

## The Pentose Phosphate Pathway of Glucose Metabolism

### MEASUREMENT OF THE NON-OXIDATIVE REACTIONS OF THE CYCLE

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Methods for the quantitative determination of ribose 5-phosphate isomerase, ribulose 5-phosphate 3-epimerase, transketolase and transaldolase in tissue extracts are described. The determinations depend on the measurement of glyceraldehyde 3-phosphate by using the coupled system triose phosphate isomerase,  $\alpha$ -glycerophosphate dehydrogenase and NADH. By using additional purified enzymes transketolase, ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase conditions could be arranged so that each enzyme in turn was made rate-limiting in the overall system. Transaldolase was measured with fructose 6-phosphate and erythrose 4-phosphate as substrates, and again glyceraldehyde 3-phosphate was measured by using the same coupled system. Measurements of the activities of the non-oxidative reactions of the pentose phosphate pathway were made in a variety of tissues and the values compared with those of the two oxidative steps catalysed by glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

The existence of the pentose phosphate pathway in a variety of tissues is now well established (Glock & McLean, 1954; Glock, 1964; see Hollman, 1964). Evidence for the non-oxidative reactions of the cycle is based on the use of such methods as ribose 5-phosphate disappearance and transformation into sedoheptulose 7-phosphate or into hexose 6-phosphate (Dische, 1938; Glock, 1952; de la Haba & Racker, 1952; Horecker & Smyrniotis, 1953; Glock & McLean, 1954; Nigam, Sie & Fishman, 1961), on studies on the isolation and distribution of the individual enzymes (see Racker, 1961*a,b*; Topper, 1961; Maxwell, 1961; Hollman, 1964), on identification of intermediates characteristic of this cycle in tissues (Peeters & Debackere, 1956; Nigam, Sie & Fishman, 1959; Bonsignori, Foranini, Segni & Antolini, 1959) and on isotopic studies with  $^{14}\text{C}$ -labelled glucose (Hiatt, 1957; Marks & Feigelson, 1957; see Dickens, Glock & McLean, 1959; Hollman, 1964).

Though there have been many reports of changes in glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in different hormonal conditions and in various nutritional states, particularly in liver (Glock & McLean, 1955; Huggins & Yao, 1959; Tepperman & Tepperman, 1963; Noltmann & Kuby, 1963; Hollifield & Parson, 1965; Leveille & Hanson, 1966), there have been only

relatively few assays under optimum conditions of the enzymes of the non-oxidative part of this cycle (Hollman, 1964; Horecker, 1964; Srivastava & Hübscher, 1966; Dreyfus, 1967). It therefore seemed of interest to establish conditions for the assay of the four non-oxidative reactions of the cycle, ribulose 5-phosphate 3-epimerase (D-ribulose 5-phosphate 3-epimerase, EC 5.1.3.1), ribose 5-phosphate isomerase (D-ribose 5-phosphate ketol-isomerase, EC 5.3.1.6), transketolase (sedoheptulose 7-phosphate-D-glyceraldehyde 3-phosphate glycolaldehydetransferase, EC 2.2.1.1) and transaldolase (sedoheptulose 7-phosphate-D-glyceraldehyde 3-phosphate dihydroxyacetone transferase, EC 2.2.1.2), and to measure these in addition to glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase [6-phospho-D-gluconate-NADP oxidoreductase (decarboxylating), EC 1.1.1.44] to determine the complete pattern of changes in enzymes of the pentose phosphate cycle in different hormonal and nutritional states. The present work is concerned with the methods of assay and relative activities of these enzymes in different tissues.

### EXPERIMENTAL

*Animals.* Adult male albino rats with body weight 150–200 g. were used; food and water were allowed *ad lib*.

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**Materials.** NADP<sup>+</sup>, NAD<sup>+</sup>, NADH, ATP (sodium salt), glucose 6-phosphate (sodium salt), 6-phosphogluconate (sodium salt), ribose 5-phosphate (barium salt) and fructose 6-phosphate (barium salt) were obtained from Boehringer Corp., London, W. 5. The barium salts of the sugar phosphates were converted into their potassium salts by treatment with K<sub>2</sub>CO<sub>3</sub>; the excess of carbonate was removed by treatment with HCl and the solutions were adjusted to pH 7.2. In certain experiments an aqueous solution of the sodium salt of erythrose 4-phosphate was used; this was also obtained from Boehringer Corp. Sedoheptulosan was a gift from Dr B. Horecker and Dr O. Tsolas (Albert Einstein College of Medicine, New York, N.Y., U.S.A.).

Glucose 6-phosphate dehydrogenase (140 units/mg.), glycerol 1-phosphate dehydrogenase-triose phosphate isomerase mixture (the relative activities of the two components being 1:6) (10 mg./ml.) and phosphoglucose isomerase (390 units/mg.) were obtained from Boehringer Corp.

**Preparation of erythrose 4-phosphate.** The erythrose 4-phosphate used mainly in these experiments was prepared by oxidation of glucose 6-phosphate with lead tetra-acetate by the procedure of Ballou (1963). The lead tetra-acetate was standardized by the method described by Baxter, Perlin & Simpson (1959); the final solution was adjusted to pH 5.0 and stored at -20°. Erythrose was determined by the colorimetric method of Dische & Dische (1958). The yield of erythrose 4-phosphate was 60% of the theoretical value and each 100 μmoles of erythrose was accompanied by 117 μmoles of total phosphate, 11 μmoles of inorganic phosphate and 7 μmoles of triose phosphate.

**Preparation of ribose 5-phosphate free from pentulose.** In certain experiments, e.g. in the measurement of pentose phosphate isomerase, it was necessary to use a ribose 5-phosphate preparation completely free from pentuloses; commercial preparations of ribose 5-phosphate contained sufficient pentulose to cause serious interference in this assay, and it was therefore removed by treatment with alkali according to the following procedure: 1 m-mole (365 mg. of the barium salt of ribose 5-phosphate) was dissolved in 9 ml. of distilled water with the aid of a small amount of 1 N-HCl, to this was added 1 ml. of 10 N-NaOH and the mixture was incubated for 10 min. at 25°. An excess (4-5 g.) of Amberlite IR-120 (H<sup>+</sup> form) was added to remove the Na<sup>+</sup> and Ba<sup>2+</sup>; the complete removal of Ba<sup>2+</sup> was tested for by use of a small amount of Na<sub>2</sub>SO<sub>4</sub> and the final solution was adjusted to pH 7.0.

**Preparation of an equilibrium mixture of ribulose 5-phosphate and xylulose 5-phosphate.** A 1 g. sample of ribose 5-phosphate was incubated for 1 hr. at 37° with a large excess of spleen enzyme preparation containing a mixture of ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase (for the preparation of this enzyme see below). The reaction was stopped by adding HClO<sub>4</sub> to give a final concentration of 0.5 N; after removal of the precipitated protein by centrifugation the supernatant was neutralized with 5 N-KOH and left in the cold for 1 hr. to allow complete precipitation of the KClO<sub>4</sub>. After removal of the KClO<sub>4</sub> by centrifugation the pentuloses were separated from ribose 5-phosphate by chromatography on a Dowex 1 (formate form) column as described by Dickens & Williamson (1956). Successive elutions with 0.15 M-ammonium formate containing 5 mM-Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> removed ribulose 5-phosphate and xylulose 5-phosphate, and this, followed by treatment

with borate-free 0.5 M-ammonium formate for elution of ribose 5-phosphate, was used to obtain a preparation of the mixed pentuloses. The elution pattern was very similar to that obtained by Dickens & Williamson (1956; see their Fig. 6).

**Purification of transketolase.** Transketolase was purified from rat liver by a modification of the method of Simpson (1960); the liver was kept deep-frozen until required for the preparation. Acetone drying caused a substantial loss of activity and therefore the preliminary steps were homogenization of liver in 5 vol. of 1 mM-tris-HCl buffer, pH 7.6. This homogenate was centrifuged at 2000g for 30 min. and the supernatant fraction used for further purification. The fraction that was precipitated at 50-60% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was collected and dissolved in 1 mM-triethanolamine-HCl buffer, pH 8.0, and dialysed overnight against 6 l. of a solution containing 15 mM-Na<sub>2</sub>CO<sub>3</sub> and 1 mM-triethanolamine-HCl buffer, pH 8.0; from this point the preparation was essentially as described by Simpson (1960). Transketolase activity was measured by following the rate of formation of glyceraldehyde 3-phosphate by using triose phosphate isomerase, α-glycerophosphate dehydrogenase and NADH as described below.

It was essential for our purposes to have a preparation of transketolase free from pentose phosphate isomerase or epimerase, and the batch treatment with 12 g. portions of DEAE-cellulose was repeated until no glyceraldehyde 3-phosphate was produced in a system containing ribose 5-phosphate (freed from pentulose 5-phosphate by treatment with alkali as described above) and the liver transketolase preparation. As can be seen from Scheme 1, this indicated only that either pentose phosphate isomerase or epimerase or both were absent. If ribose 5-phosphate isomerase, free from transketolase or epimerase, was added to the system it was possible to check that the transketolase preparation was free from epimerase. The purification of transketolase was taken as far as the treatment with basic lead acetate in the method described by Simpson (1960).

**Preparation of a mixture of ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase.** Since only ribose 5-phosphate was available in sufficient quantities to act as a substrate for the transketolase reaction, it was necessary to add supplementary enzymes to give a rapid rate of formation of xylulose 5-phosphate; therefore a mixture of ribulose 5-phosphate epimerase and ribose 5-phosphate isomerase was prepared from rat spleen for this purpose. The purification procedure followed that of Ashwell & Hickman (1957) with certain modifications. Rat spleens could be collected and stored frozen for a long period of time; but the preparation of an acetone-dried powder diminished the yields of these enzymes and therefore the spleens were homogenized in water and the protein fraction precipitated at 40-50% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation was collected. This fraction was dissolved in water and the enzyme solution was brought to 60° rapidly and maintained at this temperature for 10 min. The solution was cooled, the heavy flocculent precipitate was removed by centrifugation and the supernatant was tested for transketolase; at this stage the preparation should be completely free from transketolase. Tris-HCl buffer, pH 7.6, was added to bring the preparation to a final concentration of 1 mM and the enzyme was dialysed against a large volume of 1 mM-tris-HCl buffer, pH 7.6, overnight. This enzyme was stable for several months when stored frozen and is suitable for the



preparation of equilibrium mixtures of ribulose 5-phosphate, xylulose 5-phosphate and ribose 5-phosphate (Ashwell & Hickman, 1957).

