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Direct Oxidation of Glucose-6-phosphate, 6-Phosphogluconate and Pentose-5-phosphates by Enzymes of Animal Origin

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(*Received* 27 *March* 1951)

The existence of a direct oxidative pathway, distinct from the glycolytic route, for the oxidation of glucose-6-phosphate was demonstrated in yeast extracts by Warburg & Christian (1936, 1937), Negelein & Gerischer (1936), Lipmann (1936) and Dickens (1936, 1938*a*). Although the oxidation products were not fully identified, oxidation was considered to proceed by stepwise C-1 and C-2 oxidation and decarboxylation via 6-phosphogluconate and a pentose phosphoric ester, and Dickens (1938*a*) suggested that this was D-ribose-5-phosphate rather than D-arabinose-5-phosphate. Although the latter ester would be expected to arise from D-glucose-6-phosphate (Lipmann, 1936), of the pentose phosphates examined by Dickens (1938*a*, *b*) only D-ribose-5-phosphate was attacked by yeast enzymes at a rate justifying its assumption as an intermediate product, and the occurrence of an inversion was postulated to account for its formation.

Recent work from other laboratories, full details of which have not yet been published, tends to support this hypothesis. Cohen & McNair Scott (1950*a*), using the same yeast system as Dickens (1938*a*), identified chromatographically D-ribose-5-phosphate and possibly D-arabinose-5-phosphate among oxidation products of 6-phosphogluconate, although 50% of the pentose phosphate fraction remained unidentified. Horecker (1950) reported an enzyme preparation from yeast which converted 6-phosphogluconate quantitatively into pentose phosphate. At equilibrium (Horecker & Smyrniotis, 1950) 75% of this pentose phosphate was in the form of D-ribose-5-phosphate.

The occurrence in animal tissues of a similar oxidative pathway was indicated by preliminary experiments of Dickens (1936, 1938*a*) and the oxidation of glucose-6-phosphate by erythrocytes had already been demonstrated by Warburg & Christian (1931). Indirect physiological evidence (see Stotz, 1945) also indicated the occurrence of a non-glycolytic pathway of carbohydrate oxidation. Apart from more recent work of Barkhash & Demianovskaya (1946), Lindberg (1946) and Wainio (1947), confirming its existence, this direct oxidative pathway has not been systematically investigated in animal tissues. The present work was designed with this object in view. A preliminary account has already been published (Dickens & Glock, 1950).

MATERIALS

Hexose monophosphate (HMP). The Ba salt was prepared by the method of Ostern, Guthke & Terszakowec (1936) using starch instead of glycogen as substrate (cf. Fantl & Anderson, 1941). (Found: organic P, 7·5; inorganic P, 0·0; total hexose (Hagedorn & Jensen as modified by Robison & King, 1931), 41·6; aldose (hypoiodite method of Macleod & Robison, 1929), 29·3. Calc. for $C_6H_{11}O_5.PO_4Ba$: P, 7·8; hexose, 44·8%.)

D-*Glucose-6-phosphate* (G-6-P). The Ba salt was kindly synthesized by Dr C. T. Beer by the method of Levene & Raymond (1931). (Found: organic P, 7·5; inorganic P, 0·0; total hexose, 38·3; aldose, 39·2. Calc. for $C_6H_{11}O_5.PO_4Ba$: P, 7·8; hexose, 44·8%.)

6-*Phospho*-D-*gluconate* (6-PG) was a preparation of the Ba salt made by the late Prof. R. Robison and kindly supplied by Dr Marjorie Macfarlane. (Found: organic P, 6·7; inorganic P, 0·0; total hexose (calculated as glucose), 2·0%. Calc. for $(C_6H_{10}O_6.PO_4)_2Ba_3$: P, 6·5%.)

D-*Ribose*-5-*phosphate* (R-5-P). The Ba salt was prepared by hydrolysis of barium inosinate (Boots Pure Drug Co.) according to Levene & Jacobs (1911), and carefully freed from hypoxanthine. (Found: P, 8·9. Calc. for $C_5H_9O_4.PO_4Ba$: P, 8·5%.)

2-*Keto*-D-*gluconate*, D-*arabinose*-5-*phosphate*, *and* D-*xylose*-5-*phosphate* were preparations of the Ba salts already described (Dickens, 1938*a*, *b*).

Fructose-6-*phosphate and fructose*-1:6-*diphosphate* were kindly supplied by Dr C. S. Hanes.

Phenazine methosulphate (m.p. 160–162°) was prepared from phenazine (Light and Co.) by the method of Hillemann (1938).

Coenzyme I (*diphosphopyridine nucleotide*) (CoI) was purchased from Nutritional Biochemical Inc. Analysis (see below) showed that it contained 35·8% CoI and only 0·7% CoII.

Coenzyme II (*triphosphopyridine nucleotide*) (CoII) was prepared from horse liver by the following method kindly communicated to us by Dr C. Liébecq in 1949, and reproduced here with his permission:

'Fresh chilled horse liver (4 kg.) is finely minced through an efficient electrically driven mincer, previously cooled with ice, into cold acetone (20 l.), filtered on Büchner funnels, washed with acetone and dried in the air (yield, 1375 g. dry powder). Successive portions (300 g.) are dropped into boiling 0·5% (w/v) aqueous nicotinamide (1500 ml.), heated on a powerful gas ring so that boiling resumes within 1 min. After 2 min. further boiling, the mixture is cooled quickly, aided by the addition of 750 g. clean ice (temp. below 40° in 1 min.). The suspensions separately prepared in this way are combined, adjusted to pH 8–9 with N-NaOH and maintained at that pH while vigorously stirred for 2 hr. at room temp. The extract is separated in a press, and treated with 40 ml. 100% (w/v) trichloroacetic acid for each litre of extract. The supernatant is siphoned off and the sediment centrifuged. The combined extract is neutralized by the addition of 50% (w/v) KOH, avoiding excess.'

From the above stage Dr Liébecq's method is essentially that of Warburg, Christian & Griese (1935), being *Schritte II und III* of these authors. The final product (about 1 g. from 4 kg. liver) was still not sufficiently pure for our purpose, various batches containing from 2·8–13·8% CoI (for analytical method see below). Consequently, *Schritt III* (Warburg *et al.* 1935) was repeated on a sample which contained 25·6% CoII and 3·1% CoI, when the resulting acetone-precipitated material (0·5 g.) was almost free of CoI. (Found: 34·5% CoII, 0·6% CoI.) Unless otherwise stated, all weights of CoII given in the text refer to amounts of this specimen, and those of CoI to the sample described above. It should be noted that the precipitate of the Ba salt formed by addition of ethanol should be centrifuged immediately at room temperature, otherwise CoI is also precipitated.

Analysis of CoI *and* CoII. The reduced forms of CoI and CoII were determined spectrophotometrically at 340 mμ. in the Hilger Uvispek, using the extinction coefficients of Horecker & Kornberg (1948). CoI was determined by the method of Slater (1950) and CoII according to LePage & Mueller (1949), 'Zwischenferment' being prepared from Löwenbräu (Munich) bottom lager yeast. As an alternative method of reduction of CoII, the dehydrogenase present in liver fractions *C* (see below) obtained at 60–70% saturation with $(NH_4)_2SO_4$, together with its specific substrate, 6-phosphogluconate, proved equally suitable.

Preparation of solutions of sodium salts. The Ba salts were dissolved in the minimum of N-HCl, a slight excess of 10% (w/v) Na_2SO_4 was added, the mixture neutralized with N-NaOH, diluted to make the final substrate concentration either 0·1 or 0·05M and centrifuged. Solutions of the Na salts were stored at −10°.

METHODS

Determination of dehydrogenase activity: (*a*) *Decolorization of brilliant cresyl blue.* A modification of Friedemann & Hollander's (1942) method was adopted, methylene blue being replaced by brilliant cresyl blue which is a more effective hydrogen acceptor in these systems (Dickens & McIlwain, 1938). To 1·0 ml. 0·1M-(sodium) phosphate buffer (pH 7·4), 0·5 ml. 0·001M brilliant cresyl blue, 0·1 ml. 0·05M-substrate, 0·2 ml. (0·2 mg.) CoII and 0·7 ml. water, contained in a test tube and previously heated to 37°, were added rapidly in succession 2·5 ml. agar in phosphate buffer (Friedemann & Hollander), liquefied by warming to 42°, and 1·0 ml. enzyme solution at 37°. After mixing, the reaction mixture was solidified by placing the tubes in a freezing mixture of salt and ice and, after leaving for an additional 10 min. in a refrigerator, the decolorization times were determined at 37°. Suitable blanks (without substrate and/or coenzyme) were included in each series. The rapidity and ease of this method makes it especially useful for preliminary determinations of dehydrogenase activity in tissue extracts and also for following the CoII activity during stages in the preparation of this coenzyme.

