Activities of glucokinase and hexokinase in mammalian and avian livers

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1. A radiochemical assay for glucokinase activity was developed for use in high-speed supernatants of liver. 2. The maximum activities of glucokinase ranged from 0.4 to 3.8 $\mu\text{mol/min per g}$ fresh wt. at 30°C in some avian and mammalian livers, including pigeon, guinea pig and man, in which previous reports indicated zero activities. 3. The reported maximum rates of hepatic glycogen synthesis in livers of rat and man *in vivo* are similar to the calculated glucokinase activities at 10 mM-glucose; therefore glucokinase activity should not limit glycogen synthesis from glucose.

It is well established that glycogen is synthesized rapidly in the liver of both rat and man *in vivo* after a carbohydrate-containing meal (Postle & Bloxham, 1980; Hultman, 1978). It is generally assumed that this glycogen is derived from glucose present in the hepatic portal vein, and, since the transport of glucose into the liver cell is near-equilibrium, the conversion of glucose into glucose 6-phosphate catalysed by glucokinase (EC 2.7.1.2) plays an important role in controlling the rate of glucose uptake by the liver (Newsholme & Start, 1973). However, several lines of evidence challenge this view. First, glucose is a poor precursor for glycogen synthesis *in vitro* in the perfused rat liver (Hems *et al.*, 1972) and in isolated hepatocyte suspensions (Katz *et al.*, 1976). In contrast, lactate is a good precursor, particularly in the presence of glucose and glutamine, which stimulate glycogen synthesis without acting as sources of carbon (Katz *et al.*, 1979; Boyd *et al.*, 1981). Secondly, 3-mercaptopicolinate, a specific inhibitor of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, inhibits hepatic glycogen synthesis *in vivo* when starved rats are re-fed with glucose (Sugden *et al.*, 1983). Thirdly, the specific radioactivities of ^3H and ^{14}C in newly synthesized glycogen, after administration of $[\text{U-}^{14}\text{C}, 3\text{-}^3\text{H}]\text{glucose}$ to starved rats, suggest that most of this glycogen is derived from glucose previously metabolized to the triose level (Newgard *et al.*, 1983). Finally, more than half the absorbed glucose in rats with a porto-

caval shunt is recovered in the peripheral circulation as lactate (Shapiro & Shapiro, 1979), which is presumably derived from glucose metabolism by the mucosal cells of the small intestine (Windmüller & Spaeth, 1978). Taken together, this evidence suggests that hepatic glycogen synthesis from glucose is an indirect process: the mucosal cells metabolize glucose to lactate, which is transported via the hepatic portal vein to the liver, where it is converted into glucose 6-phosphate by reactions of the gluconeogenic pathway. This glucose 6-phosphate is then used for glycogen synthesis.

One possible explanation for low rates of hepatic glycogen synthesis from glucose is a low activity of glucokinase. An inspection of published activities of this enzyme reveals marked discrepancies. For example, human glucokinase activity is variously reported to be absent (Lauris & Cahill, 1966), lower than (Willms *et al.*, 1970) or equal to (Pilkis, 1968) that of rat liver. These differences are likely to be caused by reactions which interfere with the standard spectrophotometric assay for glucokinase. In this assay the formation of glucose 6-phosphate is coupled to the reduction of NADP^+ by glucose-6-phosphate dehydrogenase (Di Pietro & Weinhouse, 1960). Since this method measures the activities of glucokinase and hexokinase, a separate determination of hexokinase activity is necessary, which must be subtracted from the total glucose-phosphorylating activity. Contamination of the coupling enzyme glucose-6-phosphate dehydrogenase with a glucokinase-like activity causes further errors, and the presence of 6-phosphogluconate dehydrogenase in liver extracts introduces uncertainties as to the stoichiometry between NADPH formed and glucose phosphorylated

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(Newgard *et al.*, 1983). The spectrophotometric assay can be improved by using an NAD⁺-linked glucose-6-phosphate dehydrogenase as the coupling enzyme (Newgard *et al.*, 1983). We have developed a radiochemical assay for glucokinase which depends on the conversion of [U-¹⁴C]glucose into [U-¹⁴C]glucose 6-phosphate in the presence of sufficient unlabelled glucose 6-phosphate to inhibit hexokinase activity by more than 95%. This assay has been used to re-examine the activities of glucokinase in the livers of some birds and mammals, including man. Activities of hexokinase were also measured, and the results are reported and discussed.