*Preparation of ribose 5-phosphate isomerase.* A preparation of ribose 5-phosphate isomerase, free from ribose 5-phosphate epimerase, was required to generate ribulose 5-phosphate for assaying the ribulose 5-phosphate 3-epimerase in tissue extracts, since here again ribose 5-phosphate was the most readily available substrate (see Scheme 1). The mixed ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase prepared from rat spleen as described above was used as the starting material for this preparation and the two enzymes were separated by acetone fractionation as described by Ashwell & Hickman (1957). As stated by these authors, it was necessary to carry out a pilot run on this step to determine the exact amount of acetone to be added, since there was variation from batch to batch. In general, the first fraction precipitated at 35–45% (v/v) acetone contained ribose 5-phosphate isomerase free from epimerase and the fraction precipitated at 45–65% (v/v) acetone contained the bulk of the ribulose 5-phosphate 3-epimerase free from isomerase. The temperature was kept as low as possible during the acetone fractionation; the acetone was cooled to  $-10^{\circ}$  before addition to the enzyme preparation, which was kept at  $-2^{\circ}$  and was gradually cooled as the acetone concentration rose.

By using purified transketolase and ribose 5-phosphate free from ribulose 5-phosphate it was possible to show that the ribose 5-phosphate isomerase was free from ribulose 5-phosphate 3-epimerase and vice versa. Only in the presence of both enzymes was triose phosphate formed from ribose 5-phosphate as shown by the coupled system containing triose phosphate isomerase,  $\alpha$ -glycerophosphate dehydrogenase and NADH (see Scheme 1 and assay for transketolase below).

The two acetone fractions were dialysed overnight against 1 mM-tris-HCl buffer, pH 7.6. Occasionally it was necessary to repeat the acetone fractionation if the ribose 5-phosphate isomerase was contaminated with ribulose 5-phosphate 3-epimerase.

*Partial purification of transaldolase.* A preparation of transaldolase free from ribose 5-phosphate isomerase, ribulose 5-phosphate 3-epimerase, transketolase and triose isomerase, but containing some phosphoglucose isomerase, was obtained from rat liver by  $(\text{NH}_4)_2\text{SO}_4$  fractionation and treatment with calcium phosphate gel as follows.

Rat liver was homogenized in 5 vol. of 0.1 M-tris-HCl buffer, pH 7.6, and centrifuged at 8000g for 30 min. to remove the particulate fraction. The supernatant was treated with  $(\text{NH}_4)_2\text{SO}_4$  and the fraction precipitated at 60–70% saturation was collected and dissolved in a small volume of 1 mM-tris-HCl buffer, pH 7.6. This preparation was dialysed overnight against the same buffer. Calcium phosphate gel (containing 30 mg. of calcium phosphate/ml.) was added to the enzyme preparation; 0.5 vol. of gel/vol. of preparation was used. After stirring for 5 min. the mixture was centrifuged and the gel was separated and washed with 50 mM- $\text{NaHCO}_3$ . The enzyme was eluted from the gel with 0.3 M-potassium phosphate buffer, pH 7.6, and the eluate, containing the transaldolase, was dialysed overnight against 1 mM-triethanolamine-HCl buffer, pH 7.6, and was stored at  $-20^{\circ}$ .

*Colorimetric determination of sugar phosphates.* (a) Triose

phosphates. These were determined by the cysteine-carbazole reaction of Dische & Borenfreund (1951).

(b) Erythrose 4-phosphate. This was determined by the method of Dische & Dische (1958) by using the reaction of tetrose with fructose,  $\text{H}_2\text{SO}_4$  and cysteine. The presence of cysteine in the incubation medium seriously interfered with the colour reaction for erythrose 4-phosphate. If cysteine was added to the standard solution of erythrose 4-phosphate and was therefore present during the period of treatment with  $\text{H}_2\text{SO}_4$  and fructose, then there was no absorption peak at  $458\text{m}\mu$ . In the method of Dische & Dische (1958) cysteine is added in the later stages of colour development after heating with  $\text{H}_2\text{SO}_4$  and fructose, and is essential at this stage for the development of the characteristic peak at  $458\text{m}\mu$ .

(c) Pentulose phosphates. These were determined by using the modification by Ashwell & Hickman (1957) of the Dische & Borenfreund (1951) cysteine-carbazole reaction. The present results were calculated by using the standard values given by Ashwell & Hickman (1957), namely that 0.1  $\mu$ mole of ribulose 5-phosphate gave  $E_{540}$  0.140 and 0.1  $\mu$ mole of xylulose 5-phosphate gave  $E_{540}$  0.050, and the relative concentrations in equilibrium conditions found by Tabachnick, Srere, Cooper & Racker (1958).

(d) Sedoheptulose 7-phosphate. This was determined by two methods. In the first the colour developed with orcinol by using procedure 2 as described by Dische (1953) with sedoheptulosan as a standard was measured. The difference  $E_{610} - E_{530}$  was used to correct for colour due to pentoses or hexoses; these sugars show a very small absorption when the difference  $E_{610} - E_{530}$  is calculated even when they are present at concentrations double that of the sedoheptulose. As shown in Table 1 the presence of other sugars or of cysteine somewhat depressed the difference  $E_{610} - E_{530}$  of the sedoheptulosan standard, and it seemed possible that the amount of  $\text{FeCl}_3$  or of orcinol might be insufficient for the full colour development of sedoheptulose in the presence of these compounds. It was essential to increase the orcinol concentration twofold (from 0.15 ml. to 0.3 ml. of 6% orcinol) and the  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  tenfold (from 0.4 ml. of 13 mg./100 ml. reagent to 0.4 ml. of 130 mg./100 ml. reagent). As shown in Table 1 the specificity of the reaction appears unchanged, but the interference from cysteine and fructose is abolished. However, this method sometimes gave results that were not reproducible, particularly in the presence of erythrose 4-phosphate. In this method sedoheptulose 7-phosphate gives twice the extinction value of sedoheptulose (Bruns, Dünwald & Noltmann, 1958), and where this method was used the values were corrected for this factor.

The second method that was used in most of these experiments was the reaction with cysteine and  $\text{H}_2\text{SO}_4$  as described by Dische (1953). This method was preferred because it gave more reproducible results and because there was less interference from cysteine when this was present in the reaction mixture.

*Orcinol reaction for mixtures of pentoses and heptuloses.* Pentoses and heptuloses both form coloured products with orcinol and  $\text{FeCl}_3$  reagents, the pentoses yielding a product with an absorption maximum at  $670\text{m}\mu$  whereas the heptuloses have a maximum at  $580\text{m}\mu$ . The concentration of these substances in a mixture can be calculated from measurements of  $E_{670}$  and  $E_{580}$  by using the method and formula derived by Horecker (1957).

Table 1. *Effect of the concentration of orcinol and ferric chloride on the determination of sedoheptulose 7-phosphate in the presence of other sugars and cysteine*

To 2ml. of solution containing 80  $\mu$ g. of sedoheptulosan with or without the additional compounds (cysteine, erythrose, fructose, ribose 5-phosphate and a mixture of these compounds) was added 0.4ml. of conc. HCl. (sp.gr. 1.19). The mixture was heated for 1hr. at 100°. The FeCl<sub>3</sub> and orcinol reagents were added in either (1) the normal concentration (0.4ml. of 0.013% FeCl<sub>3</sub>.6H<sub>2</sub>O in 2N-HCl and 0.15ml. of 6% orcinol in ethanol plus 0.15ml. of ethanol) or (2) the increased reagent concentration (0.4ml. of 0.13% FeCl<sub>3</sub>.6H<sub>2</sub>O in 2N-HCl and 0.3ml. of 6% orcinol in ethanol). The mixtures were heated at 100° for 3min. and to these were added 2vol. of acetic acid. Readings were taken at 610m $\mu$  and 530m $\mu$ .

Additional compounds	Determination of sedoheptulosan (80 $\mu$ g.) ( $E_{610} - E_{530}$ )	
	Normal reagent concentration	Increased reagent concentration
None	0.097	0.097
Cysteine (0.3 $\mu$ mole)	0.079	0.097
Cysteine (1.5 $\mu$ moles)	0.069	0.094
Erythrose (0.3 $\mu$ mole)	0.100	0.097
Fructose (0.3 $\mu$ mole)	0.076	0.100
Fructose (1.0 $\mu$ mole)	0.057	0.098
Ribose 5-phosphate (1.0 $\mu$ mole)	0.097	0.096
Cysteine (0.3 $\mu$ mole) + erythrose (0.3 $\mu$ mole) + fructose (0.3 $\mu$ mole) + ribose 5-phosphate (1.0 $\mu$ mole)	0.051	0.099

*Preparation of tissue homogenates.* Liver homogenates were prepared either in 0.1M-tris-HCl buffer, pH7.6, or in a mixture containing 0.15M-KCl-5mM-EDTA-5mM-MgCl<sub>2</sub>-10mM-mercaptoethanol by using the proportions 1g. of liver to 3ml. of medium. The homogenate was centrifuged at 100000g for 40min. at 0° and dialysed, with stirring, for 1-2hr. against the same extraction medium.

*Assay of transketolase.* Two methods were used for this enzyme, one based on the formation of sedoheptulose and the other based on the formation of triose phosphate. The final method used for liver is given for each of these methods; modifications of these methods for specific purposes are given in the Results and Discussion section.

(a) Formation of sedoheptulose 7-phosphate. The incubation mixture contained 1ml. of 0.25M-glycylglycine, 0.5ml. of 0.1M-MgCl<sub>2</sub>, 0.1ml. of 0.1M-ribose 5-phosphate, 0.1ml. of 60mM-cysteine and 0.15ml. of rat liver dialysed supernatant fraction in a final volume of 2.5ml. This was incubated at 30°; 0.5ml. portions were removed at different time-intervals between 0 and 30min. (the usual time of incubation was 15min.) and were deproteinized by the addition of 1.5ml. of 5% (w/v) trichloroacetic acid. Por-

tions (1ml.) of the supernatant fraction were used for the determination of sedoheptulose. The reaction was linear for about 30min. No difference was found between tissue extracts prepared in 0.1M-tris or the 0.15M-KCl mixed medium described above. If only one pair of duplicate samples was needed all the components of the incubation mixture were decreased by half.

(b) Formation of triose phosphate. In this assay spleen enzyme containing ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase was used. The preparation contained 3-5units of the isomerase/ml. of enzyme preparation and a greater amount of the epimerase; 0.2ml. (approx. 0.6unit) of such a preparation was added to give a fast rate of formation of xylulose 5-phosphate, and an amount of this mixed enzyme capable of producing at least 0.1  $\mu$ mole of xylulose 5-phosphate/min. was used. The assay system contained 0.5ml. of 0.25M-glycylglycine-KOH buffer, pH7.6, 0.2ml. of 50mM-ribose 5-phosphate, ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase (approx. 0.6unit), 0.05ml. of triose isomerase- $\alpha$ -glycerophosphate dehydrogenase (Boehringer preparation, 10mg./ml., diluted 1:10 with 0.25M-glycylglycine-KOH buffer, pH7.6), 0.2ml. of NADH (2mg./ml., freshly prepared) and 0.1ml. or 0.2ml. of liver supernatant fraction diluted to be equivalent to 1 part of liver in 10 parts of medium. The reoxidation of NADH was followed at 340m $\mu$  with 1cm. light-path in the Unicam SP.800 recording spectrophotometer.

