Biochem. J. (1967) **102**, 753

Inhibition of Glucose Phosphate Isomerase by Metabolic Intermediates of Fructose

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(*Received* 23 *August* 1966)

1. Purified rabbit-muscle and -liver glucose phosphate isomerase, free of contaminating enzyme activities that could interfere with the assay procedures, were tested for inhibition by fructose, fructose 1-phosphate and fructose 1,6-diphosphate. 2. Fructose 1-phosphate and fructose 1,6-diphosphate are both competitive with fructose 6-phosphate in the enzymic reaction, the apparent K_i values being $1{\cdot}37 \times 10^{-3}$–$1{\cdot}67 \times 10^{-3}$M for fructose 1-phosphate and $7{\cdot}2 \times 10^{-3}$–$7{\cdot}9 \times 10^{-3}$M for fructose 1,6-diphosphate; fructose and inorganic phosphate were without effect. 3. The apparent K_m values for both liver and muscle enzymes at pH 7·4 and 30° were $1{\cdot}11 \times 10^{-4}$–$1{\cdot}29 \times 10^{-4}$M for fructose 6-phosphate, determined under the conditions in this paper. 4. In the reverse reaction, fructose, fructose 1-phosphate and fructose 1,6-diphosphate did not significantly inhibit the conversion of glucose 6-phosphate into fructose 6-phosphate. 5. The apparent K_m values for glucose 6-phosphate were in the range $5{\cdot}6 \times 10^{-4}$–$8{\cdot}5 \times 10^{-4}$M. 6. The competitive inhibition of hepatic glucose phosphate isomerase by fructose 1-phosphate is discussed in relation to the mechanism of fructose-induced hypoglycaemia in hereditary fructose intolerance.

Hereditary fructose intolerance in man is characterized by a deficiency of fructose 1-phosphate aldolase (EC 4.1.2.7) in the liver (Froesch, Prader, Wolf & Labhart, 1959; Froesch, Wolf & Baitsch, 1963). In this condition fructose 1-phosphate probably accumulates in the tissue and inhibitory effects of this metabolite have been reported on rabbit-muscle aldolase (EC 4.1.2.7) (Froesch *et al.* 1959) and on phosphoglucomutase (EC 2.7.5.1) (Sidbury, 1959). It was suggested that the inhibition of phosphoglucomutase may explain the severe hypoglycaemia that follows the administration of fructose to hereditary fructose-intolerant patients. However, the fructose-induced hypoglycaemia is relieved by intravenous infusion of galactose (Cornblath, Rosenthal, Reisner, Wybergt & Crane, 1963), indicating that neither phosphoglucomutase nor glucose 6-phosphatase (EC 3.1.3.9) is inhibited *in vivo*. Unpublished experiments by the present authors showed that phosphoglucomutase and glucose 6-phosphatase showed only slight inhibition by excessive concentrations of fructose 1-phosphate *in vitro*; furthermore, similar concentrations of inorganic phosphate were even more inhibitory.

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Glucose phosphate isomerase (D-glucose 6-phosphate ketol-isomerase; EC 5.3.1.9) catalyses the equilibrium between glucose 6-phosphate and fructose 6-phosphate (Lohmann, 1933), and is the only enzyme in the sequence of reactions in gluconeogenesis with a ketohexose monophosphate as substrate. In view of the structural similarity of fructose 1-phosphate and fructose 6-phosphate, experiments were designed to test fructose 1-phosphate as an inhibitor of glucose phosphate isomerase.

MATERIALS AND METHODS

Chemicals

Glucose 6-phosphate (sodium salt), fructose 1,6-diphosphate (sodium salt), fructose 1-phosphate (dicyclohexylammonium salt), phosphoenolpyruvate (tricyclohexylammonium salt), ATP (monosodium salt), NADP (sodium salt) and $NADH_2$ (sodium salt) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Fructose was obtained from British Drug Houses Ltd., Poole, Dorset. Fructose 6-phosphate (barium salt) containing approx. 5% of glucose 6-phosphate was obtained from Sigma Chemical Co. and purified by chromatography on Dowex 1 (X8) resin (borate form) as described by Diedrich & Anderson (1961). Glucose 6-phosphate was assayed enzymically in the eluent with glucose 6-phosphate dehydrogenase free of glucose phosphate isomerase, and

fructose 6-phosphate was assayed with glucose phosphate isomerase and glucose 6-phosphate dehydrogenase. Fructose 6-phosphate assayed as 42% of the weight of the anhydrous barium salt, and glucose 6-phosphate as 0·11% on the same basis.

