A Variant of Rabbit Phosphoglucose Isomerase

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An autosomally inherited variant of the rabbit enzyme phosphoglucose isomerase that differs both electrophoretically and kinetically from the usual (wild-type) rabbit enzyme has been investigated. Animals homozygous for this character have an isomerase with considerably decreased activity (in the erythrocytes), an increased K_m for fructose 6-phosphate, an increased K_i for 6-phosphogluconate and a decreased stability towards heat and urea. The variant enzyme has been demonstrated in erythrocytes, leucocytes and tissues from the affected rabbits. The kinetic evidence suggests that the mutation present in the variant enzyme affects a histidine residue.

The enzyme phosphoglucose isomerase (EC 5.3.1.9), first described by Lohmann (1933), catalyses the reversible interconversion of glucose 6-phosphate and fructose 6-phosphate. It is widely distributed in animals and plants. Purified and partially purified preparations have been reported from a variety of sources, including rat kidney (Wick, Drury, Nakada & Wolfe, 1957), baker's yeast (Klotzch & Bergmeyer, 1960), human erythrocytes (Tsuboi, Estrada & Hudson, 1958; Smith & McCants, 1969), rabbit muscle (Noltmann, 1964) and human skeletal muscle (Carter & Yoshida, 1969).

The first electrophoretic variant of phosphoglucose isomerase to be discovered was from mouse erythrocytes (Carter & Parr, 1967), and subsequently human variants were reported from this laboratory (Fitch, Parr & Welch, 1968) and from elsewhere (Detter *et al.* 1968). Deficiencies of the human erythrocyte enzyme have also been found (Baughan *et al.* 1968; Paglia, Holland, Baughan & Valentine, 1969; S. G. Welch & C. W. Parr, unpublished work), sometimes accompanied by severe haemolytic anaemia.

The present paper is concerned with an inherited variant of rabbit phosphoglucose isomerase that has been shown to possess both kinetic and electrophoretic abnormalities.

MATERIALS AND METHODS

Purified yeast glucose 6-phosphate dehydrogenase was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. The tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Disodium glucose 6-phosphate, disodium fructose 6-phosphate, trisodium 6-phosphogluconate,

NADP⁺ and phenazine methosulphate were from Wessex Biochemicals Ltd., Bournemouth, Hants., U.K. The hydrolysed starch was prepared by Connaught Medical Research Laboratories, Toronto, Ont., Canada, and the dextran (Dextraven 150) was from Fisons Pharmaceuticals Ltd., Loughborough, Leics., U.K. All other reagents were purchased from British Drug Houses Ltd., Poole, Dorset, U.K., or Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Blood samples from rabbits were collected from a peripheral ear vein, with EDTA as anticoagulant.

The erythrocytes were washed twice in 20 vol. of 0.15 M-NaCl. For starch-gel electrophoresis the cells from 0.1 ml of whole blood were lysed by the addition of 0.2 ml of water, with mixing. After 5 min the lysate was centrifuged at 2000g for 10 min, and the clear supernatant used. For erythrocyte enzyme assays the same volume of blood was lysed by the addition of 9.9 ml of water.

Leucocytes were separated from 5 ml of blood, either by sedimentation in dextran (Silber, Gabrio, Huennekens & Albrecht, 1962), or more simply by removal of the leucocyte coat from the centrifuged cells with a capillary pipette. After two washings with 0.15 M-NaCl, residual erythrocytes were lysed by adding water (3.0 ml) to the pellet. After 30 s, 0.6 M-KCl (1.0 ml) was added to restore the molarity to 0.15. The solution was centrifuged at 2000g for 10 min, and the leucocyte pellet (together with platelets) was rewashed in 0.15 M-NaCl. The cells were then suspended in 2 ml of 0.1 M-sodium phosphate buffer, pH 7.0, and lysed by rapid freezing and thawing twice. The extract was centrifuged to give a clear supernatant.

Horizontal starch-gel electrophoresis was carried out with 0.01 M-sodium phosphate buffer in the gel and 0.2 M-sodium phosphate in the bridge compartments, both at pH 6.5. The samples (about 50 μ l) were applied to inserts of Whatman no. 17 filter paper (13 mm $\times$ 5 mm) and the electrophoresis was carried out with a potential difference of about 20 V/cm of gel length for about 5 h. The gels were kept from overheating by the use of metal cooling plates through which water at 12°C was circulated. After electrophoresis the gels were sliced horizontally and the cut surface was stained for phosphoglucose isomerase.

