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Pentose Phosphate Isomerase and Epimerase from Animal Tissues

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Pentose phosphate isomerase (PPI) was first described by Horecker, Smyrniotis & Seegmiller (1951) as a yeast enzyme which interconverts D-ribose 5-phosphate and D-ribulose 5-phosphate ($R\ 5-P \rightleftharpoons Ru\ 5-P$). The presence of this enzyme in a wide variety of cells explains the formation of R 5-P from the Ru 5-P arising on the hexose monophosphate oxidative pathway (for reviews see Racker, 1954; Gunsalus, Horecker & Wood, 1955; Dickens, 1955). This is one of the key reactions of this cycle, since the R 5-P accepts 'active glycolaldehyde', formed from a pentulose 5-phosphate by transketolase, yielding sedoheptulose 7-phosphate and triose phosphate.

Although the isomerase of spinach leaves has been studied (Axelrod, Bandurski, Greiner & Jang, 1953), and the plant enzyme has been much purified from extracts of alfalfa (lucerne) leaves (Axelrod & Jang, 1954), little work has hitherto been reported on the preparation and properties of PPI of animal tissues. Since Glock & McLean (1954) have shown that R 5-P breakdown occurs rapidly in extracts of many animals cells, the PPI activity in some of these has been studied and is described in the present paper.

Until quite recently it has been generally assumed that Ru 5-P is the only pentulose phosphate concerned in the hexose monophosphate oxidative pathway (cf. Gunsalus *et al.* 1955). Although D-

xylulose 1-phosphate (Xu 1-P) has been noted by several workers as a product of aldolase action on dihydroxyacetone phosphate and glycolaldehyde (Hough & Jones, 1952; Glock, 1952; Byrne & Lardy, 1954), this substance has not been clearly shown to be converted into the 5-phosphate, and its metabolism without preliminary re-cleavage by aldolase has not yet been demonstrated (cf. Glock, 1952). The suggestion by McGeown & Malpress (1954) that guinea-pig liver extract can form a ribose phosphate from Xu 1-P needs further supporting evidence, and in attempts to confirm this conclusion we have not yet succeeded in obtaining significant amounts of ribose, such as would demonstrate its formation.

The production of a pentulose phosphate from R 5-P in crude muscle extracts was described by Sable (1952), who believed this to be Ru 5-P. Ashwell & Hickman (1954) found that a D-xylulose phosphate was formed by the action on R 5-P of a fraction from mouse-spleen homogenate; this was the first indication of participation of this ester in animal metabolism. Ashwell & Hickman (1955) also observed the presence of a substance reacting as an erythro-3-pentulose among the reaction products: they suggest that the interconversion of the pentulose phosphates may have occurred by way of a 2:3-enediol intermediate compound.

In extracts of certain micro-organisms which can utilize D-xylose (*Lactobacillus pentosus*, Lampen, 1953; Mitsuhashi & Lampen, 1953; Stumpf & Horecker, 1956; *Pseudomonas hydrophila*, Hochster, 1955), reactions have been demonstrated by which free D-xylose is converted by a pentose isomerase into D-xylulose, the last being phosphorylated by adenosine triphosphate acting together with a xylulokinase to give the 5-phosphate; this is transformed by an epimerizing enzyme into Ru 5-P and thus finally into R 5-P by pentose phosphate isomerase.

The name phosphoketopento-epimerase has been introduced by Stumpf & Horecker (1956) for the enzyme responsible for the reaction $\text{Ru 5-P} \rightleftharpoons \text{Xu 5-P}$, and is abbreviated for the purposes of this paper to epimerase. This nomenclature seems preferable to the term 'ketopentose phosphate waldenase' suggested for the same enzyme by Hochster (1955), since it implies nothing as to the mechanism of the reaction, which is not yet decided. The occurrence of this enzyme in association with PPI in extracts of skeletal muscle and in Krebs II ascites carcinoma cells is described below. Epimerase activity is shown to be very low in similar extracts of PPI from erythrocytes. A preliminary account has been published (Dickens & Williamson, 1955).

After the present work had been completed, brief reports appeared showing that the true substrate for transketolase is not Ru 5-P but Xu 5-P (Srere, Cooper, Klybas & Racker, 1955; Horecker, Hurwitz & Smyrniotis, 1956; cf. Stumpf & Horecker, 1956, footnote 4). This would indicate that epimerase, like PPI, is an essential enzyme in the hexose monophosphate oxidative cycle.

EXPERIMENTAL

Materials

Those not described earlier (Dickens, 1938; Dickens & Glock, 1951; Glock, 1952) were as follows.

D-Ribose 5-phosphate (R 5-P). The barium salt, from acid cleavage of barium inosinate, after recrystallization still contained a few per cent of alkali-labile pentulose (cf. Glock, 1952; Moffatt & Khorana, 1956). This could be reduced to about 1% by column chromatography (see below), or more conveniently by treatment with 0.15*N* barium hydroxide for 20 min. at 20°, followed by neutralization with HBr and precipitation with 80% ethanol. The product was the dihydrate, and the pentose content of its solutions was based on analysis by means of the orcinol reaction.

D-Ribulose. This compound (purified through the *o*-nitrophenylhydrazone) and D-xylulose (purified on a Dowex-1, borate-form, column) were prepared by pyridine treatment of the corresponding aldoses (Glatthaar & Reichstein, 1935; Levene & Tipson, 1936). Crystalline 2:3-isopropylidene-D-xylulose was a gift from Professor A. W. Johnson and the *o*-nitrophenylhydrazones of D- and L-ribulose were given by Professor T. Reichstein.

Acid phosphatase. This was prepared from potatoes (Schäffner, 1941), and purified by precipitation with acetone (45%, v/v) according to details kindly supplied by Dr B. L. Horecker. An 0.1 mg. quantity was an excess for the complete hydrolysis of 1 μ mole of R 5-P or pentulose 5-P at pH 5, and did not produce any interconversion of ribulose and xylulose during the hydrolysis.