Enzymes

Crystalline preparations of rabbit-muscle phosphofructokinase (EC 2.7.1.11), yeast glucose 6-phosphate dehydrogenase (EC 1.1.1.49), rabbit-muscle triose phosphate isomerase (EC 5.3.1.1) and rabbit-muscle α-glycerophosphate dehydrogenase (EC 1.1.1.8) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Crystalline rabbit-muscle pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) were obtained from Sigma Chemical Co.

Rabbit-muscle glucose phosphate isomerase was prepared by the procedure of Noltmann (1964) to fraction VII; crystallization was not attempted. The preparation was stored at 2°; after 4 weeks the specific activity was 228 i.u./mg. of protein.

Rabbit-liver glucose phosphate isomerase was prepared essentially by the method of Noltmann (1964), with the following modifications. Frozen rabbit liver (70g.) was homogenized with 210ml. of 0·01M-KCl in a chilled Waring Blendor at full speed for 2min. The homogenate was centrifuged at 25000*g* for 2hr. at 0°. The precipitate was discarded. The supernatant was treated through the zinc acetate precipitation and ammonium sulphate fractionation as described by Noltmann (1964). Ethanol fractionation according to Noltmann (1964) resulted in the precipitation of 90% of the enzyme activity in a 0–34% (v/v) fraction. Accordingly, this fraction was dissolved in 0·05M-magnesium acetate and diluted to 7mg. of protein/ml. with the same solvent. Bentonite was added (7·5mg./mg. of protein) and, after 10min. stirring, removed by centrifugation at 15000*g* at 0° for 20min. The supernatant was stored at 2°. The procedure resulted only in eightfold purification but aldolase (EC 4.1.2.7) could not be detected. The final specific activity of the enzyme was 2·5 i.u./mg. of protein.

Neither of the preparations of glucose phosphate isomerase had detectable activities of fructose 1-phosphate or fructose 1,6-diphosphate aldolase, fructose 1,6-diphosphatase (EC 3.1.3.11), fructokinase (EC 2.7.1.4), ketohexokinase (EC 2.7.1.3), glucose 6-phosphatase and 6-phosphogluconate dehydrogenase (EC 1.1.1.44), when assayed by the procedures described below.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin (Sigma Chemical Co.) as a standard.

Assay of enzymes

Glucose phosphate isomerase. Method A. Reaction cuvettes contained 1·0mM-fructose 6-phosphate, 1·0mM-NADP, 5mM-$MgSO_4$, 100mM-tris buffer, adjusted to pH7·4 with N-HCl, and 1 i.u. of glucose 6-phosphate dehydrogenase (free of glucose phosphate-isomerase activity) in a volume of 0·95ml. After preincubation at 30° for 10min., the reaction was initiated by addition of 0·05ml. of suitably diluted glucose phosphate isomerase. The rate of change of extinction at 340mμ in a 1cm. micro-cuvette was measured in a Beckman DU Spectrophotometer maintained at 30$\pm$0·5°. One unit of glucose phosphate-isomerase activity was defined as the amount that produces 1μmole of glucose 6-phosphate/min., i.e. a change in extinction at 340mμ of 6·22/min. Essentially, this procedure was also used to estimate glucose 6-phosphate (without glucose phosphate isomerase) and fructose 6-phosphate (with glucose phosphate isomerase).

Method B. Reaction cuvettes each contained 1·0mM-glucose 6-phosphate, 0·3mM-$NADH_2$, 0·5mM-phosphoenolpyruvate, 0·5mM-ATP (adjusted to pH7·4 with 0·1N-KOH before use), 5·0mM-$MgSO_4$, 10mM-2-mercaptoethanol, 50mM-tris buffer, pH7·4 (adjusted with N-HCl), 1·0 i.u. of phosphofructokinase, 1·0i.u. of pyruvate kinase and 2·0 i.u. of lactate dehydrogenase. The final volume in a 1cm. light-path silica micro-cuvette was 0·95ml. Cuvettes were preincubated at 30° for 10min., then placed in the cell compartment of a Beckman DU Spectrophotometer at 30$\pm$0·5°. A Gilford Instruments Multiple-Sample Absorbance Recorder was used to record the extinction decrease at 340mμ. Reaction was initiated by addition of 0·05ml. of glucose phosphate isomerase.