*Assay of transaldolase.* Two methods were used for this enzyme. With fructose 6-phosphate and erythrose 4-phosphate as substrate either of the products of the reaction could be measured, i.e. sedoheptulose 7-phosphate or triose phosphate.

(a) Formation of sedoheptulose 7-phosphate. The incubation mixture contained 1ml. of 0.25M-glycylglycine-KOH buffer, pH7.6, 0.1ml. of 0.16M-EDTA, 0.1ml. of 0.1M-fructose 6-phosphate, 0.1ml. of 40mM-erythrose 4-phosphate and 0.1ml. of dialysed liver supernatant fraction in a final volume of 2.5ml. The incubation period was normally 15min. at 30°, at the end of which time 0.8ml. portions of the mixture were deproteinized by the addition of 1.2ml. of 5% (w/v) trichloroacetic acid. After centrifugation, the sedoheptulose was determined in 1.0ml. portions of the supernatant fraction.

(b) Formation of triose phosphate. The assay system contained 0.5ml. of 0.25M-glycylglycine-KOH buffer, pH7.6, 0.1ml. of 4mM-erythrose 4-phosphate, 0.2ml. of 50mM-fructose 6-phosphate, 0.05ml. of triose isomerase- $\alpha$ -glycerophosphate dehydrogenase (Boehringer preparation, 10mg./ml., diluted 1:10 with 0.25M-glycylglycine-KOH buffer, pH7.6), 0.2ml. of NADH (2mg./ml., freshly prepared) and 0.05-0.10ml. of liver supernatant fraction diluted to be equivalent to 1 part of liver in 10 parts of medium. The final volume was 1.4ml. and the reaction was followed with 0.5cm. cell light-path at 340m $\mu$ . The smaller cells were used because of shortage of erythrose 4-phosphate.

*Assay of ribose 5-phosphate isomerase.* The formation of triose phosphate from ribose 5-phosphate was measured in the presence of excess of transketolase and ribulose 5-phosphate 3-epimerase. Purified transketolase, free from ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase, was used (for preparation see above). In liver, ribulose 5-phosphate 3-epimerase is already present in

great excess, some tenfold higher than ribose 5-phosphate isomerase (the relative activities of these two enzymes are 4 and 30 units/g. of liver respectively). The assay system contained 0.5 ml. of 0.25 M-glycylglycine-KOH buffer, pH 7.6, 0.3 ml. of 0.1 M-ribose 5-phosphate (treated with NaOH to remove pentuloses), 0.2 ml. of NADH (2 mg./ml.), 0.05 ml. of triose isomerase- $\alpha$ -glycerophosphate dehydrogenase (Boehringer preparation, 10 mg./ml., diluted 1:10 with 0.25 M-glycylglycine-KOH buffer, pH 7.6), 0.1 unit of transketolase and 0.1-0.2 ml. of liver supernatant fraction diluted to be equal to 1 part of liver in 10 parts of medium. The final volume was 2.5 ml. and the reoxidation of NADH was followed at 340 m $\mu$  with 1 cm. light-path at 25°.

In tissues other than liver where ribulose 5-phosphate epimerase may be considerably less active it may be necessary to add excess of this enzyme also.

**Assay of ribulose 5-phosphate 3-epimerase.** The formation of triose phosphate from ribose 5-phosphate was measured in the presence of excess of transketolase and ribose 5-phosphate isomerase. The preparation of ribose 5-phosphate isomerase free from the epimerase is given above and follows the method of Ashwell & Hickman (1957). The assay system contained 0.5 ml. of 0.25 M-glycylglycine-KOH buffer, pH 7.6, 0.5 ml. of 0.1 M-ribose 5-phosphate (treated with NaOH to remove pentuloses), 0.2 ml. of NADH (2 mg./ml.), 0.05 ml. of triose isomerase- $\alpha$ -glycerophosphate dehydrogenase (Boehringer preparation, 10 mg./ml. diluted 1:10 with 0.25 M-glycylglycine-KOH buffer, pH 7.6), 0.1 unit of transketolase, 0.1-0.2 unit of ribose 5-phosphate isomerase and 0.005 ml. of liver supernatant fraction diluted to be equal to 1 part of liver in 50 parts of medium. The final volume was 2.5 ml. and the reoxidation of NADH was followed at 340 m $\mu$  with 1 cm. light-path at 25°.

**Enzyme activity units.** A unit of enzyme activity is defined as the amount catalysing the formation of 1  $\mu$ mole of product/min. at 25°.

## RESULTS AND DISCUSSION

The determination of the activities of a sequence of enzymes in a metabolic pathway in unfractionated extracts, such as dialysed high-speed supernatant preparations of liver, presents considerable difficulties, since the products of one reaction are the substrates for the next. With the non-oxidative reactions of the pentose phosphate cycle this is particularly difficult, since the reactions are readily reversible and some of the sugar phosphate substrates are not readily available. Racker (1961*a,b*) has emphasized that, of the methods used for the assay of highly purified enzyme preparations, not all are equally suitable for crude preparations. In the present work the activities of the enzymes were checked whenever possible by more than one method of assay.

### *Transketolase*

**Measurement of transketolase by determination of sedoheptulose 7-phosphate formation.** The results in Fig. 1 show that, when ribose 5-phosphate is the

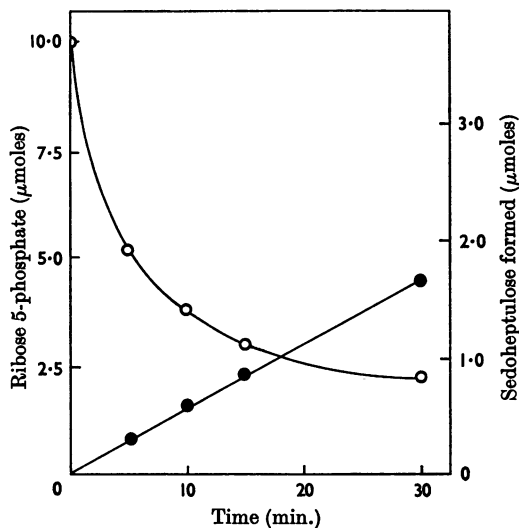


Fig. 1. Formation of sedoheptulose 7-phosphate from ribose 5-phosphate by rat liver supernatant preparations. The incubation medium contained 250  $\mu$ moles of glycylglycine-KOH buffer, pH 7.6, 50  $\mu$ moles of MgCl<sub>2</sub>, 6  $\mu$ moles of cysteine, 10  $\mu$ moles of ribose 5-phosphate and 0.15 ml. of liver supernatant preparation (1 part of liver to 3 parts of 0.1 M-tris buffer) in a total volume of 2.5 ml. This was incubated at 30° and at timed intervals 0.5 ml. samples were removed for determination of sedoheptulose 7-phosphate (as sedoheptulose) (●) and ribose 5-phosphate (○) as described in the Experimental section.

sole substrate used for the measurement of transketolase in dialysed liver extracts, the rate of formation of sedoheptulose 7-phosphate is linear with time up to 30 min. There is a very fast initial fall in ribose 5-phosphate concentration followed by a slower decline once equilibrium conditions have been established. These results suggest that ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase are present in excess over transketolase, so that the rate of formation of xylulose 5-phosphate would not appear to be rate-limiting. A more detailed study of the effects of variations in ribose 5-phosphate concentration, enzyme concentration and of pH or sedoheptulose 7-phosphate formation is shown in Figs. 2(a), 2(b) and 2(c).

No requirement for additional thiamine pyrophosphate was found in either the freshly prepared or stored liver extracts. Removal of this cofactor does not occur readily and has been shown to require precipitation of the enzyme with ammonium sulphate at a low pH (Horecker, Smyrmiotis & Klenow, 1953; Simpson, 1960). Horecker *et al.* (1953) have shown that liver preparations that had lost activity on storage in the frozen state were fully reactivated by addition of cysteine, and for

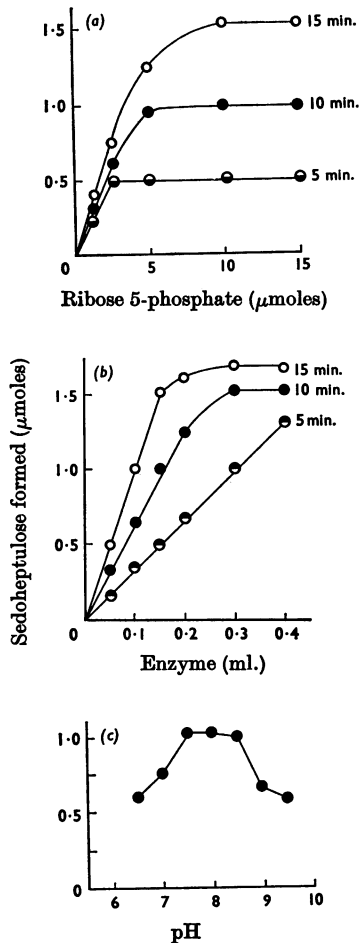


Fig. 2. Effect of variations in ribose 5-phosphate concentration, enzyme concentration and pH on the formation of sedoheptulose 7-phosphate by rat liver. (a) The incubation mixture contained 250  $\mu$ moles of glycylglycine-KOH buffer, pH 7.6, 50  $\mu$ moles of  $MgCl_2$ , 6  $\mu$ moles of cysteine, 1.25, 2.5, 5.0, 10 or 15  $\mu$ moles of ribose 5-phosphate and 0.15 ml. of liver supernatant preparation (1 part of liver to 3 parts of 0.1M-tris buffer) in a total volume of 2.5 ml. Incubation was for 5, 10 and 15 min. at 30°, and 0.5 ml. samples were used for the determination of sedoheptulose 7-phosphate (as sedoheptulose). (b) The incubation mixture contained 250  $\mu$ moles of glycylglycine-KOH buffer, pH 7.6, 50  $\mu$ moles of  $MgCl_2$ , 6  $\mu$ moles of cysteine, 10  $\mu$ moles of ribose 5-phosphate and 0.03, 0.05, 0.1, 0.2, 0.3 or 0.4 ml. of liver supernatant preparation in a total volume of 2.5 ml. Incubation was for 5, 10 and 15 min. at 30°, and 0.5 ml. samples were taken for determination of sedoheptulose 7-phosphate (as sedoheptulose). (c) The incubation mixture contained 250  $\mu$ moles of glycylglycine-KOH buffer, pH 6.5-9.5 (as indicated), 50  $\mu$ moles of  $MgCl_2$ , 6  $\mu$ moles of cysteine, 10  $\mu$ moles of ribose 5-phosphate and 0.5 ml. of liver supernatant preparation. Incubation was for 10 min. at 30°, and 0.5 ml. samples were used for the determination of sedoheptulose 7-phosphate (as sedoheptulose).

this reason cysteine was added as a component of the standard reaction mixture. Storage of liver extracts for a month at  $-20^\circ$  did not result in appreciable loss of transketolase activity either with or without cysteine.

