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# The Role of Glucokinase in the Phosphorylation of Glucose by Rat Liver

BY D. G. WALKER AND SHYAMALA RAO
*Department of Biochemistry, University of Birmingham, Edgbaston, Birmingham* 15

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In view of the long-recognized role of the liver in the control of the blood sugar concentration in animals (Soskin, Essex, Herrick & Mann, 1938; Soskin & Levine, 1952; Cahill, Ashmore, Renold & Hastings, 1959) and the appreciation of the part played by glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) in the production of glucose by the liver (for reviews, see Ashmore, 1959; Weber, 1959), the study of the nature of the enzymic system for the phosphorylation of glucose by the liver has started rather late. Various experimental procedures (see, for example, Slein, Cori & Cori, 1950; Long, 1952; Renold, Hastings, Nesbett & Ashmore, 1955; Bekina & Petrova, 1957; Vester, 1959) gave glucose-phosphorylating activities too low to account for the observed rates of conversion of glucose into carbon dioxide and glycogen (Renold *et al.* 1955) by liver slices.

DiPietro & Weinhouse (1960) described a greatly improved system for the assay of glucose phosphorylation. Using this system and dialysed samples of preparations of the supernatant fraction of liver homogenates, Walker (1962) demonstrated the presence in adult guinea-pig liver of two enzymes, both of which catalysed the phosphorylation of glucose to glucose 6-phosphate but with widely differing apparent affinities for glucose. Extending this work, Walker (1963*b*) showed by kinetic analysis that adult rat liver also contains two enzymes having apparent $K_m$ values of about 10 mM and 37 $\mu$M for glucose respectively. This has received confirmation in a preliminary report (Viñuela, Salas & Sols, 1963) of the appearance of the two enzymes in different ammonium sulphate fractions of a liver-cell supernatant preparation. These observations correlated many reports (Cahill, Hastings, Ashmore & Zottu, 1958*b*; Spiro, 1958*b*; DiPietro, Sharma & Weinhouse, 1962; Lange & Kohn, 1961) on the nature of the hepatic glucose-phosphorylating system. The two enzymes have been tentatively designated as a more-specific glucokinase (i.e. of type ATP–D-glucose 6-phosphotransferase, EC 2.7.1.2) and a non-specific hexokinase (i.e. of type ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1) (Viñuela *et al.* 1963;

Walker, 1963*b*) pending studies on more-purified enzyme preparations. The names glucokinase and hexokinase are used in the present paper for the two enzymes respectively.

Though the hepatic cell is freely permeable to glucose (Cahill, Ashmore, Earle & Zottu, 1958*a*), the initial rate of phosphorylation of glucose appears to be a rate-limiting step in glucose metabolism by the liver (DiPietro *et al.* 1962; M. A. Lea & D. G. Walker, unpublished work). The total glucose-phosphorylating activity of rat liver varies in fed, starved and diabetic rats (DiPietro & Weinhouse, 1960; Niemeyer, Clark-Turri, Garcés & Vergara, 1962*a*). The present paper reassesses such changes by indicating the effect of starvation and certain hormones on the activities of the two enzymes concerned in the phosphorylation of glucose by rat liver and underlines the important role of the more-specific glucokinase in the process. A preliminary account of this work has been given (Walker & Rao, 1963).

## MATERIALS AND METHODS

*Animals.* The male and female rats were of the Wistar albino strain. With the few exceptions noted, all the male rats weighed 150–230 g. at the start of any treatment. The six female rats were litter mates; three of them were mated within the same 24 hr. period by leaving them with separate males for this time. All animals were fed on Thompson's Diet rat cubes manufactured by Heygate and Sons Ltd., Northampton. The starved animals had their food (but not water) removed 48 hr. before being killed.

Alloxan-diabetes was induced by the intraperitoneal injection of animals previously starved overnight with alloxan monohydrate in the form of a 1·8% (w/v) solution in 0·2M-sodium citrate buffer, pH 5, at the rate of 180 mg. of alloxan/kg. body wt. Diabetes was assessed on the bases of massive glycosuria, increased food and water intake, and decreased (compared with normal animals) weight gain. Only animals that survived the injection for 14 days and had a non-starvation blood glucose concentration greater than 350 mg./100 ml. of blood were employed except where otherwise stated. The three alloxan-diabetic rats treated with insulin for 1 day only were given 6 units of Insulin Zinc Suspension B.P. (Insulin Novo Lente, Novo Industri A/S, Copenhagen) and 4 units of soluble insulin (Boots Pure Drug Co. Ltd.)/100 g. body wt., and were killed 24 hr. later. Longer-term treatment of alloxan-diabetic animals with insulin (same preparations as above) followed the schedule given by Spiro & Hastings (1958), with the same criteria of effectiveness of treatment as far as possible. Further comments are included in the Results section.

