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Note

Spontaneous changes in the ultraviolet absorption spectrum of D-ribulose (D-*erythro*-pentulose) 5-phosphate

TERRY WOOD*

Department of Biochemistry, McGill University, Montreal, Quebec (Canada)

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In 1968, Knowles and Pon[1] reported that the product of the action of spinach D-ribose 5-phosphate ketol-isomerase (EC 5.3.1.6) on D-ribose 5-phosphate had a u.v. absorption maximum at 280 nm. This initial product was converted into products having different spectral characteristics by treatment with acid or base, and further changes also occurred spontaneously in the presence of the isomerase[1,2]. Although a number of possible structures for the u.v.-absorbing intermediates have been suggested[1,2], as pointed out by Noltmann[3], none of the postulated structures correspond to that of D-ribulose (D-*erythro*-pentulose) 5-phosphate that was identified by Horecker *et al.*[4] as the product of the action of D-gluconic acid 6-phosphate:$NADP^+$ 2-oxidoreductase (dehydrogenase) (EC 1.1.1.44) on its substrate.

The present Note reports the formation by 6-phosphogluconate dehydrogenase acting on D-gluconic acid 6-phosphate of a compound which, in the absence of enzymes, gives rise to structures having spectra resembling those of the compounds generated from D-ribose 5-phosphate after treatment with D-ribose 5-phosphate ketol isomerase. When frozen solutions of this compound were thawed, a number of reversible spectral changes were observed. Under the same conditions, the spectra of D-xylulose (D-*threo*-pentulose) 5-phosphate and D-ribulose 1,5-bisphosphate remained unchanged.

Absorption spectra of ketopentose phosphates. — A solution of the freshly prepared sodium salt of D-ribulose 5-phosphate was thawed, diluted to a concentration of ~0.5mM in 50mM triethanolamine (Cl^-) buffer, pH 7.4, and the spectrum at 37° was recorded at intervals over 4 h. The earliest spectrum (3 min after thawing the solution) usually showed a small peak in the 266–276-nm region with a shoulder around 300 nm (Fig. 1). This peak rapidly disappeared with the formation of a peak at 305–307 nm that reached a maximum (ε 540)** after 20 to 30 min. This peak then began to decline with the appearance of another peak at 272–273 nm (ε 430)** and, in

*Present address: Department of Chemistry, Njala University College, Private Mail Bag, Freetown, Sierra Leone.

**Molar extinctions were calculated after correction for background absorbance, measured at 360 nm.

some experiments, an isosbestic point at 280 nm was observed (Fig. 1). After 240 min, there were no further changes. The solution was removed from the spectrophotometer and stored at −20° for 4 days. On thawing, a sequence of changes similar to those just described were noted, and, after freezing the solution a second time, could be repeated once more, 12 days later. Although the absorbance values were some 50% greater by the last run, the sequence of changes was the same, and the positions of the peaks varied by only 2 to 4 nm. The measurements were repeated on two other preparations of D-ribulose 5-phosphate, and essentially the same changes were observed.

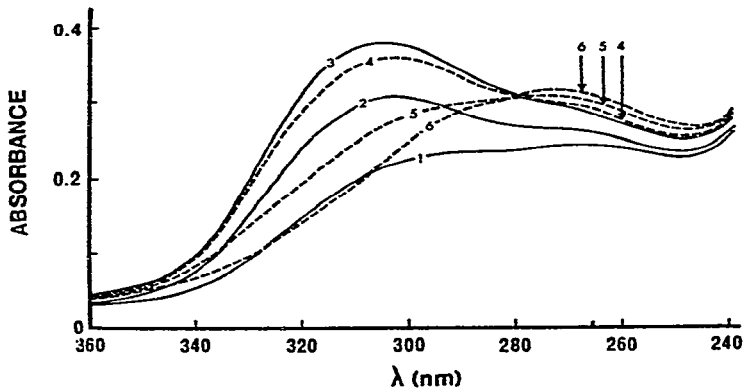

Fig. 1. Absorption spectra of 0.6mM D-ribulose 5-phosphate in 50mM triethanolamine chloride buffer, pH 7.4, at 37° in a 1-cm cell. Trace 1 taken at 3 min, trace 2 at 6 min, trace 3 at 22 min, trace 4 at 30 min, trace 5 at 60 min, and trace 6 at 105 min.