Since transketolase activity was measured with ribose 5-phosphate as the substrate it was important to establish that neither ribose 5-phosphate isomerase nor ribulose 5-phosphate 3-epimerase was rate-limiting. Sedoheptulose formation was measured (Table 2) in rat liver in the presence of a spleen enzyme preparation containing ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase but free from transketolase (Ashwell & Hickman, 1957). There was a faster rate of formation of ribulose 5-phosphate and xylulose 5-phosphate in the presence of the spleen enzyme, but the rate of production of heptulose was unchanged. These results also illustrate the high rate of activity of pentose phosphate isomerase and epimerase in rat liver supernatant preparations.

There is some disagreement in the literature on the requirement for supplementation of transketolase assay systems with these two enzymes. Mangiarotti, Calissano & Luzzatto (1960) found a faster rate of transketolase activity in liver in the presence of a spleen enzyme preparation containing ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase, but Horecker *et al.* (1953) found that with crude liver extracts the rate of pentose phosphate cleavage was as rapid with ribose 5-phosphate as with ribulose 5-phosphate, indicating that the ribose 5-phosphate isomerase was not rate-limiting. The high activity of ribose 5-phosphate isomerase in muscle, erythrocytes and ascites-tumour cells has also been demonstrated by Dickens & Williamson (1956). Similarly Ostrovsky (1963) has shown that in brain, skeletal muscle, cardiac muscle and liver of pigeons the pentose phosphate isomerase activity greatly exceeded that of transketolase. The present results are in accord with those showing that transketolase activity is the rate-limiting step in crude extracts; this is borne out by direct measurement of these enzymes, showing that under optimum conditions ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase activities were about twofold and tenfold greater respectively than that of transketolase. Since the ribose 5-phosphate isomerase activity was only about twice that of transketolase the spleen enzyme preparation, containing the pentose phosphate isomerase and epimerase, was added as a routine when transketolase was measured.

In longer-term experiments it was found that substances giving a fructose reaction were also formed from ribose 5-phosphate. It was also observed that  $Mg^{2+}$  had a pronounced effect on the products of this reaction (Fig. 3). When  $Mg^{2+}$  was

Table 2. Measurement of transketolase activity with ribose 5-phosphate as substrate in the presence and absence of a spleen enzyme preparation containing ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase

The incubation mixture contained 250  $\mu$ moles of glycylglycine-KOH buffer, pH 7.6, 50  $\mu$ moles of  $MgCl_2$ , 15  $\mu$ moles of ribose 5-phosphate and 0.15 ml. of liver supernatant preparation (1 part of liver to 3 parts of 0.1 M-tris buffer) in a total volume of 2.5 ml. In the second group excess of ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase was added (spleen enzyme approx. 0.5 unit). Incubation was for 10, 15 and 20 min. at 30°. The sugar phosphates were determined as described in the Experimental section. The results given are means of duplicate determinations at each time-interval.

	Time (min.)	Product formed ( $\mu$ moles/2.5 ml.)		
		Sedoheptulose 7-phosphate	Ribulose 5-phosphate	Xylulose 5-phosphate
Liver extract	10	0.40	1.75	4.25
	15	0.57	2.30	6.90
	20	0.82	2.53	7.59
Liver extract + spleen enzyme	10	0.41	2.12	6.36
	15	0.62	2.50	7.50
	20	0.83	2.63	7.89

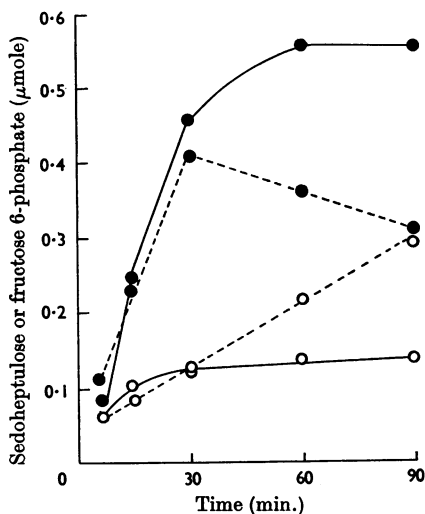


Fig. 3. Effect of  $Mg^{2+}$  on the products of the reaction of liver transketolase with ribose 5-phosphate. The incubation medium contained 250  $\mu$ moles of glycylglycine-KOH buffer, pH 7.6, 6  $\mu$ moles of cysteine, 10  $\mu$ moles of ribose 5-phosphate and 0.03 ml. of liver supernatant fraction (1 part of liver to 3 parts of 0.1 M-tris buffer), with (—) and without (---) the addition of 50  $\mu$ moles of  $MgCl_2$ , in a final volume of 2.5 ml. Incubation was for the times shown at 30°. Samples were removed and sedoheptulose 7-phosphate (as sedoheptulose) (●) and fructose 6-phosphate (○) were determined as described in the Experimental section.

omitted from the reaction mixture the initial rates of sedoheptulose and fructose formation were unchanged (0–30 min.). After this time, however, sedoheptulose showed a progressive decrease, and

more fructose accumulated. Horecker, Gibbs, Klenow & Smyrniotis (1954) first described this effect of  $Mg^{2+}$  and they ascribed this to the utilization of triose phosphate by two competing reactions. One is the formation of fructose 6-phosphate from triose phosphate alone by way of aldolase and fructose diphosphatase, the latter enzyme being active only in the presence of  $Mg^{2+}$ ; the other is the formation of fructose 6-phosphate from sedoheptulose 7-phosphate and triose phosphate by way of transaldolase, a reaction not dependent on  $Mg^{2+}$ . In the former case, sedoheptulose is not utilized, the fructose being formed only from the triose, and thus there is a parallelism between sedoheptulose and fructose formation. In the latter case, in the absence of  $Mg^{2+}$ , both sedoheptulose 7-phosphate and triose phosphate are utilized in the formation of fructose 6-phosphate and thus sedoheptulose decreases as fructose increases. It seems probable that a similar explanation holds with the present experiments. By effectively removing one of the products of the transketolase reaction in the presence of  $Mg^{2+}$  it is therefore possible to use the rate of sedoheptulose formation as a measurement of the transketolase reaction (Fig. 4).

*Determination of transketolase by measurement of triose phosphate formation.* This assay depends on the formation of glyceraldehyde 3-phosphate from xylulose 5-phosphate in the presence of an acceptor aldehyde. The formation of glyceraldehyde 3-phosphate is measured with triose phosphate isomerase (D-glyceraldehyde 3-phosphate ketol-isomerase, EC 5.3.1.1) and glycerol 3-phosphate dehydrogenase ( $\alpha$ -glycerol 3-phosphate-NAD oxidoreductase, EC 1.1.1.8) by following the oxidation of NADH spectrophotometrically (Horecker,



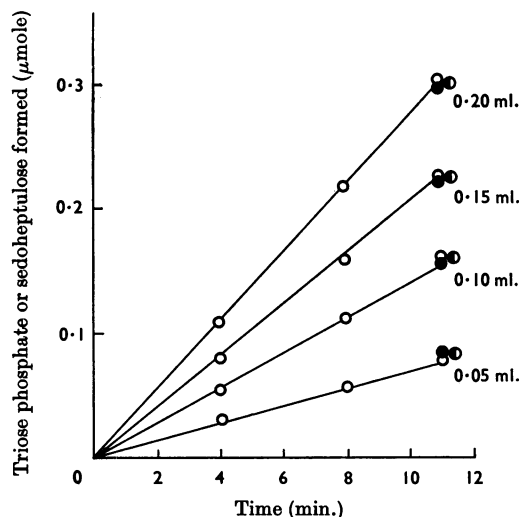


Fig. 4. Comparison of two methods of measurement of transketolase: (1) the formation of triose phosphate and (2) the formation of sedoheptulose 7-phosphate. The assay system contained 0.5 ml. of 0.25 M-glycylglycine-KOH buffer, pH 7.6, 0.2 ml. of 50 mM-ribose 5-phosphate, ribose 5-phosphate isomerase and ribulose 3-phosphate epimerase (approx. 0.6 unit), 0.05 ml. of triose phosphate isomerase- $\alpha$ -glycerophosphate dehydrogenase (diluted 1:10 with 0.25 M-glycylglycine-KOH buffer, pH 7.6), 0.2 ml. of NADH (2 mg./ml.), and 0.05–0.2 ml. of liver supernatant (as indicated), from 1:10 liver homogenate prepared in the KCl-MgCl<sub>2</sub>-EDTA-mercaptoethanol mixture; the total volume was 2.7 ml. The decrease in  $E_{340}$  was followed for 11 min. at 25° to measure triose phosphate (○). At the end of this time 0.3 ml. of 10 N-HClO<sub>4</sub> was added to each cell and, after removal of protein, the sedoheptulose 7-phosphate (as sedoheptulose) (●) was determined as described in the Experimental section. For the formation of sedoheptulose 7-phosphate when triose phosphate was not trapped (◐), the incubation system was exactly as described above except that triose phosphate isomerase- $\alpha$ -glycerophosphate dehydrogenase and NADH were omitted.

Smyrniotis & Hurwitz, 1957; Simpson, 1960; Srivastava & Hübscher, 1966) or by reduction of NAD<sup>+</sup> in the presence of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (Cooper, Srere, Tabachnick & Racker, 1958; Datta & Racker, 1961). The former method was used in the present work. This has the advantage, as pointed out by Racker (1961a), that the product of the reaction is removed as soon as it is formed and thus prevents further metabolism by way of transaldolase.

Two comparisons were made: first, in the present method it can be shown that the sedoheptulose formed during the incubation period is exactly equivalent to the triose phosphate produced. The close matching of these two measurements (see Fig. 4) shows that, despite the complexities of the

incubation system and colour determination, there is no interference or drawing-off of either metabolite. More important is the comparison of the rate of sedoheptulose formation under conditions where triose phosphate is not specifically trapped; here again exact equivalence was shown (Fig. 4). These results suggest that, though transaldolase is present in approximately equal amounts to transketolase, the products of the transketolase reaction are not, in this system *in vitro*, metabolized further to form fructose 6-phosphate and erythrose 4-phosphate because of the removal of triose phosphate by aldolase and fructose diphosphatase to give fructose 6-phosphate in the presence of Mg<sup>2+</sup>. In the absence of a rapid rate of formation of triose by the glycolytic route this might lead to conditions favouring sedoheptulose 7-phosphate accumulation, and the relatively high concentration reported for calf liver (Nigam *et al.* 1959) might be an indication that this, in fact, can happen. Thus aldolase, fructose diphosphatase and Mg<sup>2+</sup> may be considered to be among the factors controlling the pentose pathway.