Phlorrhizin-glycosuria was induced by subcutaneous injection of 20 mg. of phlorrhizin in 0·5 ml. of olive oil each morning and early evening for 48 hr. before each animal was killed; each animal received five injections, the final one 2 hr. before the experiment. All the animals showed massive glycosuria just before death. Cortisone-treated animals received intramuscular injections twice daily of 5 mg. of cortisone acetate (Injection Cortisyl Roussel; Roussel, London) for 7 days before being killed, the last injection being 2 hr. before death. Animals treated with both cortisone and insulin received similar doses of cortisone acetate and 1 unit of Insulin Zinc Suspension/100 g. body wt. daily.

The normal animals were killed for enzyme assays not in one batch but at frequent and regular intervals throughout the course of all the work described in the present paper. The normal values quoted are therefore a good estimate of the true range for this group of rats and can be used for comparison of all the results obtained with treated animals.

*Chemicals and enzyme.* ATP (disodium salt), $NADP^+$ (sodium salt) and glucose 6-phosphate dehydrogenase (Type V, from yeast) (EC 1.1.1.49) were obtained from Sigma Chemical Co. (through G. T. Gurr Ltd., London, S.W. 6). Alloxan and phlorrhizin were obtained from Hopkin and Williams Ltd. All other chemicals were of the best quality obtainable from British Drug Houses Ltd.

*Blood glucose.* This was determined, in filtrates obtained after treatment of whole blood with perchloric acid, by the glucose-oxidase method with commercial glucose-determination kits (C. F. Boehringer und Soehne G.m.b.H.; obtained through Courtin and Warner Ltd., Lewes, Sussex) used as directed by the manufacturers.

*Liver extracts.* The methods for the killing of the animals, perfusion of the livers *in situ*, preparation of homogenates [33·3% (w/v) in a medium containing: potassium chloride (0·15M), EDTA (sodium salt) (5 mM) and magnesium chloride (5 mM), pH 7·0], and preparation of the liver supernatant fraction were as described by Walker (1963*a*, *b*). Enzyme assays were performed as soon as possible after the preparation of these extracts; in particular, assays with high glucose concentrations (see below) were carried out within 3 hr. of killing the animals. Small (up to $\pm 10\%$) changes in activities were sometimes recorded when extracts were left for longer periods before the assays were performed. For this reason, a maximum of three and usually only two animals were killed on any one day to ensure that all assays on an animal could be completed on the same day.

*Determination of hexokinase and glucokinase.* Because the two enzymes have such widely differing affinities for glucose (Walker, 1963*b*) it is possible to determine the two enzymes in the presence of one another by using appropriate substrate (glucose) concentrations in the assay procedure. Glucose phosphorylation was estimated by coupling the glucose 6-phosphate formed to $NADP^+$ in the presence of exogenous glucose 6-phosphate dehydrogenase in incubation mixtures (total vol. 1·5 ml.) containing (final concentrations): glycylglycine buffer (50 mM), adjusted to pH 7·5 with sodium hydroxide; magnesium chloride (7·5 mM); ATP (5 mM); $NADP^+$ (0·5 mM); glucose 6-phosphate hydedrogenase [0·1 unit (Kornberg, 1950)]; 0·02 ml. of liver-supernatant preparation; glucose (100 mM, 10 mM, 5 mM, 0·2 mM or zero: see below). The liver supernatant fraction was added last and the production of NADPH followed at 340 mμ in silica cells having a 1 cm. light-path in a Unicam SP. 500 spectrophotometer fitted with a constant-temperature cell-housing maintained at 30°. Readings were taken every minute or half minute as necessary and reaction rate was assessed from the linear portion of the progress curve which was reached in 3–5 min. and continued for at least a further 10 min. Because the liver-supernatant fraction contains considerable 6-phosphogluconate-dehydrogenase (EC 1.1.1.44) activity, two mole.

cules of $NADP^+$ will be reduced for every molecule of glucose phosphorylated to glucose 6-phosphate (DiPietro & Weinhouse, 1960; DiPietro *et al.* 1962; Walker, 1963*b*).

The choice of glucose concentrations was based on the following properties of the tissue preparations containing both the hexokinase and the glucokinase. With fresh undialysed liver-supernatant preparations from animals whose livers contained both enzymes, almost maximum rate was obtained in the presence of 100 mM-glucose. The presence of progressively lower glucose concentrations resulted in lower rates of glucose phosphorylation, until the stage was reached where the presence of about 0·2 mM-glucose or less gave rates no higher than those in the presence of endogenous glucose only (Fig. 1.) If the liver-supernatant preparation was first dialysed for 20 hr. against a large volume of homogenizing medium (as used in previous kinetic studies by Walker, 1963*b*) and then used in a similar manner to study the effect of glucose concentration on the rate of NADPH formation, a typical reciprocal plot was obtained (Fig. 1) (cf. the results of Walker, 1963*b*). Extrapolation of the line at lower glucose concentrations would cut the ordinate (where 1/[glucose] is zero) at a point approximately midway between (*a*) the activity of both dialysed and undialysed liver-supernatant preparations with added glucose at 1 mM ($1/s = 1000 M^{-1}$) and (*b*) the activity with added glucose at 0·2 mM ($1/s = 5000 M^{-1}$), which is essentially the same as the activity of the undialysed fresh preparation in the presence of endogenous glucose only. Thus the maximum velocity of the non-specific hexokinase (with the low $K_m$) was calculated from the mean of the activities of fresh preparations with 1 mM-glucose and endogenous glucose only. In practice, the difference between these two activities was rarely more than 0·004 extinction unit/min. The mean value used for the evaluation of the hexokinase activity was corrected for the very low (less than 0·001 extinction unit/min.) activity in the absence of the liver-supernatant preparation due to a trace of glucose 6-phosphate-forming activity in the glucose 6-phosphate-dehydrogenase preparation. Glucokinase activity (i.e. the activity of the enzyme with the high $K_m$) was given by the difference between the total glucose-phosphorylating activity at 100 mM-glucose and that of the hexokinase.

