Further Studies on the Properties and Assay of Glucose 6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase of Rat Liver

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Renewed interest in the direct oxidative pathway of glucose 6-phosphate metabolism during the last few years has revealed that this pathway is by no means restricted to erythrocytes, yeast and micro-organisms. The triphosphopyridine-nucleotide-(TPN)-specific glucose 6-phosphate and 6-phosphogluconate dehydrogenases are also widely distributed in mammalian tissues (Dickens & Glock, 1950, 1951; Horecker & Smyrniotis, 1951), in a variety of lower plants and animals (Cohen, 1950) and also in higher plants (Conn & Vennesland, 1951; Gibbs, 1952). The significance of this pathway in animal tissues, its physiological control and the relative importance of the direct oxidative and glycolytic routes of carbohydrate metabolism are still, however, chiefly matters of conjecture. An essential preliminary step to such an investigation is to devise a satisfactory procedure for the assay of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in animal tissues and it was with this object in view that the present work was undertaken.

EXPERIMENTAL

Materials

D-*Glucose 6-phosphate* (G 6-P). This was prepared as the heptahydrate of the barium salt. Embden ester was dissolved in water, seeded with a few crystals of the heptahydrate, kindly given by Dr B. L. Horecker, adjusted to pH 8·0 with saturated $Ba(OH)_2$ solution and left overnight at 4°. The insoluble heptahydrate (Horecker, 1952) which crystallized out was washed with ethanol and acetone. It was 99·5% pure (analysed with Zwischenferment according to Kornberg, 1950).

Fructose 6-phosphate (F6-P). This was a preparation of the barium salt free from G 6-P.

6-*Phosphogluconate* (6-PG). A preparation of the barium salt of 6-phosphohexonate was used as a source of 6-PG in most of the experiments. Enzymic assay with a partially purified liver 6-PG dehydrogenase (see Methods section) showed that it contained 77% 6-PG. For P and sugar analysis see Dickens & Glock (1951). In later experiments a preparation of 6-PG prepared from the heptahydrate of G 6-P (barium salt) according to Robison & King (1931) was used. (Found: 6-PG, 87 (by enzymic assay); G 6-P, 0·0 (by enzymic assay); organic P, 6·4; inorganic P, 0·0%. Calc.: P, 6·47%.) Both phosphohexonate and fructose 6-phosphate were kindly provided by Dr Marjorie Macfarlane from the collection of the late Prof. R. Robison.

Ribulose 5-phosphate. An equilibrium mixture of the barium salts of ribulose 5-P and ribose 5-P (containing respectively 65 and 35% of these two esters) was used. We are indebted to Dr B. L. Horecker for this preparation.

D-*Ribose 5-phosphate* (R 5-P) was prepared from barium inosinate according to Marmur, Schlenk & Overland (1951). (Found: organic P, 8·41; inorganic P, 0·0; pentose (orcinol reaction), 41·2%. Calc.: P, 8·48; pentose, 41·1%.)

Triphosphopyridine nucleotide (TPN). This was made from horse liver by the unpublished method of Kornberg and Horecker (Horecker, 1952), involving purification of a crude preparation, obtained by Hg precipitation, on Dowex 1 formate. It contained 70% TPN (analysed with Zwischenferment according to Kornberg, 1950) and no DPN (by the alcohol-dehydrogenase method of Racker, 1950).

Cystine, o-iodosobenzoate and p-chloromercuribenzoate. These were prepared by Mr W. Lawson, Miss D. Salmony and Mr M. Woodford, respectively, to whom we are indebted.

Glutathione. This was presented by Distillers Co. Ltd.

Methods

Preparation of liver supernatants and differential centrifugation for studying intracellular distribution of dehydrogenases. Livers from rats which had previously fasted overnight were chilled to 0° and disintegrated in a Potter glass homogenizer in 8 vol. of ice-cold isotonic (0·15M) KCl containing $KHCO_3$ (8 ml. 0·02M-$KHCO_3$/l.) to maintain the pH at 7·0 (LePage, 1948). When the supernatant only was required, for the assay of the dehydrogenases, the suspension was centrifuged at 4000 *g* for 60 min. at 2–4° in an International refrigerated centrifuge (Type PR 1) and the supernatant ('supernatant I') siphoned off and dialysed overnight against the same extracting medium at 2°. For determining the intracellular distribution of the dehydrogenases, the suspension was divided into three parts, one part ('suspension') kept, one part centrifuged as above ('supernatant I') and the third part centrifuged at 20000 *g* for 60 min. at 2–4° ('supernatant II'). Supernatants I and II were then diluted with the isotonic extracting medium up to the original volume of the suspension used and all three parts dialysed as described above. Except where otherwise stated, reference to 'supernatant' in the text refers to 'supernatant I'.

Preparation of 6-phosphogluconate dehydrogenase from liver supernatants. The fraction, precipitating between 0·6 and 0·7 saturation with $(NH_4)_2SO_4$ at pH 7·3 from pooled liver supernatants at 4°, was collected by centrifuging at 4° and dialysed overnight against distilled water at this temperature. It was diluted to one-tenth of the original volume of the supernatant and stored at −15°. The protein

content, determined by the biuret method of Robinson & Hogden (1940), was 10·7 mg. protein/ml. This fraction contained a very active 6-PG dehydrogenase (approximately 10 times as active as the original liver supernatant) and no G 6-P dehydrogenase. It was used in the assay of G 6-P dehydrogenase (see below).