(*b*) *Decolorization of* 2:6-*dichlorophenolindophenol.* A modification of the method of Haas (1944) was used. 0·015M-Phosphate buffer (5 ml., pH 7·4), 0·2 ml. substrate (usually 0·1M), 0·2 ml. CoII (or CoI) solution (usually containing 0·2 mg. coenzyme), and 1·0 ml. of a 1 in 10 dilution of the stock dye solution ($2·76 \times 10^{-3}$M, prepared by dissolving 20 mg. 2:6-dichlorophenolindophenol in 25 ml. 0·015M-phosphate, pH 7·4) were introduced into a 1 cm. absorptiometer cell and mixed. At t_0 min. the enzyme solution (usually 0·25 or 0·5 ml., diluted to 1·0 ml.) was added with mixing. The optical densities were read at intervals timed with a stopwatch in a Hilger Spekker absorptiometer using an Ilford spectrum red Filter no. 608. Usually two readings per minute were taken and the course was linear for at least 10 min. Blanks were run without coenzyme and substrate for each series. This method enables the initial rate of reduction to be determined, and is therefore valuable in comparative tests of activity. It is especially useful in the determination of concentration-activity relationships and for the evaluation of specificity of coenzyme and substrate.

With this method the dehydrogenase activity is expressed as μmmol. dye reduced in 1 min. (1 μmmol. or 10^{-9} g.mol./min. is equivalent to the transfer of 1·33 μl. H_2/hr.).

(*c*) *Determination of oxygen uptake.* This was determined manometrically at 37° with O_2 as the gas phase. The main part of each Warburg manometer vessel usually contained 1·0 ml. enzyme solution, 0·1 ml. 0·25M-phosphate buffer (pH 7·0), 0·2 ml. (0·2 mg.) CoII solution, 0·2 ml. 0·05M-substrate and 0·6 ml. water. The side bulb had 0·2 ml. aqueous phenazine methosulphate (0·3 mg.) as carrier

(Dickens & McIlwain, 1938). The centre well contained 0·2 ml. 20% (w/v) KOH as CO_2 absorbent, or in experiments with cyanide a suitable KOH-KCN mixture. After equilibration, the reaction was started by tipping in the carrier.

(*d*) *Spectrophotometric determination.* The rate of reduction of Co II was followed at 340 mμ. in 2 cm. cells in the Hilger Uvispek spectrophotometer. This method was used for determining coenzyme specificity. In the case of 6-phosphogluconate dehydrogenase, the reaction mixture consisted of 0·25 ml. (approx. 2·5 mg. dry wt.) liver fraction *C* (see below), 0·1 ml. 0·1 M-6-phosphogluconate and a solution containing 1 mg. coenzyme made up to a total volume of 4·0 ml. with 0·01 M-phosphate buffer (pH 7·4). For glucose-6-phosphate dehydrogenase activity, 0·5 ml. (approx. 4 mg. dry wt.) liver fraction *B* (see below) and 0·1 ml. 0·1 M-glucose-6-phosphate were used. The differences in densities in the presence and absence of substrates were taken as a measure of dehydrogenase activity. Suitable blanks omitting coenzyme and substrate were included.

Phosphatase activity. 1·0 ml. enzyme solution, 0·2 ml. 0·05 M-substrate and 3·8 ml. Michaelis (1931) veronal acetate buffer (pH 7·4) were incubated at 37°, and samples removed at 0, 30, 60 and 120 min. for determination of inorganic phosphate (Fiske & Subbarow, 1925) after trichloroacetic acid precipitation. Although the above conditions are presumably not optimal for phosphatase action since no Mg salts were added, they were chosen since dehydrogenase activity was measured under similar conditions. Glucose-6-phosphate, fructose-1:6-diphosphate, 6-phosphogluconate and ribose-5-phosphate were used as substrates.

Aldolase activity. The method of Herbert, Gordon, Subrahmanyan & Green (1940) was employed, except that veronal acetate buffer was used instead of borate buffer and, in addition to hexose diphosphate, ribose-5-phosphate was tested as a substrate. The liberation of free phosphate was also followed.

Phosphokinase activity. This was determined manometrically with glucose, gluconate and 2-ketogluconate as substrates by measuring the anaerobic evolution of CO_2 on addition of adenosinetriphosphate (ATP) in the presence of bicarbonate with 5% (v/v) CO_2 in N_2 as the gas phase. The main part of each Warburg manometer vessel contained 1·0 ml. enzyme solution and 1·0 ml. of a solution containing 0·02 M-$MgCl_2$ and 0·06 M-$NaHCO_3$ and the side bulb 0·1 ml. 0·1 M-substrate (Na salt) and 0·2 ml. 0·05 M-ATP (Na salt). Since the adenosinetriphosphatase (ATP-ase) activity was almost negligible, fluoride was omitted because it was not known how it might affect any phosphorylation of gluconate and 2-ketogluconate.

Preparation of tissue extracts. The fresh tissue was cooled to 0° and disintegrated thoroughly in 5 vol. ice-cold 0·01 M-phosphate buffer (pH 7·4) in the Atomix or Nelco homogenizer (supplied by Measuring and Scientific Equipment Ltd., London, S.W. 1), according to the quantity of tissue used. The homogenate was centrifuged (all centrifugation of enzymes was at 0–4°), the supernatant was siphoned off and brought to pH 4·6 at 4° with 2 N-acetic acid. After removal of the precipitate (inactive in dehydrogenating glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate), the supernatant was brought to pH 7·0 in the cold with 2 N-NaOH, and the inactive precipitate which formed was again centrifuged off. The remaining clear solution, the volume of which was approximately that of the original homogenate, is called 'crude extract' below, and was used for rough comparison of dehydrogenase activities by the brilliant cresyl blue-agar method and as a starting material for the ammonium sulphate fractionations.

Fractional precipitation with ammonium sulphate. Sufficient solid $(NH_4)_2SO_4$ was stirred with the crude extract at pH 7 to give the desired percentage saturation at 4°. The resulting precipitates were collected by centrifugation and dialysed in cellophan tubing at 4° overnight against glass-distilled water. After dialysis the clear filtered solutions were diluted with water to one-third to one-fifth of the original volume of crude extract and stored at −10°.

If the crude extract contained glycogen this was removed by incubation with a little saliva at 37° followed by dialysis. Rabbit-liver extracts usually had much glycogen. Fasting the animals overnight was advantageous.

Occasionally, after the first addition of ammonium sulphate, the suspension was heated to 50° for 15 min. with a view to stabilizing the enzymes (Warburg & Christian, 1937), but as no increased stability was observed, heating was generally omitted.

Nucleic acid as enzyme precipitant. Fractional precipitation of the glucose-6-phosphate and 6-phosphogluconate dehydrogenases by yeast nucleic acid, in a final concentration of 0·33 mg./ml. (Kubowitz & Ott, 1942), was attempted over a pH range of 4·5–7·0.

RESULTS

Oxidation of glucose-6-phosphate and 6-phosphogluconate

The dehydrogenase activity of 'crude tissue extracts'. Dehydrogenase activity was tested by the brilliant cresyl blue method using as substrates hexose monophosphate and 6-phosphogluconate. Although the decolorization rates were found to be reasonably proportional to the amount of extract taken, the results are only semiquantitative because of some reduction of dye in absence of added substrate. This was always appreciable and was very marked with liver, presumably due to the enzymic breakdown of glycogen to glucose-6-phosphate. The blank values observed without substrate were considerably diminished when the livers of animals fasted for the previous 24 hr. were used. Dialysis was even more effective (Table 1). The reduction times (Table 1) show clearly that liver, liver carcinoma (induced by feeding *p*-dimethylaminoazobenzene to rats) and kidney, have very active Co II-coupled dehydrogenases for both hexose monophosphate and 6-phosphogluconate. Extracts of brain were also active, though less so than liver and kidney extracts. Skeletal muscle and the Rous fowl sarcoma were relatively feebly active towards both substrates. These estimations were of a preliminary nature, and the study of the distribution of the enzymes in normal and tumour tissue will be continued. The activities recorded in Table 1 are not due to adventitious blood (Warburg & Christian, 1931), since blood diluted to a haemoglobin content similar to that present in the above tissue extracts and carried through the same precipitations showed negligible dehydrogenase activity.

Table 1. *Hexose monophosphate and 6-phosphogluconate dehydrogenase activities of tissue extracts*

(Decolorization of brilliant cresyl blue with 1 ml. 'crude extract' and 0·2 mg. Co II. In the absence of Co II (or with Co I) reduction was almost negligible. The numbers in brackets show the number of samples of tissue tested.)

Tissue or organ	Substrate: None	Hexose monophosphate	6-Phosphogluconate
	Mean decolorization time (min.)		
Rat liver (9)	29	13	12
Rat liver (4)*	145	12	12
Rat liver (1)†	>600	19	5
Rabbit liver (4)	56	19	21
Horse liver (1)	105	39	46
Rat kidney (1)	117	67	62
Rabbit kidney (1)	150	41	36
Rat brain (1)	330	150	135
Rabbit brain (1)	Approx. 450	88	76
Rat skeletal muscle (2)	280	125	130
Rabbit skeletal muscle (1)	230	196	166
Rous fowl sarcoma (1)	Approx. 510	300–330	300–330
Rat-liver carcinoma (1)	65	40	11

* Animals previously fasted 24 hr.
† After dialysis.