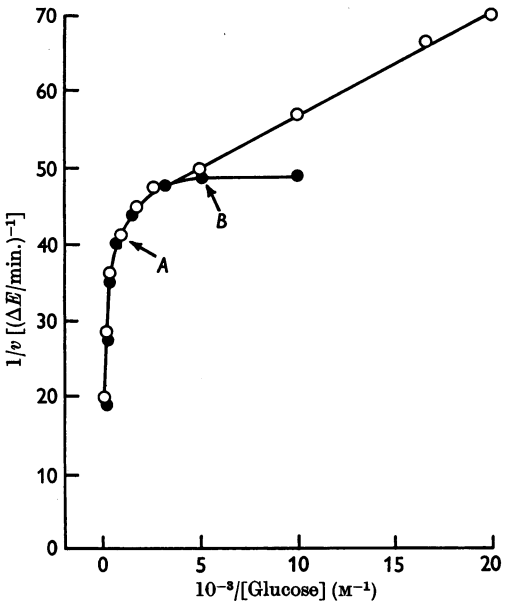


Fig. 1. Reciprocal plots for the effect of glucose concentration on the rate of glucose phosphorylation by: ●, 20 μl. of fresh liver-supernatant fraction prepared from an adult male rat starved for 48 hr. to lower the glucokinase activity; ○, 20 μl. of the same liver-supernatant fraction but dialysed for 20 hr. Substrate concentrations ranged from 100 to 0·05 mM-glucose. Experimental details are given in the text. Points *A* and *B* represent 1 mM- and 0·2 mM-glucose respectively.

Assays were performed in duplicate. The rates of glucose phosphorylation were expressed both per g. wet wt. of liver and on a nitrogen basis. Nitrogen was determined on the whole homogenate by a micro-Kjeldahl procedure. Activities were also calculated per whole liver and per 100 g. body wt. to assess the physiological significance of the results. Results are presented as means ± S.D. unless otherwise stated.

## RESULTS

*Normal values.* Table 1 gives the total glucose-phosphorylating activity and the contributions of glucokinase and hexokinase to this for normal male and normal and pregnant female rats. The male rats were divided into two equal groups on a weight basis. There were no significant differences between the results for any of the groups of normal animals of both sexes. The pregnant female rats also showed similar values.

The contribution of the glucokinase as measured at a substrate concentration of 100 mM-glucose to the total glucose-phosphorylating activity was about 75%, i.e. it represented the major portion. Because the apparent Michaelis constant, $K_m$, for the glucokinase is approximately 10 mM for glucose (Walker, 1963*b*; Viñuela *et al.* 1963) and a normal starvation blood glucose concentration of 90 mg./100 ml. of blood corresponds to 5 mM-glucose, glucokinase is unlikely to make such a high contribution towards the phosphorylation of glucose under physiological conditions as that indicated by the results in Table 1. Glucose-phosphorylating activity with 10 mM-glucose in the standard assay system was also measured. The results confirm the values for $K_m$ of glucokinase mentioned above and gave glucokinase activities 40–60% of those at 100 mM-glucose. Glucokinase activities measured with 5 mM-glucose were about 25–30% of those with 100 mM-glucose.

*Effect of starvation.* Male rats starved for 48 hr. showed a mean weight loss of 15% and their liver weights were 31% lower than those of normal fed animals of similar body weight. The total glucose-phosphorylating activity fell significantly ($P < 0·01$) (Table 1). No significant decreases were observed

Table 1. *Glucose-phosphorylating activities (total and that due to glucokinase and hexokinase) of normal, pregnant and starved rats*

Details of the assay procedures are given in the text. The results are given as means ± S.D.