*Transketolase activity with other donor and acceptor sugar phosphates.* (a) Fructose 6-phosphate. In the present work ribose 5-phosphate was used as the substrate for transketolase and it was found that with liver supernatant fractions this was rapidly converted into xylulose 5-phosphate, these then acting as the acceptor aldehyde and donor of the C<sub>2</sub> moiety respectively. When fructose 6-phosphate was added together with ribose 5-phosphate to liver preparations it was found that it was not a good donor of C<sub>2</sub> units, as can be seen from Table 3. This shows that the rate of sedoheptulose 7-phosphate formation is proportional to the ribose 5-phosphate added and is not influenced by fructose 6-phosphate even when ribose 5-phosphate becomes rate-limiting. In this context Datta & Racker (1961) found that for purified yeast transketolase the  $K_m$  for fructose 6-phosphate is  $1.8 \times 10^{-3}$  M whereas that for xylulose 5-phosphate is  $2.1 \times 10^{-4}$  M. The present results would be consistent with the view that in animal tissue, also, the  $K_m$  for fructose 6-phosphate may be considerably higher than that for xylulose 5-phosphate.

(b) Erythrose 4-phosphate. Dische & Igals (1961) reported that erythrose 4-phosphate inhibits the reaction between xylulose 5-phosphate and ribose 5-phosphate in erythrocyte haemolysates. This was confirmed in the present experiments with liver supernatant preparations with ribose 5-phosphate as a substrate from which xylulose 5-phosphate is rapidly formed (see Table 4). After a 15 min. incubation period with ribose 5-phosphate as substrate 1.0  $\mu$ mole of sedoheptulose 7-phosphate was formed, whereas in the presence of 3.2  $\mu$ moles of erythrose 4-phosphate only 0.6  $\mu$ mole of sedoheptulose 7-phosphate was produced. However,

Table 3. *Utilization of ribose 5-phosphate and fructose 6-phosphate in the transketolase reaction*

The reaction mixture contained 250  $\mu$ moles of glycylglycine-KOH buffer, pH 7.6, 50  $\mu$ moles of  $MgCl_2$ , 6  $\mu$ moles of cysteine, 0.15 ml. of rat liver supernatant (1 part of liver to 3 parts of 0.1 M-tris buffer) and substrates as shown; the total volume was 2.5 ml. and this was incubated for 20 min. at 30°. Sedoheptulose 7-phosphate was measured in the deproteinized extract by the colorimetric method of Dische (1953). Ribose 5-phosphate and xylulose 5-phosphate are rapidly interconverted under these conditions, so that in this experiment comparison was being made of the contribution of xylulose 5-phosphate and fructose 6-phosphate as  $C_2$  donors in the formation of sedoheptulose 7-phosphate by the transketolase reaction. Details of methods are given in the Experimental section.

Substrates ( $\mu$ moles/2.5 ml.)		Product ( $\mu$ mole of sedoheptulose 7-phosphate/2.5 ml.)
Ribose 5-phosphate	Fructose 6-phosphate	
10	0	0.9
5	5	1.0
5	2.5	1.0
5	1.25	1.0
2.5	5	0.7
1.25	5	0.3

Table 4. *Utilization of ribose 5-phosphate and erythrose 4-phosphate in the transketolase reaction*

The reaction mixture contained 250  $\mu$ moles of glycylglycine-KOH buffer, pH 7.6, 50  $\mu$ moles of  $MgCl_2$ , 0.15 ml. of rat liver supernatant (1 part of liver to 3 parts of 0.1 M-tris buffer) and substrates as shown; the total volume was 2.5 ml. Samples were removed for determination of sedoheptulose 7-phosphate and fructose 6-phosphate at the time-intervals shown. In this experiment comparison was being made of the utilization of ribose 5-phosphate and erythrose 4-phosphate as acceptors of the  $C_2$  unit from xylulose 5-phosphate. Details of assay methods are given in the Experimental section.

Substrates ( $\mu$ moles/2.5 ml.)			Products ( $\mu$ moles/2.5 ml.)		
Ribose 5-phosphate	Erythrose 4-phosphate	Time (min.)	Sedoheptulose 7-phosphate	Fructose 6-phosphate	Sum of products
10	0	5	0.35	0.3	0.65
		10	0.70	0.3	1.0
		15	1.1	0.4	1.5
10	3.2	5	0.2	1.4	1.6
		10	0.35	1.5	1.85
		15	0.6	1.5	2.1
0	3.2	5	0	0.05	
		10	0	0.05	
		15	0	0.07	

a large amount of fructose 6-phosphate, one product of the reaction between erythrose 4-phosphate and xylulose 5-phosphate, is synthesized in this time-period. This suggests that the reaction of xylulose 5-phosphate and erythrose 4-phosphate proceeds at a faster rate than that with xylulose 5-phosphate and ribose 5-phosphate. This is clearly seen if the products of the reactions are measured after 5 min. incubation, when about seven times as much fructose 6-phosphate is formed as sedoheptulose 7-phosphate, and the sum of the products sedoheptulose 7-phosphate plus fructose 6-phosphate is doubled when erythrose 4-phosphate is added to the ribose 5-phosphate. Since erythrose 4-phosphate may be contaminated with triose phosphate or with glucose 6-phosphate, a blank reaction was

run with erythrose 4-phosphate alone to check on the formation of sedoheptulose from erythrose 4-phosphate and triose phosphate by way of aldolase, of fructose 6-phosphate from glucose 6-phosphate by way of phosphoglucose isomerase, or of fructose 6-phosphate from triose phosphate by way of triose phosphate isomerase, aldolase and fructose diphosphatase. There was only a small blank value for fructose 6-phosphate and none for sedoheptulose 7-phosphate production (Table 4).

The effect of erythrose 4-phosphate on the transketolase reaction with ribose 5-phosphate and ribulose 5-phosphate was also investigated by following the rate of glyceraldehyde 3-phosphate production with the linked system triose phosphate isomerase- $\alpha$ -glycerophosphate dehydrogenase and

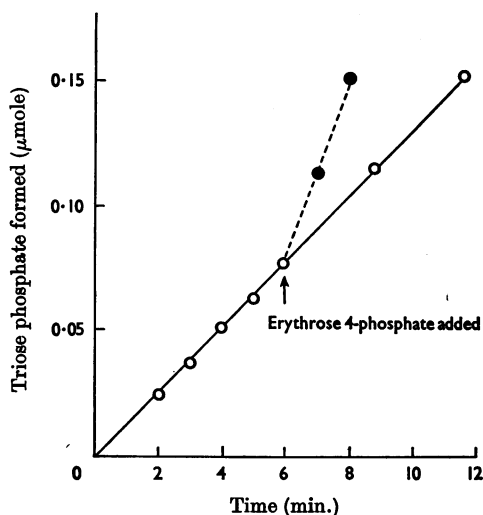


Fig. 5. Rate of formation of triose phosphate in the transketolase reaction with ribose 5-phosphate and erythrose 4-phosphate as substrates. The incubation medium was as in Fig. 4 and as described in the Experimental section, with 0.1 ml. of liver supernatant fraction from homogenate prepared in 9 vol. of KCl-EDTA-MgCl<sub>2</sub>-mercaptoethanol mixture. The rate of the transketolase reaction was measured with ribose 5-phosphate as substrate (○). At 6 min., 0.5 μmole of erythrose 4-phosphate was added to one cell and the increased rate of reoxidation of NADH was followed (●).

the reoxidation of NADH. Triose phosphate will be a product of the reaction whether ribose 5-phosphate or erythrose 4-phosphate is acting as acceptor aldehyde in the C<sub>2</sub> transfer from xylulose 5-phosphate (see Fig. 5). The results in Fig. 5 show that when erythrose 4-phosphate is added to a system containing ribose 5-phosphate and xylulose 5-phosphate there is a marked stimulation in the rate of triose phosphate production, again showing that this substrate is used rapidly in the transketolase reaction.

With a partially purified liver transketolase preparation the  $K_m$  for erythrose 4-phosphate is  $4.4 \times 10^{-5} M$  and that for xylulose 5-phosphate is  $1.6 \times 10^{-4} M$  (F. Novello & P. McLean, unpublished work); this again shows the high affinity of transketolase for erythrose 4-phosphate.

It is noteworthy that equilibrium constants for the crystalline yeast transketolase are 1.2 for the reaction between xylulose 5-phosphate and ribose 5-phosphate and 10.3 for the reaction between xylulose 5-phosphate and erythrose 4-phosphate (Racker, 1961a). The rapid removal of erythrose 4-phosphate by the transketolase reaction may be significant in view of the finding that erythrose 4-phosphate is a potent inhibitor of phosphoglucose

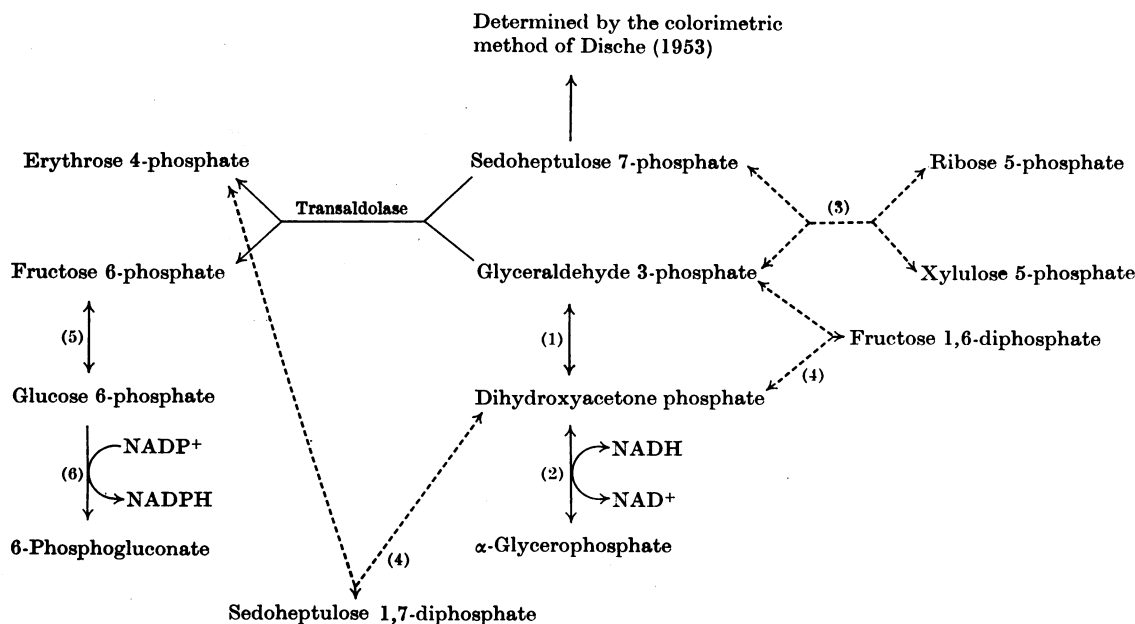
isomerase (Grazi, De Flora & Pontremoli, 1960; Venkataraman & Racker, 1961). Inhibition of phosphoglucose isomerase could have an important regulatory mechanism both in the glycolytic pathway and in the recycling of ribose 5-phosphate to glucose 6-phosphate via fructose 6-phosphate in the pentose phosphate pathway. In liver, transketolase is the rate-limiting step in the non-oxidative reactions of the cycle, so that alterations in this enzyme could have a regulatory effect on the glycolytic pathway.