Fractional precipitation of the dehydrogenases from 'crude extracts' of liver. Owing to its high dehydrogenase activity, liver was chosen for the attempted separation of the dehydrogenases. The following fractions were precipitated by successively adding ammonium sulphate to give the percentage saturation included in brackets:

Fractions *O* (0–40 %), *A* (40–50 %), *B* (50–60 %), *C* (60–70 %).

Fractions precipitated by 70–85 and 85–100 % saturation were also collected, but the 85–100 % fraction showed no activity towards either hexose monophosphate or 6-phosphogluconate, and the 70–85 % fraction was feebly active towards the latter substrate only. Fraction *O* was also almost inactive, and fractions *A*, *B* and *C*, which contained nearly all the dehydrogenase activity, were therefore used in all subsequent experiments, either singly or as a combined 40–70 % saturation fraction, which is designated (*A* + *B* + *C*) below. The mean dry weights of fractions *A*, *B* and *C* were respectively 5·5, 6·5 and 4 % of the dry weight of liver taken.

Table 2 shows the dehydrogenase activities of the various fractions towards hexose monophosphate and 6-phosphogluconate as determined by the brilliant cresyl blue-agar or the dichlorophenolindophenol method. Co II was present in all the recorded experiments, and since the blanks with substrate but without Co II all showed negligible activity they are omitted.

The results show that the distribution of the two dehydrogenases is the same for rat and horse liver, and are summarized below:

	Fraction *A*	*B*	*C*
Hexose monophosphate dehydrogenase	+	+ +	−
6-Phosphogluconate dehydrogenase	+	+ +	+ + +

Table 2. *Fractional precipitation of liver dehydrogenases from 'crude extract' with ammonium sulphate*

(HMP = hexose monophosphate; 6-PG = 6-phosphogluconate.)

Species	Method*	Substrate	Saturation with $(NH_4)_2SO_4$ (%): 40–50	50–60	60–70	70–85
			Time of decolorization (min.)			
Rat	*a*	None	∞	∞	∞	∞
		HMP	66	37	∞	∞
		6-PG	270	36	10	103
Horse	*a*	None	∞	∞	∞	∞
		HMP	69	51	∞	∞
		6-PG	88	51	39	87
			mμmol. dye reduced/min.			
Rat	*b*	None	1·0	2·3	0·8	—
		HMP	10·9	14·3	1·2	—
		6-PG	7·1	16·0	18·7	—
Rabbit	*b*	None	0	1·7	15·6†	—
		HMP	0	6·9	80·4	—
		6-PG	0	4·4	82·2	—

* Method *a*. Time of decolorization of brilliant cresyl blue. Method *b*. Rate of reduction of 2:6-dichlorophenolindophenol (see Methods section).
† This high blank was due to the presence of glycogen and fell to 4·0 after digestion with saliva.

Table 3. *Substrate specificity of rat-liver fractions*

(HMP = hexose monophosphate, G-6-P = glucose-6-phosphate, 6-PG = 6-phosphogluconate, G-1-P = glucose-1-phosphate.)

Fraction	Dry wt. enzyme (mg.)	Substrate: None	HMP	G-6-P	6-PG	Glycogen	Glucose	G-1-P	Gluconate	2-Ketogluconate
		Dehydrogenase activity at 20° (μmmol./min.)*								
C, preparation 1	2·8	1·0	1·0	—	12·4	—	—	—	1·5	1·2
C, preparation 2	2·65	0	0	—	17·2	—	0	—	0·5	0·2
A + *B* + *C*, preparation 1	11·1	1·2	20·7	21·2	84·0	6·0	1·8	4·4	4·5	6·0
A + *B* + *C*, preparation 2	8·03	2·2	19·7	20·2	60·6	2·4	2·4	2·9	2·8	2·4

* μmmol. 2:6-dichlorophenolindophenol reduced/min. (see Methods section).

Fraction *C*, though richest in 6-phosphogluconate dehydrogenase, is completely free from the hexose monophosphate dehydrogenase, showing that in animal tissues, as in yeast (Dickens, 1936), these two are different enzymes. Separation of these two dehydrogenases in fraction *B* has not yet been achieved and other methods, such as electrophoresis, are being investigated. Preliminary experiments indicated that nucleic acid does not precipitate either dehydrogenase, but since many other enzymes including animal hexokinase (Glock, 1947) are precipitated, this method may prove useful for further purification of the dehydrogenases.

Although, as has already been stated, the distribution of these two dehydrogenases is similar in the rat and horse it is somewhat different in the rabbit. In the rabbit there is no clear separation of the two dehydrogenases and fraction *C* is rich in both enzymes. For this reason, rat or horse liver was used in subsequent experiments.

Substrate specificity. The specificity of the dehydrogenase preparations was tested in the presence of 0·2 mg. Co II on the combined (*A* + *B* + *C*) fraction, which has practically the whole activity, and also on fraction *C* which attacks 6-phosphogluconate but not glucose-6-phosphate. There was negligible activity in absence of substrate. Marked substrate specificity was shown by both liver fractions (Table 3).

The whole system (*A* + *B* + *C*) vigorously oxidizes Embden ester (hexose monophosphate), glucose-6-phosphate and 6-phosphogluconate. Very weak activity was shown by preparation 1 (Table 3) towards glycogen, glucose-1-phosphate, gluconate and 2-ketogluconate, but another preparation (2) had negligible activity towards these substrates. It is thus unlikely that either glucose-6-phosphate or 6-phosphogluconate dehydrogenases attacks any of these substrates. Glucose itself was not oxidized.

Fraction *C*, although highly active towards 6-phosphogluconate, did not oxidize hexose monophosphate, glucose, gluconate or 2-ketogluconate.

Coenzyme specificity. This was determined by following the reduction of Co I and Co II spectrophotometrically at 340 mμ. (see Methods section). The results (Fig. 1) show a vigorous and steady reduction of Co II by both dehydrogenase systems. With Co I there is a very brief initial reduction period which quickly (2–3 min.) ceases. The Co II content of the Co I (0·7 %) is sufficient to account for half the slight and transient activity found in the presence of Co I. The residual activity catalysed by Co I is negligible, hence both the glucose-6-phosphate and 6-phosphogluconate dehydrogenases are completely Co II specific.

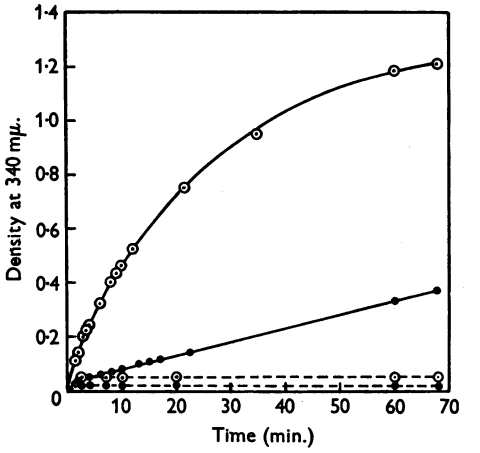

Fig. 1. Coenzyme specificity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases of horse liver measured spectrophotometrically at 340 mμ. Room temperature; for details see Methods section. ⊙, 6-Phosphogluconate; ●, glucose-6-phosphate; ——, + Co II; – – –, + Co I.

Confirmation of the Co II specificity of these two systems is provided by O_2-uptake measurements. These are shown in Fig. 2, in which similar proof of the Co II specificity of ribose-5-phosphate oxidation has been included for convenience. Co I is completely ineffective in all three systems. Fig. 2 also shows that fructose-6-phosphate is oxidized in the presence of Co II almost as rapidly as glucose-6-

phosphate, whereas fructose-1:6-diphosphate is not attacked.

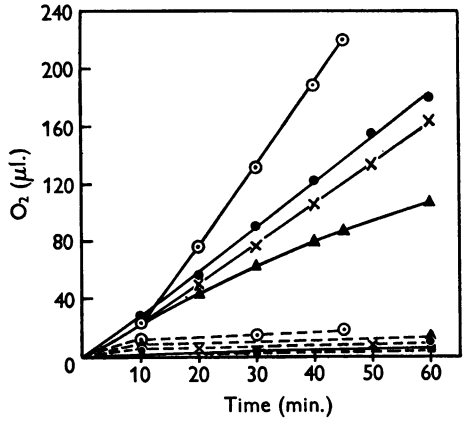

Fig. 2. Coenzyme specificity determined by O_2 uptake method. Total volume 2·5 ml. including 1 ml. horse-liver fraction ($A+B+C$), 0·2 ml. 0·05 M-substrate, with or without the addition of 0·2 mg. Co II or Co I. 37°; for details see Methods section. ⊙, 6-Phosphogluconate; ●, glucose-6-phosphate; ▲, ribose-5-phosphate; ×, fructose-6-phosphate; ■, fructose-1:6-diphosphate; ——, +Co II; – – –, +Co I.