| Animals | No. of animals | Body wt. (g.) | Liver wt. (g.) | Activity (μmoles of glucose phosphorylated/min.) (per g. of liver) Total | (per g. of liver) Hexokinase | (per g. of liver) Glucokinase | (per whole liver) Total | (per whole liver) Hexokinase | (per whole liver) Glucokinase | (per 100 g. body wt.) Total | (per 100 g. body wt.) Hexokinase | (per 100 g. body wt.) Glucokinase | (per 100 mg. of liver N) Total | (per 100 mg. of liver N) Hexokinase | (per 100 mg. of liver N) Glucokinase |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal males up to 185 g. | 10 | 168 ± 15 | 7·66 ± 1·47 | 1·73 ± 0·47 | 0·45 ± 0·10 | 1·28 ± 0·44 | 13·3 ± 4·3 | 3·3 ± 1·2 | 10·0 ± 3·7 | 7·8 ± 2·2 | 2·0 ± 0·6 | 5·8 ± 1·8 | 5·5 ± 1·4 | 1·4 ± 0·4 | 4·1 ± 1·3 |
| Normal males over 185 g. | 10 | 213 ± 18 | 9·20 ± 1·51 | 1·60 ± 0·31 | 0·41 ± 0·12 | 1·20 ± 0·32 | 14·9 ± 4·3 | 3·6 ± 1·0 | 11·3 ± 4·1 | 6·9 ± 1·8 | 1·7 ± 0·6 | 5·2 ± 1·7 | 5·6 ± 1·4 | 1·4 ± 0·5 | 4·2 ± 1·3 |
| Normal females | 3 | 227 ± 6 | 8·93 ± 1·04 | 1·63 ± 0·20 | 0·30 ± 0·03 | 1·33 ± 0·18 | 14·7 ± 3·5 | 2·7 ± 0·5 | 12·0 ± 3·0 | 6·5 ± 1·4 | 1·2 ± 0·2 | 5·3 ± 1·2 | 5·7 ± 1·0 | 1·1 ± 0·1 | 4·6 ± 0·8 |
| Pregnant females, gestational age 20–21 days | 3 | 267 ± 7 | 12·00 ± 0·62 | 1·91 ± 0·36 | 0·33 ± 0·06 | 1·58 ± 0·32 | 22·9 ± 3·9 | 3·9 ± 0·7 | 19·0 ± 3·3 | 8·4 ± 1·5* | 1·4 ± 0·2* | 7·0 ± 1·3* | 7·2 ± 1·7 | 1·2 ± 0·2 | 6·0 ± 1·5 |
| Males starved for 48 hr. | 10 | 174 ± 10† / 148 ± 11‡ | 5·31 ± 0·63‡ | 1·17 ± 0·22 | 0·48 ± 0·15 | 0·69 ± 0·25 | 6·2 ± 1·3 | 2·6 ± 1·4 | 3·6 ± 1·4 | 4·2 ± 0·8 | 1·7 ± 0·6 | 2·5 ± 0·9 | 3·6 ± 0·9 | 1·5 ± 0·6 | 2·1 ± 0·8 |

* Calculated for the whole gravid female including the conceptus.
† Weights before period of starvation.
‡ Weights after period of starvation; activities in starved animals are based on weights at the time of death.

in animals starved for only 24 hr. The decreases after starvation for 48 hr. were a little lower than those recorded by DiPietro & Weinhouse (1960), perhaps owing to the use of a different strain of rats.

The decreased total glucose-phosphorylating activity of starved rats compared with that of normal rats was due to a decrease in the glucokinase activity only. No change in the hexokinase activity occurred. Determinations of glucokinase activity with 5 mM-glucose (corresponding approximately to the starvation blood glucose concentration) on the starved animals gave values of about 0·2 μmole of glucose phosphorylated/min./g. of tissue. Expression of the results obtained with 100 mM-glucose in terms of the whole liver and per 100 g. body wt. gave rather greater decreases of glucokinase as compared with the results per g. of tissue, thus emphasizing the physiological importance of the results. The phosphorylating activity per 100 g. body wt. due to glucokinase in a starving rat measured with a glucose concentration of 5 mM was only about 12% of the glucokinase activity measured under optimum conditions for a normal fed animal. A more rapid fall in glucokinase activity during starvation to almost zero as recorded for a few animals by Viñuela *et al.* (1963) was never observed by us.

*Hepatic glucose phosphorylation in experimental diabetes.* The massive glycosuria over a period of 48 hr. brought about by the administration of phlorrhizin resulted in no changes in the activities of either of the hepatic glucose-phosphorylating enzymes as estimated *in vitro* (Table 2). The results in alloxan-diabetes were, however, different. The rats which had stabilized to the hypo-insulin state for 2 weeks, and could therefore be considered to be free of any other effects of the alloxan other than that on the insulin-producing cells of the pancreas (Spiro & Hastings, 1958), showed normal hexokinase activity but greatly diminished glucokinase activity (Table 2). The glucokinase activities ranged from 0·03 to 0·38 μmole of glucose phosphorylated/min./g. of tissue. The virtual absence of glucokinase in a severely alloxan-diabetic rat was confirmed by a study of the effect of glucose concentration with a dialysed liver-supernatant preparation. The reciprocal plot (Fig. 2) indicated the presence of only one enzyme having a low $K_m$ value. The small bend in the line at high glucose concentration is due to a very small amount of glucokinase present in the liver extract. At a glucose concentration of 25 mM (equivalent to 450 mg. of glucose/100 ml. of blood such as was found for the fed alloxan-diabetic animals) the rates of glucose phosphorylation were not significantly lower than those measured with 100 mM-glucose for those animals still possessing a little

Table 2. *Glucose-phosphorylating activities (total and that due to glucokinase and hexokinase) of normal, phlorrhizin-treated and alloxan-diabetic male rats*

