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ON THE PRESENCE OF TWO SOLUBLE GLUCOSE-PHOSPHORYLATING ENZYMES IN ADULT LIVER AND THE DEVELOPMENT OF ONE OF THESE AFTER BIRTH

D. G. WALKER

Department of Biochemistry, University of Birmingham, Edgbaston, Birmingham (Great Britain)

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SUMMARY

1. The nature of the enzymes involved in hepatic glucose phosphorylation has been examined at various stages of mammalian development (guinea-pigs and rats). Dialysed preparations of the liver soluble fraction were used to examine the effect of glucose in the presence of ATP and Mg^{2+} on glucose 6-phosphate formation, as measured by coupling to NADPH production.

2. Kinetic analysis of the results indicated that the liver of the foetal and immediately new-born animal contains one hexokinase having a high affinity for glucose. The mature adult liver contains two glucose-phosphorylating enzymes having widely different apparent K_m values.

3. Some properties of the two enzymes have been studied. These have been confined to those obtainable using the same assay procedure applied to crude tissue preparations. The low-affinity enzyme, having a high $K_{m,1}$, which develops after birth, appears to be a specific glucokinase. The high-affinity enzyme, having a low $K_{m,2}$, seems to be a non-specific hexokinase catalysing the phosphorylation of several monosaccharides.

4. The kinetic analysis of an enzyme system consisting of two enzymes catalysing identical reactions simultaneously is discussed as are some of the physiological implications of the results to the problem of the control of carbohydrate metabolism in the developing mammal.

INTRODUCTION

The role of the liver in the control of the blood glucose level in the adult animal has been recognized for many years[1]. Hepatic glucose production by both glycogenolysis and gluconeogenesis involves the action of the enzyme glucose 6-phosphatase as the final step and the level of this enzyme in the liver is influenced by a number of dietary and hormonal factors[2]. The nature of the enzyme system involved in hepatic glucose utilization has, until recently, been little studied. CRANE AND SOLS[3] mentioned that liver might contain a hexokinase having an apparent Michaelis constant, K_m, higher than that of hexokinases in other mammalian tissues. Glucose phosphorylation by the liver has been estimated by several groups of workers[4-10] using either liver slices[6] or various types of tissue extracts.

The work presented in this paper is part of a study on carbohydrate metabolism in the developing foetus and new-born mammal. The characteristics of the glucose-phosphorylation systems in the foetal and adult liver will be shown to be different. The hepatic glucokinase described whilst this work was in progress by DIPIETRO, SHARMA AND WEINHOUSE[11], which seems to have a special function in the control of glucose uptake, will be shown to appear after birth. It is one of two enzymes catalysing glucose phosphorylation by the mature adult liver in both the guinea-pig[12] and the rat.

EXPERIMENTAL

Materials

Glc-6-*P*, $NADP^+$, ATP (sodium salt) and Glc-6-*P* dehydrogenase (EC 1.1.1.49) were purchased from the Sigma Chemical Co. (obtained through Messrs. G. T. Gurr Ltd.). All other chemicals were "AnalaR", or the best quality grade from Messrs. British Drug Houses Ltd. Tris was recrystallized twice from methanol.

Animals

The male and female rats were of an albino strain and the guinea-pigs were of the Pirbright albino strain. Gestational age was assessed on the assumption of post-partum mating (rats) and a combination of post-partum mating and the tables[13] of crown-rump length and weight *versus* age (guinea-pigs). Gravid guinea-pigs were used normally during their second or third pregnancy (mean gestation period 68 days).

Preparation of liver supernatant fraction

The method was based upon those of CRANE AND SOLS[3] and DIPIETRO AND WEINHOUSE[10]. Adult and young animals were killed by dislocation of the cervical vertebrae. In order to remove the bulk of the blood in the tissue, the livers were perfused *in situ* with about 20 ml of ice-cold homogenizing medium which consisted of 0.15 M KCl, 5 mM EDTA, 5 mM $MgCl_2$, adjusted to pH 7.0 with NaOH. The perfusion step was omitted with the new-born rats. The whole liver was removed, weighed and a 33.33% (w/v) homogenate prepared in the above medium using a glass Potter–Elvehjem-type homogenizer with steel-shafted Teflon pestle for 2 min at 2000 rev./min. This and succeeding steps were all performed at 0–4°. After preliminary centrifugation at $600 \times g$ for 10 min the supernatant fraction was prepared by high-speed centrifugation at $100\,000 \times g$ for 30 min as described by DIPIETRO AND WEINHOUSE[10].

Foetal animals were obtained by maternal laparotomy, excision of the uterus and foetal membranes and cutting of the umbilical cord. The foetuses were killed by decapitation (rats) or dislocation of the cervical vertebrae (guinea-pigs). Foetal guinea-pig livers were perfused via the umbilical vein with partial success but the perfusion of foetal rat livers was not possible. In a few instances where insufficient tissue was available to make enough 33.33% homogenate to fill the centrifuge tube, a more dilute homogenate was prepared. A pad of solid fat was found in the

top of the tubes following high-speed centrifugation of homogenates of livers from late gestation animals and this had to be carefully eased aside prior to collection of the clear supernatant fraction. The foetal supernatant fractions were coloured bright red due to foetal haemoglobin and resulted in higher blank values than with adult preparations in the assay procedure.

Assay of glucose phosphorylation

This was based upon the method of DIPIETRO AND WEINHOUSE[10] involving direct coupling of the Glc-6-*P* formed with $NADP^+$ by Glc-6-*P* dehydrogenase. Any possible inhibition by the product of the phosphorylation is thus eliminated. The formation of NADPH was followed at 340 mμ in cells having a 1-cm light path in a Unicam SP. 500 spectrophotometer fitted with a constant-temperature cell housing maintained at 30°. The incubation medium (total volume 1.5 ml) contained (final concentrations): 50 mM sodium glycylglycine buffer (pH 7.5) (or 45 mM sodium Tris–maleate buffer (pH 7.5) in some of the earlier work), 7.5 mM $MgCl_2$, 5 mM ATP, 0.5 mM $NADP^+$, 0.1 Kornberg units[14] Glc-6-*P* dehydrogenase, and various concentrations of glucose and liver supernatant preparation as described in the individual experiments. The supernatant fraction containing the soluble glucose-phosphorylating system was added last and the production of NADPH followed every minute over a period of not more than 15 min. The linear section of the progress curve (see later) was used for assessment of reaction rate, the period 5–12 min normally being the most useful portion.

