of isolated rat hepatocytes

Fig. 1 shows the glucose-saturation curve obtained when the rate of the reaction was measured both spectrophotometrically and radiochemically on incubation of an extract of hepatocytes at 22°C and pH 7.4 in the presence of 5mM-MgATP. With both methods, the curves were sigmoidal, and a half-maximal velocity was obtained at a concentration of glucose ($s_{0.5}$) close to 9mM with a Hill coefficient (h) of 1.4–1.5. The mean rate of removal of ³H from

Fig. 1. ATP-glucose phosphotransferase activity in an extract of rat hepatocytes as a function of the concentration of glucose

The hepatocytes were obtained from a 12h-starved rat. The rate of glucose 6-phosphate formation (v) was measured both spectrophotometrically (○) and radiochemically (●). For this last method, values shown are for a complete removal of ³H from [2-³H]glucose. The inset shows a double-reciprocal plot of the same data.

glucose, expressed in $\mu\text{mol}/\text{min}$ per g of protein, was 76% of the values obtained spectrophotometrically; this difference is in agreement with the known incomplete release of ^3H from C-2 of glucose 6-phosphate during the reaction catalysed by hexose phosphate isomerase (Rose & O'Connell, 1961; Katz & Rognstad, 1975).

Fig. 1 also shows that the rate of phosphorylation at 0.5 mM-glucose was 1–2% of V_{max} , indicating a nearly complete absence of hexokinase. When a similar experiment was performed in an extract of total liver, the saturation curve was hyperbolic and the rate of the reaction at 0.5 mM-glucose was about 10% of V_{max} . Some 80–90% of the activity measured at 100 mM-glucose (glucokinase plus hexokinase) present in the crude extract of liver was recovered in the isolated hepatocytes; in contrast, up to 80% of the activity measured at 0.5 mM-glucose (hexokinase) was lost during the preparation of hepatocytes (results not shown).

The effects of Na^+ and K^+ on the rate of reaction were tested with both radiochemical and spectrophotometric methods; the influence of the other parameters was studied by the spectrophotometric method only. The kinetic properties of glucokinase were influenced neither by the replacement of K^+ by Na^+ in the assay nor by the presence of 5 mM-EGTA or EGTA/ Ca^{2+} buffer prepared as described by Portzehl *et al.* (1964) giving a range of concentrations of Ca^{2+} between 10 nM and 1 μM . The rate of the reaction was influenced by changes in pH in a manner

Fig. 2. Effect of substrate concentration and of ionic composition of the incubation medium on the rate of phosphorylation of glucose by isolated hepatocytes (v)

The hepatocytes were isolated from a fed rat and incubated for 30 min in either a Na^+ ($\circ$) or a K^+ medium ($\bullet$). The inset shows a double-reciprocal plot of the same data.

very similar to that previously reported by other authors (Salas *et al.*, 1965; Parry & Walker, 1966) for the purified enzyme (results not shown). It has also been checked that the Hill coefficient was the same at pH 7.3 and 7.5.

Phosphorylation of glucose by isolated hepatocytes

Ionic effect. The radiochemical method was used to measure the phosphorylation of glucose by isolated hepatocytes. Fig. 2 shows the influence of the concentration of glucose on the rate of its phosphorylation by hepatocytes incubated at 37°C in either a Na^+ or a K^+ medium. In both conditions, the saturation curve was sigmoidal and the same V_{max} was obtained. However, the saturation curve was markedly more sigmoidal in a Na^+ medium than in a K^+ medium, with a Hill coefficient and $s_{0.5}$ close to 1.8 and 20 mM respectively in the former medium and to 1.3 and 12 mM in the latter. The data shown in Fig. 2 were obtained with hepatocytes isolated from a fed rat. A higher rate of glucose phosphorylation at low glucose concentration in a K^+ medium than in a Na^+ medium was also observed with hepatocytes from rats starved for 12 or 48 h and from alloxan-diabetic rats (results not shown).