Details of the treatment of animals and the assay procedures are given in the text. The results are given as means ± S.D.

| Animals | No. of animals | Body wt. (g.) | Liver wt. (g.) | Activity (μmoles of glucose phosphorylated/min.) (per g. of liver) Total | Hexokinase | Glucokinase | (per whole liver) Total | Hexokinase | Glucokinase | (per 100 g. body wt.) Total | Hexokinase | Glucokinase | (per 100 mg. of liver N) Total | Hexokinase | Glucokinase |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal males* | 20 | 191 ± 29 | 8·43 ± 1·65 | 1·67 ± 0·39 | 0·43 ± 0·09 | 1·24 ± 0·35 | 14·1 ± 4·3 | 3·5 ± 1·2 | 10·6 ± 3·9 | 7·4 ± 1·9 | 1·9 ± 0·6 | 5·5 ± 1·8 | 5·6 ± 1·4 | 1·4 ± 0·5 | 4·2 ± 1·3 |
| Males, treated with phlorrhizin | 6 | 175 ± 10 | 8·38 ± 0·95 | 1·84 ± 0·27 | 0·53 ± 0·15 | 1·31 ± 0·29 | 15·5 ± 3·2 | 4·3 ± 0·7 | 11·2 ± 3·5 | 9·1 ± 1·5 | 2·5 ± 0·5 | 6·5 ± 1·7 | 6·1 ± 0·7 | 1·8 ± 0·4 | 4·2 ± 0·9 |
| Males, alloxan-diabetic for 2 weeks | 8 | 163 ± 27† / 150 ± 31‡ | 7·33 ± 1·84‡ | 0·62 ± 0·25 | 0·44 ± 0·16 | 0·19 ± 0·14 | 4·4 ± 1·3 | 3·2 ± 1·1 | 1·2 ± 0·8 | 3·0 ± 1·0 | 2·1 ± 0·7 | 0·9 ± 0·7 | 1·9 ± 0·7 | 1·3 ± 0·5 | 0·6 ± 0·4 |
| Males, 24 hr. after alloxan administration | 3§ | 176 ± 12 | 8·08 ± 0·85 | 1·02 ± 0·09 | 0·41 ± 0·03 | 0·61 ± 0·11 | 8·3 ± 1·5 | 3·3 ± 0·2 | 5·0 ± 1·0 | 4·7 ± 1·2 | 1·9 ± 0·2 | 2·8 ± 0·9 | 3·2 ± 0·5 | 1·3 ± 0·1 | 1·9 ± 0·5 |

* These values are for the 20 normal male rats presented in two groups in Table 1.
† Weights before treatment with alloxan.
‡ Weights after treatment with alloxan; these values are used in the calculation of activities.
§ The blood glucose concentrations were 350, 453 and 700 mg./100 ml. of whole blood.

glucokinase activity. There was no obvious inverse correlation between the low glucokinase activity and the severity of alloxan-diabetes as indicated by the blood glucose concentration at the time of death. The difficulty in obtaining uniformly diabetic animals and the doubtful value of the blood glucose concentration as a criterion of severity are familiar (Lequin & Steyn-Parve, 1962; Wool, 1963), and it could be that the three rats that gave the appreciable glucokinase activities were not so severely diabetic.

Three rats were killed 24 hr. after the administration of alloxan. Whereas other effects of alloxan cannot be eliminated at this stage after the onset of the diabetic state, the results (Table 2) indicated that the glucokinase activity fell by about 50% during that time. Alloxan had no effect on the enzyme assay system *in vitro*.

The insulin-treatment programme detailed by Spiro & Hastings (1958) was used to follow the ability of insulin therapy to restore hepatic glucokinase activity to normal. Insulin therapy was inaugurated in a group of six animals that had been alloxan-diabetic for 14 days, and two animals were killed on the first, second and third days after the start of the treatment. The glucose-phosphorylating activities (Table 3) were quickly restored, normal glucokinase activities being found within 1 or 2 days of the first injection of insulin. As previously noted (Spiro & Hastings, 1958; Steiner & Williams, 1959), the ratio of liver wt. to body wt. increased considerably during this period (cf. a mean value of 4·4 g. of liver/100 g. body wt. for the normal male rats). The large initial doses of insulin required to




Fig. 2. Reciprocal plot for the effect of glucose concentration on the rate of glucose phosphorylation by 70 μl. of a dialysed liver-supernatant fraction prepared from an alloxan-diabetic rat. Substrate concentrations ranged from 100 to 0·02 mM-glucose. Other details are as given in Fig. 1.