The liver supernatant fraction contains phosphogluconate dehydrogenase[15] (EC 1.1.1.44) so that, for every molecule of Glc-6-*P* formed, two molecules of $NADP^+$ will be reduced[10,11]. The validity of this assumption was assessed in several ways. (a) Direct estimations of $NADP^+$ reduction in the presence of 5 mM sodium 6-phosphogluconate showed that the phosphogluconate dehydrogenase activity was always in considerable excess of that of the glucose-phosphorylation system. (b) There are some changes in the activity of the hexose monophosphate-shunt dehydrogenases during development[16,17] but these changes are unlikely to effect statement (a). (c) A few experiments were performed in which a preparation of phosphogluconate dehydrogenase[15], essentially free of hexokinase activity, was added to the assay system. No significant enhancement of activity was observed. (d) NADPH production was always proportional to enzyme concentration whenever this was to be expected.

The main limitation of the assay procedure for studying the effect of glucose concentration upon reaction rate is that a change of absorbancy, ΔA, greater than about 0.07 per min is difficult to determine with accuracy. This limits the range over which rates can be spread. A maximum use of the most accurate portion of the spectrophotometer scale was obtained by including in the blank cuvette a small quantity of stable red dye (methyl red) so that the initial absorbancy of the test cuvette was only a little above zero.

Relevant theoretical kinetic considerations

The results of the present study will demonstrate the presence, in adult liver tissue, of two enzymes catalysing the same reaction simultaneously. Theoretical

treatments have been given for this type of situation by DIXON AND WEBB[18] and REINER[19]. Using the usual abbreviations, and letting the subscripts 1 and 2 refer to the two enzymes having the high and low apparent Michaelis constants, $K_{m,1}$ and $K_{m,2}$, respectively, the observed velocity, v, is given by

$$v = v_1 + v_2 = \frac{V_1 S}{S + K_{m,1}} + \frac{V_2 S}{S + K_{m,2}}$$

Taking reciprocals and simplifying,

$$\frac{1}{v} = \frac{\left(1 + \frac{K_{m,1}}{S}\right)\left(1 + \frac{K_{m,2}}{S}\right)}{V + \frac{V_1 K_{m,2} + V_2 K_{m,1}}{S}} \qquad (1)$$

where $V = V_1 + V_2$, the sum of the maximum velocities of the two enzymes. In general, the shape of the curve represented by this Eqn. 1 will be hyperbolic in form[18] when $1/v$ is plotted *versus* $1/S$.

Also V_1 and V_2 must be of a similar order of magnitude for two components to be recognized[18]. If $K_{m,1} = K_{m,2}$, Eqn. 1 reduces to that of a straight line so that the behaviour of the system cannot be distinguished from that of one enzyme. At the other extreme (as is the case for the present results), if the ratio of the two Michaelis constants is large, the shape of the curve will approximate to two straight lines of different slope joined by a curved portion. This follows from the following considerations:

(A) In the range of concentrations of substrate where S is large, the second enzyme, having $K_{m,2}$, will be at maximum activity, *i.e.* giving V_2 (assuming no inhibition by excess substrate concentration). Following the treatment of REINER[19] and ignoring power terms in S, Eqn. 1 becomes

$$\frac{1}{v} = \frac{1}{V} + \frac{1}{S}\left(\frac{V_1 K_{m,1} + V_2 K_{m,2}}{V^2}\right) \qquad (2)$$

This Eqn. 2 represents a LINEWEAVER–BURK[20] linear plot having a slope equal to the bracketed term and cutting the $1/v$ ordinate at $1/V$. By putting $1/v = 0$, the intercept on the $1/S$ abscissa is at

$$-\frac{V}{V_1 K_{m,1} + V_2 K_{m,2}}.$$

In the special case where $K_{m,1}$ is much greater than K_{m_2}, the intercept on the abscissa becomes $\frac{V_1 + V_2}{V_1 K_{m,1}}$. This takes into account the contribution V_2 of the $K_{m,2}$ enzyme to the observed velocities.

(B) When S is very low, the contribution, v_1, of the higher-K_m enzyme will be negligible and terms including S^2 may be ignored. Eqn. 1 then becomes

$$\frac{1}{v} = \frac{K_{m,1} + K_{m,2}}{V_1 K_{m,2} + V_2 K_{m,1}} + \frac{1}{S}\left(\frac{K_{m,1} K_{m,2}}{V_1 K_{m,2} + V_2 K_{m,1}}\right). \qquad (3)$$

In the special case where $K_{m,2} \ll K_{m,1}$, the intercept on the $1/v$ ordinate will correspond to $1/V_2$. By putting $1/v = 0$ in the general case, the intercept on the abscissa equals

$$-\frac{K_{m,1} + K_{m,2}}{K_{m,1}K_{m,2}}$$

or, when $K_{m,2} \ll K_{m,1}$, $-1/K_{m,2}$. Thus, graphical representation of the results by the method of LINEWEAVER AND BURK[20] can make possible the direct determination of V $(= V_1 + V_2)$, V_2 and thence by difference V_1, and $K_{m,2}$. The value of $K_{m,1}$ may be calculated from the intercept on the abscissa as given above.

RESULTS AND INTERPRETATION

The first results were obtained using the guinea-pig because of the larger amount of liver tissue available from the foetuses of one gravid female of this species. Using the standard assay system, including 0.1 M glucose, a series of glucose-phosphorylation measurements on animals at various stages of pre- and post-natal development were made and are recorded in Table I. The values indicate smaller glucose-phos-

TABLE I

GLUCOSE-PHOSPHORYLATING ACTIVITY OF GUINEA-PIG LIVER AT VARIOUS STAGES OF DEVELOPMENT

Activities were measured by the standard procedure in the presence of 0.1 M glucose and 50-μl samples of supernatant fractions, prepared from 33% homogenates, in a total volume of 1.5 ml. Results are expressed as μmoles glucose phosphorylated per min either per g wet tissue or per 100 g animal and are the means of duplicate determinations. The figures quoted represent means ± S.D. for the group of animals.