Table 1 shows an experiment, performed with hepatocytes from 12h-starved rats, in which it appears that the faster rate of phosphorylation of glucose in a K^+ medium is reflected in a higher concentration of glucose 6-phosphate in the cell and also in a faster rate of metabolite recycling between glucose and glucose 6-phosphate; the rate of recycling was measured by the decrease in $^3\text{H}/^{14}\text{C}$ ratio in [$2\text{-}^3\text{H}, \text{U-}^{14}\text{C}$]glucose used as substrate (Clark *et al.*, 1973).

Inhibition by *N*-acetylglucosamine. *N*-Acetylglucosamine is a strong competitive inhibitor of hepatic glucokinase, with a K_i of 0.5 mM (Parry & Walker, 1966). Accordingly, we show in Fig. 3 that *N*-acetylglucosamine caused a marked inhibition of the phosphorylation of glucose in isolated hepatocytes incubated in either a Na^+ medium or a K^+ medium. The double-reciprocal plot of percentage inhibition against the concentration of inhibitor had an upward curvature and also indicated that the phosphorylation of glucose could be completely inhibited in both Na^+ and K^+ media at saturating concentrations of the inhibitor.

Relationship with glucokinase and glucose 6-phosphatase content of the cells. It was decided to check to what extent the phosphorylation of glucose in isolated hepatocytes is proportional to the activity of glucokinase and whether it is reasonable to assume that glucose 6-phosphatase can take over glucose phosphorylation in the absence of activity or low activity of glucokinase.

Fig. 4 shows that there exists a linear relationship between the phosphorylation of glucose measured at

Table 1. *Effect of the ionic composition of the medium on the concentration of glucose 6-phosphate and on the rate of recycling of glucose in isolated hepatocytes*

The hepatocytes were isolated from 12h-starved rats and were incubated with 5mM-[2-³H,U-¹⁴C]glucose. The amount of glucose that has been recycled is the number of μmol present in the incubation medium multiplied by the fraction that had lost its ³H relative to ¹⁴C. This value is minimal because of incomplete removal of ³H from [2-³H]-glucose 6-phosphate by hexose phosphate isomerase. Values shown are means $\pm$ S.E.M. for three experiments. The difference between the values obtained in Na⁺ and K⁺ medium are statistically significant ($P < 0.001$).

Incubation medium	[Glucose 6-phosphate] at 30 min (nmol/g of protein)	³ H/ ¹⁴ C ratio of [2- ³ H,U- ¹⁴ C]glucose at 60 min of incubation (fraction of value at zero time)	Minimal recycling of glucose ($\mu\text{mol}/60\text{min}$ per g of protein)
Na ⁺	430 $\pm$ 31	0.913 $\pm$ 0.008	28.7 $\pm$ 3.8
K ⁺	769 $\pm$ 62	0.859 $\pm$ 0.007	47.6 $\pm$ 2.5

Fig. 3. *Inhibition by N-acetylglucosamine of the phosphorylation of glucose by isolated hepatocytes*
Hepatocytes were obtained from a 12h-starved rat and incubated for 30min in a Na⁺ (○) or in a K⁺ medium (●) containing 5mM-glucose and various concentrations of N-acetylglucosamine. The inset shows a double-reciprocal plot of the same data, expressed as percentage inhibition.

20mM-glucose and the glucokinase content of the cells. A low rate of phosphorylation was observed in hepatocytes from newborn rats despite a normal activity of glucose 6-phosphatase, and also from diabetic animals, in which glucose 6-phosphatase is markedly increased. The values of glucose 6-phosphatase in these different preparations are shown in Table 2.

In the livers of diabetic or starved rats, the saturation curve of glucose remained sigmoidal, with the same Hill coefficient as in control cells (results not shown). With hepatocytes obtained from newborn rats, the saturation curve tended to be more hyperbolic when the activity of glucokinase was low. Examples of such curves are shown in Fig. 5. The

Fig. 4. *Correlation between the rate of phosphorylation of glucose by isolated hepatocytes and their content in glucokinase*

Hepatocytes were isolated from fed (○), 48h-starved (●), diabetic (▲) or newborn (■) rats and were incubated for 60min in a Na⁺ medium containing 20mM-[2-³H]glucose. Glucokinase activity was also measured spectrophotometrically by using 100mM-glucose in extracts of the same preparation of hepatocytes and was calculated as indicated in Table 3. The correlation coefficient was 0.957 ($P < 0.001$).