A particulate form of glucokinase has been found in rat liver (Berthillier *et al.*, 1976) and the liver of two species of birds (Wals & Katz, 1981). However, there are several differences between particulate and soluble glucokinase. Rat liver particulate glucokinase does not cross-react immunologically with an antiserum to soluble glucokinase, nor does its activity change in the livers of diabetic or neonatal rats, unlike the activity of soluble glucokinase (Berthillier *et al.*, 1976). Furthermore, full particulate glucokinase activity is only obtained when liver extracts are sonicated or treated with detergents (Berthillier *et al.*, 1976; Wals & Katz, 1981). Since it has been shown that particulate glucokinase is involved in a pathway for the synthesis of UDP-glucose for glucosyltransferase reactions in the Golgi apparatus (Berthillier & Got, 1974), we have confined our studies to measurements of the activity of soluble glucokinase.

Materials and methods

Animals, chemicals and enzymes were obtained from sources given previously (Cooney & Newsholme, 1982).

Preparation of homogenates and assay of enzyme activities

Animals were killed by cervical fracture and the liver was removed. Weighed samples of liver were chopped into small pieces and homogenized in 10 vol. of extraction medium by using either a manually operated glass homogenizer or a Polytron set at position 4 for 10 s at 0°C. The extraction medium for glucokinase was 50 mM-triethanolamine/0.3 M-sucrose/1 mM-EDTA, pH 7.5. For glucokinase the extract was centrifuged at 100000g for 60 min and the supernatant layer used for assays. Human liver extracts were obtained from Dr. P. J. Snodgrass and Dr. P. Lund of the Metabolic Research Laboratory, Radcliffe Infirmary, Oxford, who obtained samples of liver during operation which were extracted in 220 mM-mannitol/70 mM-sucrose/2 mM-Hepes[4-(2-

hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4 (see Snodgrass & Lund, 1984). The extract was centrifuged at 100000g for 60 min and the supernatant used for assay of glucokinase as described below. The extraction medium for hexokinase was 50 mM-triethanolamine/2 mM-MgCl₂/1 mM-EDTA, pH 7.5.

Glucokinase was assayed by measuring the rate of formation of radioactively labelled glucose 6-phosphate from radioactively labelled glucose. The assay medium contained 100 mM-Tris/HCl, 10 mM-MgCl₂, 5 mM-ATP, 10 mM-glucose 6-phosphate, 100 mM-glucose and [U-¹⁴C]glucose (4 µCi/ml) in a final volume of 125 µl and at a final pH of 8.0. Samples of assay medium (95 µl) were pipetted into small glass tubes in a Griffin Dri-block unit, and these samples together with centrifuged liver extract were preincubated for 10 min at 30°C. The reaction was initiated by the addition of 30 µl of extract to each assay-medium sample, and incubations were carried out for 12 min. The reaction was stopped by the addition of 250 µl of industrial methylated spirits, and the mixture was placed on ice. Two 20 µl samples of each mixture were pipetted on to the centre of separate acid-washed DEAE-cellulose paper discs. The discs were allowed to dry at room temperature for 30 min. All the control discs and one disc corresponding to each incubation were then washed on a Pyrex Hydrosol micro-analysis filter holder with 5 litres of distilled deionized water. During the washing procedure a single unused DEAE-cellulose paper disc was placed underneath each radioactive disc. Unwashed and washed discs were then placed in glass scintillation vials and dried in a 60°C oven overnight. The radioactivity retained on each DEAE-cellulose paper disc was determined by using a Beckman model LS-233 scintillation counter and 5 ml of a toluene scintillant containing 3 g of 2,5-diphenyloxazole/l and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l.

Hexokinase was assayed by a similar radiochemical assay, except that glucose 6-phosphate was omitted and the glucose concentration was decreased to 1 mM (2 µCi of [U-¹⁴C]glucose/ml). An ATP-regenerating system consisting of 10 mM-phosphocreatine and creatine kinase (100 µg/ml) was necessary to maintain linearity in the assay for hexokinase, but not in that for glucokinase.

Results and discussion

The problems of the coupled spectrophotometric assay for glucokinase are described in the introduction. To overcome these problems, a radiochemical assay was developed which depends on the incorporation of ¹⁴C from [U-¹⁴C]glucose into

glucose 6-phosphate and their subsequent separation on DEAE-cellulose paper discs. The following preliminary experiments were carried out, using rat liver extracts, to demonstrate that incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ into a component that was bound by DEAE-paper discs represented not only a glucose-phosphorylating activity but glucokinase activity.