#### *Transaldolase*

This enzyme was assayed in liver extracts either by the measurement of sedoheptulose 7-phosphate formation or by the measurement of triose phosphate production from fructose 6-phosphate plus erythrose 4-phosphate (Scheme 2). The methods of assay thus have some close similarities to those used for transketolase, and were used partly because of the more ready availability of the substrates fructose 6-phosphate and erythrose 4-phosphate and also because measurement in the opposite direction, i.e. the formation of fructose 6-phosphate from sedoheptulose 7-phosphate and triose phosphate, is complicated by the marked inhibition of phosphoglucose isomerase by both sedoheptulose 7-phosphate and erythrose 4-phosphate (Venkataraman & Racker, 1961; Grazi *et al.* 1960). Venkataraman & Racker (1961) have found that even a 1000-fold excess of phosphoglucose isomerase gave values for transketolase activity a little below maximum.

*Measurement of transaldolase by determination of sedoheptulose 7-phosphate formation.* Fig. 6 shows the volume of liver supernatant fraction and the time-period over which a linear response (sedoheptulose 7-phosphate formation) is obtained. The effect of longer incubation periods on the accumulation of products and disappearance of fructose 6-phosphate is shown in Fig. 7. Under the conditions of this experiment 3 μmoles of fructose 6-phosphate are utilized with the formation of 3 μmoles of sedoheptulose 7-phosphate and only 0.04 μmole of pentose phosphate is formed.

Two processes could interfere in the assay of transaldolase by measurement of sedoheptulose 7-phosphate formation. These are the formation of sedoheptulose 7-phosphate by reactions other than that catalysed by transaldolase and the removal of sedoheptulose 7-phosphate and triose phosphate in the transketolase reaction. The direct conversion of fructose 6-phosphate into sedoheptulose 7-phosphate and xylulose 5-phosphate by the combined action of transketolase and transaldolase has been demonstrated by Bonsignore, Pontremoli, Mangiarotti, De Flora & Mangiarotti (1962). However, in the present experiments only a very small



Scheme 2. Methods for the assay of transaldolase and some possible interfering reactions. The enzymes involved in this scheme of reactions are: (1) triose phosphate isomerase; (2)  $\alpha$ -glycerophosphate dehydrogenase; (3) transketolase; (4) aldolase; (5) phosphoglucose isomerase (inhibited by sedoheptulose 7-phosphate and erythrose 4-phosphate); (6) glucose 6-phosphate dehydrogenase. The broken lines represent pathways of metabolism that could interfere with the assay of transaldolase by measurement of either sedoheptulose 7-phosphate or triose phosphate.

amount of sedoheptulose could be detected when the liver extracts were incubated with fructose 6-phosphate in the absence of erythrose 4-phosphate. The preparation of erythrose 4-phosphate might be contaminated by triose phosphate (a further product formed by lead tetra-acetate oxidation), which could form sedoheptulose 1,7-diphosphate by a reaction catalysed by aldolase (Horecker, Smyrniotis, Hiatt & Marks, 1955). This possibility was eliminated by the finding that no sedoheptulose was formed when liver extracts were incubated with either of the two preparations of erythrose 4-phosphate described in the Experimental section.

Transaldolase activity might be overestimated if one of the substrates, erythrose 4-phosphate, combined with one of the products, triose phosphate, to give sedoheptulose 1,7-diphosphate by the aldolase reaction (Horecker *et al.* 1955). This would result in more than 1 equivalent of sedoheptulose being formed and this could rise to as much as a twofold increase if the reaction occurred rapidly. This process is probably not important since it was shown that there were equal amounts of fructose 6-phosphate lost and sedoheptulose 7-phosphate formed (Fig. 7). This equivalence also suggests that

triose phosphate is not being rapidly metabolized to give fructose 1,6-diphosphate by the aldolase reaction. This latter is of some importance when the transaldolase reaction is measured by the rate of formation of triose phosphate by the linked  $\alpha$ -glycerophosphate dehydrogenase assay.

Another factor that could cause difficulties is the removal of sedoheptulose 7-phosphate and triose phosphate by the transketolase reaction. The magnitude of this effect may be judged by the very small amount of pentose phosphate accumulating in the reaction medium even when the incubation is continued for as long as 90 min. (Fig. 7). The reason why so little is converted into pentose phosphate is not at present clear, but it may be related to removal of triose phosphate by side reactions and to the fact that transketolase is less active than transaldolase in rat liver. It is possible that other controlling factors are operating that impose a certain direction or restriction on reactions that appear to be otherwise readily reversible.

The inhibition of phosphoglucose isomerase by erythrose 4-phosphate is advantageous in this present assay since it inhibits the formation of glucose 6-phosphate from fructose 6-phosphate and

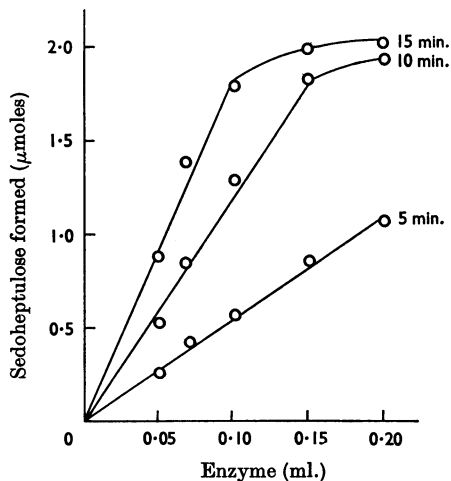


Fig. 6. Effect of time of incubation and concentration of enzyme on the determination of transaldolase by measuring sedoheptulose 7-phosphate formation. The incubation mixture contained 1.0 ml. of 0.25 M-glycylglycine-KOH buffer, pH 7.6, 0.1 ml. of 0.16 M-EDTA, 0.1 ml. of 0.1 M-fructose 6-phosphate and 0.1 ml. of 40 mM-erythrose 4-phosphate in a final volume of 2.5 ml.; 0.005–0.20 ml. (as indicated) of dialysed liver supernatant fraction was used. Incubation was for 5, 10 and 15 min. at 30°. At the end of this time 0.8 ml. portions of the mixture were deproteinized by addition of 1.2 ml. of 5% trichloroacetic acid. After deproteinization sedoheptulose 7-phosphate (as sedoheptulose) was determined in 1.0 ml. portions of the supernatant fraction.

thus maintains the substrate at a high concentration.

*Determination of transaldolase by measurement of triose phosphate formation.* Direct comparison was made between the above method and one based on the formation of glyceraldehyde 3-phosphate, which is measured with triose phosphate isomerase and glycerol 3-phosphate dehydrogenase by following the oxidation of NADH. There is an exact correspondence between these two methods (Fig. 8a). Interference by aldolase appeared to be negligible, since addition of excess of purified aldolase (3.6 units of Boehringer aldolase) did not result in any decrease in the rate of reoxidation of NADH, no doubt owing to the rapidity of the reactions involved in the  $\alpha$ -glycerophosphate route. The  $K_m$  for dihydroxyacetone phosphate is lower for  $\alpha$ -glycerophosphate dehydrogenase than that for aldolase,  $4.6 \times 10^{-4}$  M and  $1 \times 10^{-3}$  M respectively (Baranowski, 1963; Rutter, 1961), and the equilibrium of  $\alpha$ -glycerophosphate dehydrogenase is far on the side of glycerol 3-phosphate formation at neutral pH (Baranowski, 1963).

Srivastava & Hübscher (1966) used this method

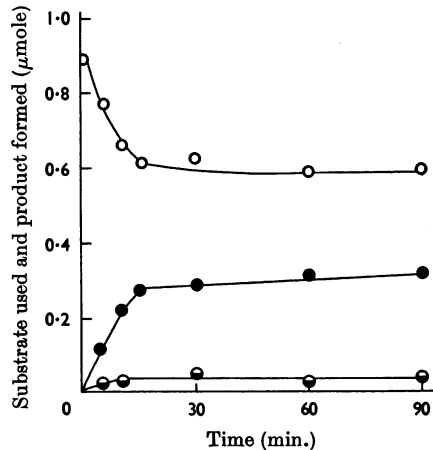


Fig. 7. Changes in concentrations of fructose 6-phosphate, sedoheptulose 7-phosphate and pentose phosphate with time when liver supernatant fraction is incubated with fructose 6-phosphate and erythrose 4-phosphate. The incubation medium was as described in the Experimental section and in Fig. 6, except that 0.03 ml. of liver supernatant fraction was used. At the time-intervals shown samples were removed for the determination of fructose 6-phosphate (○), sedoheptulose 7-phosphate (as sedoheptulose) (●) and ribose 5-phosphate (◐). The extent of interference by transketolase will be indicated by measurement of the pentose phosphate formed.

for determining transaldolase activity in intestinal mucosa and they found that the rate of the reaction appeared much faster, about threefold, in the direction of sedoheptulose 7-phosphate and triose phosphate formation than in the reverse direction. As pointed out by these authors this may be related to the inhibition of phosphohexose isomerase by erythrose 4-phosphate as shown by Venkataraman & Racker (1961) for yeast preparations. However, unlike the yeast preparation, the intestinal-mucosa transaldolase activity was not increased by addition of cysteine to the assay system. These findings again dictated the choice of method for transaldolase activity determination.

Since erythrose 4-phosphate has such a marked inhibitory effect on phosphoglucose isomerase it seemed important to test the effect on triose phosphate isomerase. It was found that at the concentrations used in the present assay there was only a small inhibition (approx. 20%; F. Novello & P. McLean, unpublished work). Further, addition of triose phosphate isomerase to the assay system did not in any way increase the rate of reoxidation of NADH.

The pH-activity curve, shown in Fig. 8(b), varied only slightly from pH 7.0 to 8.0, a result

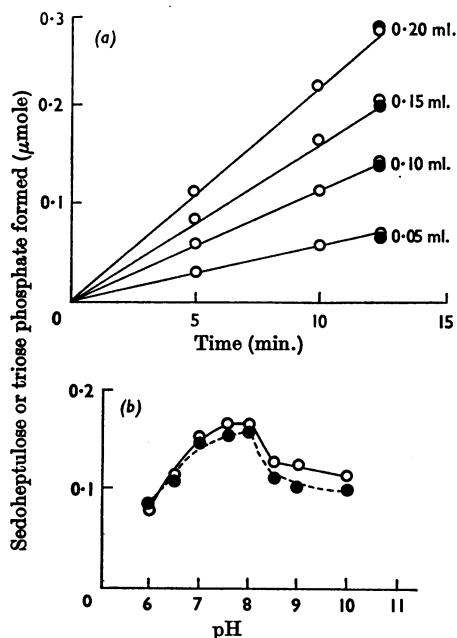


Fig. 8. Comparison of two methods of measurement of transaldolase: (1) the formation of triose phosphate and (2) the formation of sedoheptulose 7-phosphate. The effects of time of incubation, enzyme concentration and pH on the activity of transaldolase are shown. (a) The assay system contained 0.5 ml. of 0.25 M-glycylglycine-KOH buffer, pH 7.6, 0.1 ml. of 4 mM-erythrose 4-phosphate, 0.2 ml. of 50 mM-fructose 6-phosphate, 0.05 ml. of triose isomerase- $\alpha$ -glycerophosphate dehydrogenase (Boehringer preparation, 10 mg./ml., diluted 1:10 with 0.25 M-glycylglycine-KOH buffer, pH 7.6), 0.2 ml. of NADH (2 mg./ml.) and 0.05–0.2 ml. (as indicated) of liver supernatant fraction (1 part of liver in 10 parts of medium); the final volume was 1.4 ml. The reaction was followed at 340 m $\mu$  with 0.5 cm. cells to measure triose phosphate (O). After 12.5 min. the reaction was stopped by addition of 1 ml. of 1.0 N-HClO<sub>4</sub> and the deproteinized extract was used for determination of sedoheptulose 7-phosphate (as sedoheptulose) (●) as described in the Experimental section. (b) The incubation mixture was as described above but with the 0.25 M-glycylglycine-KOH buffer in the pH range shown. Partially purified transaldolase was used (approx. 0.02 unit). Activity was determined by measuring triose phosphate (O) and the formation of sedoheptulose 7-phosphate (as sedoheptulose) (●).