Throughout this work the isomerase activity has been measured by the 'backward' reaction, in which fructose 6-phosphate is converted into glucose 6-phosphate, and this is oxidized by an excess of added purified glucose 6-phosphate dehydrogenase, with the concomitant reduction of added NADP⁺. NADPH was measured either spectrophotometrically at 340nm or, for the histochemical detection of the enzyme, was coupled with phenazine methosulphate to reduce a tetrazolium salt. Details are as follows.

For the histochemical staining, agar (100mg), tris-HCl buffer (8ml, 0.3M), pH8.0, and MgCl₂ (2ml, 0.1M) were mixed in a boiling tube, heated just to boiling, and placed in a water bath to cool to about 50°C. At this temperature a mixture containing the MTT (2mg), phenazine methosulphate (1mg), fructose 6-phosphate (6mg), NADP⁺ (3mg) and purified glucose 6-phosphate dehydrogenase (5 units) in a total volume of about 3ml was added, mixed, and the mixture poured over the gel. The gel was allowed to develop in the dark at 37°C for about 30min and then photographed.

Spectrophotometric assays for the enzyme were carried out by recording the rate of increase of E_{340} in an assay mixture containing (final concns.): tris-HCl buffer (100mM), pH8.0, MgCl₂ (30mM), NADP⁺ (0.2mM), disodium fructose 6-phosphate (1.5mM) and purified glucose 6-phosphate dehydrogenase (0.33 unit/ml). An amount of enzyme giving a rate of increase of E_{340} of about 0.1 in 5min was added (0.2ml of a 1:100 haemolysate was suitable). The final volume was 3.0ml. The activity of the erythrocyte enzyme was expressed as units (μ mol of NADPH formed/min) per g of haemoglobin at 30°C, assuming ϵ_{340} 6.22×10^3 for NADPH (Horecker & Kornberg, 1948). For measurement of haemoglobin the extinctions of the test cells were measured at 540nm against a water blank, its having been ascertained that no component of the reaction mixture other than the haemoglobin gave any appreciable extinction at this wavelength, and calculations were made on the basis that haemoglobin has $E_{1\%}^{1\text{cm}}$ 8.58 at 540nm (Donaldson, Sisson, King, Wootton & Macfarlane, 1951).

Michaelis constants were measured by determining the initial reaction velocities at concentrations of substrate ranging from 0.01mM to 1.0mM and making Lineweaver & Burk (1934) plots of the results. Inhibitor constants (K_i) for the competitive inhibition of the enzyme by 6-phosphogluconate were determined by the method of Dixon (1953b), with 6-phosphogluconate concentrations ranging from 0.01mM to 1.0mM, at the two substrate concentrations of 0.066mM and 0.33mM.

The effect of pH on K_m , K_i and V_{max} was investigated by using tris-histidine buffers (final concn. of each component 0.15M) adjusted to a range of pH values between 6.0 and 10.0 with HCl or NaOH. All determinations were made in duplicate. Concentrations of fructose 6-phosphate ranging from 0.06mM to 2.64mM were used for the determinations of K_m and V_{max} , and 6-phosphogluconate concentrations ranging from 0.01mM to 1.0mM were employed, at two fixed fructose 6-phosphate concentrations of 0.066mM and 0.33mM, for the determinations of K_i .

Urea inhibition experiments were carried out by determining the percentage residual activity after incubating the enzyme in the presence of 3.0M-urea (twice re-

crystallized from methanol) at 30°C for 10min. Heat-inactivation experiments were performed by incubation of samples of enzyme at 47°C, in 0.3M-tris-HCl buffer, pH9.0, over periods of 1-60min.

RESULTS AND DISCUSSION

Electrophoretic variants of rabbit phosphoglucose isomerase. Fig. 1 shows the zymogram normally obtained after starch-gel electrophoresis of rabbit haemolysates and leucocyte extracts and specific staining for the enzyme. There is a single strongly staining major band, and one or more weaker components anodic to the major band.

During a survey of rabbits for phosphoglucose isomerase, a female New Zealand White rabbit (designated B9) was found, one of the breeding stock of a smallholder in Dorset, in which the

Fig. 1. Starch-gel electrophoresis at pH6.5 of rabbit erythrocyte (1) and leucocyte (2) lysates, showing the usual (wild-type) phosphoglucose isomerase zymogram.

Fig. 2. Starch-gel electrophoresis at pH6.5 of haemolysates from rabbits of wild-type phosphoglucose isomerase phenotype (1, 2, 3, 5 and 6) and from the B9 variant (4).

major enzyme band migrated further towards the cathode than was the case with the other rabbits (Fig. 2).