Source of liver	*No. of observations*	*Activity per g tissue*	*Activity per 100 g animal*
Adult*, males	2	0.64 ± 0.03	2.76 ± 0.31
Adult*, gravid females	6	0.87 ± 0.18	—
Foetuses, gestational age 50–59 days	4	0.55 ± 0.02	2.33 ± 0.81
Foetuses, gestational age 60–68 days	3	0.42 ± 0.09	1.97 ± 0.44
New-born, 0–5 days	7	0.33 ± 0.07	1.17 ± 0.15

* Adult animals were 6–9 months old.

phorylating potential in the foetal livers, compared to the adult, and lower values still in the new-born animals. At a later stage it was found that use of glycylglycine buffer instead of Tris–maleate buffer gave slightly higher values (up to 10% higher) than those recorded above but this made no essential difference to the pattern of activities recorded.

As a first approach to the possible reasons for the observed changes, activities were measured in the absence of added glucose. Endogenous glucose was still present in these assays (from the liver supernatant fraction) but direct estimations of the glucose level in the incubation medium indicated that this endogenous glucose represented a substrate level of not more than $2 \cdot 10^{-4}$ M glucose. Compared to the activity in the presence of endogenous glucose only, addition of 0.1 M glucose in-

creased the adult liver enzymic activity by over 100% but foetal liver systems by not more than 12%. This difference suggested that the apparent Michaelis constants of the glucose-phosphorylating systems were different in foetal and adult liver. For further examination of the system it was necessary to lower the activity due to endogenous glucose.

Effects of dialysis upon assay system and preliminary investigation of enzyme systems involved

The progress curve of NADPH production using freshly prepared (undialysed) liver supernatant fractions usually showed a fast initial rate which quickly (within 5 min) settled to a steady rate (see DiPIETRO *et al.*[11] for typical progress curves). That this high initial rate was due to endogenous Glc-6-P[11] is strongly supported by the fact that dialysis of the fraction for as short a period as 30 min was sufficient to remove its cause, virtually linear rates of NADPH production being found with such preparations. Longer periods of dialysis resulted in variable losses of maximum glucose-phosphorylating activity (measured in presence of 0.1 M glucose), the loss being from 5 to 30% in 24 h for adult liver preparations and not more than 15% for foetal liver preparations. Attempts to stabilize the enzyme systems were made by including in the dialysing medium such substances as cysteine, ATP and various concentrations of EDTA higher than 5 mM, but these additions gave no better stability than that obtained using a standard dialysing technique as follows. Dialysis was performed at 0–4° using 8/32-in Visking tubing, previously soaked in distilled water, against the same medium as that used to prepare the homogenate (see EXPERIMENTAL). From 3 to 4 ml of supernatant fraction was dialysed for 4 h against 1.5 l of medium followed by 16 h against a similar volume of the medium. A slight precipitate sometimes formed during dialysis and this was removed by centrifugation at 20 000 × g for 10 min. Some of the loss of activity was not necessarily due to

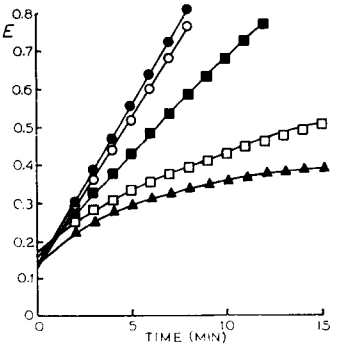

Fig. 1. Progress curves showing formation of NADPH by measurement of absorbancy at 340 mμ. All incubations contained 50 μl of supernatant fraction, prepared from a 33% homogenate of adult male guinea-pig liver, dialysed for 1 h. ●—●, full system (see text) including 0.1 M glucose; ○—○, full system, including 0.1 M glucose, plus 10 mM 2-deoxyglucose; ■—■, full system minus added glucose; □—□, full system minus added glucose plus 10 mM 2-deoxyglucose; ▲—▲, full system including 0.1 M glucose but minus ATP, or full system minus glucose and minus ATP. Readings taken before 2 min are omitted for clarity.

dialysis, for storage of the supernatant fraction at 0° or −18° also resulted in smaller losses of activity over similar time periods.

Two other main effects resulted from the dialysis of both adult and foetal preparations. First, the activity due to endogenous glucose was now not more than 15% of that under optimum conditions (0.1 M glucose). Secondly, the increase in absorbancy at 340 mμ was slow over the first few minutes and increased to a steady linear rate within 5 min. The reason for this lag phase is not understood as yet in spite of attempts to determine its cause.

Further properties of the assay systems are illustrated in Fig. 1 for a preparation from adult liver dialysed for 1 h. Omission of glucose from this preparation still gave approx. 50% of maximum activity. Addition of 10 mM 2-deoxyglucose to the incubation mixture reduced the activity of the full system by less than 10% but had a much greater effect upon the system in the absence of added glucose. The lowest curve of Fig. 1 suggests that the endogenous activity in the absence of added ATP falls off very gradually as the traces of endogenous substrate are utilized.

Enzyme preparations dialysed for longer periods (*e.g.* 20 h as in the standard procedure) behaved similarly except that the activity in the absence of added glucose was much lower and was only a little higher than that in the absence of added ATP.

Comparison of the glucose-phosphorylating systems in foetal and adult guinea-pig liver

Using foetal liver preparations dialysed for 20 h, linear plots of $1/v$ *versus* $1/S$ for varying glucose concentrations are obtained. Fig. 2 shows a typical result giving an apparent K_m of $2 \cdot 10^{-5}$ M. Similar plots were obtained with preparations of foetal livers ranging from 45 to 68 days gestational age. Such plots are typical of that expected for one enzyme having a low K_m value.