None of the indicator enzymes used in the assays A and B described above had detectable glucose phosphate-isomerase activity. They were also assayed for the enzyme activities described below with negative results.

Aldolase. This was assayed by the method of Leuthardt & Wolf (1955) as modified by Dahlquist & Crane (1964).

Fructose 1,6-diphosphatase. This was assayed by the method of Taketa & Pogell (1963).

Fructose phosphorylation. This was assayed by ADP production from ATP and fructose in the assay B described above except that glucose 6-phosphate was replaced by 10mM-fructose and phosphofructokinase was omitted from the reaction mixture. Blanks contained no fructose and showed no activity of adenosine triphosphatase.

Glucose 6-phosphate dehydrogenase. This was assayed as described by Ballard & Oliver (1964).

Glucose-6-phosphatase. This was assayed by the method of Schull, Ashmore & Mayer (1956); the resulting inorganic phosphate was estimated by the method of Taussky & Shorr (1953).

6-Phosphogluconate dehydrogenase. This was assayed by the method of Horecker & Smyrniotis (1955).

The concentrations of stock solutions of glucose 6-phosphate and fructose 6-phosphate used in the kinetic experiments were determined by complete oxidation of diluted samples with excess of glucose 6-phosphate dehydrogenase alone and in combination with excess of glucose phosphate isomerase respectively. The total extinction change at 340mμ was used to calculate the concentrations.

Inhibition tests

The effects of fructose, fructose 1,6-diphosphate, fructose 1-phosphate and cyclohexylammonium phosphate on the initial rate of both forward and back reactions catalysed by glucose phosphate isomerase were determined in preliminary experiments.

Substances found to be inhibitory were then tested in kinetic experiments to determine the nature of the inhibition. The results of kinetic experiments were plotted by the method of Lineweaver & Burk (1934) and the Michaelis constants and inhibitor constants determined graphically by standard procedures (Dixon & Webb, 1958).

Table 1. *Effect of fructose and intermediates on the rate of glucose 6-phosphate formation by glucose phosphate isomerase*

Assay method A was used as described in the Materials and Methods section, except for the concentrations of substrate and inhibitors, which are given below. The results represent the means of at least three separate experiments. Each rate determination was carried out in duplicate.

Inhibitor	Concn. of fructose 6-phosphate (mM)	Molar ratio inhibitor/fructose 6-phosphate	Inhibition (%) (liver enzyme)	Inhibition (%) (muscle enzyme)
Fructose (100mM)	1·00	100	0	1
	0·10	1000	2	1
Fructose 1,6-diphosphate (10mM)	1·00	10	25	22
	0·10	100	49	48
Fructose 1-phosphate (10mM)	1·00	10	40	40
	0·10	100	74	78
Cyclohexylammonium phosphate, pH 7·4 (20mM)	1·00	20	3	3
	0·10	200	4	6

RESULTS

In the preliminary experiments, significant inhibition of the glucose phosphate isomerase conversion of fructose 6-phosphate into glucose 6-phosphate was observed in the presence of fructose 1-phosphate and fructose 1,6-diphosphate. Similar concentrations of cyclohexylammonium phosphate had no significant effect (see Table 1). This latter finding rules out an 'anion effect' as the cause of inhibition. Fructose gave no significant inhibition.

The Lineweaver–Burk plots (Figs. 1 and 2) show clear evidence of competitive inhibition. Both fructose 1-phosphate and fructose 1,6-diphosphate brought about a change in the slope of the lines, but were without effect on the intercepts on the reciprocal velocity axis. The values of K_m and K_i are given in Table 3.

Table 2 shows that fructose and its phosphorylated intermediates do not significantly inhibit the conversion of glucose 6-phosphate into fructose 6-phosphate by either the muscle or liver enzyme. The K_m values for glucose 6-phosphate were $5{\cdot}5 \times 10^{-4}$–$5{\cdot}8 \times 10^{-4}$M for the liver enzyme and 8×10^{-4}M for muscle glucose phosphate isomerase.

Glucose 6-phosphate dehydrogenase (0·01i.u.) assayed with 0·01mM- and 0·10mM-glucose 6-phosphate and in the presence of 10mM-fructose 1-phosphate was not inhibited significantly.