Estimation of glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities. Dehydrogenase activity was determined spectrophotometrically by following the rate of reduction of TPN at 340 mμ. in 1 cm. cells at room temperature. In the final assay procedure, the reaction mixture consisted of 0·05–0·10 ml. liver supernatant (1·3–1·6 mg. N/ml.), 0·5 ml. 0·1 M-$MgCl_2$, 0·5 ml. 0·25 M-glycylglycine buffer (pH 7·6 for G 6-P dehydrogenase and pH 9·0 for 6-PG dehydrogenase) and 0·2 mg. TPN in a total volume of 2·4 ml. The reaction was started by the addition of 0·1 ml. 0·05 M substrate to both cells, the blank cell being devoid of TPN.

The activity measured at pH 7·6 with G 6-P as substrate is the summation of G 6-P and 6-PG dehydrogenase activities. In order to determine G 6-P dehydrogenase activity only, two alternative procedures were adopted. In one method, dehydrogenase activity of liver supernatants was determined in the presence of a large excess of 6-PG dehydrogenase (0·1 ml. dialysed $(NH_4)_2SO_4$ fraction described above). Under these conditions, 50% of the total activity is due to G 6-P dehydrogenase. In the second method, the G 6-P dehydrogenase activity was taken as the difference between the activities measured separately at pH 7·6 in the presence of 0·1 ml. 0·05 M-6-PG and 0·1 ml. of a solution containing G 6-P and 6-PG (0·05 M in respect to each).

The increase in density at 340 mμ. during the first 5 min. was taken as a measure of dehydrogenase activity. All the activities are expressed at 20° by applying an experimentally determined temperature coefficient of 1·7 (for the 10° temperature range between 20 and 30°) for both dehydrogenases.

A unit of enzyme activity is defined as the quantity of enzyme which, at 20° and the given pH, reduces 0·01 μmole TPN/min., based on the readings over the first 5 min.

With the whole suspension, it was not possible to measure dehydrogenase activity directly in the spectrophotometer cells on account of the opacity of the reaction mixtures, and for this reason the reaction was allowed to proceed at room temperature, and at 1 min. intervals 2·5 ml. samples were removed and introduced into stoppered centrifuge tubes containing 2·5 ml. ethanol, and 0·1 ml. 10% (w/v) Na_2SO_4 solution added to aid flocculation. After 30 min. at room temperature to allow for complete precipitation of the protein, the tubes were spun for 5 min. and the clear supernatant used for spectrophotometric assay of reduced TPN. The suitability of this ethanol-precipitation procedure was tested on liver supernatants, and identical results were obtained for the activity of both dehydrogenases by direct and precipitation procedures.

In most of the experiments when the action of metal ions was being studied, dehydrogenase activities were measured at pH 8·4 in a final concentration of 0·01 M-tris(hydroxymethyl)aminomethane (Tris) buffer.

Isomerase. Isomerase activity was determined by substituting F 6-P for G 6-P.

Phosphatase. Phosphatase activity towards G 6-P and 6-PG was tested under the conditions of the G 6-P and 6-PG dehydrogenase assay procedure previously described, by determining the liberation of inorganic phosphate.

Enzymic breakdown of TPN including conversion to diphosphopyridine nucleotide (DPN). Enzymic breakdown of TPN resulting in the formation of free nicotinamide was determined in the whole suspension and in supernatants I and II at pH 7·6 under the conditions of the dehydrogenase assay procedure. Protein and unchanged TPN were precipitated with $Zn(OH)_2$ (McIlwain & Rodnight, 1949) and any liberated nicotinamide determined colorimetrically by the procedure of Melnick & Field (1940). Conversion of TPN to DPN by liver supernatants was determined by allowing 0·1 ml. liver supernatant to react with 0·2 mg. TPN at room temperature for 30 min. and then determining DPN with alcohol dehydrogenase (Racker, 1950).

Action of compounds reacting with sulphydryl groups. Iodoacetamide, *o*-iodosobenzoate, *p*-chloromercuribenzoate, *p*-aminophenylarsenoxide and cystine were used as inhibitors and cysteine and glutathione as reactivators. All experiments were carried out anaerobically in N_2 at 30° in Warburg manometers, the liver supernatant (1 ml.), neutralized inhibitor (0·1 ml.) and veronal buffer, pH 7·3, being introduced into the main chamber and neutralized reactivator (0·1 ml.) into the side arm, the total volume in each case being 2·0 ml. The inhibitors were allowed to react with the enzyme for 10 min. (in the case of *p*-chloromercuribenzoate) or 30 min. (with the other inhibitors) before the reactivator was added and incubation then continued for a further 30 min. After cooling rapidly to room temperature G 6-P and 6-PG dehydrogenase activities were measured in veronal buffer at pH's 8·5 and 9·8, respectively. G 6-P dehydrogenase activity was measured by the method employing two substrates. Suitable incubated controls were included.

Competitive inhibition of dehydrogenase by adenosine triphosphate (ATP). The G 6-P and 6-PG dehydrogenase activities of liver supernatants were determined as in the assay procedure, but with four different levels of TPN (0·05, 0·07, 0·1 and 0·2 mg.) and, at each level of TPN, without ATP and with the addition of 0·2 ml. or 0·5 ml. 0·06 M-ATP.

Nitrogen contents of liver fractions. These were determined by the micro-Kjeldahl procedure.