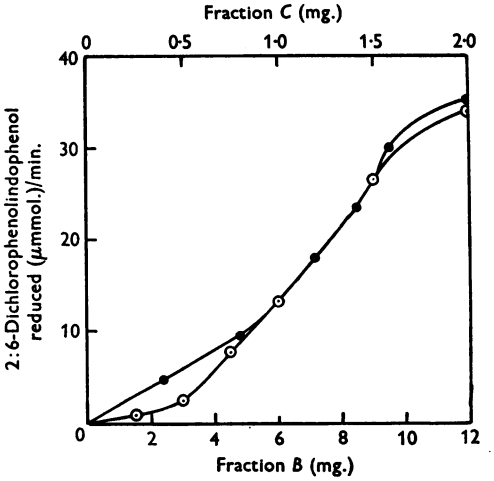

Fig. 3. Effect of enzyme concentration on the rate of dehydrogenation of glucose-6-phosphate and 6-phosphogluconate. Rate of reduction of 2:6-dichlorophenolindophenol (µmmol./min.) at 20°; total volume 7·4 ml. including 1 mg. Co II, 0·2 ml. 0·05 M-substrate and varying amounts of rat-liver fractions B and C. For details see Methods section. ●—●, Fraction B; substrate glucose-6-phosphate. ⊙—⊙, fraction C; substrate 6-phosphogluconate.

Enzyme kinetics. The 2:6-dichlorophenolindophenol reduction method was used since this permits readings of the initial rates of reaction. The concentrations of substrate, Co II and enzyme were varied, and the results are shown graphically in Figs. 3–5.

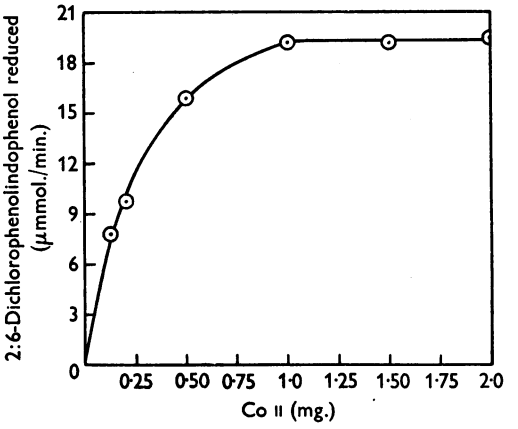

Fig. 4. Effect of Co II concentration on 6-phosphogluconate dehydrogenase activity. Rate of reduction of 2:6-dichlorophenolindophenol at 20°; 0·25 ml. rat-liver fraction ($A+B+C$), 0·1 ml. 0·05 M-6-phosphogluconate and varying amounts of Co II (purity 34·5 %). Total vol. 7·4 ml. For details see Methods section.

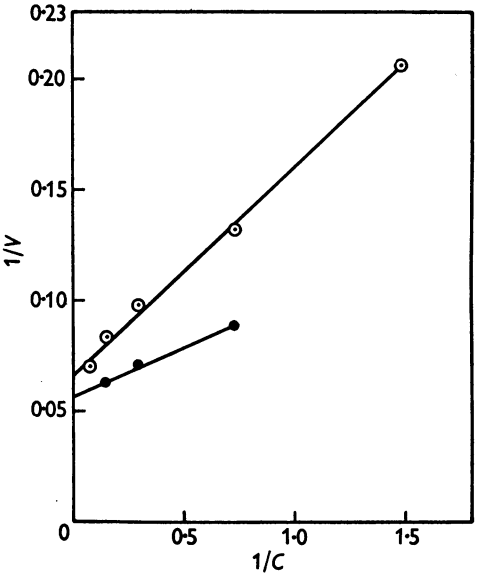

Fig. 5. Effect of substrate concentration on glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities. Rate of reduction of 2:6-dichlorophenolindophenol at 20°. Total vol. 7·4 ml. including 0·2 mg. Co II, and the following: ●—●, glucose-6-phosphate dehydrogenase (1 ml. rat-liver fraction B); ⊙—⊙, 6-phosphogluconate dehydrogenase (0·25 ml. rat-liver fraction C). V = Initial reaction velocity (µmmol. 2:6-dichlorophenolindophenol reduced/min.). C = Molar concentration of substrate. Values for $1/C$ on abscissa to be multiplied by 10^4 for glucose-6-phosphate and by 10^5 for 6-phosphogluconate dehydrogenase systems.

The enzyme concentration-activity curves (Fig. 3) are closely similar for the hexose monophosphate and 6-phosphogluconate dehydrogenases, although the activity per mg. of preparation is six times as high for the latter in the example shown. Calculated on the dry weight of enzyme taken, the Q_{H_2} values at 20° are respectively 3·4 and 22·5 μl. H_2 transferred/mg./hr., which are reasonably high considering that these preparations are certainly impure.

The effect of changes in Co II concentration on the activity of 6-phosphogluconate dehydrogenase (Fig. 4) shows that 1·0 mg. coenzyme of purity 34 % is sufficient to saturate the system contained in a total vol. of 7·4 ml., under the conditions stated.

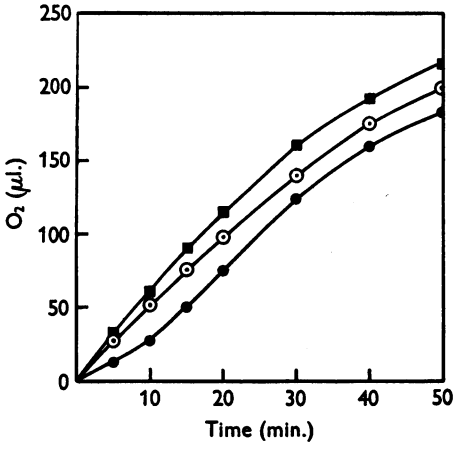

Fig. 6. Effect of cyanide on the oxidation of 6-phosphogluconate. 1 ml. rat-liver fraction *C*, 0·2 mg. Co II and 0·1 ml. 0·1 M-6-phosphogluconate. Total volume 2·5 ml.; O_2 uptake measured at 37°. ■—■, Final concentration of cyanide 0·01 M; ⊙—⊙, final concentration of cyanide 0·004 M; ●—●, no cyanide.

The effect of changes in substrate concentration on the initial reaction rates with both dehydrogenases is shown in Fig. 5. Under the conditions of these experiments 50 % of maximum initial velocity is reached with $7·5 \times 10^{-5}$ M-hexose monophosphate and $1·5 \times 10^{-5}$ M-6-phosphogluconate.

Time course of oxidation of glucose-6-phosphate and 6-phosphogluconate. Whereas the O_2 uptake with glucose-6-phosphate was always approximately linear, a slight lag period of about 15 min. duration was generally evident with 6-phosphogluconate oxidation (see Fig. 2). In presence of cyanide (0·01–0·004 M neutralized NaCN) this lag period is abolished, so that the initial rate was almost linear with 6-phosphogluconate and in presence of cyanide the O_2 uptake in the first 10 min. was often double that of the control without cyanide (Fig. 6). The addition of cyanide strongly promotes 6-phosphogluconate oxidation by the similar enzyme system from yeast (Dickens, 1938*a*), an effect possibly due to cyanhydrin formation with a ketonic oxidation product.

In presence or absence of cyanide, the rate of 6-phosphogluconate oxidation falls off steadily with time, and when the concentration of substrate is kept low in relation to enzyme, the end point (without cyanide) approximates to 1·5 mol. O_2/mol. phosphogluconate present (Fig. 7). This suggests that the enzyme fraction *C* is capable of oxidizing phosphogluconate to a stage beyond a pentonic acid under these conditions, the above O_2 uptake corresponding with that required for formation of a tetrose. However, the end point is not very definite and this problem can only satisfactorily be investigated by further fractionation of *C*.

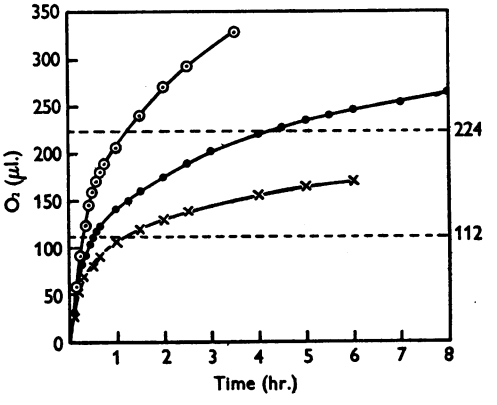

Fig. 7. Prolonged experiment on the course of oxygen uptake by the 6-phosphogluconate dehydrogenase system. Total volume 2·5 ml. including 1 ml. rat-liver fraction *C*, 1·0 mg. Co II and 6-phosphogluconate. O_2 uptake measured at 37°. ●—●, 0·1 ml. 0·1 M-substrate (calc. for 1 mol. O_2/mol. substrate = 224 μl.); ×—×, 0·05 ml. 0·1 M-substrate (calc. for 1 mol. O_2/mol. substrate = 112 μl.); ⊙—⊙, 0·1 ml. 0·1 M-substrate (a more active enzyme).