The anodically sited weaker components seen in normal rabbit haemolysates (and sometimes, faintly, in the B9 animal) have yet to be fully explained. However, it has been observed that the second band and part of the third band (the first band being the major band), both of which develop in intensity with increasing age of the blood, can be apparently converted back into the major band by the addition of 2-mercaptoethanol to the lysate. Similar reversible aging effects have been observed by others (Yoshida & Carter, 1969).

The six progeny resulting from a cross between a male rabbit with the usual or wild-type phosphoglucose isomerase and the female B9 all gave identical erythrocyte zymograms in which the major band was a broad one extending over the whole of the region occupied by the two (different) major parental bands (one from each parent). Even after electrophoresis for 8 h at 24 V/cm there was insufficient resolution to show whether the wide band was composed of just the two parental bands alongside each other, or whether there was also an additional band of intermediate mobility, as was found in mice with a heterozygous phosphoglucose isomerase (Carter & Parr, 1967) and attributed to an enzyme structure comprising two, or multiples of two, randomly assorting subunits. However, when leucocyte extracts were submitted to electrophoresis at 24 V/cm of gel length for 20 h, sufficient separation of the electrophoretic components was obtained for a triplet pattern to be clearly discernible (Fig. 3).

The lack of coincidence of the major bands in the normal and B9 animals, the demonstration of a triplet pattern in the progeny from a B9 × wild-type mating, and the fact that all six of the progeny showed identical electrophoretic patterns, which were different from those of either of the parents, led us at this stage to assume, as a working hypothesis, that the B9 female was homozygous and

Fig. 3. Starch-gel electrophoresis at pH 6.5 of rabbit leucocyte phosphoglucose isomerase from the wild-type (3), from the B9 variant (1) and from one of the progeny from a mating of these two (2). The offspring clearly shows three bands of enzyme activity in the region of the major cathodal bands. This result required a long period of electrophoresis (20 h) at a relatively high voltage (24 V/cm of gel length). Because of the distance the major band has migrated, the origin is not shown on the photograph: it is about an equal distance further back, towards the anode.

Table 1. *Activity of and effect of two types of stress on three types of rabbit phosphoglucose isomerase*

This table demonstrates how the heterozygous progeny, which cannot be differentiated from the wild-type rabbits by phosphoglucose isomerase (PGI) assay, can nevertheless be detected by their greater lability to heat and urea. Results in columns 2 and 3 are means of duplicate determinations; those in columns 4 and 5 are means $\pm$ s.d. of 12 determinations.

	Erythrocyte PGI activity (units/g of haemoglobin at 30°C)	Percentage residual PGI activity		
		Heat treatment (10 min at 50°C)	Heat treatment (10 min at 47°C)	Urea treatment (3.0M for 10 min)
Wild-type (male)	125.2	66.6	87.3 $\pm$ 4.2	72.8 $\pm$ 4.4
Progeny 1 (male)	104.9	31.3	—	—
Progeny 2 (female)	116.6	31.5	—	—
Progeny 3 (male)	120.0	33.6	55.4 $\pm$ 2.2	56.2 $\pm$ 2.3
Progeny 4 (female)	118.0	30.6	—	—
Progeny 5 (female)	127.0	33.4	—	—
Progeny 6 (female)	121.0	34.1	—	—
B9 (female)	49.6	3.5	5.3 $\pm$ 2.8	30.1 $\pm$ 1.8

the progeny were heterozygous for an allelic form of the normally occurring phosphoglucose isomerase in the rabbit.

Activity, heat stability and urea stability. In addition to the difference in electrophoretic mobility, the erythrocyte enzyme from rabbit B9 was distinguishable from the normal enzyme in that it possessed less than half the normal activity (Table 1). The presumably heterozygous progeny, however, all possessed erythrocyte phosphoglucose isomerase activities that were normal, or only slightly less than normal, and could not on this account be distinguished from wild-type rabbits (Table 1). A similar difficulty in distinguishing heterozygotes from normals was encountered by Kalow & Genest (1957) in their classical work on the human plasma cholinesterases, but they were able to achieve reliable differentiation by measuring the percentage inhibition brought about by the competitive inhibitor dibucaine under set standard assay conditions. The use of a competitive inhibitor of the isomerase (6-phosphogluconate) was therefore investigated, but appreciably different inhibitory effects in the three isomerase phenotypes were not found. Experiments were tried in which the three types were subjected to stress, either by incubation at an elevated temperature or by exposure to high concentrations of urea. This technique has been successfully applied for the differentiation of variants of human phosphogluconate dehydrogenase (Parr & Parr, 1965; Carter, Gould, Parr & Walter, 1966). The heat-inactivation experiments were carried out at temperatures ranging from 45°C to 55°C, for periods of 1–60 min, and at pH values 6.0–9.5. Optimum differentiation was obtained by