RESULTS

The direct oxidative pathway of G 6-P metabolism proceeds in a cycle via 6-PG, ribulose phosphate and probably sedoheptulose phosphate (Horecker, Smyrniotis & Seegmiller, 1951; Horecker & Smyrniotis, 1952) back to hexosemonophosphate (see Dische, 1951). Of these various stages only the oxidation of G 6-P and the oxidative decarboxylation of 6-PG are TPN-dependent. It thus follows that when the rate of reduction of TPN is taken as a measure of dehydrogenase activity, the activity determined with G 6-P as substrate represents a summation of G 6-P and 6-PG dehydrogenase activities. Moreover, the assay of G 6-P and 6-PG dehydrogenases by such a method is only valid if there is no detectable formation of G 6-P from 6-PG. To establish this point, an equilibrium mixture of ribulose 5-phosphate and R 5-P was used as substrate and reduction of TPN measured under the conditions of the assay procedures for G 6-P and 6-PG dehydrogenases, in glycylglycine at pH's 7·6

and 9·0 respectively. In neither case was there any reduction of TPN, thus indicating that no G 6-P was formed (Fig. 1). These results do not necessarily mean that the complete system for resynthesis of G 6-P from ribulose phosphate is not present in the liver supernatants used for the assay of G 6-P and 6-PG dehydrogenases, but rather that the conditions are not optimal particularly in respect to

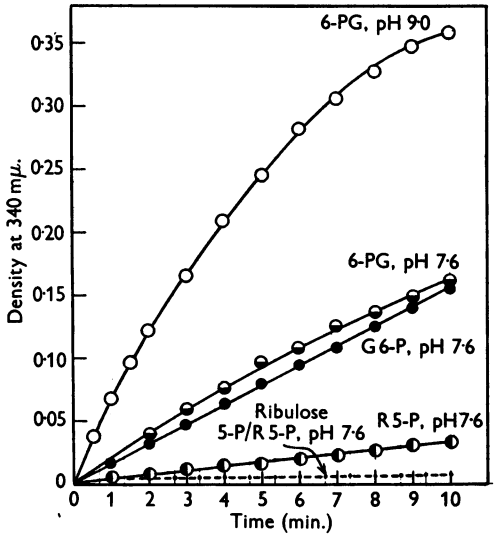

Fig. 1. Oxidation of 6-phosphogluconate, glucose 6-phosphate, ribose 5-phosphate and ribulose 5-phosphate by rat-liver supernatant. Rate of reduction of TPN measured at 340 mμ. in 0·05 M-glycylglycine buffer, at pH 7·6 or 9·0, in the presence of 0·1 ml. liver supernatant, 0·5 ml. 0·1 M-$MgCl_2$, 0·2 mg. TPN and 0·1 ml. substrate (0·05 M in respect to 6-PG, G 6-P and R 5-P and 0·001 M in respect to an equilibrium mixture of ribulose 5-P/R 5-P). Concentration of ribulose 5-P used approximately equivalent to the G 6-P oxidized under the conditions of the assay procedure. Total volume, 2·5 ml. ○—○, 6-PG, pH 9·0; ◒—◒, 6-PG, pH 7·6; ●—●, G 6-P, pH 7·6; ◐—◐, R 5-P, pH 7·6; – – · – – ·, ribulose 5-P/R 5-P, pH 7·6.

temperature, time of reaction and substrate concentration. Fig. 1 does indeed show that with a large excess of R 5-P there is some reduction of TPN at room temperature, although with the concentration of R 5-P which could be formed from 6-PG under the conditions of the assay of 6-PG dehydrogenase, this is negligible.

In order to determine G 6-P dehydrogenase activity as distinct from the summation of G 6-P and 6-PG dehydrogenases, two alternative procedures have been adopted. In one method, dehydrogenase activity of liver supernatants was determined in glycylglycine buffer at pH 7·6 with G 6-P as substrate in the presence of a large excess of 6-PG dehydrogenase. Under these conditions the true G 6-P dehydrogenase activity is 50 % of the total measured activity. In the second method, the G 6-P dehydrogenase activity was taken as the difference between the activities measured at pH 7·6 with 6-PG only and with both substrates together. There was good agreement between the results obtained by these two methods. The mean values for G 6-P dehydrogenase activity in units/g. liver (1 unit being defined as the quantity of enzyme which reduces 0·01 μmole TPN/min. at 20°) for a group of six female rats, together with the standard errors, were 98 ± 15 by the method using two substrates and 96 ± 14 by the method with excess 6-PG dehydrogenase. The corresponding uncorrected value for G 6-P dehydrogenase activity was 160 ± 21, indicating that, at least in the livers of normal rats, approximately 60 % of the dehydrogenase activity measured at pH 7·6 with G 6-P as substrate is due to G 6-P dehydrogenase and the remaining 40 % to 6-PG dehydrogenase.

For routine estimation of G 6-P dehydrogenase activity the method using excess 6-PG dehydrogenase is preferable, since only one measurement has to be made and the reaction rate is linear. The method employing two substrates, however, was found to have special application in connexion with the inhibition by heavy metals and sulphydryl compounds.

pH-*Activity curves of glucose 6-phosphate and 6-phosphogluconate dehydrogenases*

The pH-activity curves for both dehydrogenases in 0·05 M glycylglycine and 0·006 M veronal, using liver supernatants as the source of the dehydrogenases, are given in Figs. 2 and 3. The pH optimum for 6-PG dehydrogenase is consistently higher than for G 6-P dehydrogenase. In glycylglycine buffer, the pH optima are 9·0 for 6-PG dehydrogenase and 7·6 for G 6-P dehydrogenase, although in the latter case the activity falls off only slightly on the alkaline side of the optimum. In veronal buffer, the corresponding pH optima are at 9·8 and 8·5 respectively. The pH optima in glycylglycine were unaffected by the addition of magnesium chloride in a final concentration of 0·02 M or in the case of the G 6-P dehydrogenase by the addition of excess 6-PG dehydrogenase.