Table 3. *Effect of insulin on the hepatic glucose-phosphorylating enzymes in alloxan-diabetic rats*

Details of the treatment of the animals and the assay procedures are given in the text. The results are given as means ± S.D.

| Period of treatment with insulin | Animal no. | Body wt. (g.) | Liver wt. (g.) | Liver wt. (g./100 g. body wt.) | Liver N (mg./g. of liver) | Concn. of blood glucose (non-starvation) (mg./100 ml. of whole blood) | Activity (μmoles of glucose phosphorylated/min.) (per g. of liver) Total | (per g. of liver) Hexokinase | (per g. of liver) Glucokinase | (per whole liver) Total | (per whole liver) Hexokinase | (per whole liver) Glucokinase | (per 100 g. body wt.) Total | (per 100 g. body wt.) Hexokinase | (per 100 g. body wt.) Glucokinase | (per 100 mg. of liver N) Total | (per 100 mg. of liver N) Hexokinase | (per 100 mg. of liver N) Glucokinase |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 hr. | 64 | 211 | 10·46 | 5·0 | 30·5 | 523 | 0·70 | 0·31 | 0·39 | 7·3 | 3·2 | 4·1 | 3·5 | 1·5 | 2·0 | 2·3 | 1·0 | 1·3 |
| | 65 | 203 | 14·15 | 7·0 | 21·8 | 416 | 1·12 | 0·36 | 0·76 | 15·9 | 5·1 | 10·8 | 7·8 | 2·5 | 5·3 | 5·1 | 1·6 | 3·5 |
| 48 hr. | 66 | 179 | 18·84 | 10·5 | 16·1 | 212 | 1·57 | 0·81 | 0·76 | 29·5 | 15·2 | 14·3 | 16·5 | 8·5 | 8·0 | 9·8 | 5·0 | 4·8 |
| | 67 | 233 | 18·45 | 7·9 | 17·5 | 354 | 1·63 | 0·25 | 1·38 | 30·0 | 4·6 | 25·4 | 12·9 | 2·0 | 10·9 | 9·3 | 1·4 | 7·9 |
| 72 hr. | 68 | 214 | 11·48 | 5·4 | 22·5 | 137 | 2·19 | 0·58 | 1·61 | 25·1 | 6·7 | 18·4 | 11·7 | 3·1 | 8·6 | 9·7 | 2·6 | 7·1 |
| | 69 | 240 | 19·50 | 8·1 | 19·8 | 240 | 1·81 | 0·39 | 1·42 | 35·3 | 7·6 | 27·7 | 14·7 | 3·2 | 11·5 | 9·1 | 2·0 | 7·1 |
| 14 days | 78 | 223 | 11·35 | 5·1 | 24·6 | 97 | 2·29 | 0·58 | 1·71 | 26·0 | 6·6 | 19·4 | 11·6 | 3·0 | 8·6 | 9·3 | 2·4 | 6·9 |
| | 79 | 180 | 11·36 | 6·3 | 28·8 | 101 | 2·52 | 0·38 | 2·14 | 28·7 | 4·3 | 24·4 | 15·9 | 2·4 | 13·5 | 8·7 | 1·3 | 7·4 |
| | 80 | 227 | 11·63 | 5·1 | 27·7 | 220 | 3·24 | 0·32 | 2·92 | 37·7 | 3·7 | 34·0 | 16·6 | 1·6 | 15·0 | 11·7 | 1·2 | 10·5 |

restore the blood glucose concentrations to the normal range resulted in glucokinase activities, estimated *in vitro*, that were higher than normal. After the first 3–5 days of treatment, doses of 0·8–1·5 units/100 g. body wt./day were required to keep the animals in a state such that they showed no glycosuria, normal food and water intake, and normal weight gain. Glucokinase activities were still somewhat above the normal range when the animals were killed 14 days after the start of the insulin treatment. The blood glucose concentrations of these animals did not indicate a hyper-insulin state.

*Effects of cortisone treatment.* Ilyin & Shanygina (1960) reported that cortisone lowered hepatic glucose-phosphorylating activity and that insulin partly protected against this change. Their procedure for estimation of total glucose-phosphorylating activity used conditions (lower substrate concentration and liver preparations which included microsomes) that were unlikely to be optimum. Our results (Table 4) show that cortisone treatment did cause a significant ($P < 0·01$) decrease in total glucose-phosphorylating activity when expressed per g. of tissue. These decreases were not significant when expressed per whole liver, per 100 g. body wt. or on a nitrogen basis because of the enlarged livers of the cortisone-treated animals and a lower tissue nitrogen content. (The four untreated control animals of similar weight gave results within the normal ranges given in Table 2, so the normal values in Table 2 were used for the assessment of the statistical significance of the changes.) The decreases on the per g. of tissue basis were due to significant falls in the activities of both glucokinase ($0·02 < P < 0·05$) and hexokinase ($P < 0·01$). Simultaneous treatment of animals with insulin in a dose sufficient to maintain an alloxan-diabetic animal in normal condition (i.e. 1 unit/day/100 g. body wt.) had no significant effect on the changes caused by cortisone.