Since the slowing of oxidation in these experiments might have been due to accumulation of intermediates, e.g. a ketonic acid, the effect of cocarboxylase was tested. However, the addition of 1 mg. cocarboxylase, together with 0·1 mg. Mg^{++} and 0·1 mg. Mn^{++} had no effect on the course of oxidation of 6-phosphogluconate. The rate of reaction was also unaffected by the addition of Mg and Mn alone, contrary to the experience of Horecker (1950) with the yeast enzyme.

Oxidation of pentose phosphates

The experiments in this section are only preliminary and for brevity we have referred to the enzyme preparation which brings about the reduction of dyes in presence of pentose phosphate as pentose

Table 4. *Distribution of* D-*ribose-5-phosphate dehydrogenase and a comparison of its activity with that of glucose-6-phosphate and 6-phosphogluconate dehydrogenases*

(Brilliant cresyl blue reduction times at 37°; see Methods section.)

	Substrate				
	None	HMP	6-PG	R-5-P	R-5-P
	Coenzyme				
	Co II	Co II	Co II	None	Co II
Source of enzyme	Decolorization time (min.)				
'Crude extract' of					
Rat liver*	—	—	—	>240	97
Horse liver	105	39	46	>180	68
Rat skeletal muscle	240	115	105	>300	160
Rat kidney	—	—	—	>360	245
'Fraction $(A+B+C)$' from:					
Rat liver	>350	16	12	>350	46
Horse liver	∞	49	51	∞	70

* Average of seven batches.

phosphate dehydrogenase, although it is recognized that a more complex mechanism of reduction, possibly involving preliminary fission of the 5-carbon chain, is a possibility.

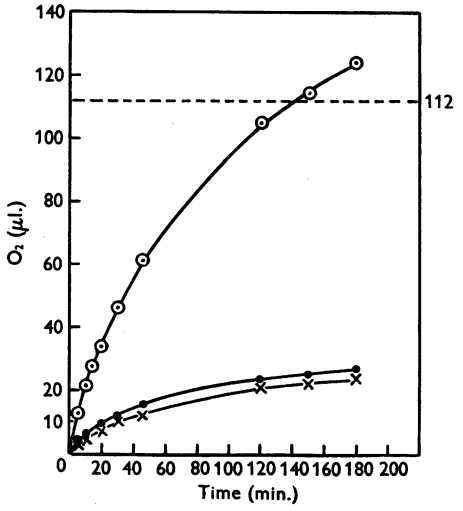

Fig. 8. Oxidation of pentose phosphates. Total volume 2·5 ml. including 1·5 ml. rat-liver fraction $(A+B+C)$, 0·2 mg. Co II and 0·1 ml. 0·05 M-substrate. O_2 uptake measured at 37°. ⊙—⊙, D-Ribose-5-phosphate; ●—●, D-arabinose-5-phosphate; ×—×, D-xylose-5-phosphate. (Calc. for 1 mol. O_2/mol. substrate = 112 μl.)

Distribution of ribose-5-phosphate dehydrogenase in animal tissues. Table 4 shows the results of a number of assays by brilliant cresyl blue reduction, made either on 'crude extract' or the $(A+B+C)$ fraction. Liver again appears to be the richest source of enzyme. In all cases the dehydrogenating activity towards ribose-5-phosphate is somewhat less than that for glucose-6-phosphate and 6-phosphogluconate, determined on the same preparations. This liver fraction $(A+B+C)$ contains most of the ribose-5-phosphate dehydrogenating activity and gives a low reduction rate in absence of Co II.

The ribose-5-phosphate dehydrogenating system is less stable than glucose-6-phosphate and 6-phosphogluconate dehydrogenases. Even when stored at −10°, the ability of the enzyme to oxidize ribose-5-phosphate falls steadily and is lost in a few weeks.

Specificity towards pentose phosphates. Among the pentose-5-phosphates tested, only D-ribose-5-phosphate was oxidized rapidly, the oxidation of D-arabinose-5-phosphate and D-xylose-5-phosphate being comparatively slight (Fig. 8). This agrees with the behaviour of yeast enzymes (Dickens, 1938*a*, *b*). Unphosphorylated D-ribose was not appreciably attacked in the presence of Co II by liver fractions $(A+B+C)$ or C.

Coenzyme specificity of ribose-5-phosphate oxidation. This was determined both spectrophotometrically and by measuring the O_2 uptake. The results of the O_2 uptake measurement are shown in Fig. 2 and indicate that oxidation of ribose-5-phosphate is completely Co II specific.

Fig. 9 shows the results of the spectrophotometric measurement of reduction of Co II at 20 and 37°. For the 37° results, the reaction mixture was incubated at 37° for 15, 30 and 50 min. and then rapidly cooled to room temperature (20°) for the readings. It will be seen that the reduction of Co II is very slow at 20°, only approx. 0·2 mg. being reduced after 17 hr. This is in marked contrast to the rapid reduction of Co II by the glucose-6-phosphate and 6-phosphogluconate systems even at room temperature (Fig. 1). At 37°, however, the rate of reduction of Co II by the ribose-5-phosphate de-

hydrogenase system is rapid. Co I was not reduced by this system.

Time course of ribose-5-phosphate oxidation. Although no true end point was reached, more than 1 mol. O_2/mol. substrate was consumed (see Fig. 8).

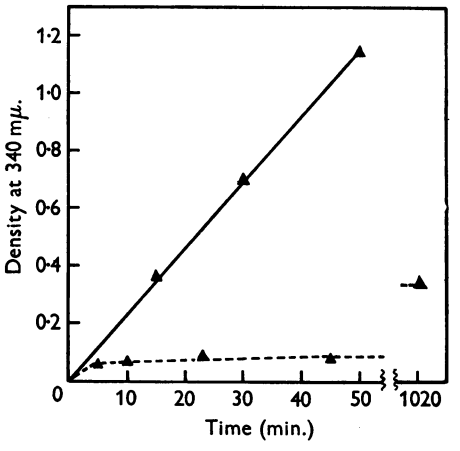

Fig. 9. Rate of reduction of Co II by ribose-5-phosphate dehydrogenase system measured spectrophotometrically at 340 mμ. Total volume 4·0 ml. including 1 ml. horse-liver fraction ($A+B+C$), 1 mg. Co II and 0·2 ml. 0·05 M-ribose-5-phosphate. Optical density at 340 mμ. measured at room temperature (20°) after incubation for different times at either 20° (▲ - - - ▲) or 37° (▲—▲).

Distribution of ribose-5-phosphate oxidizing activity in liver fractions. The activity was determined by measurement of the O_2 uptake with horse-liver fractions *O*, *A*, *B* and *C* (see p. 84) and also with combinations of these fractions, after it was found that more than one fraction was necessary for pentose phosphate oxidation (Table 5). This is clearly shown by the fact that with preparations I and II no O_2 was taken up by fractions *O*, *A*, *B* or *C* separately, but a fairly vigorous oxidation occurred in the presence of combined extracts *A*, *B* and *C*. By repeating this experiment with the combinations ($A+B$), ($A+C$) and ($B+C$), it was found that ($B+C$) together had most of the activity of ($A+B+C$).

The above experiments distinguish this dehydrogenase from those for 6-phosphogluconate (oxidized by fractions *B* and *C* separately) and glucose-6-phosphate (oxidized by fraction *B*).

In contrast to these results it was found that with another horse-liver preparation (III) fractions *A* and *C* alone were inactive, all the activity residing in fraction *B*. Since the results with preparations I and II indicated that more than one factor is necessary for the oxidation of ribose-5-phosphate, fraction *B* of Exp. III was reprecipitated with $(NH_4)_2SO_4$. The precipitates separating between 50–55, 55–60 and 60–65% saturation (called, respectively, *B*1, *B*2 and *B*3) were collected separately and their activities tested. Table 5 shows that *B*1, *B*2 and *B*3 were all inactive when tested alone. All combinations except (*B*2+*B*3), however, were active, most of the activity being found in (*B*1+*B*3). This indicates that in this experiment *B*1 is essential for ribose-5-phosphate oxidation and, in addition, another factor present in both *B*2 and *B*3 but chiefly in *B*3. Fraction *B*1 of Exp. III thus corresponds with fraction *B* of Exps. I and II and (*B*2+*B*3) of Exp. III with *C* of Exps. I and II. There is evidently a narrow range of $(NH_4)_2SO_4$ concentration required to give adequate separation of these fractions.

Table 5. *Enzyme fractions necessary for the oxidation of* D-*ribose-5-phosphate*

(O_2 uptake measurements at 37°. Total volume 2·5 ml. including 0·5 ml. horse-liver fractions (see text, p. 84), 0·2 mg. Co II and 0·1 ml. 0·1 M-ribose-5-phosphate.)