The final assay procedure was carried out in glycylglycine in order to remove traces of inhibitory heavy metals. The high pH optimum for 6-PG dehydrogenase is in contrast to the value of 7·6 found for purified yeast 6-PG dehydrogenase by Horecker & Smyrniotis (1951).

The effect of metal ions on dehydrogenase activities

The activation of both dehydrogenases by Mg^{2+} and Ca^{2+} in glycylglycine is shown in Fig. 4. Although the degree of activation is very variable with different enzyme preparations, the optimal

concentration of Mg^{2+} and Ca^{2+} for both dehydrogenases is approximately 2×10^{-2}M. Magnesium in this final concentration was used as the activating ion in the assay procedure. Cohen (1951) also found

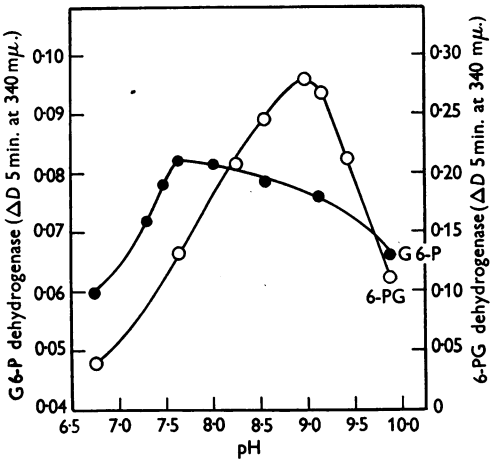

Fig. 2. pH-Activity curves of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in glycylglycine buffer. Dehydrogenase activities determined as described in the Methods section with 0·1 ml. liver supernatant, without the addition of Mg^{2+}. ●—●, G 6-P dehydrogenase; ○—○, 6-PG dehydrogenase.

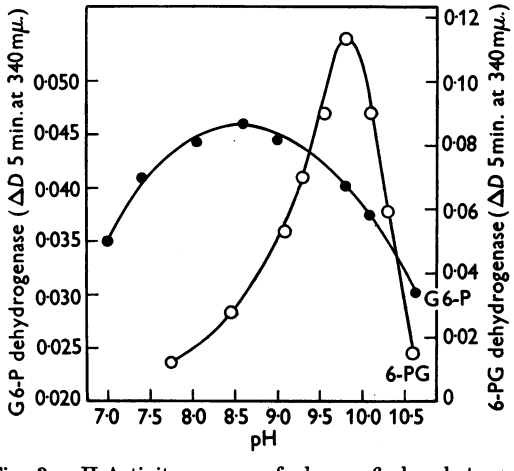

Fig. 3. pH-Activity curves of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in veronal buffer. Dehydrogenase activities determined with 0·1 ml. liver supernatant in 0·006M veronal (veronal acetate/HCl or veronal/Na_2CO_3/HCl). ●—●, G 6-P dehydrogenase; ○—○, 6-PG dehydrogenase.

activation of both dehydrogenases of *Chaetopterus* by Mg^{2+} and Ca^{2+}, the optimal concentration of both ions for both dehydrogenases being at a final concentration of 3×10^{-2}M. With purified yeast 6-PG dehydrogenase, Horecker & Smyrniotis (1951) found that a concentration of 5×10^{-3}M Mg^{2+} produced maximal activation.

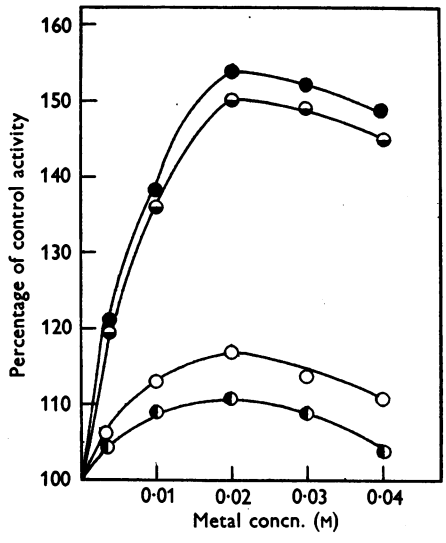

Fig. 4. Activation of glucose 6-phosphate and 6-phosphogluconate dehydrogenases by Ca^{2+} and Mg^{2+}. Dehydrogenase activities determined in glycylglycine buffer with 0·1 ml. liver supernatant as described in the Methods section. G 6-P dehydrogenase determined with excess 6-PG dehydrogenase. ●—●, G 6-P dehydrogenase, Mg^{2+}; ◒—◒, G 6-P dehydrogenase, Ca^{2+}; ○—○, 6-PG dehydrogenase, Mg^{2+}; ◐—◐, 6-PG dehydrogenase, Ca^{2+}.

Table 1. *Influence of metals on glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities*

Metal ion	Final metal concn. (M)	Dehydrogenase activity as percentage of control* G 6-P	6-PG
K^+	1×10^{-2}	117	101
Mg^{2+}	1×10^{-2}	152	120
Ca^{2+}	1×10^{-2}	154	120
Mn^{2+}	1×10^{-2}	152	128
Co^{2+}	1×10^{-4}	94	106
Al^{3+}	1×10^{-4}	109	104
Fe^{3+}	1×10^{-4}	93	91
Fe^{2+}	1×10^{-4}	100	94
Zn^{2+}	1×10^{-4}	82	3
Cu^{2+}	1×10^{-4}	103	18
Hg^{2+}	1×10^{-4}	0	0

* Dehydrogenase activity determined as described in the Methods section with 0·1 ml. liver supernatant in Tris buffer pH 8·4 (final concentration, 0·01M) G 6-P dehydrogenase activity determined by the method employing two substrates.