DISCUSSION

The apparent Michaelis constants of glucose phosphate isomerase for fructose 6-phosphate and glucose 6-phosphate reported in this paper are higher than those of Kahana, Lowry, Schulz, Passonneau & Crawford (1960), but are of the same order as those reported for fructose 6-phosphate by Grazi, de Flora & Pontremoli (1960) and Hines & Wolf (1963). Although differences in assay procedures and conditions may account for the differences it should be noted that the data of Kahana *et al.* (1960) are computed values corrected for (*a*) a substantial contamination of fructose 6-phosphate with glucose 6-phosphate (8–15%) and (*b*) apparent inhibition of glucose phosphate isomerase by 6-phosphogluconate that would accumulate in the assay system. It is noteworthy that the Sigma Chemical Co. (1966) have reported contamination of synthetic 6-phosphogluconate with 'potent inhibitors' that may also be inhibitory to the isomerase. The K_i values for synthetic 6-phosphogluconate used by Kahana *et al.* (1960) to correct their values may thus be substantially incorrect.

Inhibition of glucose phosphate isomerase by inorganic phosphate, as reported by Kahana *et al.* (1960), was not confirmed in the present work; Parr (1957) also reported negative findings in this respect.

Previous reports of the competitive inhibition of glucose phosphate isomerase by 6-phosphogluconate and sorbitol 6-phosphate (Parr, 1956, 1957) implied that these effects may be of some physiological significance, although other authors have queried this notion (Racker, 1959; Kahana *et al.* 1960) and inhibitory results obtained with synthetic 6-phosphogluconate must now be suspect. Erythrose 4-phosphate is also a powerful competitive inhibitor and a regulatory role has been proposed for this effect by Grazi *et al.* (1960).

These results show that fructose 1-phosphate and fructose 1,6-diphosphate are competitive inhibitors of both liver and muscle glucose phosphate isomerase. The physiological significance of the inhibition of the muscle enzyme is difficult to assess, since ketohexokinase is not detectable in muscle (Hers, 1957) and fructose 1,6-diphosphate would probably not reach concentrations in excess of

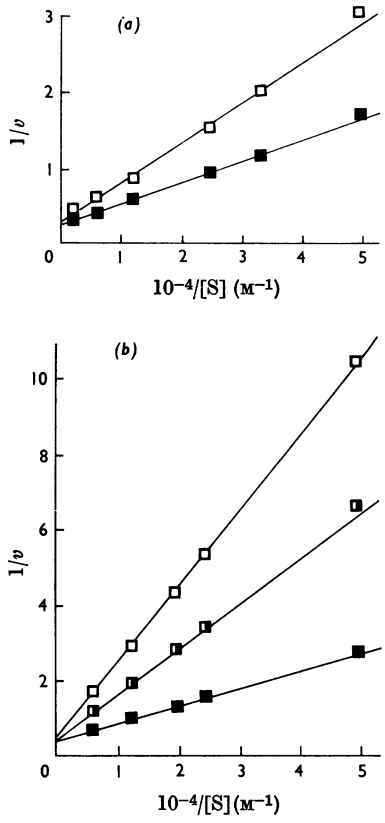

Fig. 1. Kinetics of inhibition of liver glucose phosphate isomerase. Assay conditions are described in the Materials and Methods section. The concentration of fructose 6-phosphate was varied between 0·02 and 0·40mM. Each point represents the mean of two or three determinations of the rate under identical conditions. Velocity units are arbitrary. (*a*) Inhibition by fructose 1,6-diphosphate. ■, Control: fructose 6-phosphate only. □, Test: fructose 6-phosphate plus 5mM-fructose 1,6-diphosphate. (*b*) Inhibition by fructose 1-phosphate. ■, Control: fructose 6-phosphate only. ◨, Test: fructose 6-phosphate plus 2·5mM-fructose 1-phosphate. □, Test: fructose 6-phosphate plus 5·0mM-fructose 1-phosphate.