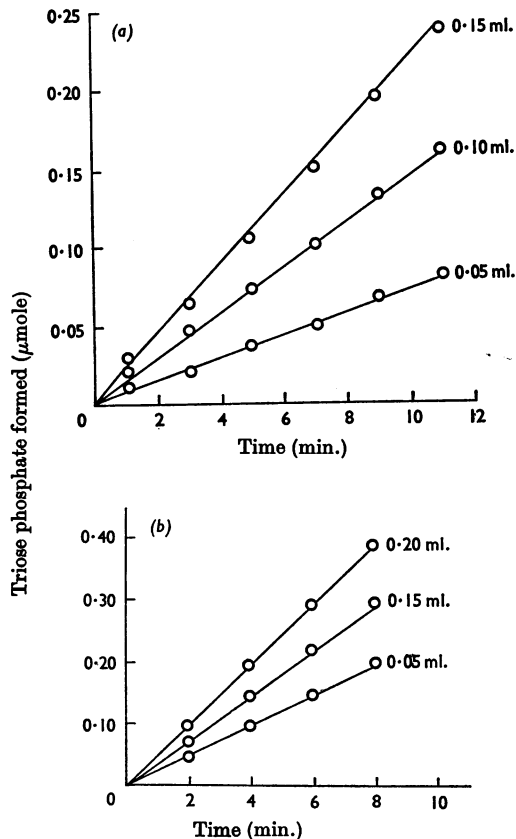


Fig. 9. Effect of variation in enzyme concentration and in the time of incubation on the determination of ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase in rat liver supernatant fractions. (a) The assay system for ribose 5-phosphate isomerase contained 0.5 ml. of 0.25 M-glycylglycine-KOH buffer, pH 7.6, 0.3 ml. of 0.1 M-ribose 5-phosphate (treated with NaOH to remove pentuloses), 0.2 ml. of NADH (2 mg./ml.), 0.05 ml. of triose phosphate isomerase- $\alpha$ -glycerophosphate dehydrogenase (Boehringer preparation, 10 mg./ml., diluted 1:10 with 0.25 M-glycylglycine-KOH buffer, pH 7.6), 0.1 unit of transketolase and 0.05–0.15 ml. (as indicated) of liver supernatant fraction prepared with 1 part of liver in 10 parts of medium; the final volume was 2.5 ml. The reoxidation of NADH was followed at 340 m $\mu$  with 1 cm. light-path at 25°. (b) The incubation medium for ribulose 5-phosphate 3-epimerase contained the same components as for the isomerase assay given above except that this system was supplemented with 0.1–0.2 unit of ribose 5-phosphate isomerase and that smaller portions of liver supernatant fraction were used, i.e. 0.01–0.02 ml. (as indicated) of liver supernatant fraction prepared with 1 part of liver in 10 parts of medium.

similar to that reported by Racker (1961b) for the yeast enzyme.

#### *Ribulose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase*

The assay of these enzymes was dependent on the addition of excess of transketolase, triose

isomerase and  $\alpha$ -glycerophosphate dehydrogenase and, for ribulose 5-phosphate 3-epimerase, of excess of ribose 5-phosphate isomerase. In each

case the substrate used was ribose 5-phosphate and the product measured was triose phosphate (see Scheme 1). In liver it was not necessary to supplement the ribose 5-phosphate isomerase assay with ribulose 5-phosphate 3-epimerase, since the latter enzyme is extremely active in this tissue. However, each tissue needs to be examined for the relative activities of these two enzymes to determine the necessity for supplementation. The results in Figs. 9(a) and 9(b) show the proportionality between enzyme concentration and time of incubation with the rate of reoxidation of NADH.

Ribose 5-phosphate free from ribulose 5-phosphate was required for the determination of ribose 5-phosphate isomerase, and therefore ribose 5-phosphate treated with alkali as described in the Experimental section was used. In addition, the substrate was tested with the complete system minus the tissue extract, i.e. with ribulose 5-phosphate 3-epimerase, transketolase, triose phosphate isomerase- $\alpha$ -glycerophosphate dehydrogenase mixture and ribose 5-phosphate, and readings were continued at 340m $\mu$  until there was no further extinction change. At this point the tissue extract was added and the reoxidation of NADH was followed spectrophotometrically. There was no

necessity to use the specially purified ribose 5-phosphate in the epimerase assay.

Kinetic studies with ribose 5-phosphate and erythrose 4-phosphate indicated that erythrose 4-phosphate was a competitive inhibitor of pentose phosphate isomerase (F. Novello & P. McLean, unpublished work). It also inhibits ribose 5-phosphate isomerase and triose phosphate isomerase. Such inhibitions must be kept in mind when transketolase is studied with erythrose 4-phosphate as the aldehyde acceptor and also when ribose 5-phosphate is used as the acceptor and the xylulose 5-phosphate is generated by adding ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase. The inhibition of isomerase reactions by erythrose 4-phosphate is most marked for the hexose phosphates and less so with the pentose phosphates and the triose phosphates.

#### *Enzyme activities in tissues*

The activities of the enzymes of the non-oxidative part of the pentose phosphate pathway were measured in some normal rat tissues and these results together with values for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydro-

Table 5. *Activities of the enzymes of the pentose phosphate pathway in rat tissues*

Tissue extracts were prepared in a medium containing 0.15M-KCl and 0.16M-KHCO<sub>3</sub>. The whole homogenate was centrifuged at 100000g for 40min. and the enzyme activities and protein content were determined on the dialysed supernatant fraction. Values are given in units defined as the amount of enzyme catalysing the formation of 1 $\mu$ mole of product/min. at 25°. The results are expressed as units/g. of tissue or milliunits/mg. of protein in the supernatant fractions; mean values  $\pm$  S.E.M. are given, with numbers of values in parentheses.

Tissue	Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase	Ribose 5-phosphate isomerase	Ribulose 5-phosphate 3-epimerase	Transketolase	Transaldolase
Activity (units/g. of tissue)						
Liver (6)	1.5 $\pm$ 0.1	2.8 $\pm$ 0.13	3.8 $\pm$ 0.2	28 $\pm$ 2.1	1.3 $\pm$ 0.03	1.2 $\pm$ 0.04
Adipose tissue (6)	1.18 $\pm$ 0.05	1.0 $\pm$ 0.12	1.2 $\pm$ 0.12	3.9 $\pm$ 0.2	0.4 $\pm$ 0.03	0.5 $\pm$ 0.02
Kidney (1)	0.70	0.62			1.1	1.4
Brain (1)	0.32	0.22			0.24	0.31
Adrenal gland (2)	5.9, 6.9	7.7, 6.7	4.3, 3.9	30, 31	1.3, 1.2	2.9, 2.4
Mammary gland:						
Pregnancy (5)	0.86 $\pm$ 0.09	0.92 $\pm$ 0.07			0.45 $\pm$ 0.05	1.0 $\pm$ 0.12
Lactation (16th day) (6)	50 $\pm$ 5	11 $\pm$ 2			5.2 $\pm$ 0.4	2.8 $\pm$ 0.3
Novikoff hepatoma (6)	2.8 $\pm$ 0.3	1.3 $\pm$ 0.15	3.2 $\pm$ 0.4	16 $\pm$ 1.4	0.7 $\pm$ 0.07	1.3 $\pm$ 0.2
Activity (milliunits/mg. of protein)						
Liver (6)	17 $\pm$ 0.7	33 $\pm$ 1.4	47 $\pm$ 2.2	330 $\pm$ 23	14 $\pm$ 0.3	13 $\pm$ 0.4
Adipose tissue (6)	72 $\pm$ 3	61 $\pm$ 7	74 $\pm$ 7	240 $\pm$ 12	24 $\pm$ 2	30 $\pm$ 1.5
Kidney (1)	9	8			15	18
Brain (1)	8	5.5			6	8
Adrenal gland (2)	132, 154	172, 150	96, 88	670, 690	30, 27	64, 53
Mammary gland:						
Pregnancy (5)	21 $\pm$ 2	23 $\pm$ 2			13 $\pm$ 1.5	27 $\pm$ 3
Lactation (16th day) (6)	350 $\pm$ 35	73 $\pm$ 10			35 $\pm$ 3	19 $\pm$ 2
Novikoff hepatoma (6)	56 $\pm$ 6	26 $\pm$ 3	63 $\pm$ 7	330 $\pm$ 28	14 $\pm$ 2	25 $\pm$ 4

genase are given in Table 5. Outstandingly high values for transketolase and transaldolase are found in adrenal gland, in lactating mammary gland and in adipose tissue, tissues that have previously been shown to have a high content of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Glock & McLean, 1954; Weber, Banerjee & Ashmore, 1960; Weber, Hird, Stamm & Wagle, 1965). In all tissues so far studied ribulose 5-phosphate 3-epimerase activity is considerably higher, about tenfold, than the ribose 5-phosphate isomerase activity.

It is of interest to see how far the enzymes of this pathway behave as a constant-proportion group of enzymes, a concept developed by Pette, Klingenberg & Bücher (1962) and by Pette, Luh & Bücher (1962), and which has been valuable in revealing the importance of certain enzymes that, in tissues with specialized function, do not conform to the basic pattern of the group. Examination of the results given in Table 5 show that though most tissues have an approximately equal activity of the enzymes of the cycle (ribulose 5-phosphate 3-epimerase being the exception in this case) lactating rat mammary gland and adrenal gland both have outstandingly high values for the oxidative enzymes of this cycle.

Though transketolase is, in many cases, the rate-limiting enzyme of the cycle when the enzymes are studied under optimum conditions *in vitro*, the overall rate of the cycle is probably limited by the supply of NADP<sup>+</sup>, the rate of reoxidation of NADPH in reductive synthetic reactions being an important factor in the control of the cycle. The stimulation of the formation of <sup>14</sup>C<sub>2</sub> from [1-<sup>14</sup>C]-glucose by mammary gland and adipose tissue in the presence of artificial electron acceptors such as phenazine methosulphate provides evidence for the limitation of this cycle by NADP<sup>+</sup> (McLean, 1960).