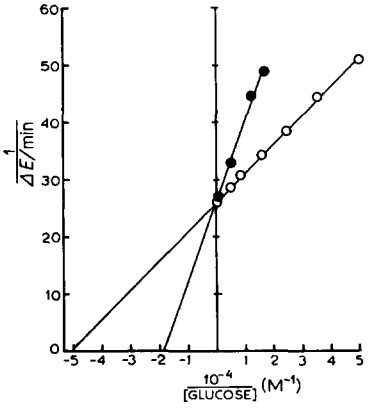

Fig. 2. Plot of reciprocal velocity *versus* reciprocal glucose concentration for glucose-phosphorylating system of foetal guinea-pig liver (from 2 animals pooled), gestational age 65 days. All incubations contained 100 μl of supernatant fraction, prepared from a 33% homogenate, dialysed 20 h, and glucose concentrations ranging from 0.1 M to $2 \cdot 10^{-5}$ M. ○—○, full system; ●—●, full system plus 1 mM 2-deoxyglucose. For details of incubation system, see text.

Typical plots obtained with adult liver enzyme preparations are given in Figs. 3a and b. Fig. 3a suggests that two enzymes are present catalysing the same reaction for the plot contains two straight lines having widely differing slopes. The points obtained at the higher glucose concentrations are plotted on an extended $1/S$ scale in Fig. 3b from which the slope of the line at high glucose concentrations may be determined. Visual inspection of the slopes clearly indicates that the two enzymes have widely differing apparent affinities for glucose and justify the theoretical simplifications already noted (see theoretical discussion in EXPERIMENTAL section)

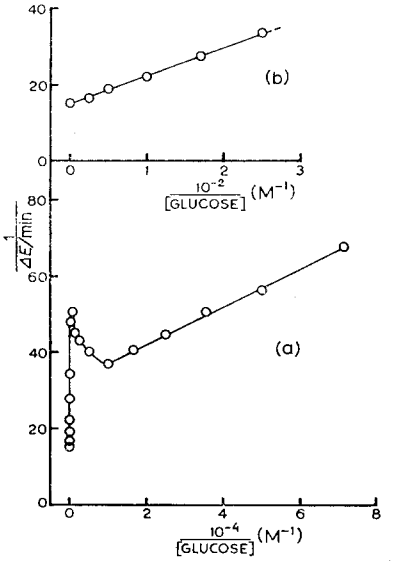

Fig. 3. Plots of reciprocal velocity *versus* reciprocal glucose concentration for glucose-phosphorylating system of adult guinea-pig liver. All incubations contained 50 μl of supernatant fraction, prepared from a 33% homogenate, dialysed 20 h, and glucose concentrations ranging from 0.1 M to $1.4 \cdot 10^{-5}$ M (a) and 0.1 M to $4 \cdot 10^{-3}$ M (b). The six points shown in plot (b) are the same as the first six points (highest glucose concentrations) of plot (a). For details of incubation system, see text.

for the situation when $K_{m,2}$ is much less than $K_{m,1}$. Fig. 3a also shows a deviation from the expected type of curve[18] in the region between $2 \cdot 10^{-3}$ and $1 \cdot 10^{-4}$ M glucose. Such a deviation might be explained as being due to inhibition of the low-K_m enzyme at high substrate concentration[21]. From the theoretical considerations given earlier, an extension of the line through the points obtained at low glucose concentrations will cut the abscissa at $-1/K_{m,2}$. In the case of Fig. 3a, $K_{m,2} = 1.6 \cdot 10^{-5}$ M, which is very similar to that obtained with the foetal liver preparation. This type of experiment has been repeated several times and similar plots obtained on all occasions. Both Tris–maleate and glycylglycine buffers give analogous results. The comparison between the plots can leave little doubt that the high-K_m enzyme is absent from foetal liver. The possibility that the enzyme with high K_m is more labile in the foetal liver than in adult liver and is lost during dialysis is excluded by the observations on undialysed foetal liver preparations. It is also necessary to

postulate that the inhibition of the $K_{m,2}$-enzyme at high substrate concentrations is a property which also develops after birth, for the plot of $1/v$ *versus* $1/S$ for the foetal liver preparation (Fig. 2) is a straight line up to 0.1 M glucose. The intercepts in Fig. 3 on the ordinate, where $1/S = 0$, will be $1/V$ and $1/V_2$, ($V = V_1 + V_2$), for the lines drawn through the points obtained at high and low glucose concentrations, respectively. The maximum rates of the two guinea-pig enzymes appear to be very similar.

Comparison of the glucose-phosphorylating system in foetal and adult rat liver

Preliminary experiments on adult rat liver indicated that omission of glucose from the assay system, using undialysed tissue preparations, resulted in a much greater lowering (up to 80%) of activity (compared to the activity in presence of 0.1 M glucose) than had been observed with the guinea-pig. Comparison of the behaviour of preparations from the two species therefore suggested that, if adult rat liver also contains two enzymes as in the guinea-pig, the activity of the low-K_m enzyme represented a considerably smaller proportion of the total glucose-phosphorylating activity in this species. The theoretical calculations of DIXON AND WEBB[18] indicate that the hyperbolic type of $1/v$ *versus* $1/S$ plot for a two-enzyme system such as that under consideration is only obtained when the two enzymes have comparable maximum activities and they pointed out that a second enzyme might be missed if this condition were not fulfilled.

Using an adult rat-liver preparation dialysed for 20 h, a plot of $1/v$ *versus* $1/S$ gave strong indication that a second low-K_m enzyme is present. The limitations of the assay procedure, however, made the study of the effect of changes of glucose concentration below $1 \cdot 10^{-4}$ M very difficult. The total glucose-phosphorylating activity of adult rat is lowered by at least 40% by starvation for 48 h (ref. 10). Preliminary experiments indicated that this was due to a fall in the activity of enzyme having the high $K_{m,1}$ rather than the enzyme having the low $K_{m,2}$. Use of a starved adult rat might, therefore, provide a preparation in which the maximum activities of the two enzymes were much closer to one another than in the fed animal.

Fig. 4 gives results obtained using an adult rat which had been starved for 48 h. Fig. 4a is a plot of the points obtained throughout the whole glucose concentration range while Fig. 4b gives only the points at the higher glucose concentrations. The presence of two enzymes is clearly indicated. From the intercepts of the two straight lines on the ordinate where $1/S = 0$, the activity of the low-K_m enzyme is now approx. one-third of the total activity. Fig. 4a also indicates a slight inhibition of the low-K_m enzyme by high glucose concentrations but the effect is much less than that observed for the guinea-pig.