Methods

Pentuloses (present as the free sugars) were assayed as described by Dische & Borenfreund (1951) with colour development at room temperature for 2 hr. By this method ribulose (regenerated quantitatively from the *o*-nitrophenylhydrazone by benzaldehyde), ribulose combined as *o*-nitrophenylhydrazone, xylulose and 2:3-isopropylidenexylulose gave identical molar absorption at 540 $m\mu$.

2-Ketopentose phosphates. These were estimated according to Axelrod & Jang (1954) with an incubation period of 30 min. at 37°. This method, a modified Dische & Borenfreund (1951) cysteine-carbazole reaction for keto sugars, is convenient, but is open to criticism on the following grounds, which make it necessary to control the results by estimation of the free sugars after phosphatase hydrolysis. Whereas with free ribulose the colour development with the Dische & Borenfreund reagent rises rapidly to a steady value after a few minutes, with free xylulose (cf. Stumpf & Horecker, 1956), and with the pentulose phosphates at 37°, about 2 hr. are needed for full colour development (Fig. 1). Nevertheless the values, read after precisely 30 min. at 37°, followed by rapid cooling, are linearly related to the ketopentose content. An empirical correction factor of 2.2 must be applied to the readings when the pentuloses are present as phosphate

Fig. 1. Rate of colour development in the Dische & Borenfreund (1951) pentulose reaction. ●, Free ribulose, 0.175 μ mole, 20°; ○, free xylulose, 0.180 μ mole, 20°; ■, pentulose phosphates from muscle preparation, 0.175 μ mole, 37°; □, D-xylulose 1-phosphate, 0.180 μ mole, 37°. The temperatures are those for colour development (see text).

esters, and if the reading is taken after this standard 30 min. period. The method has been used only as a rough guide in following ketopentose phosphate formation.

Phosphatase hydrolysis of sugar phosphates. For analytical purposes, 1 ml. total volume, containing pentose phosphates equivalent to not more than 100 μ g. of pentose together with 0.2M acetate, pH 5, and 0.01M-MgCl₂, was incubated with 0.1 mg. of acid phosphatase for 2 hr. at 37°. Samples were then used for determination of pentulose (Dische & Borenfreund, 1951) or for the orcinol test.

Orcinol reaction. This was as described by Meijbaum (1939), with a 45 min. heating period. The loss of absorption at 670 m μ . in the orcinol reaction after 10 min. contact with N-NaOH at 20° gave the alkali-labile pentose phosphate. With these conditions, the destruction of pentuloses combined as the 5-phosphates is virtually complete, whereas the aldopentose 5-phosphates and the pentulose 1-phosphates are stable, the orcinol reaction being unaffected.

The molar absorption at 670 m μ . was the same for free aldopentose and aldopentose 5-phosphate. The pentulose 5-phosphates gave only 70% of the absorption due to equivalent aldopentose, and the free pentuloses, or their derivatives (ribulose *o*-nitrophenylhydrazones, isopropylidenexylulose) gave only 50% of that due to equivalent aldopentose (cf. Horecker *et al.* 1951), with the standard conditions. The use of these factors is not suitable for accurate determination, which was based on analyses before and after phosphatase action, determination of alkali-labile pentulose phosphate, and comparison with the Dische & Borenfreund (1951) reaction, as well as column chromatography.

The ratio of absorption, in the orcinol reaction, at 540/670 m μ . is a useful aid in identifying pentuloses (cf. Horecker *et al.* 1951); the ratios found by us were: D-ribulose, 0.85-1.00; D-xylulose, 0.35-0.46; 3-pentulose (see below), 1.15. The ranges given cover the variations observed, the lower values being given by larger amounts of pentulose in the standard test.

Bromine oxidation for removal of aldopentose. Aldopentose was selectively removed, in the presence of ketopentose, by adding to the solution (4 ml. of water containing up to 15 μ moles of pentose) 40 mg. of BaCO₃ and 0.2 ml. of saturated bromine water. After magnetic stirring at 20° for 1 hr. the barium salts were removed by centrifuging and the excess of bromine by a stream of nitrogen. Pentulose recovery in the final solution was 95% of that originally present, as shown by the Dische & Borenfreund (1951)

reaction. For larger quantities proportional amounts of reagents were taken. The use of a large excess of bromine must be avoided.

Deionization. Woolf (1953) has pointed out that the loss of sugars during deionization by mixed-bed resins may be avoided if the strongly basic component is first partially neutralized with CO₂. Dr T. R. E. Kressman of Permutit Ltd. kindly supplied a special (Biodemineralit G) mixed-bed resin, containing the less basic Deacidite G instead of the usual Deacidite FF, which has proved completely satisfactory for deionizing sugar solutions with nearly quantitative recovery. This step was regularly included after phosphatase treatment and before fractionation on either analytical or preparative columns.

Chromatography

Column chromatography. This was done on Dowex-1 resin (Dow Chemical Co., Midland, Michigan; 200-mesh, 10% cross-linked), used in the borate form for free sugars (Lampen, 1953), and in the formate form for sugar phosphoric acids (Horecker *et al.* 1951). Analytical columns, 1 cm. diam. $\times$ 15 cm., were used with small test samples (50-100 μ moles) of pentose or pentose phosphate. Fractions of 10 ml. at a flow rate of 1.5-2 ml./min. were received in an automatic collector (Towers and Co. Ltd.) and analysed by the methods described.

For free sugars the eluents were 0.02M sodium borate (Na₂B₄O₇; removes xylulose) and 0.04M sodium borate (removes ribulose) (cf. Fig. 7). For pentose phosphates elution was with 0.1M formic acid containing 0.1M sodium formate and from the pooled fractions, cautiously neutralized with Ba(OH)₂, the barium salts were precipitated by addition of 4 vol. of ethanol. This precipitation was usually repeated after discarding water-insoluble material. Examples are given below.

Separation of phosphates of aldopentoses and pentuloses. Separation was effected by a column of Dowex-1 (chloride form), 1 cm. diam. $\times$ 14 cm. Pentulose 5-phosphates were eluted, without separation of the xylulose- and ribulose-5-phosphates, by 0.03M-NH₄Cl containing 0.0005M-Na₂B₄O₇. This does not elute ribose 5-phosphate (cf. Khym & Cohn, 1953), which appears as a second well-separated peak when 0.05M-NH₄Cl without added borate is run through the column (cf. Fig. 6).