## DISCUSSION

The recognition of the effect of glucose concentration on glucose phosphorylation by liver slices (Cahill *et al.* 1958*b*) and the use of a high substrate concentration in assays on a cell-free extract (DiPietro & Weinhouse, 1960) led to values for glucose-phosphorylating activity measured *in vitro* which are in essential agreement with the results for liver slices (Renold *et al.* 1955). A glucose-phosphorylation rate of 1·6 μmoles/min./g. of liver measured at 30° under optimum conditions corresponds to a rate of glycogen formation of 15·5 mg./hr./g. of liver. This compares favourably with rates *in vivo* without taking into account the higher activities expected at 37°, and is still

Table 4. *Effect of cortisone and cortisone plus insulin on hepatic glucose phosphorylation in male rats*

Details of the treatment of animals and the assay procedures are given in the text. The results are given as means ± S.D.

| | Activity (μmoles of glucose phosphorylated/min.) | | |
|---|---|---|---|
| | Cortisone | Cortisone plus insulin | Normal controls |
| No. of animals ... ... ... ... ... | 4 | 5 | 4 |
| Body wt. (g.) before treatment | 141±15 | 142±12 | 153±15 |
| Body wt. (g.) after treatment | 130±13 | 128±17 | — |
| Liver wt. (g.) | 8·78±0·69 | 8·14±1·92 | 6·91±0·65 |
| Concn. of blood glucose (mg./100 ml. of blood) | 170±31 | 151±41 | — |
| Total | | | |
| Per g. of liver | 0·96±0·31 | 1·05±0·30 | 1·73±0·38 |
| Per whole liver | 8·5±2·8 | 8·4±2·2 | 12·0±3·9 |
| Per 100 g. body wt. | 6·5±1·9 | 6·6±1·7 | 7·8±1·9 |
| Per 100 mg. of liver N | 3·4±1·1 | 3·8±1·5 | 5·7±0·5 |
| Hexokinase | | | |
| Per g. of liver | 0·21±0·06 | 0·17±0·08 | 0·45±0·11 |
| Per whole liver | 1·8±0·5 | 1·4±0·5 | 3·1±1·1 |
| Per 100 g. body wt. | 1·4±0·5 | 1·1±0·4 | 2·0±0·7 |
| Per 100 mg. of liver N | 0·7±0·2 | 0·7±0·3 | 1·4±0·5 |
| Glucokinase | | | |
| Per g. of liver | 0·77±0·34 | 0·87±0·24 | 1·28±0·34 |
| Per whole liver | 6·4±2·5 | 7·1±1·8 | 8·9±3·5 |
| Per 100 g. body wt. | 5·1±2·1 | 5·5±1·4 | 5·8±1·8 |
| Per 100 mg. of liver N | 2·7±1·2 | 3·1±0·9 | 4·3±1·2 |

sufficient even if such potential maximum rates are not achieved because glycogen synthesis takes place *in vivo* at glucose concentrations not greater than the apparent $K_m$ for glucokinase.

If the absence of glucokinase in the foetal rat liver (Walker, 1963*a*) is due to the effect of some factor causing repression of glucokinase synthesis, then lower rates of glucose phosphorylation could be expected in the maternal liver. Normal values were recorded. This type of behaviour is different from that recorded (Combes & Stakelum, 1962) for the hepatic enzyme system which catalyses the conjugation of sulphobromophthalein with glutathione, where an unidentified factor results in decreased maternal hepatic activity in the third trimester of pregnancy. In the present case repression of the production of glucokinase in the foetal liver could still occur if such a responsible agent can only exert its effect for some reason in the foetal animal or has a distribution confined to the conceptus to the exclusion of the maternal organism. Another type of repression mechanism may, however, be operating here.

Because it is unlikely that the foetal liver parenchymal cells would be without a soluble glucose-phosphorylating enzyme, it seems reasonable to assume that, in the absence of evidence to the contrary, the hexokinase is present in such cells (Walker, 1963*b*) and is not confined to the non-parenchymal tissue cells. Further, unless some sort of damage to organelles or enzyme release has occurred during the preparation of the supernatant fraction, the presence of both glucokinase and hexokinase in this soluble fraction suggests that the two enzymes are equally available to glucose. Hence both enzymes are involved simultaneously in the phosphorylation of glucose by the mature liver and could exert control over carbohydrate metabolism within this organ. Though glucokinase accounts for the major portion of the total glucose-phosphorylating activity of normal liver measured under optimum conditions, under physiological conditions the respective contributions of glucokinase and hexokinase may be very similar because of the relationship between the cell glucose concentration and the $K_m$ for glucokinase. Several means of metabolic control thence arise owing to both the varying relative contributions of the two enzymes and their respective properties. For example, the hexokinase is likely to be inhibited by glucose 6-phosphate but glucokinase is not thus inhibited by the product of its own reaction (Viñuela *et al.* 1963). It is also known that changes in the tissue concentration of glucose 6-phosphate (Steiner & Williams, 1959) influence the further metabolism of this compound (Steiner, Rauda & Williams, 1961), and considerable variations in its rate of production are now apparent. If some form of spatial separation between the hexokinase and glucokinase existed within the liver cell, differential 'channelling' of glucose 6-phosphate into one of several metabolic pathways might occur. Factors such as these may operate in a complex way to control the further metabolism of glucose

6-phosphate and might explain the type of result obtained with liver slices (Cahill *et al.* 1959; Felts, Doell & Chaikoff, 1956), where the range of concentrations of glucose over which marked changes in the rate of formation of the various end products of the metabolic pathways leading from glucose 6-phosphate occur showed distinct differences.