(1) The incubations were stopped by addition of HClO_4 and neutralized with KOH . Glucose 6-phosphate was isolated by t.l.c. on polyethyleneimine sheets as described by Conyers *et al.* (1976). When losses were taken into account, the amount of radiolabelled hexose monophosphate produced gave a glucose-phosphorylating activity within 15% of that measured by the radiochemical assay procedure for glucokinase described in the Materials and methods section (results not shown).

(2) Liver extracts were always centrifuged at $100\,000g$ for 60 min before use of the supernatant for assay. This procedure should sediment particulate glucokinase activity (Berthillier *et al.*, 1976) and also glucose-6-phosphatase (Hers *et al.*, 1951). The latter can catalyse the phosphorylation of glucose with pyrophosphate as a phosphate donor (Nordlie & Lygre, 1966). Apparent activities of glucokinase were twice as high in crude extracts of rat liver, owing to the presence of particulate glucokinase and the phosphotransferase activity of glucose-6-phosphatase. If the crude extracts were dialysed to remove endogenous pyrophosphate (Veech *et al.*, 1980) and ATP was omitted, the activity decreased to almost zero, but could be increased to half the original activity by addition of 2 mM-pyrophosphate. This activity was presumably due to the phosphotransferase activity of glucose-6-phosphatase and, as expected, was completely removed by centrifugation at $100\,000g$ for 60 min.

(3) The glucose 6-phosphate concentration at the beginning of the incubations was $9.6 \pm 0.3 \text{ mM}$ (mean $\pm$ S.E.M. of three independent observations) and at the end of the 12 min incubations was $8.8 \pm 0.7 \text{ mM}$ (mean $\pm$ S.E.M. of three independent

observations). Sufficient glucose 6-phosphate remains to cause almost total inhibition of hexokinase.

(4) The reported value of K_m of soluble glucokinase for glucose measured in crude rat liver extracts is 12–15 mM (Parry & Walker, 1966). By using the radiochemical method and rat liver supernatant, a K_m value for glucose of 12.7 mM was obtained.

(5) The activity of soluble rat liver glucokinase decreases after 48 h starvation and after the induction of diabetes with alloxan (Di Pietro & Weinhouse, 1960) and increases in the livers of genetically obese mice (Newsholme *et al.*, 1979). Glucokinase activity measured radiochemically decreases (by 34%) after 48 h starvation, decreases (by 19%) after induction of diabetes with streptozotocin and increases (by 23%) in obesity.

(6) Glucokinase is reported to be inhibited by mannoheptulose (Coore & Randle, 1964) and by *N*-acetylglucosamine (Parry & Walker, 1966). Glucokinase activity measured radiochemically was inhibited by 15 mM-mannoheptulose (by 50%) and 10 mM-*N*-acetylglucosamine (by 26%). Glucokinase also phosphorylates mannose, but not galactose (Parry & Walker, 1966). We found that glucokinase activity was inhibited by 100 mM-mannose (by 24%), whereas 100 mM-galactose had no effect (see Table 1). With $[\text{U-}^{14}\text{C}]\text{mannose}$, the rate of phosphorylation of 100 mM-mannose was $1.88 \pm 0.11 \mu\text{mol/min per g fresh wt.}$ (mean $\pm$ S.E.M. of six independent observations); in the presence of 100 mM-glucose the rate of mannose phosphorylation fell to $1.25 \pm 0.12 \mu\text{mol/min per g fresh wt.}$ (mean $\pm$ S.E.M. of six independent observations).

For the reasons given above, the glucose-phosphorylating activity measured by our radiochemical method is considered to be glucokinase. Significant activities of glucokinase were found in high-speed liver supernatants of the three birds and five mammals investigated. The activities of glucokinase ranged from 0.40 to $3.8 \mu\text{mol/min per g fresh wt.}$, whereas the activities of hexokinase ranged from 0.11 to $0.52 \mu\text{mol/min per g fresh wt.}$ (Table 2). The highest activities of hexokinase

Table 1. *Effects of inhibitors or alternative substrates on glucokinase activity measured radiochemically*
Glucokinase activity was measured as described in the Materials and methods section with additions as stated. Means that are significantly different by Student's *t* test from those of controls are shown by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Other differences are not significant. Numbers of observations are given in parentheses.