For preparative purposes the presence of Cl⁻ ions was undesirable, owing to the resulting contamination of the

Table 1. *Paper chromatography of free pentoses*

Phenol-water (4:1); front movement 300 mm.; 17 hr. on Whatman no. 1 paper (descending chromatogram). The last three columns give the colours on spraying; for conditions see text.

Pentose	R_F	R_F (Hochster, 1955)	Orcinol-TCA alone*	Aniline phosphate	
				Alone*	As overspray†
D-Ribose	0.59	0.63	None	Red	None
D-Ribulose	0.66	0.70	Brownish yellow	Yellowish brown	Bright pink
D-Xylose	0.44	0.49	None	Red-brown	None
D-Xylulose	0.59	0.63	Purple	Yellowish brown	Grey-purple
Ribulose + xylulose	0.64; 0.58	—	—	—	—
3-Pentulose	0.63	—	Brownish yellow	—	Orange

* With heating at 100° after spraying.

† Without subsequent heating after second spraying (first sprayed with orcinol-trichloroacetic acid (TCA) and heated to 100°).

barium salts with BaCl_2 . It was found preferable to use Dowex-1 (formate form) and perform successive elutions with 0.15 M ammonium formate containing 0.005 M $\text{Na}_2\text{B}_4\text{O}_7$ (removes pentulose phosphates), followed by borate-free 0.5 M ammonium formate for elution of ribose 5-phosphate. This separation was entirely satisfactory: an example is shown in Fig. 6.

Paper chromatography. Descending chromatograms were made on Whatman no. 1 paper with phenol-water (4:1, v/v) which, in agreement with Hochster (1955), was found best of several systems tried for the separation of free pentuloses (Table 1). The dried chromatogram was sprayed, first with trichloroacetic acid–orcinol (Klevstrand & Nordal, 1950), then after drying at 100° oversprayed (Hochster, 1955) with aniline phosphate (Bryson & Mitchell, 1951). The latter reagent (1.3 ml. of aniline and 0.6 ml. of orthophosphoric acid, sp.gr. 1.75, in 100 ml. of acetic acid) has proved advantageous over the aniline phthalate used by Hochster (1955), in that after heating to 100° it will detect clearly both aldopentoses and pentuloses (cf. Table 1). Also, on keeping the papers after overspraying with this reagent, aldose spots slowly develop at room temperature; the ketose spots appear at once, even without reheating.

Melting points. Those quoted are uncorrected.

RESULTS

Preparation of tissue extracts and activity test

Acetone-dried tissue. Freshly excised rabbit muscle was chilled and disintegrated in acetone previously cooled to -5° , in an Atomix or Nelco blender (Measuring and Scientific Equipment, Ltd., London). The filtered powder was washed with cold acetone and dried *in vacuo* over CaCl_2 . Erythrocytes of the horse, and Krebs II carcinoma ascites cells of the mouse, were centrifuged, washed with isotonic saline, and similarly treated with acetone. The acetone-dried powders stored at -10° retained their activity for months.

Determination of enzymic activity. Pentose phosphate isomerase (PPI) activity was determined as described by Axelrod & Jang (1954). An example is shown in Fig. 2, which also illustrates the unit of activity adopted. Since the extracts usually contained also xylulose phosphate epimerase, the colorimetric standard was normally the pentulose phosphate (equilibrium mixture of ribulose 5-phosphate and xylulose 5-phosphate) freed from aldopentose and isolated as barium salt, as described below. The total pentulose of the preparation was determined after phosphatase hydrolysis by the analytical methods already described.

Preparation of enzyme extracts. Centrifuging and other operations were at $0-4^\circ$. The acetone-dried tissue was stirred with 10 times its weight of 0.05 M aminotrihydroxymethylmethane (tris) buffer, pH 7.4, for 15 min. The extract from muscle was strained through muslin; extracts from erythrocytes and ascites cells were centrifuged. The residue was re-extracted similarly, and the combined

extracts were then rapidly heated to $55-60^\circ$, held at this temperature for 3 min. and cooled immediately to 0° . The clear filtrate obtained by filtering through a fluted agar-filter paper was brought to 0.3 saturation by addition of solid ammonium sulphate, and the centrifuged precipitate discarded. To the supernatant further solid ammonium sulphate was added to 0.5 saturation. After about 2 hr. at 0° , the precipitate was collected by centrifuging, suspended in water and dialysed at 0° overnight against water. The insoluble globulins were centrifuged off and the supernatant fluid, which contained the enzyme, was usually freeze-dried. The dry preparation was quite stable when stored at -10° .

Table 2 shows this purification, as applied to muscle extract. Similar results were obtained with erythrocytes and ascites tumour cells.

Further purification can be obtained by adsorption on calcium phosphate gel, followed by elution of the active material with successive portions of tris buffer (0.1 M, pH 7.0), whereby the units/mg. of protein are increased twofold. Little fractionation was obtained by precipitation at pH 4.6 (slight removal of inactive material) or by fractionation with ammonium sulphate at pH 5.

Activity of the muscle-enzyme preparation. The PPI activity, measured as the ketopentose formation from barium R 5-P (cf. Axelrod & Jang, 1954), is related to the quantity of enzyme, as shown in Fig. 2. Essentially similar results were obtained when the sodium salt was used instead of the barium salt. Fig. 2 also shows the unit of enzymic activity adopted: for assay purposes enzyme strength was adjusted by suitable dilution to fall on the linear portion of Fig. 2.

The enzyme from animal tissues is fairly thermostable, like that from lucerne (Axelrod & Jang, 1954). Heating in tris buffer at pH 7 for up to 3 min. at 70° yielded solutions having 90% of the initial PPI activity.

At other stages used in the purification losses are greater, particularly on dialysis. Attempts at reactivation by Mg^{2+} ions or cysteine, or protection by cysteine during dialysis, were not successful.