Table 2. *Glucose 6-phosphatase activity in isolated rat hepatocytes*

Hepatocytes were those used in the experiment shown in Fig. 4. Values shown are means $\pm$ S.E.M. with the numbers of observations given in parentheses.

Metabolic state	Glucose 6-phosphatase (μmol of P _i formed/min per g of protein)
Fed	94 $\pm$ 5 (4)
48h-starved	138 $\pm$ 3 (3)
Diabetic	214 $\pm$ 20 (3)
Newborn	142 $\pm$ 5 (6)

activities of hexokinase and of glucokinase measured in the hepatocytes of newborn rats are shown in Table 3.

Fig. 5. Effect of substrate concentration on the rate of phosphorylation of glucose (v) by hepatocytes isolated from newborn and adult rats

Hepatocytes isolated from rats aged 13 days ($\bullet$), 15 days ($\circ$), 20 days ($\blacksquare$), 24 days ($\blacktriangle$) or from an adult rat ($\square$) were incubated for 60 min in a Na^+ medium. (a) Direct plot; (b) double-reciprocal plot of some of the curves shown in (a). A straight line was also obtained at 13 days.

Discussion

Phosphorylation of glucose in extract of isolated hepatocytes

Our investigation of the properties of glucokinase in a crude extract of isolated hepatocytes indicates that there is little or no hexokinase in this preparation. Indeed, the activity measured at 0.5 mM-glucose was only of the order of 1–2% of V_{max} , and this activity could obviously be attributed to glucokinase itself; by contrast, this activity is 5–10% of V_{max} when measured in an extract of whole liver. Furthermore, the sigmoidal shape of the glucose-saturation curve was quite similar to those reported by Niemeyer *et al.* (1975) and by Storer & Cornish-Bowden (1976); this sigmoidicity would not be apparent if any important amount of low- K_m hexokinase was present. Our results are therefore in agreement with the previous conclusion of Sapag-Hagar *et al.* (1969) and of Crisp & Pogson (1972) about the location of glucokinase in the parenchymal cells of the liver and of hexokinase in non-parenchymal liver cells. However, they contrast with other reports (Morrison, 1967; Werner *et al.*, 1972; Bonney *et al.*, 1973). Although the reasons for this discrepancy are not clear, the simple fact that hepatocytes have been obtained free of low- K_m hexokinase is a demonstration that the latter enzyme is not normally present in hepatocytes, at least in adult rats.

There was no important loss of glucokinase during the preparation of cells, and an extract of isolated hepatocytes is therefore a satisfactory preparation in which to investigate the properties of glucokinase in its native form. The pH-activity curve was similar to that previously reported for purified liver glucokinase (Salas *et al.*, 1965; Parry & Walker, 1966). The kinetics of the enzyme were not influenced by the concentration of Na^+ , K^+ or Ca^{2+} in the incubation medium, and the sigmoidal shape of the curve was not modified by a change in pH from 7.3 to 7.5.

Table 3. Activities of hexokinase and glucokinase in extracts of hepatocytes isolated from newborn and adult rats. Activities were measured spectrophotometrically. Values shown for adult rats are means $\pm$ S.E.M. for seven experiments.

Age (days)	Hexokinase(A) phosphorylation measured by using 0.5 mM-glucose ($\mu\text{mol/min per g of protein}$)	Hexokinase+glucokinase(B) phosphorylation measured by using 100 mM-glucose ($\mu\text{mol/min per g of protein}$)	B-A (Glucokinase) ($\mu\text{mol/min per g of protein}$)
13	0.20	0.46	0.26
14	0.25	0.38	0.13
15	0.41	0.57	0.16
20	0.39	1.42	1.03
22	0.29	1.49	1.20
24	0.25	3.69	3.44
Adult	0.07 ± 0.02	3.93 ± 0.21	3.86 ± 0.20

The measurement of glucokinase activity by the radiochemical method gives results similar to those obtained with the more usual spectrophotometric procedure, except for a difference in $V_{\max}$, due to incomplete removal of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$ 6-phosphate by hexose phosphate isomerase in the liver (Rose & O'Connell, 1961; Katz & Rognstad, 1975). Therefore the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$ is a good tool for a qualitative study of the properties of glucokinase.