The action of other metal ions is summarized in Table 1. Manganese produced marked activation of both dehydrogenases and in this respect was at least as active as Mg^{2+}. Al^{3+}, Co^{2+}, Fe^{2+}, and Fe^{3+} in a final concentration of 1×10^{-4}M were almost

ineffective. Hg^{2+}, in a final concentration of 1×10^{-4} M, inhibited both dehydrogenases completely. 6-PG dehydrogenase was markedly inhibited by Zn^{2+} and Cu^{2+} at this concentration.

The previous findings of Dickens (1938) and Dickens & Glock (1951) that cyanide activates both dehydrogenases can probably be accounted for by the removal of inhibitory heavy metals.

Variations in substrate concentration

The effect of variations in substrate concentration on the initial rates of reaction of G 6-P dehydrogenase at pH 7·6 and of 6-PG dehydrogenase both at pH's 7·6 and 9·0 is shown in Fig. 5, these results being plotted according to Lineweaver & Burk (1934). The Michaelis constant for the G 6-P

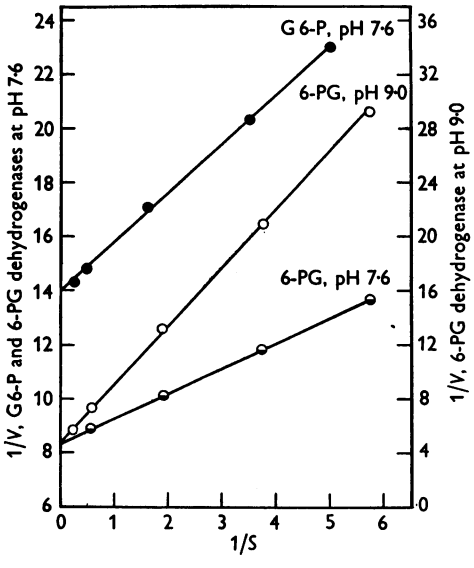

Fig. 5. Effect of variations in substrate concentration on glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities. Dehydrogenase activities determined in glycylglycine buffer, as described in the Methods section, with 0·1 ml. liver supernatant. Values for $1/S$ to be multiplied by 10^4. O—O, 6-PG dehydrogenase at pH 9·0; ◒—◒, 6-PG dehydrogenase at pH 7·6; ●—●, G 6-P dehydrogenase at pH 7·6 (determined with excess 6-PG dehydrogenase).

dehydrogenase is $1{\cdot}3 \times 10^{-5}$ M and for 6-PG dehydrogenase 9×10^{-5} M at pH 9·0 and approximately 1×10^{-5} M at pH 7·6, indicating a much greater affinity of 6-PG for its dehydrogenase at the lower pH. The value of the constant for purified yeast 6-PG dehydrogenase at pH 7·6 is 5×10^{-5} M (Horecker & Smyrniotis, 1951).

Variations in TPN concentration

The effect of variations in TPN concentration on the initial reaction rates of both dehydrogenases is shown in Fig. 6. Approximate values for the Michaelis constant, K_{TPN}, are $1{\cdot}3 \times 10^{-5}$ M for G 6-P dehydrogenase, $2{\cdot}4 \times 10^{-5}$ M for 6-PG dehydrogenase at pH 9·0 and $2{\cdot}8 \times 10^{-5}$ M for 6-PG dehydrogenase at pH 7·6.

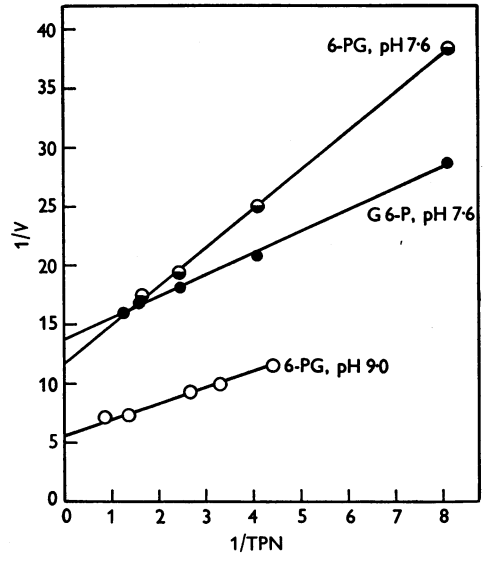

Fig. 6. Effect of variations in TPN concentration on glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities. Dehydrogenase activities determined in glycylglycine buffer, as described in the Methods section, with 0·1 ml. liver supernatant. Values for 1/TPN to be multiplied by 10^4. O—O, 6-PG dehydrogenase at pH 9·0; ◒—◒, 6-PG dehydrogenase at pH 7·6; ●—●, G 6-P dehydrogenase at pH 7·6 (determined with excess 6-PG dehydrogenase).

Variation in enzyme concentration

The effect of variations in enzyme concentration on the initial rates of reaction of both dehydrogenases is shown in Fig. 7. The reaction rate (rate of reduction of TPN) is directly proportional to enzyme concentration provided that not more than 40 % of the TPN is reduced during the first 5 min., which, under the conditions of the assay of both G 6-P and 6-PG dehydrogenases, amounts to the reduction of 0·08 μmole TPN. The range of proportionality can be extended by increasing the TPN concentration. In the case of G 6-P dehydrogenase, the rate of reaction is proportional to enzyme concentration over a slightly wider range using the method with excess 6-PG dehydrogenase than by the method

employing two substrates, owing to the fact that the total reduction of TPN is greater in the latter case.