Table 2. *Purification of pentose phosphate isomerase from rabbit skeletal muscle*

Rabbit muscle (470 g.) gave 100 g. of acetone-dried powder, which was extracted with the following results:

Stage	Amount	Units
Tris-buffer extract	1700 ml.	6400
Heated, centrifuged	1520 ml.	5750
Ammonium sulphate fraction (0.3-0.5 sat.)	40 ml.	1720
Dialysed, centrifuged, freeze-dried	133 mg.	880

The average relative activity of PPI in the crude extracts from acetone-dried powders prepared from the various tissues was: rabbit muscle 55, horse erythrocytes 280, ascites tumour cells 800 units/g. of acetone-dried powder. The very high activity in the Krebs II ascites carcinoma is noteworthy. An active PPI was also detected in rat intestinal mucosa. After purification by the method described, the freeze-dried preparation from erythrocytes was the most active (10 000–16 000 units/g.), that from muscle having 4000–7000 units/g.

Fig. 2. Activity curve of a PPI preparation from muscle. Incubation, 10 min. at 37° with 1.25 μmole of R 5-P (barium salt); pH 7.4, tris buffer 0.1M. Corrected for initial pentulose, which here was 0.11 μmole. One enzyme unit is here 1 mg. of protein = 10 × the amount producing 0.1 μmole of pentulose (cf. Axelrod & Jang, 1954).

Fig. 3. pH-Activity curve for the muscle enzyme. ●, Acetate-veronal buffer; ○, tris buffers.

The optimum activity of the muscle enzyme occurs over the pH range 7–9 (Fig. 3).

The specificity was high: no pentulose was formed from any of the following: D-xylose 5-phosphate, D-arabinose 5-phosphate, D-xylose, D-arabinose or D-ribose. Since the purest preparations still contained an active aldolase, D-xylulose 1-phosphate, like fructose 1:6-diphosphate, was attacked with disappearance of keto sugar, but no alkali-labile ketopentose phosphate [tested for by the orcinol reaction and Dische & Borenfreund (1951) reaction] was produced. This indicates that no pentulose 5-phosphate was formed, since these compounds are alkali-labile, and the xylulose 1-phosphate is stable to alkali under the usual conditions (see Methods). Addition of Mg^{2+} ions and thiamine pyrophosphate during incubation did not affect this result.

Aldolase and hexose phosphate isomerase were present in the extracts from muscle, as these enzymes are precipitated in the region of 0.5 saturation with ammonium sulphate, and are not removed during the purification procedure. Table 3 gives a comparison of aldolase and PPI activity for three tissues. Unlike the extracts from muscle and ascites tumour cells, that from erythrocytes is free of aldolase activity. This important point will be discussed later.

Inhibitors of PPI activity. The following gave no significant inhibition with muscle PPI when the purified enzyme was treated in tris buffer (0.1M, pH 7.4) for 10 min. at 37° with the substance: iodoacetic acid (10^{-2} M), fluoride (5×10^{-3} M), arsanilic acid (10^{-3} M), cystine (2×10^{-4} M). Inhibitions of from 15 to 60% were given by rather high concentrations of iodosobenzoate (10^{-3} M), iodoacetamide (10^{-2} M), hydrazine (10^{-2} M), ethylenediaminetetraacetic acid (EDTA; 5×10^{-3} M), and also by $CuSO_4$ (1.6×10^{-4} M). 5-Phosphoribonic acid (2×10^{-2} M) inhibited 45%; a similar but stronger inhibition was observed for lucerne PPI by Axelrod & Jang (1954).

Table 3. Aldolase activity compared with pentose phosphate-isomerase activity of extracts

Period of incubation: for aldolase, 15 min. at 30° with fructose 1:6-diphosphate (FDP) in 0.1M tris buffer, pH 8.6 (method of Sibley & Lehninger, 1949). For PPI, usual assay conditions (see Methods); 10 min. at 37°, pH 7.4.

Enzyme preparation	Triose-P from FDP (μ-moles/mg./hr.)	PPI activity (μ-moles of pentulose/mg./hr.)
Purified, from rabbit muscle	152	26.4
Purified, from horse erythrocytes	0	72.0
Crude, from acetone-dried Krebs II ascites carcinoma	12	4.8

The most striking inhibition, which can be completely reversed by cysteine, is caused by mercurial compounds (Table 4). The enzyme may presumably be considered, therefore, to belong to the iodoacetate-resistant sulphydryl type, which

Table 4. *Inactivation of pentose phosphate isomerase from muscle by mercury and reactivation by cysteine*

Thunberg tubes contained, in the hollow stoppers, cysteine to give a final concn. of 10^{-3} M. The main part contained rabbit muscle PPI (50 μ g.) in 0.1M tris buffer, pH 7.4, to make the total volume 1 ml., with or without *p*-chloromercuribenzoate (final concn., 10^{-4} M). After evacuation and incubation for 5 min. at 37°, the cysteine was tipped in and incubation continued for a further 10 min. at 37°. The PPI activity was then determined by opening the tubes, adding 1.25 μ mole of R 5-P, incubating for 10 min. at 37°, and estimating pentulose formation as usual.

Tube no.	...	...	1	2	3	4
<i>p</i> -Chloromercuribenzoate	—	—	—	—	+	+
Cysteine	—	—	+	+	—	—
Pentulose formed (μ -mole)	0.209	0.206	0.000	0.206		
Per cent of original PPI activity	100	99	0	99

Fig. 4. Rate of formation and removal of pentulose phosphate by muscle enzyme. O, Substrate, 90% of Ru 5-P; 10% of Xu 5-P; ●, substrate, R 5-P. Substrate concn. 1.25 μ mole/ml.; at 37° containing 50 μ g. of enzyme protein. Samples were removed for analysis at the times shown, heat-inactivated and hydrolysed by acid phosphatase, and the pentulose was determined (see Methods).

probably includes also two other enzymes (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; Glock & McLean, 1953) of the hexose monophosphate oxidative pathway.