Phosphorylation of glucose in isolated hepatocytes

Sigmoidal shape of the glucose-saturation curve and ionic effects. The glucose-saturation curve measured in intact hepatocytes displayed a sigmoidal shape very reminiscent of that observed with glucokinase in a cell extract. It is therefore not necessary to invoke mechanisms other than the kinetic properties of glucokinase to explain the sigmoidicity of glucose utilization first reported by Katz *et al.* (1975). A puzzling observation was that the Hill coefficient and $s_{0.5}$ varied according to the ionic composition of the incubation medium. In a Na^+ medium, $s_{0.5}$ and the Hill coefficient were greater than in the cell extract, whereas in a K^+ medium these two values were more comparable with those observed in the cell-free preparation. We have checked that this increased sensitivity of the cells to glucose as well as their increased content of glucose 6-phosphate in a K^+ medium is not due to an inhibition of glucose 6-phosphatase, but to a stimulation of glucokinase, since it was accompanied by an increased rate of recycling between glucose and glucose 6-phosphate.

We have no explanation for the differences in the sigmoidicity of the glucose-saturation curve with the different ionic compositions of the media. As indicated above, this cannot be explained by changes in Na^+ , K^+ or Ca^{2+} concentrations in the cell or by modifications of the intracellular pH.

Inhibition by N-acetylglucosamine. The marked inhibition of glucose phosphorylation by *N*-acetylglucosamine is consistent with that of the conversion of glucose into glycogen by liver slices, previously reported by Spiro (1958); an inhibition by glucosamine of the release of ^3H from $[2\text{-}^3\text{H}]\text{glucose}$ by isolated hepatocytes has also been reported by Rognstad *et al.* (1973). The double-reciprocal plot of the percentage inhibition against the concentration of inhibitor displayed an upward curvature, indicating co-operative binding of the inhibitor at the active site of the enzyme. As with glucose, the co-operativity was greater in a Na^+ medium than in a K^+ medium. In both media, the inhibition seems to be complete at saturating concentrations of the inhibitor. At non-saturating concentrations of the *N*-acetylglucosamine, this inhibition was, however, markedly less than predicted from a K_i of 0.5 mM as reported by Parry & Walker (1966). The possibility

that this discrepancy could be due to an incomplete penetration of *N*-acetylglucosamine into the hepatocytes has been discarded by Spiro (1958). By analogy with the differences in the sigmoidal binding of glucose, one could consider that glucokinase binds the *N*-acetylglucosamine differently in the cell and in cell-free systems.

The exclusive role of glucokinase in the phosphorylation of glucose in isolated hepatocytes. The excellent correlation shown in Fig. 4, between the phosphorylation of glucose and the content of glucokinase in the hepatocytes, measured in various conditions, including newborn, diabetic and starved rats, is a strong argument in favour for the exclusive role of glucokinase in glucose phosphorylation. It is important to recall that in the past such a correlation has never been established within the same preparation. Indeed, several groups of authors (Friedmann *et al.*, 1967; McCraw *et al.*, 1967) have argued that glucose uptake by the diabetic liver was larger than expected from the glucokinase content, which, however, was not measured in the same preparation but was assumed to be low on the basis of other reports. As mentioned in the introduction, it is remarkable that residual activity of glucokinase in the liver of starved and diabetic animals is quite variable and that only a few authors have reported extremely low values.

As indicated above, the virtual absence of hexokinase excludes the participation of that enzyme in the phosphorylation of glucose by isolated hepatocytes from adult rats. However, in the hepatocytes obtained from 15-day-old rats, the saturation curve was nearly hyperbolic and the slow phosphorylation that occurred in these cells could therefore be partially due to a hexokinase. The glucose 6-phosphatase content of hepatocytes was either normal or elevated (see also Ashmore & Weber, 1959) in conditions in which glucose uptake was decreased in proportion to the glucokinase content. Glucose 6-phosphatase is not inhibited by *N*-acetylglucosamine (Hass & Byrne, 1960; Lygre & Nordlie, 1969), and clearly this enzyme was therefore not able to compensate for the diminished activity of glucokinase in the presence of the inhibitor. All these observations allow one to conclude that glucose 6-phosphatase does not take over the role of glucokinase when this latter enzyme is deficient. This experimental evidence, added to the theoretical objection developed by Hers (1976), allows us to reject safely a hypothesis advocated by Nordlie (1971, 1974) that glucose 6-phosphatase could play a role in glucose phosphorylation.