incubating haemolysates at 47°C and pH 9.0 for 10 min, and these conditions were adopted as standard. Similarly, urea concentrations of 1.0–5.0 M were tried, for various periods of time, and a concentration of 3.0 M for a 10 min period was found to be the most effective for purposes of differentiation. Table 1 summarizes the results of these two types of stress on the three rabbit erythrocyte phosphoglucose isomerase phenotypes. Selective denaturation by heat or urea gave a clear differentiation of the three phenotypes that the activity determinations alone could not do, and these two methods were subsequently adopted for characterizing the offspring of further rabbit breeding experiments, as well as for screening purposes.

Before the successful resolution of the electrophoretic phosphoglucose isomerase pattern of leucocyte extracts of heterozygotes into three components, some evidence for the presence of three isoenzymic components in this phenotype was adduced from the following heat-stability experiment.

Haemolysates of the three rabbit isomerase phenotypes, together with a mixture of haemolysates from the wild-type and the B9 variant, were incubated at 47°C in 0.3 M-tris buffer, pH 9.0. Samples were removed at intervals over 60 min and the percentage residual activity was determined by the standard assay. Fig. 4 shows the result of such an experiment, with residual activity plotted against time. The mixture gave a sharp biphasic decay curve, whereas the heterozygote gave a curve that followed a triphasic pattern.

Effect of pH on K_m , K_i and V_{max} . The K_m for phosphoglucose isomerase of the B9 variant (with fructose 6-phosphate as substrate) was greater than the K_m for the normal rabbit enzyme by a factor of about three (Table 2).

Fig. 4. Residual activities, after various periods of time, of erythrocyte phosphoglucose isomerase from the three rabbit phenotypes. ○, Wild-type; ▲, B9 type; △, offspring from a wild-type × B9 cross; ●, mixture of B9 and wild-type.

Table 2. K_m and K_i values for wild-type and B9 rabbit phosphoglucose isomerase

The various phosphoglucose isomerase rate determinations required for the calculation of the K_m and K_i values were made under the conditions of the standard assay, except that a range of substrate concentrations (and a range of inhibitor concentrations for K_i) were employed (see the Materials and Methods section). The K_i determinations were carried out at pH 7.0 instead of pH 8.0, because (Geewater *et al.* 1965) the inhibitory effect of 6-phosphogluconate is greater at lower pH values.

	K_m fructose 6-phosphate (pH 8.0)	K_i 6-phosphogluconate (pH 7.0)
Wild-type	2.08×10^{-4} M	0.75×10^{-4} M
B9	7.40×10^{-4} M	1.95×10^{-4} M

Parr (1957) reported that 6-phosphogluconate is a competitive inhibitor of phosphoglucose isomerase. Table 2 also gives the K_i values (for 6-phosphogluconate) for the wild-type and B9 enzymes. Again, the value found for the B9 enzyme is greater than for the wild-type enzyme by a factor of about three.

Gewater, Hanshaw, Martin & Parr (1965) and Salas, Viñuela & Sols (1965) observed that the effectiveness of 6-phosphogluconate as a competitive inhibitor of phosphoglucose isomerase diminished as the pH of the reaction mixture rose.

The effect of pH on the kinetic parameters of enzyme-catalysed reactions was first examined by Walker & Schmidt (1944) for the enzyme histidine ammonia-lyase (EC 4.3.1.3). These workers described the general manner in which the ionization of the three components of their system would be expected to affect the K_m . Dixon (1953a) gave a more general treatment, with a number of rules for interpreting the data that apply equally well to K_m and K_i values. Briefly, an inflexion on a plot of K_m against pH or of K_i against pH is taken to indicate the participation in enzyme catalysis of a component with a pK value corresponding to the pH of the inflexion.

Dyson & Noltmann (1968), who have examined the effects of pH on the kinetic parameters of purified rabbit muscle phosphoglucose isomerase, chose to use plots of $\log(V_{\max}/K_m)$ versus pH and (using 6-phosphogluconate as competitive inhibitor) of $\log(1/K_i)$ (i.e. pK_i) versus pH, since such plots showed clearer inflexions than the simpler plots (K_m against pH and K_i against pH) discussed above. They obtained inflexions near pH 7 for both plots, which they interpreted as indicating that possibly the non-protonated nitrogen atom of the imidazole group of a histidine residue participates in the attachment of the straight-chain enediol form of the substrate (either fructose 6-phosphate or glucose 6-phosphate), or alternatively of the straight-chain inhibitor 6-phosphogluconate. An additional inflexion on the plot of $\log(V_{\max}/K_m)$ versus pH occurred near pH 9, but there was no corresponding inflexion in the plot of pK_i versus pH, and this was thought to indicate the participation of a lysine residue in the binding and opening of the ring forms of fructose 6-phosphate and glucose 6-phosphate.