Level of pentulose phosphate formation and reversibility. When R 5-P is treated with an excess of pentose phosphate isomerase, the content of pentulose rises and reaches a steady state after about 1 hr. at 37° (Fig. 4). Expressed as the molar percentage conversion of total pentose (aldopentose and pentulose) present into pentulose, the 'equilibrium' value varied slightly with different preparations, but was 36–47% for muscle, 32–37% for erythrocytes (cf. Dickens & Williamson, 1955) and 35–37% for the ascites tumour cells. These values represent a considerably higher percentage conversion of total pentose into pentulose than that earlier recorded by Horecker *et al.* (1951), who found for the yeast enzyme 25% of pentulose. They are also higher than for the lucerne enzyme (Axelrod & Jang, 1954; 24.4% at 37°). The reverse reaction was demonstrated by incubating the muscle enzyme with the barium salt of the pentulose phosphate, prepared (as described below) from the incubation of the erythrocyte enzyme with R 5-P. The product is approximately 90% pure D-rubulose 5-phosphate, as will be shown later. On incubation with the muscle enzyme (Fig. 4) 55% of the pentulose present disappears (being transformed into ribulose 5-phosphate), at the same rate and to the same end-point as the transformation of added ribulose 5-phosphate into pentulose 5-phosphate (see Fig. 4).

The incubation, if prolonged, is accompanied by a small loss (10%) of total pentose phosphate present. The fact that this loss is so low even with crude muscle extracts, or even when aqueous extracts of fresh minced muscle are used (Fig. 5), indicates that very little transformation of pentose beyond the pentulose stage occurs in muscle (cf. Sable, 1952). Direct estimations of hexose monophosphate, by *Zwischenferment*, showed that even after incubation for 2 hr. this amounted to only 2% of the pentose phosphate. Similarly, heptulose (estimated by the difference in absorption at 610 and 530 m μ . in the orcinol reaction of Dische, 1953) accounted for only 5%, and triose phosphate (estimated by reduction of diphosphopyridine nucleotide by glycerophosphate dehydrogenase containing triose phosphate isomerase) for less than 2%, of the total products. Thus although skeletal muscle (of rat and mouse) does contain some transketolase-transaldolase activity (Glock & McLean, 1954), this system was exceedingly weak in our extracts of rabbit skeletal muscle.

Erythrocyte preparations also showed a similar stable end-point in the transformation of R 5-P into ribulose 5-phosphate. The reducing power measured by the method of Nelson (1944) showed no change

during this incubation. Liver extracts, on the other hand, prepared from acetone-dried rat liver in the usual way, had an active transketolase (cf. Horecker, Smyrniotis & Klenow, 1953), as shown by the high proportion of triose phosphate detected in liver extracts after incubation with R 5-P. These preparations from liver, therefore, gave bluish colours in the cysteine carbazole test for ketopentose, quite unlike the clear permanganate colour obtained with muscle, erythrocytes and ascites tumour cells.

Nature of the ketopentose

When this work was begun, it was commonly assumed that the only ketopentose formed enzymically from R 5-P was ribulose 5-phosphate (Horecker *et al.* 1953; Axelrod & Jang, 1954). It was thought necessary (Dickens & Williamson, 1955) to separate the aldopentoses and pentuloses and identify them, since our experiments had revealed very marked

Fig. 5. Incubation of crude muscle extract with R 5-P. Fresh rabbit muscle was extracted in a Nelco blender with 9 vol. of 0.15 M-KCl containing 0.002 M-KHCO₃. The dialysed extract was incubated at 30°, pH 7.6, with 0.005 M R 5-P (sodium salt) exactly as described by Glock & McLean (1954) in the presence of 0.01 M-MgCl₂; 0.06 M glycylglycine buffer. Samples (1 ml.) were deproteinized at intervals. ●, Pentulose phosphate; ○, alkali-stable (aldo-)pentose phosphate (orcinol reaction); ■, total orcinol-reacting pentose. The apparent decrease in total orcinol-reacting pentose is largely due to the fact that pentulose phosphates give only approx. 70% of the colour given by an equivalent amount of R 5-P (see Methods).

differences in properties of the 'equilibrium mixture' from the action of muscle and erythrocyte preparations on R 5-P.

For isolation of the products, quantities of 1 g. of barium R 5-P dissolved in 25 ml. of water adjusted to pH 7.0 were incubated at 37° with approximately 200 units (30 mg.) of various preparations of pentose phosphate isomerase. The incubation period (60–120 min.) extended 30 min. beyond that at which the pentulose formation reached a steady level. The reaction was stopped and the solution deproteinized by addition of 1.5 ml. of perchloric acid, followed by cautious neutralization to pH 7 by KOH. The filtrate was shaken with 2 g. of BaCO₃ and 25 ml. of freshly saturated bromine water at 20° for 1 hr. to remove aldoses. The clear filtrate, together with the washings (90 ml.), was aerated to remove excess bromine and adsorbed on a Dowex-1 (borate form) column, 3 cm. diam. × 12 cm., washed through with 100 ml. of water and eluted with 0.1 M formic acid containing 0.1 M sodium formate. Fractions (50 ml.) were collected and analysed; fractions 15–18, containing the pentulose phosphate, were combined and the barium salt was precipitated from them as usual (see Methods). The yield of crude barium salt (A) was 435 mg. from the rabbit-muscle enzyme preparation. The purity, based on ketopentose content, was 71%, corresponding to 31% of the total R 5-P taken, or 80% recovery of the total pentulose. At least 90% of the pentose phosphate was alkali-labile (i.e. less than 10% of aldopentose was present). The impurities (mainly BaCO₃ and barium formate) could be reduced by redissolving in a small volume of water and reprecipitation by ethanol, after filtration. Normally the crude salt (A) was used.

A similar preparation was made with the enzyme from horse erythrocytes. After incubation for 90 min. of the same quantities a yield of 300 mg. of barium pentulose phosphate (B) of similar purity was obtained.

In some experiments column-adsorption analysis was used instead of bromine oxidation to separate successfully the aldopentose and pentulose phosphates. The results (e.g. Fig. 6) were similar to those described, but no separation of individual pentulose phosphates was obtained by this method, and recovery of the total pentulose present tended to be lower.