Preparation no.	Liver fraction	O_2 uptake (μl./60 min.)
I	*O*	0
	A	0
	B	0
	C	0
	($A+B+C$)	55
II	($A+B$)	29
	($A+C$)	55
	($B+C$)	73
	($A+B+C$)	87
III	*A*	0
	B	103
	C	0
	*B*1	0
	*B*2	0
	*B*3	0
	(*B*1+*B*2)	50
	(*B*1+*B*3)	84
	(*B*2+*B*3)	0
	(*B*1+*B*2+*B*3)	90

Further studies on the liver fractions

Aldolase activity. The aldolase activity of horse-liver fractions was tested towards both fructose-1:6-diphosphate and ribose-5-phosphate, using the liberation of alkali-labile phosphate as a measure of activity. It was found necessary to prolong the preliminary incubation period beyond the 4 min. used by Herbert *et al.* (1940) in order to obtain measurable rates of activity since, in accordance with Sibley & Lehninger (1949), we found the aldolase activity of liver to be considerably less than that of skeletal muscle. In addition to determining the alkali-labile phosphate, the liberation of free phosphate was also followed, since this is presumably due to phosphatase activity and should be subtracted from the 'total' phosphate (alkali-labile + free phosphate) in order to obtain a true

value for alkali-labile phosphate. Fig. 10 shows the liberation of 'total' and free phosphate from fructose-1:6-diphosphate and ribose-5-phosphate by a horse-liver fraction ($A+B+C$). This liver fraction shows marked aldolase activity with fructose-1:6-diphosphate as substrate, but the results are less clear cut with ribose-5-phosphate. After a 10 min. incubation period it would appear that the aldolase activity towards ribose-5-phosphate is approximately the same as towards fructose-1:6-diphosphate, but after this the liberation of phosphate from ribose-5-phosphate can be accounted for entirely by liberation of free phosphate.

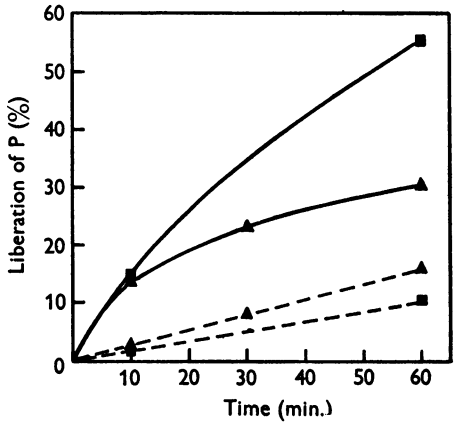

Fig. 10. Aldolase and phosphatase activity of horse-liver fraction ($A+B+C$). 2 ml. horse-liver fraction ($A+B+C$), 1 ml. veronal-acetate buffer (pH 7·4), 1 ml. 0·25 M neutralized sodium cyanide and 1 ml. 0·05 M-substrate incubated at 37° and alkali-labile and free phosphate determined at intervals. ■, Fructose-1:6-diphosphate; ▲, ribose-5-phosphate; ——, total phosphate (alkali-labile + free); – – –, free phosphate.

It was shown in the previous section that oxidation of ribose-5-phosphate by liver is dependent on the presence of at least two factors. Since it is possible that ribose-5-phosphate is first split by aldolase into a C_3 and a C_2 fragment before any oxidation can occur, the aldolase activities of liver fractions $B1$, $B2$ and $B3$ (see Table. 5) were determined. These results are shown in Fig. 11. It is obvious that $B3$ contains most of the aldolase activity with fructose-1:6-diphosphate as substrate. In the case of ribose-5-phosphate, however, most phosphate is liberated by $B1$ and almost the whole of this can be accounted for by the direct liberation of free phosphate. It would thus appear that if indeed ribose-5-phosphate is split at all by a liver aldolase, this enzyme is different from that acting on fructose-1:6-diphosphate.

To exclude the possibility that what was measured as 'free' phosphate, in the case of ribose-5-phosphate, was in fact due to the formation of an acid-labile ester (e.g. ribose-1-phosphate), 'free' phosphate was estimated both by the method of Fiske & Subbarow (1925) and at pH 4 according to Lowry & Lopez (1946). The results, however, were identical.

Phosphatase activity. Fraction ($A+B+C$) of rat liver, which contains almost all the dehydrogenase activity towards glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate, was tested (see Methods section) and found to contain no phosphatase capable of hydrolysing any of these substrates under the conditions of the dehydrogenase estimations, i.e. at the same pH (7·4) and substrate

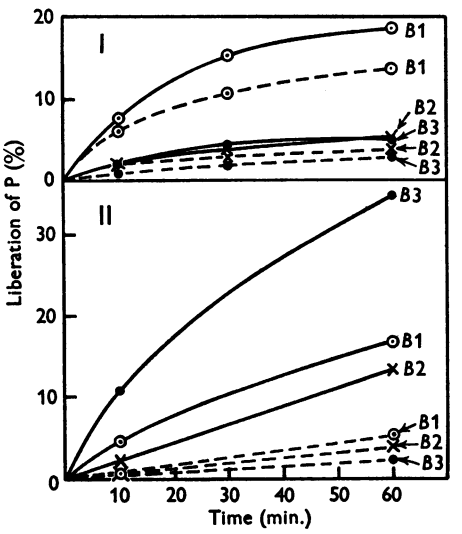

Fig. 11. Aldolase and phosphatase activity of fractions $B1$, $B2$ and $B3$. 2 ml. horse-liver fraction $B1$, $B2$ or $B3$, 1 ml. veronal-acetate buffer (pH 7·4), 1 ml. 0·25 M-cyanide and 1 ml. 0·05 M-substrate incubated at 37° and alkali-labile and free phosphate determined at intervals. I, Ribose-5-phosphate; II, fructose-1:6-diphosphate; ⊙, fraction $B1$ (50–55% saturation with $(NH_4)_2SO_4$); ×, fraction $B2$ (55–60% saturation with $(NH_4)_2SO_4$); ●, fraction $B3$ (60–65% saturation with $(NH_4)_2SO_4$); ——, alkali-labile + free phosphate; – – –, free phosphate.

concentration. No inorganic phosphate was liberated after 120 min. incubation at 37°. A similar horse-liver fraction, however, showed some activity towards both ribose-5-phosphate and fructose-1:6-diphosphate (see Fig. 10).

Since glucose-6-phosphatase has been shown to be precipitated from liver homogenates at pH 5·0 (Duve, Berthet, Hers & Dupret, 1949), this enzyme was presumably removed with the precipitate formed at pH 4·6 during the preparation of our liver extracts. Fructose-1:6-diphosphatase activity, however, is known to be stable and to remain in solution at this pH (Roche & Bouchilloux, 1950).

The effect of inorganic phosphate on the oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate. To study the need for inorganic phosphate the oxidative (or dehydrogenase) activity was measured at pH 7·4 (*a*) in Michaelis veronal-acetate buffer, (*b*) in phosphate buffer. The results (Table 6) do not indicate that added phosphate is essential in these reactions; veronal buffer in high concentrations inhibits the 6-phosphogluconate dehydrogenase. This lack of dependence on added phosphate is in contrast to the glycolytic breakdown of glucose-6-phosphate by yeast and animal tissues. The ethanolic fermentation of ribose-5-phosphate by yeast enzymes also requires the addition of inorganic phosphate (Dickens, 1938*b*).

Table 6. *Inorganic phosphate and the oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate*

(Phosphate-free liver fractions at pH 7·4 in (*a*) phosphate buffer, (*b*) veronal buffer, of concentrations as stated.)

Rate of reduction of 2:6-dichlorophenolindophenol by 6-phosphogluconate system (fraction *C*)

	(μmmol./min.) at 20°				
Veronal (M)	0·01	0·02	0·02	—	—
Phosphate (M)	—	—	0·0015	0·0015	0·005
Activity	7·7	5·8	5·9	9·3	9·1

Substrate	Liver fraction	O_2 uptake (μl./60 min.) at 37° Veronal (0·006 M)	Phosphate (0·01 M)
Glucose-6-phosphate*	(*A* + *B* + *C*)	210	202
6-Phosphogluconate*	*C*	208	195
D-Ribose-5-phosphate†	(*A* + *B* + *C*)	172	224

* Free from inorganic phosphate.

† Concentration of free phosphate initially present = 6×10^{-5} M. Calculated increase due to phosphatase action during experiment = 4×10^{-4} M-phosphate.

The effect of adenosinetriphosphate. The addition of ATP did not accelerate glucose-6-phosphate dehydrogenase activity as measured by the rate of reduction of 2:6-dichlorophenolindophenol (Table 7) or the rate of oxidation of ribose-5-phosphate by liver fraction (*A* + *B* + *C*) (Table 8). With 6-phosphogluconate there may be some inhibition of O_2 uptake (Table 9). Since the ATP-ase activity of these liver fractions was very feeble, the lack of activity of ATP is not due to its destruction by enzymic hydrolysis.