There have been relatively few quantitative measurements of enzymes of the non-oxidative part of the pentose phosphate cycle in animal tissues, though overall rates of the transformation of ribose 5-phosphate to hexose monophosphate have been reported and the disappearance of ribose 5-phosphate has also been used as an index of the activity of certain of the non-oxidative reactions of the cycle (see Hollman, 1964; Glock & McLean, 1954). The formation of sedoheptulose 7-phosphate from ribose 5-phosphate has been measured in a wide variety of tissues and organisms by Nigam *et al.* (1961). In most situations transketolase is likely to be the slowest reaction in the sequence of enzyme reactions taking part in this transformation. Nigam *et al.* (1961) also measured heptulose formation from ribose 5-phosphate in Ehrlich ascites-tumour cells, which have an activity comparable with that of normal tissues, and in Novikoff

hepatoma, in which this sequence of reactions is of lower activity than in normal liver. Colajacomo, Missale, Vergnano & Luzzatto (1957) also observed non-oxidative heptulose formation in sarcoma 181 ascites and Ehrlich solid carcinoma form. Measurements have been made of transketolase in erythrocytes and in different regions of the brain from normal and thiamine-deficient rats (Brin, 1967; Dreyfus, 1967), and the activity of ribose 5-phosphate isomerase has been reported for erythrocytes and a number of other tissues such as heart, skeletal muscle, liver and kidney (Bruns, Noltmann & Vahlhaus, 1958). The presence of a highly active ribulose 5-phosphate 3-epimerase from muscle and tumour cells and its almost complete absence from erythrocytes was observed by Dickens & Williamson (1956). One of the most extensive studies was that of Srivastava & Hübscher (1966) on the pentose phosphate cycle in intestinal mucosa.

The concentrations of intermediates of the pentose phosphate pathway have not been widely studied and it is difficult at the present time to make any correlation with enzyme activities and endogenous substrate concentrations. Glucose 6-phosphate concentration in liver is relatively high, 0.25  $\mu\text{mole/g.}$  of liver (Bücher, Krejci, Rüssmann, Schnitger & Weseman, 1964), whereas that of 6-phosphogluconate is only about one-tenth of this value; Arese (1964) gives a value of 0.027  $\mu\text{mole/g.}$  of liver. Sedoheptulose 7-phosphate appears to be present in liver in rather high concentration; both Nigam *et al.* (1959) and Bonsignore *et al.* (1959) reported values of about 0.1  $\mu\text{mole/g.}$  of liver. Sedoheptulose 7-phosphate is a competitive inhibitor of phosphoglucose isomerase, the  $K_i$  for the yeast enzyme being  $8.6 \times 10^{-6} \text{M}$  (Venkataraman & Racker, 1961). If the liver enzyme has a similar inhibitor constant the sedoheptulose 7-phosphate content of the liver might well have a powerful inhibitory action on the phosphoglucose isomerase.

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## REFERENCES

- Arese, P. (1964). *Biochem. Z.* **340**, 345.  
 Ashwell, G. & Hickman, J. (1957). *J. biol. Chem.* **226**, 65.  
 Ballou, C. E. (1963). In *Methods in Enzymology*, vol. 6, p. 479. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press Inc.  
 Baranowski, T. (1963). In *The Enzymes*, 2nd ed., vol. 7, p. 85. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York and London: Academic Press Inc.



- Baxter, J. N., Perlin, A. S. & Simpson, F. J. (1959). *Canad. J. Biochem. Physiol.* **37**, 199.
- Bonsignore, A., Foranini, G., Segni, G. & Antolini, C. (1959). *Ital. J. Biochem.* **8**, 106.
- Bonsignore, A., Pontremoli, S., Mangiarotti, G., De Flora, A. & Mangiarotti, M. (1962). *J. biol. Chem.* **237**, 3597.
- Brin, M. (1967). In *Ciba Found. Study Group no. 28: Thiamine Deficiency*, p. 87. Ed. by Wolstenholme, G. E. W. & O'Connor, M. London: J. and A. Churchill Ltd.
- Bruns, F. H., Dünwald, E. & Noltmann, E. (1958). *Biochem. Z.* **330**, 483.
- Bruns, F. H., Noltmann, E. & Vahlhaus, E. (1958). *Biochem. Z.* **330**, 483.
- Bücher, Th., Krejci, K., Rüssmann, W., Schnitger, H. & Weseman, W. (1964). In *Rapid Sampling Techniques in Biochemistry*, p. 255. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.
- Colajacomo, A., Missale, G., Vergnano, C. & Luzzatto, L. (1957). *Boll. Soc. ital. Biol. sper.* **33**, 1761.
- Cooper, J., Srere, P. A., Tabachnick, M. & Racker, E. (1958). *Arch. Biochem. Biophys.* **74**, 306.
- Datta, A. G. & Racker, E. (1961). *J. biol. Chem.* **236**, 617.
- de la Haba, G. & Racker, E. (1952). *Fed. Proc.* **11**, 201.
- Dickens, F., Glock, G. E. & McLean, P. (1959). In *Ciba Found. Symp.: Regulation of Cell Metabolism*, p. 150. Ed. by Wolstenholme, G. E. W. & O'Connor, M. London: J. and A. Churchill Ltd.
- Dickens, F. & Williamson, D. H. (1956). *Biochem. J.* **64**, 567.
- Dische, Z. (1938). *Naturwissenschaften*, **26**, 252.
- Dische, Z. (1953). *J. biol. Chem.* **204**, 983.
- Dische, Z. & Borenfreund, E. (1951). *J. biol. Chem.* **192**, 583.
- Dische, Z. & Dische, M. R. (1958). *Biochim. biophys. Acta*, **27**, 184.
- Dische, Z. & Igals, D. (1961). *Arch. Biochem. Biophys.* **93**, 201.
- Dreyfus, P. M. (1967). In *Ciba Found. Study Group no. 28: Thiamine Deficiency*, p. 103. Ed. by Wolstenholme, G. E. W. & O'Connor, M. London: J. and A. Churchill Ltd.
- Glock, G. E. (1952). *Biochem. J.* **52**, 575.
- Glock, G. E. (1964). *Hoppe-Seyler/Thierfelder*, no. 10, vol. 6A, p. 414.
- Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.
- Glock, G. E. & McLean, P. (1955). *Biochem. J.* **61**, 390.
- Grazi, E., De Flora, A. & Pontremoli, S. (1960). *Biochem. biophys. Res. Commun.* **2**, 121.
- Hiatt, H. H. (1957). *J. biol. Chem.* **229**, 725.
- Hollifield, G. & Parson, W. (1965). In *Handbook of Physiology: Adipose Tissue*, p. 393. Ed. by Renold, A. E. & Cahill, G. F., jun. Washington, D.C.: American Physiology Society.
- Hollman, S. (1964). In *Non-Glycolytic Pathways of Metabolism of Glucose*, p. 58. Ed. by Touster, O. New York and London: Academic Press Inc.
- Horecker, B. L. (1957). In *Methods in Enzymology*, vol. 3, p. 105. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Horecker, B. L. (1964). In *Comprehensive Biochemistry*, vol. 15, p. 48. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.
- Horecker, B. L., Gibbs, M., Klenow, H. & Smyrniotis, P. Z. (1954). *J. biol. Chem.* **207**, 293.
- Horecker, B. L. & Smyrniotis, P. Z. (1953). *J. Amer. chem. Soc.* **75**, 2021.
- Horecker, B. L., Smyrniotis, P. Z., Hiatt, H. H. & Marks, P. A. (1955). *J. biol. Chem.* **212**, 827.
- Horecker, B. L., Smyrniotis, P. Z. & Hurwitz, J. (1957). *J. biol. Chem.* **223**, 1009.
- Horecker, B. L., Smyrniotis, P. Z. & Klenow, H. (1953). *J. biol. Chem.* **205**, 661.
- Huggins, G. & Yao, F. (1959). *J. exp. Med.* **110**, 889.
- Leveille, G. A. & Hanson, R. W. (1966). *J. Lipid Res.* **7**, 46.
- McLean, P. (1960). *Biochim. biophys. Acta*, **37**, 296.
- Mangiarotti, G., Calissano, P. & Luzzatto, L. (1960). *Boll. Soc. ital. Biol. sper.* **36**, 1221.
- Marks, P. A. & Feigelson, P. A. (1957). *Proc. Soc. exp. Biol., N.Y.*, **95**, 376.
- Maxwell, E. S. (1961). In *The Enzymes*, vol. 5, p. 443. Ed. by Boyer, P. S., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Nigam, V. W., Sie, H.-G. & Fishman, W. H. (1959). *J. biol. Chem.* **234**, 1955.
- Nigam, V. W., Sie, H.-G. & Fishman, W. H. (1961). *Canad. J. Biochem. Physiol.* **39**, 1367.
- Noltmann, E. A. & Kuby, S. A. (1963). In *The Enzymes*, vol. 7, p. 223. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York and London: Academic Press Inc.
- Ostrovsky, Y. (1963). *Biokhimiya*, **28**, 22.
- Peeters, G. & Debackere, M. (1956). *Arch. int. Physiol. Biochim.* **64**, 527.
- Pette, D., Klingenberg, M. & Bücher, Th. (1962). *Biochem. biophys. Res. Commun.* **7**, 425.
- Pette, D., Luh, W. & Bücher, Th. (1962). *Biochem. biophys. Res. Commun.* **7**, 419.
- Racker, E. (1961a). In *The Enzymes*, vol. 5, p. 397. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York and London: Academic Press Inc.
- Racker, E. (1961b). In *The Enzymes*, vol. 5, p. 407. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York and London: Academic Press Inc.
- Rutter, W. J. (1961). In *The Enzymes*, vol. 2, p. 341. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York and London: Academic Press Inc.
- Simpson, F. J. (1960). *Canad. J. Biochem. Physiol.* **38**, 115.
- Srivastava, L. M. & Hübscher, G. (1966). *Biochem. J.* **101**, 48.
- Tabachnick, M., Srere, P. A., Cooper, J. & Racker, E. (1958). *Arch. Biochem. Biophys.* **74**, 315.
- Tepperman, H. M. & Tepperman, J. (1963). In *Advances in Enzyme Regulation*, vol. 1, p. 121. Ed. by Weber, G. London: Pergamon Press Ltd.
- Topper, Y. J. (1961). In *The Enzymes*, vol. 5, p. 429. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York and London: Academic Press Inc.
- Venkataraman, R. & Racker, E. (1961). *J. biol. Chem.* **236**, 1876.
- Weber, G., Banerjee, G. & Ashmore, J. (1960). *Biochem. biophys. Res. Commun.* **2**, 182.
- Weber, G., Hird, H. J., Stamm, N. B. & Wagle, D. S. (1965). In *Handbook of Physiology: Adipose Tissue*, p. 225. Ed. by Renold, A. E. & Cahill, G. F. Washington, D.C.: American Physiology Society.