Identification of the pentuloses present in the barium salts. The barium salts (A and B, above) of the pentulose phosphates obtained from muscle and erythrocyte preparations were hydrolysed by incubation with acid phosphatase. For this purpose, barium was first removed by a slight excess of Na₂SO₄, and after the phosphatase treatment the solution was deionized by a column of Biode-mineralit G (see Methods), the free sugars being

washed through with water. After concentration *in vacuo* the sugar solutions were first made 0.01M in $\text{Na}_2\text{B}_4\text{O}_7$ and then adsorbed on a column of Dowex-1 (borate form). Typical experiments for column analysis of muscle and erythrocyte preparations are described.

Muscle preparation. Fig. 7 (A) illustrates the separation of pentuloses obtained from the barium salt (20 mg. of prep. A, equivalent to 30 μmoles of total pentulose). The first sharp peak (I) eluted by 0.02M- $\text{Na}_2\text{B}_4\text{O}_7$ contained 11.7 μmoles of pentulose (by the Dische & Borenfreund reaction), and peak II, eluted by 0.04M- $\text{Na}_2\text{B}_4\text{O}_7$, had 9.3 μmoles ; total recovery of pentulose was 70%. Each of the combined fractions was freed from cations by a column of Amberlite IR 120 (H^+ form) and the borate removed by distillation with methanol (Khym & Zill, 1952).

Peaks I and II were qualitatively identified as xylulose and ribulose respectively as follows (their larger-scale identification is described in the next

section): (a) The positions of the fractions on elution were identical with those of pure xylulose and ribulose run on the same column. (b) The characteristic absorption spectra in the orcinol test plotted between 500 and 700 $\text{m}\mu$. were identical with those given by the pure sugars. The ratios of absorption ($D_{540}/D_{670 \text{ m}\mu}$) in this test (see Methods) were for three different muscle preparations: peak I, 0.35, 0.44, 0.42; peak II, 0.88, 0.90, 1.00. (c) Paper chromatography (cf. Table 1) gave identical movement and colour reactions with those of the authentic sugars (peak I, R_F 0.60; peak II, R_F 0.67).

It is concluded that the muscle preparation produces from R 5-P a mixture of pentulose phosphates consisting of 45% of ribulose and 55% of xylulose esters. The rate of acid hydrolysis ($\text{N-H}_2\text{SO}_4$ at 100°) corresponded closely over the whole range from 10 to 70% hydrolysis with a unimolecular hydrolysis constant $k = 5.2 \times 10^{-3}$. This agrees with that calculated from $t_{0.5} = 50$ min. for nearly pure ribulose 5-phosphate (Horecker *et al.* 1951, fig. 4), which would give $k = 5 \times 10^{-3}$. Presumably, therefore, k is nearly the same for xylulose 5-phosphate and ribulose 5-phosphate. On the other hand, these values are not consistent with

Fig. 6. Column chromatography of mixed aldopentose and pentulose phosphates. The incubation mixture consisted of the purified muscle enzyme with R 5-P after 3 hr. at 37° . The extract (deproteinized with HClO_4) was run on a Dowex-1 (formate) column, 3 cm. diam. $\times$ 15 cm., and eluted as shown, 25 ml. fractions being collected. The initial total pentose phosphate was 1570 μmoles . Pooled fractions of peak I contained 383 μmoles of pentulose phosphates (xylulose and ribulose); peak II, 900 μmoles of aldopentose phosphate (R 5-P). Recovery 82%.

Fig. 7. Column chromatography of free pentuloses. Analysis of mixed barium pentulose phosphates: A, from the muscle enzyme; B, from the erythrocyte enzyme. Peak I, D-xylulose; peak II, D-ribulose. (For details see Text.)

Axelrod & Jang's (1954) statement that virtually complete hydrolysis of their pentulose phosphate (believed by them to be ribulose 5-phosphate) appeared to be obtained in 30 min. in *N*-HCl at 100°. The value obtained by us clearly differentiates the product from xylulose 1-phosphate, for which $k = 86 \times 10^{-3}$ (Glock, 1952), and it is concluded from the mode of formation that the pentulose phosphates described here are all 5-phosphates. The possibility that a 1-phosphate or other labile ester may have been present in Axelrod & Jang's product of the lucerne enzymes acting on R 5-P does not seem to be excluded by their evidence, since they did not separate the pentulose phosphates from the equilibrium mixture before determining the rate of acid hydrolysis of phosphate from the mixed esters.

Erythrocyte preparation. Fig. 7 (B) shows the entirely different composition found for the pentuloses similarly obtained by use of the preparation from erythrocytes, instead of that from muscle. Barium salt B (10.0 mg. equivalent to 18.5 μ moles of pentulose) gave: peak I, 0.9 μ mole of xylulose; peak II, 8.5 μ moles of ribulose. The identification of these sugars was just as described for the muscle fractions.

Optical activity and derivatives of the pentuloses

Larger batches of barium salts of the pentulose phosphates (300 mg.) were hydrolysed and chromatographed as described above, but on larger columns (2 cm. diam. $\times$ 15 cm.). After being freed from ions and decolorized with a little charcoal at the methanol stage, the free separated pentuloses, corresponding to peaks I and II, were evaporated to dryness *in vacuo*, dissolved in a few millilitres of water and the optical rotation was determined in a Hilger Microptic polarimeter with a 2 dm. microtube.

The results showed: peak I, $[\alpha]_D^{20} = -31.5 \pm 2^\circ$ (c 0.54 in water, $\alpha = -0.34^\circ$); peak II, $[\alpha]_D^{20} = -18 \pm 2^\circ$ (c 0.49 in water, $\alpha = -0.18^\circ$). These values are close to those expected for D-xylulose ($[\alpha]_D^{20} = -33.2^\circ$; Levene & Tipson, 1936) and D-ribulose ($[\alpha]_D^{20} = +15.8^\circ$ for L-ribulose; Reichstein, 1934), respectively.

Further characterization was by preparation of the phenylosazone of peak I material: m.p. (crude) 146°, recryst. from aqueous ethanol, m.p. 156°; mixed m.p. with D-xylosazone of m.p. 157–158° = 154–155°; mixed m.p. with L-xylosazone of m.p. 158–159° = 191°. Mixed m.p. of L- and D-xylosazones prepared from L- and D-xylulose, 193°. Peak II material gave an *o*-nitrophenylhydrazone, m.p. 165–167°; D-ribulose-*o*-nitrophenylhydrazone had m.p. 166–167°; mixed m.p. 166–167°. Hence peak I is characterized as D-xylulose and peak II as D-ribulose.