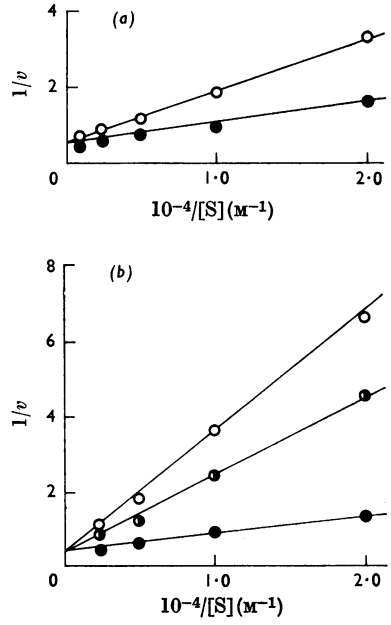

Fig. 2. Kinetics of inhibition of skeletal-muscle glucose phosphate isomerase. Assay conditions are described in the Materials and Methods section. The concentration of fructose 6-phosphate was varied between 0·05 and 1·0mM. Each point represents the mean of two or three determinations of the rate under identical conditions. Velocity units are arbitrary. (*a*) Inhibition by fructose 1,6-diphosphate. ●, Control: fructose 6-phosphate only. ○, Test: fructose 6-phosphate plus 10mM-fructose 1,6-diphosphate. (*b*) Inhibition by fructose 1-phosphate. ●, Control: fructose 6-phosphate only. ◑, Test: fructose 6-phosphate plus 5mM-fructose 1-phosphate. ○, Test: fructose 6-phosphate plus 10mM-fructose 1-phosphate.

5–10mM in muscle (cf. Hohorst, Reim & Bartels, 1962).

Fructose 1-phosphate accumulates in the liver of fructose-fed rabbits to a concentration of about 10mM (Kjerulf-Jensen, 1942). Similar concentrations have now been shown to result in substantial inhibition of glucose phosphate isomerase, and, since inhibition is competitive, even lower concentrations would be effective *in vivo*, since intracellular concentrations of fructose 6-phosphate in the liver of normal rats can be calculated, from the data of Hornbrook, Burch & Lowry (1965), Young (1966) and Hohorst *et al.* (1962), to be about 0·02mM. Thus molar ratios of inhibitor and substrate could reach values as high as 500 on this basis, and at a ratio of 100 the enzyme is inhibited over 70% *in vitro*. Fructose 1-phosphate must also accumulate in the liver of hereditary fructose-intolerant patients owing to the lack of fructose 1-phosphate aldolase (Froesch *et al.* 1963). The profound hypoglycaemia, which follows fructose loading of such patients, has been explained by Froesch *et al.* (1963) in terms of the failure of glycogenolysis catalysed by phosphorylase consequent upon a lack of inorganic phosphate. Hypophosphataemia is observed concurrently with the fructose-induced hypoglycaemia, and Froesch *et al.* (1963) considered that the phosphorylation of fructose and accumulation of fructose 1-phosphate promotes a lack of ATP and eventually of inorganic phosphate. It appears

Table 2. *Effect of fructose and intermediates on the rate of fructose 6-phosphate formation by glucose phosphate isomerase*

Assay method B was used as described in the Materials and Methods section, except for the concentrations of substrate and inhibitors, which are given below. The results represent the means of at least three experiments. Each rate determination was carried out in duplicate and simultaneous recordings of the control and test (with added inhibitor) were obtained.

Inhibitor	Concn. of glucose 6-phosphate (mM)	Molar ratio inhibitor/glucose 6-phosphate	Inhibition (%) (liver enzyme)	Inhibition (%) (muscle enzyme)
Fructose	0·10	1000	0	0
(100mM)	1·00	100	1	0
Fructose 1,6-diphosphate	0·10	100	−4	−1
(10mM)	1·00	10	0	−1
Fructose 1-phosphate	0·10	100	5	7
(10mM)	1·00	10	5	4
Cyclohexylammonium phosphate, pH7·4	0·10	200	6	8
(20mM)	1·00	20	5	2

Table 3. *Apparent Michaelis and inhibitor constants of glucose phosphate isomerase*

The apparent K_m values were determined for fructose 6-phosphate at concentrations between 0·02 and 0·40mM (liver enzyme) and 0·05 and 1·0mM (muscle enzyme). The apparent K_m values for glucose 6-phosphate were determined at concentrations between 0·1 and 2·0mM for both enzymes. Two different preparations of both enzymes gave similar values of K_m for fructose 6-phosphate. The results were all obtained on the second preparation of each enzyme.

Expt. no.	Source of enzyme	$10^4 K_m$ (M-fructose 6-phosphate)	$10^3 K_i$ (M-fructose 1-phosphate)	$10^3 K_i$ (M-fructose 1,6-diphosphate)
1	Liver	1·29	—	7·2
2	Liver	1·11	1·43, 1·67	—
3	Muscle	1·14	—	7·9
4	Muscle	1·25	1·37, 1·63	—

significant in this respect that glucagon does not relieve the hypoglycaemia.