Glucose phosphorylation against glucose uptake

It is important to recall that glucose phosphorylation may occur in the absence of net glucose uptake by the hepatocytes and can even be concomitant with

Table 4. *Effect of the ionic composition of the medium on glucose uptake*

The hepatocytes were obtained from 12h-starved rats and incubated in the presence of 5 mM-[2-³H,U-¹⁴C]glucose in a Na⁺ or in a K⁺ medium. The values for minimal recycling are those shown in Table 1. The minimal phosphorylation is the amount of glucose detritiated and values shown are from a series of three similar other experiments. The glucose uptake is calculated by difference. Values shown are means $\pm$ S.E.M. for three experiments.

Incubation medium	Minimal phosphorylation (μ mol/60min per g of protein)	Minimal recycling (μ mol/60min per g of protein)	Uptake of glucose (μ mol/60min per g of protein)
Na ⁺	39.6 $\pm$ 1.8	28.7 $\pm$ 3.8	10.9
K ⁺	90.0 $\pm$ 5.4	47.6 $\pm$ 2.5	42.4

glucose output. Indeed, as discussed in detail in a survey (Hue & Hers, 1974), glucose uptake represents the difference between the phosphorylation of glucose and the dephosphorylation of glucose 6-phosphate. Any increase in the rate of phosphorylation of glucose will result in an increase in concentration of glucose 6-phosphate, which will in turn lead to an increased rate of hydrolysis, since the latter is a first-order reaction. The consequence of this operation is an enhanced rate of metabolite recycling.

As indicated above, a recycling of glucose was observed in our preparation, and this recycling was greater in a K⁺ medium than in a Na⁺ medium. The calculation made in Table 4 shows, however, that glucose uptake also was increased in a K⁺ medium. This results from the fact that the increase in glucose 6-phosphate concentration was less important than that in glucose phosphorylation and is easily explained by the great enhancement of glycogen synthesis that we have previously reported to occur in hepatocytes incubated in a K⁺ medium (Hue *et al.*, 1975). Glucose and K⁺ ions therefore exert a concerted control both on the rate of phosphorylation of glucose and on the activation of glycogen synthase, which results in an increased uptake of glucose, of which the major part is stored as glycogen.

This work was supported by the Fonds de la Recherche Médicale and by National Institutes of Health Grant AM 9235. F. B. is a Fellow of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture. L. H. is Chercheur Qualifié of the Fonds National de la Recherche Scientifique.