Course of formation of D-ribulose 5-phosphate and D-xylulose 5-phosphate

At intervals during incubation of the muscle preparation with R 5-P, the amounts of ribulose and xylulose were determined after phosphatase hydrolysis (Table 5). D-Ribulose 5-phosphate is first formed by the action of PPI and is more slowly converted into D-xylulose 5-phosphate by epimerase. The activity of epimerase is high in muscle, and very low in erythrocytes, despite the higher activity of PPI in the latter (cf. p. 571). This evidence shows clearly that PPI and epimerase are two distinct enzyme systems.

Krebs II carcinoma ascites cells of the mouse

The high activity of PPI in these cells has already been described. That they also contain an active epimerase is shown by the following experiment. The acetone-dried powder (300 mg.) was extracted four times with 5 ml. portions of 0.05M tris buffer, pH 7.4; the extract was diluted to 25 ml. and centrifuged. The extract (1 ml., containing 13.2 units of PPI) was incubated at 37° with 125 μ moles of barium R 5-P in a total volume of 10 ml. of 0.01M tris, pH 7.4. Pentulose formation became steady at approx. 45–50 μ moles, after 60 min. The reaction mixture, brought to pH 5 with 2.5 ml. of 0.2N acetic acid, was heated for 3 min. in a bath at 100°, cooled, and treated with potato phosphatase as usual. The supernatant was freed from aldopentose by stirring for 1 hr. with 200 mg. of BaCO₃ and 1.5 ml. of freshly saturated bromine water. The pentulose content before bromine oxidation was 48 μ moles (38% of total R 5-P taken) and after removal of aldoses and ions, 39 μ moles of total pentulose. Recovery on the Dowex-1 (borate) analytical column was 90%, with clear separation of a xylulose peak (20 μ moles or 57% of total pentulose) followed by a ribulose peak (15 μ moles or 43% of total pentulose).

Table 5. *Relative rates of formation of the pentulose phosphates by muscle enzyme*

A portion (100 μ moles) of R 5-P (barium salt) in 10 ml. of water with 5 mg. of freeze-dried muscle preparation was incubated at 37°. Samples (2.5 ml.) were removed at intervals, and differential analysis of pentulose content after phosphatase was carried out on Dowex-1 (borate) analytical columns (see Methods).

Time (min.)	Total pentulose phosphate (μ moles)	Ribulose 5-phosphate (μ moles)	Xylulose 5-phosphate (μ moles)
15	32	22	10
60	39	19.5	19.5
180	42	19	23

After the usual removal of ions, including borate, the residual aqueous sugar solutions were concentrated *in vacuo* to 0.5 ml., and 20 μ l. portions were chromatographed on paper in the phenol-water system and identified as xylulose and ribulose respectively by R_F and colour reactions.

Formation of a 3-pentulose in the muscle incubation mixture

The crude barium salt of the pentulose phosphates (334 mg.; equivalent to 580 μ moles of pentulose) was hydrolysed by potato phosphatase, and the sugars were fractionated on Dowex-1 (borate). After removal of peaks I and II (xylulose and ribulose) with 0.02 and 0.04 M borate as usual, a third peak was eluted by 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$ as described by Ashwell & Hickman (1954, 1955) for extracts of mouse spleen after incubation with R 5-P. After deionization and concentration *in vacuo*, the solution (5 ml.) gave a colour with the cysteine and carbazole reagents of Dische & Borenfreund (1951) which on long standing became deep blue; when this was read at the normal time of 2 hr. the absorption

curve shown in Fig. 8 was obtained. Fig. 8 also includes the results of the orcinol reaction (see Methods) and of the specific 3-pentulose test with cysteine and sulphuric acid (Dr G. Ashwell, personal communication, 1955). The last test indicates that the total amount of 3-pentulose present in the eluate was only 7.4 μ moles, or 1.3 % of the total pentulose taken. This small yield, and the absence at the time of details of Dr Hickman's test, explains why no 3-pentulose was detected by Dickens & Williamson (1955).

Paper chromatography in phenol-water (4:1) showed a single spot (R_F 0.61) which gave an orange colour on spraying with orcinol-trichloroacetic acid followed by aniline phosphate (see Methods). The 3-ketopentulose was clearly distinguished from ribulose (pink spot, R_F 0.63) and xylulose (purple, R_F 0.57), which were run simultaneously on the same paper.

DISCUSSION

Axelrod & Jang (1954) have obtained the most active preparation of pentose phosphate isomerase yet reported. Their enzyme, from lucerne, resembles in many respects the animal preparations described here. The main difference in properties concerns the equilibrium position. The lucerne enzyme, like that from yeast obtained earlier by Horecker *et al.* (1951), yields a reported ratio of Ru 5-P:R 5-P of 0.32-0.33 at 37°. The value for our erythrocyte preparation is much higher (approx. 0.54). It is likely, however, that these are not true equilibrium values, since we have shown that even with our erythrocyte enzyme nearly 10 % of the total pentulose phosphate is D-xylulose 5-phosphate. Yeast extracts of PPI, even after purification, have recently been found to contain epimerase (Srere *et al.* 1955), and consequently the yeast-enzyme equilibrium mixture would also be expected to contain Xu 5-P. This would not necessarily have been revealed by the column fractionation of pentulose phosphate used by Horecker *et al.* (1951), which in our experience does not clearly separate Xu 5-P from Ru 5-P. Whether Axelrod & Jang's preparation also produced Xu 5-P is not known, as the fractionation of the products has not been described.