Extrapolation of the line through the points at low glucose concentrations in Fig. 4a gives a $K_{m,2}$ of $3.8 \cdot 10^{-5}$ M for glucose. This result is of the same order of magnitude as that indicated in Fig. 5 for a preparation of foetal rat liver where the apparent K_m for glucose is $5.5 \cdot 10^{-5}$ M. The presence of two enzymes catalysing the phosphorylation of glucose to Glc-6-*P* in adult rat liver is, therefore, also demonstrated.

In other experiments, the apparent K_m for ATP of adult rat liver, which gave a linear LINEWEAVER–BURK[20] plot with varying ATP concentrations, was approx.

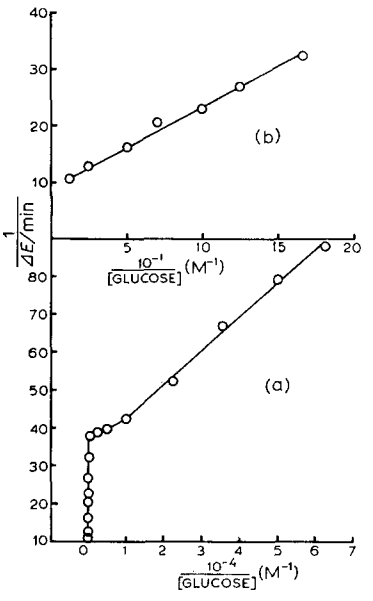

Fig. 4. Plots of reciprocal velocity *versus* reciprocal glucose concentration for glucose-phosphorylating system of adult rat liver, the animal having been starved for 48 h. All incubations contained 20 μl of supernatant fraction, prepared from a 33% homogenate, dialysed 20 h, and glucose concentrations ranging from 0.1 M to $1.6 \cdot 10^{-5}$ M (a) and 0.1 M to $6 \cdot 10^{-3}$ M (b). The seven points shown in plot (b) are the same as the first seven points (highest glucose concentrations) of plot (a). For details of incubation system, see text.

$3 \cdot 10^{-4}$ M. A value of $2 \cdot 10^{-4}$ M ATP was obtained using a foetal rat-liver preparation. These values, considered together with the absence of any obvious sign of two parts to the reciprocal plot with adult liver, suggest that the two hexokinases in adult liver have similar affinities for ATP. Variation of the pH of the assay system between 6.5 and 8.0 gave only slight changes in activity with both adult and foetal rat-liver preparations. Both hexokinases therefore have broad optimum pH ranges of a similar nature.

Some properties of the enzyme catalysing glucose phosphorylation having a low apparent Michaelis constant ($K_{m,2}$)

The straight-line plots of $1/v$ *versus* $1/S$ in Figs. 2 and 5 indicate the presence of one enzyme catalysing glucose phosphorylation by foetal liver (although the theoretical considerations indicate that a kinetic analysis of this kind cannot exclude the possibility that even here there are more than one enzyme having very similar affinities for glucose). Fig. 5 indicates that the activity of this enzyme (in a foetal rat-liver preparation) is proportional to enzyme concentration over a wide range of glucose concentrations. Similar results were obtained with preparations of foetal guinea-pig liver. In adult liver preparations the contribution of the high-K_m enzyme is likely to be negligible at glucose concentrations of approx. $1 \cdot 10^{-4}$ M and less. This was confirmed in experiments using both rat and guinea-pig adult liver pre-

parations when activities at glucose concentrations of $1 \cdot 10^{-4}$ M and lower were strictly proportional to the concentration of tissue preparation used, the lines converging on a point on the abscissa in a manner similar to that in Fig. 5. Because the adult tissue contains enzymes having such widely differing apparent Michaelis constants, therefore, the values of $K_{m,2}$ determined in both foetal and adult tissue preparations may be pooled. This argument was verified by the finding that there was no significant difference between the $K_{m,2}$ values for glucose with adult tissue

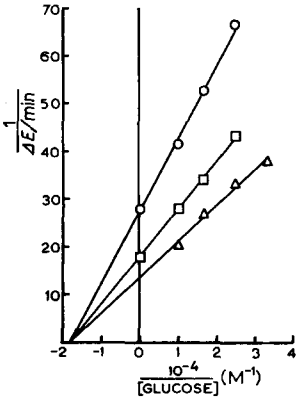

Fig. 5. Plot of reciprocal velocity *versus* reciprocal glucose concentration for glucose-phosphorylating system of foetal rat liver (gestational age 18 days) showing the effect of enzyme concentration on the system. The incubations contained various volumes of supernatant fraction, prepared from a 7% homogenate (using all livers of litter), dialysed 20 h, and glucose concentrations ranging from 0.1 M to $3 \cdot 10^{-5}$ M. Volumes of enzyme preparation used were: ●—●, 80 μl; ■—■, 120 μl; ▲—▲, 160 μl. For details of incubation system, see text.

preparations and the one K_m value obtained with foetal tissue preparations for the given species. Based on 8 separate determinations, the mean $\pm$ S.D. of $K_{m,2}$ for guinea-pig liver was found to be $(2.6 \pm 1.2) \cdot 10^{-5}$ M and 10 estimations on rat liver gave a mean $K_{m,2}$ of $(3.7 \pm 1.4) \cdot 10^{-5}$ M glucose.

Studies on the specificity of the enzyme having this very low apparent Michaelis constant have so far been restricted to those possible using the same assay technique which measures Glc-6-*P* formation. Three kinds of evidence suggest that the enzyme is a non-specific hexokinase (*i.e.* of the type EC 2.7.1.1).

(a) Fig. 2 includes results obtained with a foetal guinea-pig liver preparation in the presence of 2-deoxyglucose. This analogue of glucose appears to act as an inhibitor of glucose phosphorylation. The product of the phosphorylation of 2-deoxyglucose, 2-deoxyglucose 6-phosphate, does not react with Glc-6-*P* dehydrogenase[22], so that competitive inhibition is indicated. The extreme sensitivity of the system to 2-deoxyglucose and the limitation of the range of activities which can be measured by this procedure have made the results with 2-deoxyglucose very unsatisfactory. The inhibitor constant, K_i, appears to be of the order of $6 \cdot 10^{-4}$ M 2-deoxyglucose. This estimate is based on the data of Fig. 2 and other similar experiments.