On the basis of these proposals a mutation affecting the lysine residue might be expected to possess an altered and probably decreased affinity for substrate (i.e. an increased K_m) but an unaltered K_i for the competitive inhibitor 6-phosphogluconate. On the other hand, if an amino acid change had occurred at or around the histidine residue at the active site, such that the straight-chain form of the substrate as well as of the competitive inhibitor was

unable to approach this site so readily, then one would expect to find that both K_m and K_i were increased. The latter alternative appears to be the case for the B9 mutant, since (see Table 2) both the K_m and K_i are elevated about threefold.

Experiments similar to those of Dyson & Noltmann (1968), to determine the effects of pH on K_m , K_i and $V_{\max}$, were carried out in the present study on both the normal and the B9 variant form of rabbit phosphoglucose isomerase. With respect to the normal enzyme the results are in general agreement with those of Dyson & Noltmann (1968). In the discussion that follows the behaviour of the

Fig. 5. Plot of $\log(V_{\max}/K_m)$ values as a function of pH for the reverse reaction of erythrocyte phosphoglucose isomerase. $\circ$, Wild-type; $\bullet$, B9 type.

Fig. 6. Plot of pK_i (for 6-phosphogluconate) for the reverse reaction of erythrocyte phosphoglucose isomerase reaction with rabbit erythrocyte enzyme. $\circ$, Wild-type; $\bullet$, B9 type.

Table 3. *Progeny from the mating of various rabbit phosphoglucose isomerase phenotypes*

Type of mating	Phosphoglucose isomerase phenotype of offspring		
	Wild-type	Intermediate character (intermediate in stability to heat and urea)	B9 phenotype
Wild-type × B9	0	6	0
Intermediate × intermediate	5	9	6

normal enzyme is compared with that of the B9 variant.

Fig. 5 gives plots of $\log(V_{\max.}/K_m)$ against pH for each enzyme type. Each plot shows two inflexion points, the first near pH 7, and the second about 2 pH units higher. There is very good agreement between the second inflexion points for each enzyme (both at pH 8.9), but there is a difference of about 0.5 pH unit between the normal and the B9-type enzymes with respect to their first inflexion points (which occur at pH 7.3 and pH 6.8 respectively).

Fig. 6, in which pK_i is plotted against pH for each enzyme type, shows that both isomerase types gave single inflexions: that for the B9 variant (at pH 6.5) was about 0.6 pH unit lower than that for the wild-type enzyme. Dyson & Noltmann (1968) also found a single inflexion on their plot of pK_i versus pH, and interpreted this as indicating only a single binding site for the straight-chain competitive inhibitor.

Other tissues. The mutant form of phosphoglucose isomerase, present in the erythrocytes of rabbit B9, was also found in all other rabbit tissues examined.

CONCLUSIONS

The electrophoretic results on wild-type rabbits, on rabbit B9 and on the progeny of a wild-type × B9 mating suggested that the wild-type and the B9 variant were homozygous, and that the progeny were heterozygous, for an autosomal phosphoglucose isomerase gene locus. A more extensive breeding programme (summarized in Table 3) has confirmed these conclusions.

Both types of enzyme appear to be at least dimeric in structure, and heterozygotes possess at least three enzyme species, one apparently being a hybrid enzyme containing a subunit from each allele.

The value of differential inactivation techniques is demonstrated: the differential stabilities to incubation and to exposure to urea were the only practical means of screening for rabbits with the heterozygous phenotype.

When plots of $\log(V_{\max.}/K_m)$ against pH were

made both normal and variant forms of rabbit phosphoglucose isomerase showed identical second ionization points at pH 8.9, attributed by Dyson & Noltmann (1968) to the presence of a lysine residue. However, the B9 variant was found to possess a first ionization point, attributed by Dyson & Noltmann (1968) to a histidine residue, that was about 0.6 pH unit lower than the corresponding ionization point for the normal enzyme in plots both of $\log(V_{\max.}/K_m)$ against pH and of pK_i against pH. This constitutes further evidence that the B9 variant has a mutation affecting a histidine residue.

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