The changes in total glucose phosphorylation recorded by DiPietro & Weinhouse (1960) and Niemeyer *et al.* (1962*a*) indicated that the enzyme system involved is adaptive to various physiological changes. This adaptation now appears to be due to changes in glucokinase activity and not in that of the hexokinase. The unchanged activities in rats treated with phlorrhizin indicate that the large loss of body glucose in itself does not have any effect on glucokinase in spite of the much lower rate of glucose oxidation by the whole rat thus treated (Stetten, Welt, Ingle & Morley, 1951). Though the cortisone-treated animals showed lower total glucose-phosphorylating activity on a per g. of liver basis, this was due to a decrease in both enzyme activities rather than in that of glucokinase only. Lowered glucose utilization (reviewed by Fajans, 1961) and increased gluconeogenesis (Landau *et al.* 1962) in hyperadrenocorticalism are considered to be due to an action of the adrenocortical hormones on carbohydrate metabolism in the liver (Winternitz, Dintzis & Long, 1957). The effect of cortisone on glucose phosphorylation would now appear to be secondary in nature as compared with the evidence for a more direct effect of insulin.

The very marked change in the glucokinase activity in the alloxan-diabetic rat provides an explanation at the enzyme level of many well-established facts concerning hepatic carbohydrate metabolism in diabetes mellitus (Chernick & Chaikoff, 1950, 1951; de Duve, 1956). A series of reports (Renold, Teng, Nesbett & Hastings, 1953; Renold *et al.* 1955; Cahill *et al.* 1958*b*; Spiro, Ashmore & Hastings, 1958; Spiro, 1958*a*) led to the conclusion that the changes in glucose phosphorylation are a direct effect of insulin deficiency. The time-course of the decline in glucokinase activity after the administration of alloxan and its recovery after treatment of the stabilized diabetic animal with insulin is consistent with all the results with liver slices.

Possible mechanisms by which such changes in glucokinase activity are effected include adsorption and release of glucokinase by some cellular particles such as the microsomes, or some other type of activation–inactivation process. Steiner & Williams (1959) suggested that the increased glucose phosphorylation after treatment with insulin might be a secondary effect resulting from the stimulation of pathways involving glucose 6-phosphate utilization. This seems less likely if glucokinase is not inhibited by glucose 6-phosphate (Viñuela *et al.* 1963). The low hepatic tissue concentration of glucose 6-phosphate in starvation does not stimulate glucokinase activity.

The results can be also explained if the measured activities of glucokinase represent an equilibrium resulting from the relative rates of enzyme synthesis and degradation and insulin affects the synthesis of glucokinase. This hypothesis will also explain the lower activity during starvation, when insulin concentrations are lower. That insulin does have a direct effect on the liver is now generally accepted (for review, see Krahl, 1961; also Migliorini & Chaikoff, 1962). The many changes in liver enzymes in diabetes must be explained either by a direct effect of insulin on all the enzymes concerned or, more likely, to some more fundamental action (Krahl, 1957, 1961). Korner (1960*a*, *b*) has demonstrated that insulin *in vivo* has an effect on the ability of rat-liver microsomes to incorporate amino acids into protein *in vitro*, and Manchester & Young (1961) have collected a considerable weight of evidence for a direct effect of insulin on protein synthesis in cell-free systems from rat liver. The adaptive changes in total glucose-phosphorylating activity resulting from dietary changes are prevented by ethionine and puromycin (Niemeyer, Pérez, Garcés & Vergara, 1962*b*; Niemeyer, Clark-Turri & Rabajille, 1963). These agents inhibit protein synthesis. Perhaps the synthesis of hepatic glucokinase is particularly susceptible to control by insulin.

Since the preparation of the present paper, Dr S. Weinhouse, Philadelphia, Pa., U.S.A., has kindly sent us a manuscript of a paper recently submitted to *J. biol. Chem.* by C. Sharma, R. Manjeshwar & S. Weinhouse, describing results which overlap some of our work and lead to similar general conclusions.

## SUMMARY

1. Determination of the activities of the two hepatic glucose-phosphorylating enzymes, glucokinase and hexokinase, in the presence of one another is possible because of their very different apparent affinities for glucose. A method for their determination based on this is described.

2. Glucokinase accounts for the major portion of the total glucose-phosphorylating activity in the fed rat. Normal values are given.

3. Glucokinase activity is decreased by starvation but is unchanged during phlorrhizin-glucosuria. Alloxan-diabetes is characterized by negligible or very low glucokinase activity. Insulin treatment of alloxan-diabetic rats restores this to normal levels. Hexokinase activity is unaffected during all such changes.

4. Cortisone treatment results in lowered activities of both enzymes, and simultaneous treatment with insulin does not prevent this less-specific change.

5. The results provide an enzymic basis for many previous observations on liver slices. Possible ways in which changes in the two enzymes may effect control of carbohydrate metabolism are discussed. It is suggested that insulin may control glucokinase synthesis in the liver.

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