(b) The enzyme also phosphorylates fructose. Adult liver tissue contains a specific fructokinase which catalyses the formation of Fru-1-*P* (ref. 23). Glucose has no

effect upon this fructokinase, which has been shown to be absent from the foetal livers of both rat and guinea-pig[24]. The further metabolism of Fru-1-*P* by liver involves the action of an aldolase so that Fru-6-*P* is only formed after several further enzymic stages[25]. The phosphorylation of fructose by foetal liver preparations is shown in Fig. 6 (lower plot). These results were obtained by measuring Glc-6-*P* formation by the standard procedure. The liver supernatant fraction possesses a high phosphoglucoseisomerase activity which is in large excess over the other enzymes involved throughout the whole of pre- and post-natal life[17]. Hence these results indicate the presence in foetal liver of an enzyme catalysing phosphorylation of fructose to Fru-6-*P* which is rapidly isomerized to Glc-6-*P*. The maximum rate

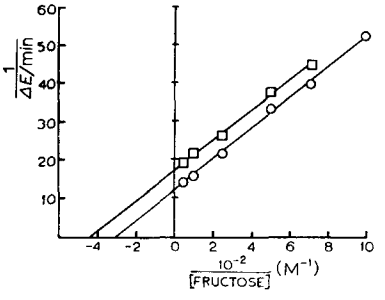

Fig. 6. Plot of reciprocal velocity *versus* reciprocal fructose concentration for the low-K_m hexokinase present in both foetal and adult rat liver. ○—○, 100 μl of foetal liver enzyme preparation (supernatant fraction from 15% homogenate of all livers from litter, gestational age 19 days, and dialysed 20 h); □—□, 40 μl of adult liver enzyme preparation (supernatant fraction from 33% homogenate dialysed 20 h). Fructose concentrations ranged from $2 \cdot 10^{-2}$ M to $1 \cdot 10^{-3}$ M. For details of incubation system, see text.

of fructose phosphorylation is 20–40% higher (in several experiments) than glucose phosphorylation. The addition of very low concentrations of glucose (say $4 \cdot 10^{-5}$ M) to the assay system has a marked effect upon the rate of apparent Glc-6-*P* formation in the presence of fructose. The presence of a non-specific hexokinase catalysing both glucose and fructose explains these results.

The effect of fructose on the system in adult liver gave analogous results (Fig. 6, upper plot) and indicates a similar order for the value of the apparent K_m for fructose. This type of result on adult liver was not expected but must indicate that, under the assay conditions used, fructose phosphorylation by the specific fructokinase (whose K_m is $< 5 \cdot 10^{-4}$ M according to HERS[23]) does not lead to Fru-6-*P* even indirectly. Pooling results obtained from seven liver specimens from foetal, newborn and adult rats, the K_m for fructose of the hexokinase was $(2.4 \pm 0.8) \cdot 10^{-3}$ M. Similar behaviour was exhibited by guinea-pig liver preparations. These results with fructose as substrate are similar to those with other mammalian hexokinases[3].

(c) A non-specific hexokinase is likely to phosphorylate mannose to Man-6-*P* (ref.23). If phosphomannoseisomerase[26] is also present in excess in the liver supernatant preparations, the Man-6-*P* should be converted via Fru-6-*P* and by phosphoglucoseisomerase to Glc-6-*P*. The results obtained with mannose as substrate proved unsatisfactory and this is tentatively assumed to be due to low phosphomannose-

isomerase activity. The indications were that mannose had an effect upon glucose phosphorylation so that mannose may be another substrate for the hexokinase.

Some properties of the enzyme catalysing glucose phosphorylation in adult liver having a high apparent Michaelis constant ($K_{m,1}$)

The foregoing results could be obtained primarily because only one glucose-phosphorylating enzyme is present in the foetal liver, so that the $K_{m,2}$-enzyme can be studied alone. This has not proved possible for the $K_{m,1}$-enzyme so far. This latter enzyme is very labile and attempts to study many of its properties have failed because of this. The following observations were made, therefore, in the presence of the low-K_m enzyme.

DIPIETRO *et al.*[11] showed with adult rat preparations (in which the $K_{m,2}$-enzyme contributed not more than 20–25% of the total activity as judged from present results) that a LINEWEAVER–BURK[20] plot through points obtained with glucose concentrations of $5 \cdot 10^{-3}$ M upwards gave a straight line which, extrapolated, cut the abscissa at points indicating apparent K_m values in different experiments ranging from $1 \cdot 10^{-2}$ M to $4 \cdot 10^{-2}$ M glucose. The theoretical considerations presented earlier show that this interpretation is not permissible when two enzymes are contributing to the total activity, and that the intercept on the abscissa gives a value for $-\frac{V}{V_1 K_{m,1}}$ in the special case where $K_{m,2} \ll K_{m,1}$.

Ignoring, temporarily, these considerations and using the intercept on the abscissa to obtain a value for S, similar variations of this property of the enzyme system of adult rat liver for glucose have been found to those recorded by DIPIETRO *et al.*[11], values varying from $6 \cdot 10^{-3}$ M to $3 \cdot 10^{-2}$ M glucose. For adult guinea-pig liver, the values of S when $1/v = 0$ ranged from $3 \cdot 10^{-3}$ M to $1.5 \cdot 10^{-2}$ M glucose, *i.e.*, a slightly lower range of values compared to the rat. These values varied not only from animal to animal but also with treatment of the tissue preparation. In one experiment, a fresh undialysed preparation of adult rat liver indicated $S = 2.3 \cdot 10^{-2}$ M glucose when $1/v = 0$, whereas after dialysis for 20 h the value fell to $1.2 \cdot 10^{-2}$ M glucose. This change is in the direction predicted from the theory that

$$-\frac{1}{S} = \frac{V_1 + V_2}{V_1 K_{m,1}}$$

when $1/v = 0$, if the total loss of activity at 0.1 M glucose (the observed decrease in this case was 27%) is due essentially to a fall during dialysis in V_1 rather than V_2.