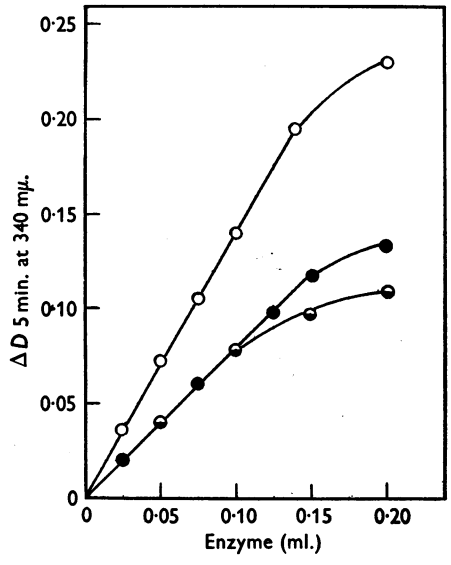

Fig. 7. Enzyme-concentration-activity curves for glucose 6-phosphate and 6-phosphogluconate dehydrogenases. Dehydrogenase activities determined in glycylglycine buffer with varying concentrations of liver supernatant (1·4 mg. N/ml.) as described in the Methods section. ○—○, 6-PG dehydrogenase; ●—●, G 6-P dehydrogenase (determined with excess 6-PG dehydrogenase); ◒—◒, G 6-P dehydrogenase (determined by method employing two substrates).

Table 2. *Levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in rat liver*

	Δ_D at 340 mμ. in 5 min.* at 20°/ml. liver supernatant	Units/g. liver†
G 6-P dehydrogenase, pH 7·6		
Male rats (18)	0·64±0·04	46±3
Female rats (12)	1·44±0·16	104±12
6-PG dehydrogenase, pH 7·6		
Male rats (18)	0·82±0·11	59±8
Female rats (12)	1·80±0·16	130±12
6-PG dehydrogenase, pH 9·0		
Male rats (18)	2·03±0·14	147±10
Female rats (12)	3·99±0·22	290±16

* Δ_D of 1·0 corresponds to the reduction of 0·403 μmoles TPN.

† A unit of activity is defined as the amount of enzyme which reduces 0·01 μmole TPN/min. at 20°, based on the readings over the first 5 min. Results are expressed as means±S.E. of means.

Levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities in the liver of rat

Mean values for the G 6-P and 6-PG dehydrogenase activities in the livers of both male and female rats are given in Table 2. It is of interest that the levels of both enzymes are consistently higher in females than in males.

Intracellular distribution of dehydrogenases

Preliminary intracellular distribution experiments in isotonic sucrose indicated that all the G 6-P and 6-PG dehydrogenase activities were in the soluble fraction of disintegrated liver. Further distribution experiments were designed solely to confirm this finding. Isotonic potassium chloride was chosen as the suspending medium since cellular components are more readily agglutinated than in sucrose and lower speeds of centrifugation are required. The preparation of suspension, supernatant I (after spinning for 60 min. at 4000 *g*) and supernatant II (after spinning for 60 min. at 20 000 *g*) is described in the Methods section.

Table 3. *Distribution of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in rat-liver suspensions*

(Method I, direct spectrophotometric estimation. Method II, ethanol-precipitation procedure. For experimental details and preparation of liver fractions see Methods section. Activity expressed as Δ_D/1 cm./340 mμ. in 5 min. at 20°.)

	G 6-P dehydrogenase activity		6-PG dehydrogenase activity	
Liver fraction	Method I	Method II	Method I	Method II
Suspension	—	0·148	—	0·142
Supernatant I	0·144	0·150	0·160	0·144
Supernatant II	0·146	0·140	0·146	0·140

Table 3 shows the results of a typical experiment, the dehydrogenase activities being determined both by the ethanol-precipitation procedure and by the direct spectrophotometric method. The results indicate that all three liver fractions have identical G 6-P and 6-PG dehydrogenase activities and that these dehydrogenases are therefore located exclusively in the soluble fraction. It is of interest that the glycolytic enzymes have the same intracellular distribution (LePage & Schneider, 1948).

Hydrolysis of substrates and TPN

Breakdown of substrates by phosphatases and of TPN by nucleotidase, as determined by the liberation of free nicotinamide, were negligible both in the suspensions and the supernatants when measured at room temperature under the conditions of the dehydrogenase assay. The dephosphorylation of TPN to DPN by liver supernatant was also very small, amounting to only 1 % conversion in 10 min. at room temperature.

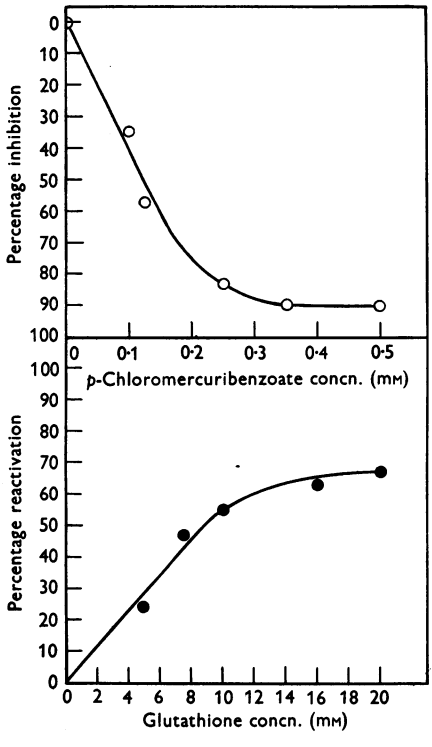

Fig. 8. Inhibition of 6-phosphogluconate dehydrogenase by *p*-chloromercuribenzoate and reversal by glutathione. Upper curve shows inhibition of 6-PG dehydrogenase by varying concentrations of *p*-chloromercuribenzoate and lower curve the reversal of the inhibition produced by $2{\cdot}5 \times 10^{-4}$ M mercurial with varying concentrations of glutathione. Incubations with mercurial allowed to proceed for 10 min. and in reactivation experiments followed by further incubation for 30 min. with glutathione. All incubations carried out in N_2 at 30° in veronal buffer, pH 7·3. 6-PG dehydrogenase activity determined in veronal-carbonate buffer at pH 9·8.