Froesch *et al.* (1959) showed that the cleavage of fructose 1,6-diphosphate by muscle aldolase was competitively inhibited by fructose 1-phosphate. This enzyme shares with the 'residual liver aldolase' found in the hereditary fructose-intolerant patient a high ratio of fructose 1,6-diphosphate/fructose 1-phosphate activity. However, the conclusion that the effect of fructose 1-phosphate on the enzyme activity resulted in inhibition of glyconeogenesis is not justified unless it can be shown that the formation of fructose 1,6-diphosphate from triose phosphates is inhibited by fructose 1-phosphate.

In a critical analysis of the causes of fructose-induced hypoglycaemia, Cornblath *et al.* (1963) suggested that gluconeogenesis may be blocked, but in experiments with glucose phosphate isomerase and hexose diphosphatase (EC 3.1.3.11) they found no significant inhibition by fructose or fructose 1-phosphate. Their findings with hexose diphosphatase have been confirmed (J. Zalitis & I. T. Oliver, unpublished work), but, as the present results show, inhibition of glucose phosphate isomerase by fructose 1-phosphate is both powerful and competitive when the conversion of fructose 6-phosphate into glucose 6-phosphate is studied. Fructose 1-phosphate does not inhibit the reverse reaction and it must be concluded that Cornblath *et al.* (1963) studied the reaction only in the direction of fructose 6-phosphate formation from glucose 6-phosphate.

Cornblath *et al.* (1963) have shown that a dramatic rise in the concentration of serum glucose occurs after galactose administration during fructose-induced hypoglycaemia, and it may be deduced that sufficient intracellular ATP is available for the phosphorylation of galactose and its subsequent conversion into glucose. The finding also further emphasizes that phosphoglucomutase, which is an essential enzyme in the interconversion of galactose and glucose, is not inhibited under these conditions.

Unpublished work by the authors has shown that the inhibition *in vitro* of phosphoglucomutase by fructose 1-phosphate is only achieved at high concentrations, and may be assigned to an 'anion effect' (Klenow, 1955) since inorganic phosphate is a more powerful inhibitor.

Dubois *et al.* (1961), using labelled glucose, showed that glucose release to the blood was almost completely inhibited during fructose-induced hypoglycaemia. Since the liver is the major organ of gluconeogenesis (Krebs, 1964), the inhibition of this process at the glucose phosphate isomerase step by fructose 1-phosphate could partly explain the fructose-induced hypoglycaemia and elevation of serum lactate and pyruvate in hereditary fructose-intolerant patients (Froesch *et al.* 1963; Cornblath *et al.* 1963). It may be noted also that glucose phosphate isomerase catalyses a reaction common to both gluconeogenesis from trioses and to interconversion of fructose and glucose via the hexokinase pathway.

The major difficulty with the interpretation above is the fact that published data on the activity of liver enzymes concerned in gluconeogenesis (Weber, 1963) indicated that glucose phosphate isomerase is not likely to be rate-limiting, since the enzyme is reported to be present in a 20–40-fold excess over hexose diphosphatase. However, procedures for the assay of hexose diphosphatase based on determinations of inorganic phosphate production (Pogell & McGilvery, 1954) underestimate the enzyme maximal activity, since fructose 1,6-diphosphate is inhibitory at the high concentrations used (Krebs, 1963; Weber, 1964; Underwood & Newsholme, 1965). The spectrophotometric procedures of Taketa & Pogell (1963) and Underwood & Newsholme (1965) give activities 3–5 times as great even at substrate concentrations of 0·1 mM or less (J. Zalitis & I. T. Oliver, unpublished work). The K_m of hexose diphosphatase for fructose 1,6-diphosphate is only 2·0 μM (Underwood & Newsholme, 1965) and hence maximal activity is reached at substrate concentrations that occur physiologically in the liver (13 μM; Hornbrook *et al.* 1965). The intracellular concentration of fructose 6-phosphate in rat liver is about 0·02 mM as previously stated, and the velocity of the glucose phosphate-isomerase reaction can be calculated from the kinetic data presented in this paper to be only about one-eighth the maximum velocity determined at saturating substrate concentration. Hence under physiological conditions the velocities of the reactions catalysed by the two enzymes may be roughly equal. Accumulation of the competitive inhibitor fructose 1-phosphate could then markedly lower the rate of the glucose phosphate-isomerase reaction and thus inhibit hepatic gluconeogenesis in fructose loading of the hereditary fructose-intolerant patient.

J. Z. is indebted for a Commonwealth Post-Graduate Award. The study was supported by monies provided by the Medical School Research Grants Committee of the University of Western Australia and by the National Health and Medical Research Council of Australia.

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