The muscle preparation, which is rich in epimerase and transforms slightly more than 50 % of the Ru 5-P into Xu 5-P, gives an 'equilibrium' ratio of total ketopentulose 5-P:aldopentulose 5-P of 0.68. A greater increase might have been expected corresponding to the removal of 50 % of the reaction product; possibly the xylulose 5-phosphate itself affects the equilibrium. This ester has now been isolated in small quantities by Stumpf & Horecker (1956), so that this question, and also that

Fig. 8. Colour reactions of 3-pentulose fraction. From muscle enzyme incubated with R 5-P; details as in text. ●, Ashwell reaction with cysteine and sulphuric acid (0.2 μ mole of 3-pentulose*); ○, orcinol reaction and ■, Dische & Borenfreund (1951) reaction read after 2 hr. at 20° (0.3 μ mole of 3-pentulose*).

* The amounts of 3-pentulose taken in these tests are those calculated from the intensity of the Ashwell reaction at 450 mμ. (see text).

of its hydrolysis rate in N acid, could be tested directly.

The presence of a highly active epimerase in extracts from muscle and tumour cells, and its almost complete absence from erythrocytes, is further evidence of the separate nature of PPI and epimerase. This distribution is similar to that of aldolase, in which the erythrocytes are exceptionally weak (Sibley & Lehninger, 1949; see also Table 4); it is interesting to note the tightly bound association of epimerase with aldolase, which persists on re-crystallization, reported by Horecker *et al.* (1956).

The preparative details given by these authors for the bacterial epimerase are almost identical with those adopted independently by us (cf. Dickens & Williamson, 1955), so that the behaviour on purification appears closely the same for the two. Very recently Horecker *et al.* (1956) have separated the bacterial epimerase activity from pentose phosphate isomerase, and Srere *et al.* (1955) mention also the preparation of muscle PPI free from epimerase. The ratio of ketopentose phosphates given by Stumpf & Horecker's (1956) bacterial epimerase was 45:55 (Xu 5-P:Ru 5-P), whereas our muscle preparation gave rather more of the xylulose ester (55:45) in relation to ribulose phosphate in the final mixture.

Although the essential part played by epimerase in providing the Xu 5-P needed as the substrate of transketolase has been revealed by this recent work, the occurrence of the latter enzyme in animal tissues needs further study. It appears probable that the transketolase activity of muscle varies in different species, for Glock & McLean (1954) report fairly active utilization of R 5-P, and resynthesis of hexose monophosphate from it, in extracts of rat and mouse skeletal muscle. On the other hand, we find, in agreement with Sable (1952), that even with crude rabbit-muscle extracts (cf. Fig. 5) the metabolism of pentose phosphate beyond the stage of pentulose is very slow. The question arises whether the formation in the animal body of xylulose phosphate might have another additional function. A metabolic pathway for ingested D-xylulose in animals, similar to that described in the Introduction for micro-organisms, has not been described; and there is doubt (Sols, 1956) if the evidence of the primary intestinal phosphorylation of xylulose described by Hele (1953) can be considered as convincing. From the plant root system, a substance closely resembling D-xylulose is exuded, and this substance, like D-xylulose, powerfully stimulates germination of vegetable seeds and promotes root growth (Brown, Johnson, Robinson & Todd, 1949; Brown, Robinson & Johnson, 1949). The possibility of a growth-promoting effect of D-xylulose in animal cells is now being tested in our Laboratory.

SUMMARY

1. Preparations of pentose phosphate isomerase (PPI) have been made from rabbit skeletal muscle, horse erythrocytes and Krebs II ascites carcinoma cells of the mouse. The enzyme has been partially purified, and its properties have been studied.

2. The enzyme preparation from erythrocytes is virtually free from aldolase and contains very little phosphoketopentose epimerase (epimerase) activity. The pentulose phosphates formed from D-ribose 5-phosphate (R 5-P) have been identified as ribulose 5-phosphate (Ru 5-P; 90%) and xylulose 5-phosphate (Xu 5-P; 10%). This enzyme provides a convenient preparation of nearly pure Ru 5-P.

3. The similar preparation from muscle is rich in epimerase and yields a mixture of pentulose phosphates containing 55% of Xu 5-P and 45% of Ru 5-P. The free ketopentoses liberated by phosphate hydrolysis were separated by column adsorption and characterized as D-xylulose and D-ribulose by optical rotation and preparation of crystalline derivatives.

4. The carcinoma-cell 'equilibrium' mixture of pentulose phosphates is similar to that from muscle. Both of these preparations also contained aldolase.

5. The extent of conversion of R 5-P into pentulose phosphate at 37° was about 40%, a higher value than that (25%) reported for other PPI preparations. Possible reasons for this are discussed.

6. With the aid of preparations containing both PPI and epimerase acting on R 5-P, the rate of transformation of the first product, Ru 5-P, into Xu 5-P has been studied.

7. The presence in the final product of a 3-pentulose, as described by Ashwell & Hickman (1955) for spleen extracts, has been observed for the muscle preparation also; the amount present is, however, very small (1.3%).

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The Metabolism of Tryptophan

2. THE METABOLISM OF TRYPTOPHAN IN PATIENTS SUFFERING FROM CANCER OF THE BLADDER*

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Observations on cancer of the bladder, among dye workers and other groups susceptible to the action of external carcinogens, suggest that a certain proportion of tumours previously considered to be spontaneous may in fact be produced by chemical carcinogens derived either from the environment or from the metabolic processes of the organism itself (Boyland, Wallace & Williams, 1955*a, b*).

The investigations of Hueper & Wolfe (1937), Hueper, Wiley & Wolfe (1938), Bonser (1943), Bonser, Clayson, Jull & Pyrah (1952) and others have shown that a number of *o*-aminophenols can

induce cancer of the bladder in mice and dogs. The *o*-aminophenols 3-hydroxykynurenine and 3-hydroxyanthranilic acid are metabolites of tryptophan and are present in human urine both in the free state and conjugated as sulphuric and glucosiduronic esters. Boyland & Watson (1956) have shown that insertion of pellets containing 3-hydroxyanthranilic acid into the urinary bladder of mice induces cancer in a statistically significant proportion of animals. The present work deals with a system which has been evolved for the extraction of six metabolites of tryptophan from urine and their estimation. Preliminary reports of this work have already been published (Boyland & Williams, 1955*a, b*).

* Part 1: Boyland, Sims & Williams (1956).