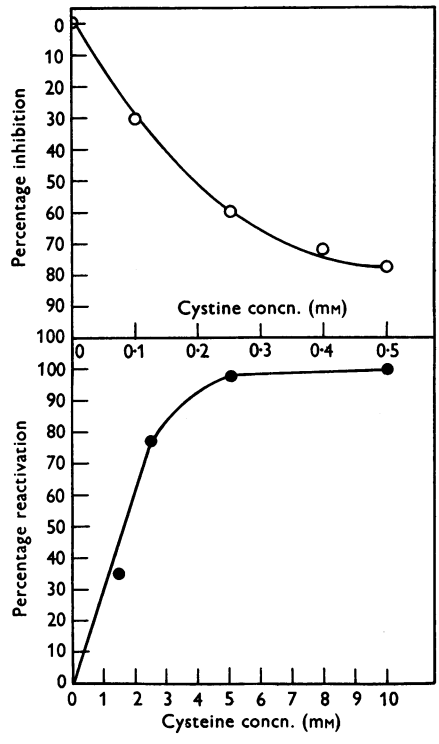

Fig. 9. Inhibition of 6-phosphogluconate dehydrogenase by cystine and reversal by cysteine. Upper curve shows inhibition of 6-PG dehydrogenase by varying concentrations of cystine and lower curve the reversal of the inhibition produced by $2{\cdot}5 \times 10^{-4}$ M-cystine with varying concentrations of cysteine. Incubations with cystine allowed to proceed for 30 min. and in reactivation experiments followed by further incubation for 30 min. with cysteine. All incubations carried out in N_2 at 30° in veronal buffer at pH 7·3. 6-PG dehydrogenase activity determined in veronal-carbonate buffer at pH 9·8.

Table 4. *Inhibition of glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities by sulphydryl-combining compounds*

(For experimental details see Methods section. G 6-P dehydrogenase activity determined by method employing two substrates.)

	Inhibition of dehydrogenase activity (%)			Reactivation of dehydrogenase activity (%)	
Inhibitor	G 6-P	6-PG	Reactivator	G 6-P	6-PG
p-Chloromercuribenzoate, $2{\cdot}5 \times 10^{-4}$ M	91	83	Cysteine, $2{\cdot}5 \times 10^{-3}$ M	10	60
			Glutathione, 2×10^{-2} M	21	67
o-Iodosobenzoate, $2{\cdot}5 \times 10^{-4}$ M	62	76	Cysteine, $2{\cdot}5 \times 10^{-3}$ M	0	23
			Glutathione, 1×10^{-2} M	15	45
Cystine, $2{\cdot}5 \times 10^{-4}$ M	6	60	Cysteine, $2{\cdot}5 \times 10^{-3}$ M	0	78
p-Aminophenylarsenoxide, $2{\cdot}5 \times 10^{-4}$ M	0	0	—	—	—
Iodoacetamide, $2{\cdot}5 \times 10^{-3}$ M	12	16	—	—	—

Isomerase

Liver supernatants were found to have a very active isomerase since the dehydrogenase activities were identical whether F 6-P or G 6-P was used as substrate.

Effect of compounds reacting with sulphydryl groups on glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities

The inhibition of both G 6-P and 6-PG dehydrogenase activities by low concentrations of Hg^{2+} and of 6-PG dehydrogenase by Cu^{2+} suggested that these enzymes might possess active SH groups. von Euler & Adler reported as early as 1935 that hexosemonophosphate dehydrogenase was inhibited by Cu^{2+} and bromoacetate. Horecker & Smyrniotis (1951) also observed marked inhibition of a purified yeast 6-PG dehydrogenase by Cu^{2+}.

In the present study, iodoacetamide, cystine, *o*-iodosobenzoate, *p*-chloromercuribenzoate and *p*-aminophenylarsenoxide were used as inhibitors and cysteine and glutathione as reactivators. Representative results are given in Table 4. Both dehydrogenases were inhibited by *p*-chloromercuribenzoate and *o*-iodosobenzoate, slightly greater inhibition being produced by the mercurial. The 6-PG dehydrogenase was, in addition, inhibited by cystine. Neither dehydrogenase was affected by the arsenical. Purified yeast G 6-P dehydrogenase (Kornberg, 1950) was also inhibited by the mercurial and by *o*-iodosobenzoate, but not by the arsenical.

Inhibition of 6-PG dehydrogenase by cystine, *p*-chloromercuribenzoate or *o*-iodosobenzoate was partially or completely reversed by cysteine and glutathione, but there was no striking reversal of G 6-P dehydrogenase inhibition by either reactivator. Inhibition of 6-PG dehydrogenase by *p*-chloromercuribenzoate and cystine and reversal by glutathione and cysteine, respectively, is shown in Figs. 8 and 9. Failure to obtain inhibition with arsenicals has also been reported for other enzymes, e.g. urease, which are known to contain active SH groups (Barron & Singer, 1945).