The activities at 0.1 M glucose with adult liver preparations were not proportional to enzyme concentration, especially with guinea-pig liver. This is illustrated in Fig. 7 where the intercepts on the abscissa vary over a range of values of S from $4.2 \cdot 10^{-3}$ M to $7.4 \cdot 10^{-3}$ M for guinea-pig adult liver depending upon the amount of supernatant fraction used in the assay. The four values thus obtained were equated to $\frac{V_1 + V_2}{V_1 K_{m,1}}$. For this purpose, the values for V were evaluated from Fig. 7 and the values for V_2 were obtained from the results at lower glucose concentrations using 40 μl of enzyme preparation (not shown in Fig. 7) and making use of the fact, already demonstrated (Fig. 5), that the activity of the $K_{m,2}$-enzyme is proportional to enzyme

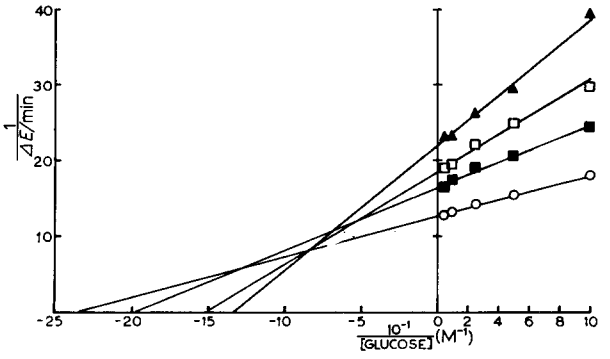

Fig. 7. Plot of reciprocal velocity *versus* reciprocal glucose concentration for glucose-phosphorylating system of adult guinea-pig liver showing the effect of enzyme concentration on the system. The incubations contained various volumes of supernatant fraction, prepared from a 33% homogenate, dialysed 20 h, and glucose concentrations ranging from 0.2 M to $1 \cdot 10^{-2}$ M. Volumes of enzyme preparation used were: ▲—▲, 20 μl; □—□, 25 μl; ■—■, 30 μl; ○—○, 40 μl. For details of incubation system, see text.

concentration. In this way the values of $K_{m,1}$ ranged from $9.9 \cdot 10^{-3}$ M to $14.9 \cdot 10^{-3}$ M. These results are very approximately double those quoted above for S when $1/v = 0$ and this is what may be expected for the guinea-pig liver where V_1 and V_2 are similar in magnitude. The reason for the calculations still leading to a range of values is mainly due to inhibition of the $K_{m,2}$-enzyme by higher glucose concentrations (Fig. 3). As a result, the contribution of the $K_{m,2}$-enzyme to the measured activity is somewhat less than predicted but this cannot be estimated quantitatively from the present results.

A similar type of experiment to that of Fig. 7 was also performed on adult rat liver. The range of values of S when $1/v = 0$ obtained was much narrower. The calculated values of $K_{m,1}$, after allowing for the contribution of V_2 to V, were approx. 20% above those deduced from the intercepts. These two statements correlate respectively with the observations that both the inhibition of the $K_{m,2}$-enzyme at high glucose concentration and the contributions of V_2 to V are much smaller in the rat than in the guinea-pig. The effect of these calculations is to bring the observed values of $K_{m,1}$ for both guinea-pig and rat adult liver, derived from more than ten observations on each species, into the range of $0.8 \cdot 10^{-2}$ M to $3 \cdot 10^{-2}$ M.

A few tests were performed in which various concentrations of fructose, mannose and 2-deoxyglucose were added to preparations from adult livers. The effects were no greater than what could be predicted from their effects upon the $K_{m,2}$-enzyme. The implication is that the $K_{m,1}$-enzyme is probably not effected by the other sugars and may be a specific glucokinase (*i.e.* of type EC 2.7.1.2).

The development of hepatic glucokinase after birth

There must be some stage in the development of the liver during which the $K_{m,1}$-enzyme, a glucokinase, appears. Dialysed preparations from livers of new-born guinea-pigs and rats were made and the standard kinetic analyses performed.

Two typical plots are shown in Fig. 8 for young guinea-pigs. Both plots show

some inhibition of the $K_{m,2}$-enzyme by high glucose concentrations. With the 3-day-old animal the contribution of the $K_{m,1}$-enzyme is not sufficient to bring the value of $1/V$ below the imaginary value of $1/V_2$ that would be obtained by the extrapolation indicated by the dotted line. With the 5-day-old animal, the contribution of V_1 is greater. Other experiments on guinea-pigs may be summarized as follows. One examination of a very late gestational age foetal liver (68 days) gave some indication of a very little $K_{m,1}$-enzyme being present. This was not seen in two

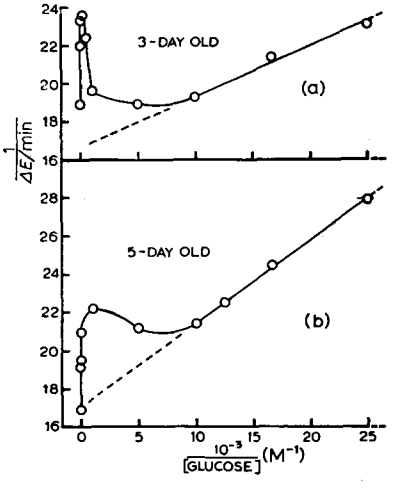

Fig. 8. Plots of reciprocal velocity *versus* reciprocal glucose concentration for glucose-phosphorylating system of livers of new-born guinea-pigs. The plots show only the results for glucose concentrations ranging from 0.1 M to $4 \cdot 10^{-5}$ M, but the straight lines drawn through the points representing glucose concentrations from $1 \cdot 10^{-4}$ M downwards take into account unplotted points ranging down to $1.4 \cdot 10^{-5}$ M glucose. (a) 3-day-old guinea-pig: 100 μl of supernatant fraction from 33% homogenate and dialysed 20 h. (b) 5-day-old guinea-pig: 50 μl of supernatant fraction from 33% homogenate and dialysed 20 h. For details of incubation system, see text.

other tests on late-term foetuses. Tests on a total of 7 new-born guinea-pigs ranging from 1 to 22 days old indicated a gradually increasing contribution of the $K_{m,1}$-enzyme to the total velocity, V, starting within a day or two of birth. Adults levels were recorded about 3 weeks after birth and thereafter. More precise information on the development is difficult to obtain because of the peculiar excess substrate-inhibition effect on the $K_{m,2}$-enzyme. The existence of this inhibitory effect explains the low values for glucose phosphorylation by the new-born guinea-pig liver preparations recorded in Table I. The fall in activity from late-term foetuses to new-born animals may not be real under physiological conditions.