Phosphokinase activity. Rat-liver fraction (*A* + *B* + *C*) was found incapable of phosphorylating glucose, gluconate or 2-ketogluconate in the presence of ATP. Liver hexokinase was presumably inactivated or precipitated at pH 4·6 during the preparation of the extracts. No animal phosphokinase capable of phosphorylating gluconate or 2-ketogluconate has yet been described, although Cohen & McNair Scott (1950*b*) have reported the existence of a specific 'gluconokinase' in gluconate-adapted *Escherichia coli*.

Table 7. *Effect of adenosinetriphosphate on glucose-6-phosphate dehydrogenase*

(2:6-Dichlorophenolindophenol method with liver fraction (*A* + *B* + *C*); temp. 20°.)

Addition: Co II (0·2 mg.)	ATP (0·0013 M)	NaF (0·01 M)	Reduction rate (μmmol. dye/min.)
–	–	–	1·2
–	+	–	2·5
–	+	+	3·9
+	–	–	17·1
+	+	–	14·6
+	+	+	14·0

Table 8. *Effect of adenosinetriphosphate on oxidation of ribose-5-phosphate*

(Liver fraction (*A* + *B* + *C*); O_2 uptake at 37°.)

Addition: Co II	ATP	Oxygen uptake (μl./45 min.)
–	–	5
+	–	21
–	+	7
+	+	21

Table 9. *Effect of adenosinetriphosphate on oxidation of 6-phosphogluconate*

(O_2 uptake method at 37°, liver fraction *C*.)

Addition: 6-Phosphogluconate (0·01 M)	Co II (0·2 mg.)	ATP (0·01 M)	O_2 uptake (μl./120 min.)
–	+	–	3
+	–	–	1
+	+	–	121
+	–	+	7
+	+	+	91

Action of enzyme inhibitors

The activity of various enzyme inhibitors was investigated either on the dehydrogenase activities, determined by the 2:6-dichlorophenolindophenol method or on the O_2 uptakes of the dehydrogenase systems.

Cyanide and azide. Cyanide (0·01 M) produced a considerable increase (89 %) in glucose-6-phosphate dehydrogenase activity and some increase (up to 31 %) of 6-phosphogluconate dehydrogenase activity. The rate of O_2 uptake by the 6-phosphogluconate system was also increased by cyanide (0·004–0·01 M), particularly the initial stages of oxidation (Fig. 7). On the other hand, the rate of

oxidation of ribose-5-phosphate by liver fraction ($A+B+C$) was either unaffected or only slightly inhibited (< 20 %).

Azide (0·01 M) had no effect on glucose-6-phosphate or 6-phosphogluconate dehydrogenase activities, although it produced a slight increase (10–20 %) in the rate of O_2 uptake by the 6-phosphogluconate system. These facts are more in keeping with cyanohydrin formation than with inhibition of catalase as a possible explanation of the increased O_2 uptake.

Fluoride. Sodium fluoride (0·01 M) did not inhibit either glucose-6-phosphate or 6-phosphogluconate dehydrogenase activity, and the O_2 uptake of the 6-phosphogluconate and ribose-5-phosphate systems was also unaffected.

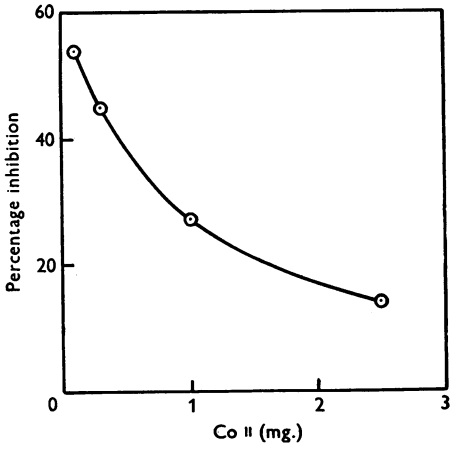

Fig. 12. Inhibition of 6-phosphogluconate dehydrogenase by nicotinamide. Rate of reduction of 2:6-dichlorophenolindophenol at 20°. Total volume 7·4 ml. including 0·5 ml. rat-liver fraction ($A+B+C$), 0·1 ml. 0·05 M-6-phosphogluconate, 40 mg. nicotinamide and varying amounts of Co II (purity 21·3 % for this experiment). Molar ratio of nicotinamide/Co II for 50 % inhibition under the above conditions = 7000/1.

Iodoacetamide. High concentrations of iodoacetamide (0·01 M) produced only a slight inhibition (< 20 %) in glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities and also of the O_2 uptakes by the 6-phosphogluconate and ribose-5-phosphate systems.

Insulin. In a few preliminary experiments, one of three samples of crystalline insulin tested (40–400 μg./ml.) was found to cause some inhibition of the glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities. Experiments are in progress to determine whether this effect can be attributed to the hyperglycaemic factor.

The effect of nicotinamide on 6-phosphogluconate dehydrogenase activity. In some preliminary experiments, estimations of enzyme activity were carried out in the presence of nicotinamide in order to diminish the enzymic breakdown of Co II. This practice was discontinued when it was observed that in the 6-phosphogluconate dehydrogenase system, nicotinamide competitively inhibited the effect of Co II (Fig. 12). Under conditions where nicotinamide produced 60 % inhibition of the above dehydrogenase system, the glucose-6-phosphate dehydrogenase system was not affected.

DISCUSSION

The present work establishes beyond doubt the existence in animal tissues of a direct oxidative pathway, distinct from the glycolytic route, for the oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate. In common with similar systems present in yeast, all these oxidations are Co II-specific and phenazonium salts (Dickens & McIlwain, 1938) serve as suitable carriers for aerobic oxidation. All these oxidations proceed independently of the glycolytic route since they are Co II-specific, are not inhibited by fluoride or iodoacetamide, and are not dependent on the presence of inorganic phosphate.

The mechanism of oxidative breakdown, however, requires further investigation. Since for animal tissues, as had previously been shown for yeast (Dickens, 1938*a*, *b*), D-ribose-5-phosphate is the pentose phosphate preferentially oxidized, this would support the view that D-ribose-5-phosphate is formed from D-glucose-6-phosphate by a two-stage oxidation and decarboxylation. The occurrence of D-ribose instead of the expected D-arabinose involves stereochemical inversion at the C-3 of glucose and, as was previously pointed out, 'it is recognized that inversion may occur in an asymmetrically disposed C atom with an attached H atom and an adjacent =CO group. A type of Walden inversion could theoretically occur either as a result of decarboxylation or by the introduction and subsequent removal of a second phosphoric acid group' (Dickens, 1938*a*). The occurrence of a phosphorylation appears unlikely, since in the present work we have shown that inorganic phosphate and ATP are not essential, and it is therefore more probable that inversion accompanies decarboxylation.

Fig. 13 represents a hypothetical course of oxidative formation of pentose from hexose which, being based on the intermediate formation of an enediol common to both 2- and 3-ketogluconate, appears to be consistent with the published data of Dickens (1938*a*), Cohen & McNair Scott (1950*a*, *b*), Horecker (1950), and Horecker & Smyrniotis (1950). Confirmation of this route awaits the preparation of the proposed intermediates, and it also remains uncertain whether the open-chain carboxylic acids or, as in the case of oxidation of glucose by notatin

(Bentley & Neuberger, 1949), the δ-gluconolactone (Fig. 13) is the primary oxidation product formed from glucose-6-phosphate. By analogy with free gluconic acid, 6-phosphogluconate would be expected to be in equilibrium with its lactone in neutral solution.

Moreover, less alkali-labile phosphate is split from ribose-5-phosphate by a liver fraction which splits fructose-1:6-diphosphate rapidly than by one which splits the latter substrate feebly (see Fig. 11). Thus our preliminary experiments do not favour the view that liver aldolase first splits ribose-5-phosphate

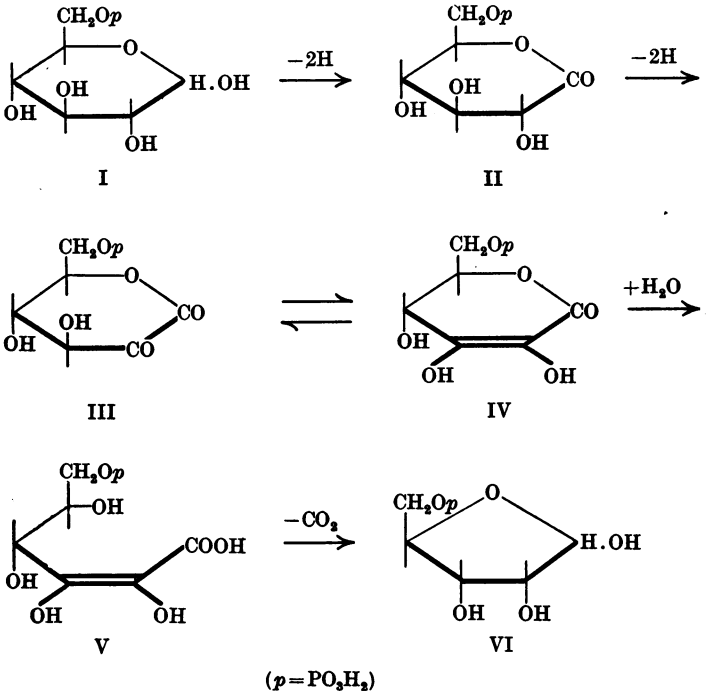

Fig. 13. Suggested route for oxidative and decarboxylative transformation of D-glucose into D-ribose. I, D-glucose-6-phosphate; II, 6-phospho-D-gluconolactone; III, 6-phospho-2-(or 3-) keto-D-gluconolactone; IV, enolic form of III; V, open-chain form of III; VI, D-ribose-5-phosphate. For the corresponding open-chain acids see Text.