References

- Ashmore, J. & Weber, G. (1959) *Vitam. Horm. (N.Y.)* **17**, 91–132
- Bonney, R. J., Walker, P. R. & Potter, V. R. (1973) *Biochem. J.* **136**, 947–954
- Bontemps, F., Hue, L. & Hers, H. G. (1977) *Abstr. FEBS Meet. 11th abstr. no. A1-8 055*
- Clark, D. G., Rognstad, R. & Katz, J. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1141–1148
- Crisp, D. M. & Pogson, C. I. (1972) *Biochem. J.* **126**, 1009–1023
- DiPietro, D. L. & Weinhouse, S. (1960) *J. Biol. Chem.* **235**, 2542–2545
- Friedmann, B., Goodman, E. H., Jr. & Weinhouse, S. (1967) *Endocrinology* **81**, 486–496
- Gonzalez, C., Ureta, T., Babul, J., Rabajille, E. & Niemeyer, H. (1967) *Biochemistry* **6**, 460–468
- Grossman, S. H., Dorn, C. G. & Potter, V. R. (1974) *J. Biol. Chem.* **249**, 3055–3060
- Hammerstedt, R. H. (1973) *Anal. Biochem.* **56**, 292–293
- Hass, L. F. & Byrne, W. L. (1960) *J. Am. Chem. Soc.* **82**, 947–954
- Hers, H. G. (1964) *Adv. Metab. Disord.* **1**, 4–44
- Hers, H. G. (1976) *Annu. Rev. Biochem.* **45**, 167–189
- Hohorst, H. J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer H. U., ed.), pp. 134–138, Academic Press, London and New York
- Hue, L. & Hers, H. G. (1972) *Eur. J. Biochem.* **29**, 268–275
- Hue, L. & Hers, H. G. (1974) *Biochem. Biophys. Res. Commun.* **58**, 540–548
- Hue, L., Bontemps, F. & Hers, H. G. (1975) *Biochem. J.* **152**, 105–114
- Huggett, A. St. G. & Nixon, D. A. (1957) *Biochem. J.* **66**, 12P
- Katz, J. & Rognstad, R. (1975) *Curr. Top. Cell. Regul.* **10**, 237–289
- Katz, J., Wals, P., Golden, S. & Rognstad, R. (1975) *Eur. J. Biochem.* **60**, 91–101
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lygre, D. G. & Nordlie, R. C. (1969) *Biochim. Biophys. Acta* **178**, 389–391
- McCraw, E. F., Peterson, M. J. & Ashmore, J. (1967) *Proc. Soc. Exp. Biol. Med.* **126**, 232–236
- Migliorini, R. H. (1971) *Biochim. Biophys. Acta* **244**, 125–128
- Morrison, G. R. (1967) *Arch. Biochem. Biophys.* **122**, 569–573
- Murphy, E. D. & Anderson, J. W. (1974) *Endocrinology* **94**, 27–34
- Niemeyer, H., Clark-Turri, L., Garces, E. & Vergara, F. E. (1962) *Arch. Biochem. Biophys.* **98**, 77–85
- Niemeyer, H., Cardenas, M. L., Rabajille, E., Ureta, T., Clark-Turri, L. & Penaranda, J. (1975) *Enzyme* **20**, 321–333

- Nordlie, R. C. (1971) *Enzymes 3rd Ed.* **4**, 543–610
- Nordlie, R. C. (1974) *Curr. Top. Cell. Regul.* **8**, 33–118
- Parry, M. J. & Walker, D. G. (1966) *Biochem. J.* **99**, 266–274
- Parry, M. J. & Walker, D. G. (1967) *Biochem. J.* **105**, 473–482
- Patterson, M. S. & Greene, R. C. (1965) *Anal. Chem.* **37**, 854–857
- Pilkis, S. J. (1972) *Arch. Biochem. Biophys.* **149**, 349–360
- Portzehl, H., Caldwell, P. C. & Rügg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591
- Rognstad, R., Clark, D. G. & Katz, J. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1149–1159
- Rose, I. A. & O'Connell, E. L. (1961) *J. Biol. Chem.* **236**, 3086–3092
- Ryman, B. E. & Whelan, W. J. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol.* **34**, 285–443
- Salas, M., Vinuela, E. & Sols, A. (1963) *J. Biol. Chem.* **238**, 3535–3538
- Salas, J., Salas, M., Vinuela, E. & Sols, A. (1965) *J. Biol. Chem.* **240**, 1014–1018
- Sapag-Hagar, M., Marco, R. & Sols, A. (1969) *FEBS Lett.* **3**, 68–71
- Sharma, C., Manjeshwar, R. & Weinhouse, S. (1963) *J. Biol. Chem.* **238**, 3840–3845
- Spiro, R. G. (1958) *J. Biol. Chem.* **233**, 546–550
- Storer, A. C. & Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 7–14
- Walker, D. G. (1966) *Essays Biochem.* **2**, 33–67
- Walker, D. G. & Holland, G. (1965) *Biochem. J.* **97**, 845–854
- Walker, D. G. & Rao, S. (1964) *Biochem. J.* **90**, 360–368
- Weichselbaum, T. E. & Somogyi, M. (1941) *J. Biol. Chem.* **140**, 5–20
- Weinhouse, S. (1976) *Curr. Top. Cell. Regul.* **11**, 1–50
- Werner, H. V., Bartley, J. C. & Berry, M. N. (1972) *Biochem. J.* **130**, 1153–1155