The inhibition effect was always much less marked for rat liver. A series of examinations on young rats ranging from 1 to 30 days was made. No $K_{m,1}$-enzyme could be detected until at least one week after birth. Thereafter increasing amounts were recorded (see Fig. 9 for three typical examples of the $1/v$ *versus* $1/S$ plots obtained). Adult levels of V_1 compared to V_2 were not found until animals about 1 month old were used. Precise information on the point is difficult to obtain because some total

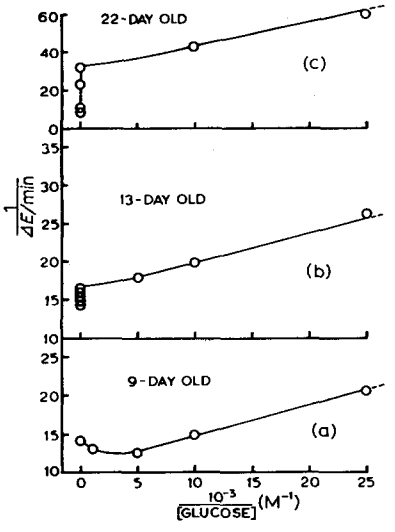

Fig. 9. Plots of reciprocal velocity *versus* reciprocal glucose concentration for glucose-phosphorylating system of livers (pooled samples from several litter mates) of new-born rats. The details are similar to those of Fig. 8. (a) 9-day-old rats: 20 μl of supernatant fraction from 25% homogenate and dialysed 20 h. (b) 13-day-old rats: 100 μl of supernatant fraction from 20% homogenate and dialysed 20 h. (c) 22-day-old rats: 80 μl of supernatant fraction from 25% homogenate and dialysed 20 h.

glucose-phosphorylating activity is always lost during dialysis. This loss is primarily due to inactivation of the $K_{m,1}$-enzyme.

DISCUSSION

This paper appears to be the first time that a physiological system consisting of two enzymes catalysing the same reaction simultaneously has been analysed by such kinetic methods. Had the compound $1/v$ *versus* $1/S$ plots not been complicated by inhibition of the low-K_m enzyme at high substrate concentrations, it might have been possible to use the hepatic glucose-phosphorylation system to distinguish between two differing theoretical treatments[18,19]. An accurate kinetic analysis of this type of system needs to be performed on a mixture of two purified enzymes. An assessment of the physiological implications of the present results, however, necessitates the use of crude tissue preparations. These clearly indicate the presence of two glucose-phosphorylating enzymes in adult liver tissue. The more specific glucokinase develops after birth in both the rat and guinea-pig.

DIXON AND WEBB[18] state that the right-hand side of Eqn. 1 is equal to zero when S equals $-K_{m,1}$ or $-K_{m,2}$, and that the linear portions of the plot will cut the abscissa at $-1/K_{m,1}$ and $1/K_{m,2}$. The two enzymes act independently, however, and at high S values the low-K_m enzyme will be functioning at maximum rate (ignoring substrate inhibition). The theoretical treatment of REINER[19], already outlined, leads to a different set of results which can be calculated from the reciprocal plots. The present results tend to substantiate the validity of the analysis of REINER[19]

and give values for $K_{m,1}$ approximately double those quoted earlier[12] which were based upon the DIXON AND WEBB[18] treatment.

Many physiological functions of liver have been shown to require an elevated blood glucose level[27,1] and liver-slice experiments[28-30] show a high dependence upon glucose concentrations of several processes involving glucose phosphorylation. Those results are given direct enzymic interpretation by the work of DIPIETRO *et al.*[11] and the present study. Hepatic glucokinase has quite different affinity characteristics from other mammalian hexokinases such as that of brain[3,31,32]. The values quoted by DIPIETRO *et al.*[11] for the K_m for hepatic glucose phosphorylation were obtained using undialysed liver extracts and the activity at low glucose concentration was subtracted from the total phosphorylating activity for calculation purposes. Recalculation of their results (Fig. 2 of ref. 11) in the light of the present study leads to an apparent K_m value approximately double that quoted[11]. The hitherto apparently anomalous K_m value of $4 \cdot 10^{-5}$ M glucose for hepatic glucose phosphorylation given by LANGE AND KOHN[23] is rationalized by the demonstration of the second high-affinity enzyme although it is not clear why their methods did not detect the glucokinase.

The apparent K_m values now quoted do not necessarily indicate the affinity of the enzymes for the substrate but the glucose requirements for saturation of the two enzymes are almost certainly quite different. The presence of an enzyme with an apparent affinity $K_{m,1}$ in the physiological range of glucose concentrations offers a basis for enzymic control of the first stage of hepatic glucose utilization. The $K_{m,2}$-enzyme may represent the hexokinase of tissue cells forming a minor component of the whole liver, *e.g.* cells of the reticulo–endothelial system. However, in view of it being the only glucose-phosphorylating enzyme in foetal liver, it is more likely to be concerned with the endogenous metabolism of the liver itself. Because the foetal liver lacks glucokinase and the placenta is permeable to glucose in all species examined[34], the foetus may depend upon maternal systems for control of its carbohydrate metabolism. The dramatic appearance of the more specific glucokinase after birth follows the development of glucose 6-phosphatase in the late pre-natal stage[35,36,17] and a rapid fall in liver glycogen after parturition[37] associated with high immediately post-natal hepatic glucose 6-phosphatase activity[35,16].

Before the glucokinase develops, the new-born animal may be unable to control its blood sugar level as well as the more mature animal. Hypoglycaemia has been reported to be a frequent occurrence in the new-born human being[38]. Glycolysis, the hexose monophosphate pathway, glucose oxidation and glycogen biosynthesis all depend upon glucose phosphorylation as the first enzymic step and normal functioning presumably follows glucokinase development.

The present studies clarify the nature of the glucose-phosphorylation systems of foetal, new-born and adult hepatic tissue. Some further properties of the enzymes involved and some of the factors affecting their activity are under further investigation.

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