The enzyme system oxidizing ribose-5-phosphate differs markedly, in some respects, from the systems oxidizing glucose-6-phosphate and 6-phosphogluconate. Thus, in the case of ribose-5-phosphate, at least two factors are necessary, which are precipitated by ammonium sulphate at different percentage saturations, both being required to effect any oxidation. In addition, the glucose-6-phosphate and 6-phosphogluconate dehydrogenase systems reduce Co II rapidly, even at room temperature, whereas with the ribose-5-phosphate dehydrogenase system, reduction of Co II is very slow at room temperature although rapid at 37°. The activity of liver aldolase has been shown by Dounce, Barnett & Beyer (1950) to decline rapidly at temperatures below 37° and extrapolation of these authors' data indicate that it would be very feeble at 20°. Although liver fractions active in oxidizing ribose-5-phosphate contain aldolase capable of splitting fructose-1:6-diphosphate, the amount of alkali-labile phosphate split from ribose-5-phosphate is very small (see Fig. 10). into a C_2 fragment and glyceraldehyde-3-phosphate before oxidation can occur, although these results remain to be substantiated by the use of other methods of determining aldolase activity.

Recent work from other laboratories, however, suggests that aldolase is involved in the metabolism of ribose-5-phosphate. Dische (1938, 1949) found that when adenosine was incubated with laked erythrocytes, pentose disappeared and was replaced by triose phosphate, hexose diphosphate and hexose monophosphate. Schlenk & Waldvogel (1946, 1947*a*) showed that under similar conditions, ribose-5-phosphate might be formed from adenosine via ribose-1-phosphate. The same authors (Schlenk & Waldvogel, 1947*b*; Waldvogel & Schlenk, 1947; Schlenk, 1949) also showed that extracts of acetone-dried rabbit liver converted ribose-5-phosphate into Robison ester. Our own experiments exclude the possibility that glucose-6-phosphate could be formed from ribose-5-phosphate via fructose-1:6-diphosphate, since this is not oxidized by liver fractions

which oxidize both ribose-5-phosphate and glucose-6-phosphate (see Fig. 2). It is possible, however, that glucose-6-phosphate could be synthesized by another route. Racker (1948, 1951) has obtained evidence of both synthesis and breakdown of a deoxypentose phosphate by an aldolase-like enzyme from *Escherichia coli*.

There seem to be two likely mechanisms of oxidation of ribose-5-phosphate by animal tissues. The first would involve splitting of ribose-5-phosphate by an aldolase, different from the enzyme splitting fructose-1:6-diphosphate, into glyceraldehyde-3-phosphate and a C_2 fragment (glycollic aldehyde?), synthesis of glucose-6-phosphate or fructose-6-phosphate from two molecules of triose and subsequent oxidation of the hexose-6-phosphate through the Co II specific systems described. The second is the oxidation of ribose-5-phosphate to 5-phospho-D-ribonic acid, with subsequent oxidative degradation as suggested by Dickens (1936, 1938*a*) for yeast. The present evidence is insufficient to decide between these alternative pathways.

SUMMARY

1. A direct oxidative pathway, distinct from the glycolytic route, for the oxidation of D-glucose-6-phosphate, 6-phospho-D-gluconate and D-ribose-5-phosphate, has been demonstrated in animal tissues and shown to resemble closely that previously described for yeast.

2. Hexose monophosphate and 6-phosphogluconate are actively oxidized by extracts of liver (horse, rat and rabbit), kidney (rat and rabbit), brain (rat and rabbit) and rat-liver carcinoma. Skeletal muscle (rat and rabbit) and Rous sarcoma of the fowl possess weak activity.

3. Ribose-5-phosphate has been shown to be oxidized by liver and kidney and less actively by skeletal muscle.

4. Oxidation of all three substrates is coenzyme II-specific.

5. These oxidations proceed independently of the glycolytic route, since inorganic phosphate is not essential for activity and high concentrations (0·01 M) of fluoride and iodoacetamide do not inhibit.

6. Partial separation of these systems from rat and horse liver has been effected by fractional ammonium sulphate precipitation. Fraction *C* (60–70 % saturation) specifically oxidizes 6-phosphogluconate. Fraction *B* (50–60 % saturation) oxidizes glucose-6-phosphate and fructose-6-phosphate but not fructose-1:6-diphosphate. Fractions *B*+*C* are generally necessary for D-ribose-5-phosphate oxidation and this is oxidized about five times as rapidly as D-arabinose-5-phosphate and D-xylose-5-phosphate.

7. At least two liver factors are necessary for ribose-5-phosphate oxidation. One of these is a coenzyme II-specific dehydrogenase and the other possibly a type of aldolase.

8. The dehydrogenation of ribose-5-phosphate is very slow at 20° but rapid at 37° and thus differs from the dehydrogenation of both glucose-6-phosphate and 6-phosphogluconate which is rapid at 20°.

9. Cyanide (0·01 M) does not inhibit the oxidation of these substrates and may accelerate the early stages of oxidation.

10. The substrate concentrations producing 50 % of the maximum initial velocities are approximately $7{\cdot}5 \times 10^{-5}$ M for hexose monophosphate and $1{\cdot}5 \times 10^{-5}$ M for 6-phosphogluconate dehydrogenases.

11. A mechanism is suggested for the oxidative formation of D-ribose-5-phosphate from D-glucose-6-phosphate.

We wish to thank Dr C. Liébecq for unpublished details of Co II preparation; Sir Jack Drummond, F.R.S., Dr M. Macfarlane, Dr C. S. Hanes, F.R.S., and Dr C. T. Beer for gifts of phosphoric esters, and Miss P. Gregory for technical assistance. Mrs C. Hoch-Ligeti and Dr P. T. J. C. P. Warner kindly gave us tumour material. This work was in part supported by a grant to this Medical School from the British Empire Cancer Campaign.

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The Digestibility of Carotene by the Cow and the Goat as Affected by Thyroxine and Thiouracil

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(*Received* 5 *April* 1951)

It has been shown at this Institute that thyroxine affects the amounts of certain nutrients secreted in milk (Owen, 1948; Chanda & Owen, 1949; Chanda, McNaught & Owen, 1949) and that thiouracil produces opposite effects. Cama & Goodwin (1949) showed that thyroxine inhibited whilst thiouracil enhanced the loss of ingested carotene in the faeces of the rat. The present experiments were made to ascertain whether such effects were reproducible in the lactating cow and the lactating goat.

EXPERIMENTAL

(1) An experiment was carried out with six cows. It had already been shown in the goat (Chanda, Clapham, McNaught & Owen, 1951) that the Cr ratio method is as accurate as the direct method for determining the digestion of carotene. Cr_2O_3 was therefore mixed with the food fed, so as to measure the digestibility of carotene in the cows without removing them from the byre. Representative samples (1 to 2 lb.; 1 lb. = 0·45 kg.) of faeces voided between the morning milking and 11 a.m. were taken every 2nd day and analysed immediately for carotene. The cows ate only the experimental diet and were fed alternately on carotene-containing and carotene-free concentrate mixtures (compositions shown in Table 1) at the rate of 21 lb. of mixture/day in two equal portions with 10 lb. oat straw/day as roughage, irrespective of which mixture was being fed. The sole source of carotene in the rations was the dried grass meal in mixture 1 which also contained 0·1% Cr_2O_3 dispersed, as described by Chanda *et al.* (1951). Mixture 2 (designed to be free from carotene) contained blood meal, potato starch and ground straw so as to simulate the content of protein, carbohydrate and fibre in the grass meal.

Table 1. *Composition of carotene-containing and carotene-free diets for cows*

	Parts by weight	
Components	Mixture 1* (containing carotene)	Mixture 2 (carotene-free)
Oats (bruised)	9	9
Bean meal	6	6
Grass meal	6	—
Potato starch	—	2·5
Blood meal	—	0·9
Ground straw	—	2·5

* Mixture 1 contained 2100 g. Cr_2O_3 in 31·5 cwt.

There were five periods. During periods 1 and 3 the carotene-free mixture was fed, and during periods 2, 4 and 5 the mixture containing grass meal was fed. During period 4