The results obtained with 6-PG dehydrogenase indicate that the activity of this enzyme is dependent on the presence of active sulphydryl groups, but it is uncertain whether the same applies to G 6-P dehydrogenase.

Competitive inhibition of 6-PG dehydrogenase by ATP

In a previous paper (Dickens & Glock, 1951) it was shown that nicotinamide acts as a competitive inhibitor of TPN utilization by 6-PG dehydrogenase but not of G 6-P dehydrogenase, and in addition that ATP caused some inhibition of 6-PG dehydrogenase activity. Feigelson, Williams & Elvehjem (1951) meanwhile found that nicotinamide behaved similarly in connexion with the DPN utilization by malic dehydrogenase, and this work was extended by Williams (1952) to include adenine, adenosine and ATP, which were all found to be more potent inhibitors than nicotinamide.

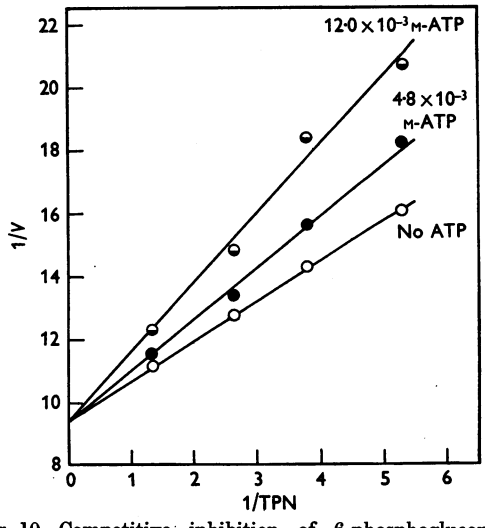

Fig. 10. Competitive inhibition of 6-phosphogluconate dehydrogenase by ATP. Activity determined as in Methods section with 0·1 ml. liver supernatant at four TPN concentrations. Positions of the lines calculated by the method of least squares. Values for 1/TPN to be multiplied by 10^4. ○—○, No ATP; ●—●, in the presence of 0·2 ml. 0·06 M ATP; ◒—◒, in the presence of 0·5 ml. 0·06 M ATP.

The action of ATP on the G 6-P and 6-PG dehydrogenase activities of liver supernatants has been studied and the results with 6-PG dehydrogenase, plotted according to Lineweaver & Burk (1934), are shown in Fig. 10. This indicates that ATP acts as a competitive inhibitor of 6-PG dehydrogenase since the slopes of the three curves (without ATP and with two different levels of ATP) are different but all cut the $1/V$ axis at the same intercept. These experiments were carried out at pH 9·0 as in the 6-PG dehydrogenase assay procedure. At pH 7·6 there was no evidence of competitive inhibition with G 6-P dehydrogenase. It would appear from these experiments that TPN is more firmly bound to G 6-P dehydrogenase than to 6-PG dehydrogenase.

SUMMARY

1. Both glucose 6-phosphate and 6-phosphogluconate dehydrogenases are present exclusively in the soluble fraction of disintegrated liver.

2. Conditions for the assay of these dehydrogenases in rat liver are described, and a unit of

activity defined as the quantity of enzyme which at 20° reduces 0·01 μmole triphosphopyridine nucleotide per minute.

3. The mean values of glucose 6-phosphate dehydrogenase activity, determined at pH 7·6, and of 6-phosphogluconate dehydrogenase activity, determined at pH's 7·6 and 9·0, were respectively 46 ± 3, 59 ± 8 and 147 ± 10 units/g. liver in male rats. All these levels were consistently higher in females.

4. Both dehydrogenases are activated by magnesium, calcium and manganese and inhibited by mercury. 6-Phosphogluconate dehydrogenase is also inhibited by cupric and zinc ions.

5. Evidence is presented that the activity of 6-phosphogluconate dehydrogenase is dependent on the presence of active sulphydryl groups.

6. Adenosine triphosphate acts as a competitive inhibitor of 6-phosphogluconate dehydrogenase but not of glucose 6-phosphate dehydrogenase.

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The Oxidation of Tryptophan in Pea-seedling Tissues and Extracts

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Tryptophan is known to be a source of nicotinic acid in animals (literature reviewed by Dalgliesh, 1951). There is evidence (Gustafson, 1949; Galston, 1949; Nason, 1950) that this conversion occurs also in some higher plants, but few of the intermediate compounds found in animals have been detected. One intermediate, anthranilic acid, accumulates in the anthers and seedling leaves of a maize mutant (Teas & Anderson, 1951).

Tryptophan may also be a precursor of indolylacetic acid in spinach leaves (Wildman, Ferri & Bonner, 1947), in tobacco ovaries (Wildman & Muir, 1949), in pineapple leaves (Gordon & Nieva, 1949) and in crown-gall tissue of sunflower (Henderson & Bonner, 1952). Conversion to indole, as by *Escherichia coli* (Happold, 1950) has not been reported in higher plants.

In the present work with pea seedlings, no conversion to indolylacetic acid was observed, but some experiments on oxidation of indolylacetic acid are reported. Certain similarities were found to the early stages of tryptophan breakdown in animal tissue.

Most studies of the mechanism of tryptophan oxidation have been made with cells of bacteria or animals previously exposed to an unusually high concentration of tryptophan (Stanier & Tsuchida, 1949; Knox & Mehler, 1950). In such cells adaptive enzymes were produced and tryptophan was