Additions	Glucokinase activity ($\mu\text{mol/min per g fresh wt. at } 30^\circ\text{C}$)
None	2.59 ± 0.13 (8)
15 mM-Mannoheptulose	$1.30 \pm 0.09^{***}$ (4)
10 mM- <i>N</i> -Acetylglucosamine	$1.91 \pm 0.09^{**}$ (5)
100 mM-Mannose	$2.04 \pm 0.16^*$ (5)
100 mM-Galactose	2.73 ± 0.24 (5)

Table 2. *Activities of hexokinase and glucokinase in livers of birds and mammals*
Activities were measured as described in the Materials and methods section.

	Enzyme activity ($\mu\text{mol/min per g fresh wt. at } 30^\circ\text{C}$)	
	Hexokinase	Glucokinase
Chicken (<i>Gallus gallus</i>)	0.52 ± 0.07 (3)	0.40 ± 0.10 (4)
Pigeon (<i>Columba livia</i>)	0.33 ± 0.04 (3)	0.60 ± 0.29 (6)
Mallard (<i>Anas platyrhynchos</i>)	0.34 ± 0.01 (3)	0.45 ± 0.06 (3)
Laboratory mouse (<i>Mus musculus</i>)	0.14 ± 0.02 (4)	3.8 ± 0.3 (4)
Laboratory rat (<i>Rattus norvegicus</i>)	0.17 ± 0.02 (4)	3.2 ± 0.3 (4)
Guinea pig (<i>Cavia porcellus</i>)	0.15 ± 0.01 (5)	0.90 ± 0.10 (4)
Rabbit (<i>Oryctolagus cuniculus</i>)	0.11 ± 0.01 (4)	2.1 ± 0.2 (4)
Man	—	1.01 ± 0.16 (5)

were found in the livers of the birds, which possessed the lowest activities of glucokinase. This suggests that the low- K_m enzyme may play some role in the uptake of glucose by the avian liver. An important finding is that glucokinase is present in the livers of man, guinea pig and pigeon; it had been reported previously that these livers possessed zero activities (Lauris & Cahill, 1966; Ureta *et al.*, 1973). This suggests that the livers of these animals possess the enzymic capacity to remove glucose from the hepatic portal blood, and therefore that this glucose could act as a precursor for glycogen. In addition, we find greater activities of glucokinase in high-speed supernatants of chicken liver than those reported by Wals & Katz (1981).

The rate of hepatic glycogen synthesis *in vivo* in rats fed on a standard laboratory diet is $1.4 \mu\text{mol/min per g fresh wt.}$ (Postle & Bloxham, 1980). Assuming that the K_m for glucokinase is 12.7 mM (see above), that the V_{max} is $3.2 \mu\text{mol/min per g fresh wt.}$ (see Table 2) and that the portal-vein and hence intracellular hepatic glucose concentration is 10 mM (Newgard *et al.*, 1983), it can be calculated that glucokinase activity in rat liver would be $1.4 \mu\text{mol/min per g fresh wt. at } 30^\circ\text{C}$, so that, at the physiological temperature, the activity of glucokinase should be greater than the rate of glycogen synthesis. Hultman (1978) has measured the rate of glycogen synthesis *in vivo* in the liver of man after feeding or infusion of glucose; it is about $0.5 \mu\text{mol/min per g fresh wt.}$ On the basis of the V_{max} reported in Table 2, and by using the same values for the K_m and intracellular glucose concentration as above, it can be calculated that glucokinase activity in human liver would be $0.44 \mu\text{mol/min per g fresh wt. at } 30^\circ\text{C}$, so that, at the physiological temperature, the activity of glucokinase should be greater than the rate of glycogen synthesis.

Our results suggest therefore that the rate of glycogen synthesis from glucose should not be limited by the activity of glucokinase in human or rat liver. This conclusion is in contrast with that of

Newgard *et al.* (1983), who used a spectrophotometric method to assay glucokinase. Since in some experiments it has been shown that lactate is a good precursor and glucose a poor precursor for glycogen synthesis in the liver (see above), it is possible that as yet unidentified factors control the activity of glucokinase *in vivo*. This possibility, together with the high activities of glucokinase reported in this paper, suggest that in some conditions glucose will be an important precursor for glycogen synthesis in the liver.

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