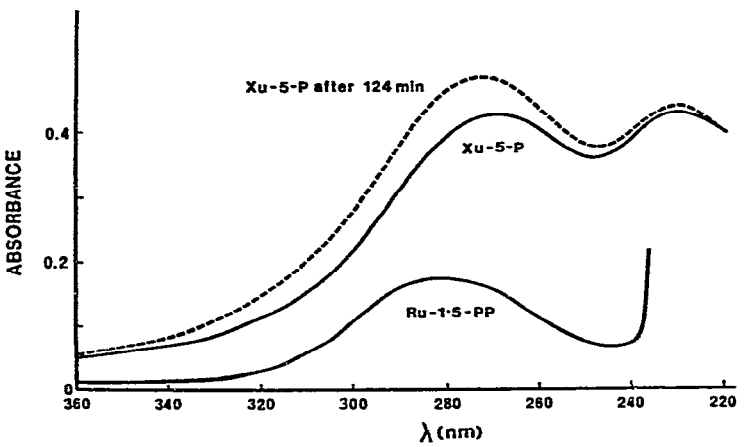

Fig. 2. Absorption spectra of: (a) 3.6mM D-xylulose 5-phosphate (Xu-5-P) at pH 7.4, in a 0.2-cm cell at 37°; and (b) 4.4mM D-ribulose 1,5-bisphosphate (Ru-1.5-PP) in 50mM triethanolamine chloride buffer, pH 7.4, at 37° in a 1-cm cell.

A solution of the sodium salt of D-xylulose 5-phosphate, under the same conditions, gave a spectrum (Fig. 2) that changed only slightly with time, and these changes were attributed to the approximately 10% of D-ribulose 5-phosphate contaminating the sample. Similar results were obtained with two other preparations. The extinction coefficient (ε_{268} 400)* was in the same range as that measured for D-ribulose 5-phosphate prepared from D-gluconic acid 6-phosphate, purified, and stored as a frozen solution. For purposes of comparison, the spectrum of the tetrasodium D-ribulose 1,5-bisphosphate was recorded (Fig. 2). The λ_{max} at 280 nm and ε 73 were close to the values of λ_{max} 282 nm and ε 85.4 reported for this compound by Gray and Barker[5], and the spectrum did not change with time.

D-Ribulose 5-phosphate was prepared by adding 2 units/ml of D-ribose 5-phosphate ketol-isomerase to a 10mM solution of D-ribose 5-phosphate at 37° and pH 7.4. The formation of the chromophore with λ_{max} 275–280 nm, ε 85**, was complete[6] after 10 min. When the absorbance at 290 nm was monitored, it began to increase after 65 min in 50 mM triethanolamine (Cl^-) buffer, and after 25 min in 50mM phosphate (Na^+) buffer. Mixtures were prepared in both buffers and, after the isomerase equilibrium had been established, the incubation at 37° was either continued and the spectra recorded, or the isomerase was removed by ultrafiltration and the spectrum of the ultrafiltrate at 37° was recorded at intervals. The pattern of changes summarised in Table I was established. In phosphate buffer, a marked bathochromic shift to λ_{max} 304–306 nm with an increase in absorbance was observed, followed by a hypsochromic shift with a fall in absorbance, followed later by an increase in absorbance at 280 nm. Phosphate ions appeared to catalyse or participate in changes that occurred only minimally in triethanolamine buffer. These changes were similar to those reported previously[2,9] for a mixture in phosphate buffer at pH 7.4 with isomerase present.

Concentration-dependence of the increase in absorbance at 280 nm. — After the D-ribose 5-phosphate ketol-isomerase equilibrium had been established in 50mM triethanolamine (Cl^-) buffer, pH 7.4, the solution was filtered and the ultrafiltrate diluted twice, and then four times with buffer. The increases in absorbance at 280 nm and 37° of the original and diluted ultrafiltrate were recorded simultaneously in a Gilford 240 spectrophotometer fitted with a 6040 Recorder and Automatic Cuvette Positioner. A plot of the initial rate of change against the square of the D-ribulose 5-phosphate concentration was linear (correlation coefficient, 1.00). This suggested that a dimerisation, analogous to that observed by Sieben *et al.*[10] with D-erythrose 4-phosphate, was responsible for the increase in absorbance.

C.d. and o.r.d. spectra of the isomerase product. — After the isomerase equilibrium had been established in 50mM triethanolamine (Cl^-) buffer, the solution was ultrafiltered at 37°, and the ultrafiltrate was incubated for 3 h at 37°, during which

*Molar extinctions were calculated after correction for background absorbance, measured at 360 nm.
**Extinction coefficients were calculated assuming that the absorbing species was 2.5mM D-ribulose 5-phosphate formed at equilibrium[7,8] from 10mM D-ribose 5-phosphate by the isomerase.

TABLE I

CHANGES IN THE ULTRAVIOLET ABSORPTION SPECTRA OF D-RIBOSE 5-PHOSPHATE–D-RIBULOSE 5-PHOSPHATE MIXTURES AT 37° AND pH 7.4[a]

Isomerase present			*Isomerase removed*		
Time (min)	λ_{max} *(nm)*	ε	*Time (min)*	λ_{max} *(nm)*	ε
50mM Triethanolamine (Cl^-) buffer					
11	275	92	40	275	110
42	275	100	95	281	135
88	275	120	185	277	200
305	275	174	310	280	274
50mM Phosphate (Na^+) buffer					
18	275	104	23	275	100
63	306	370	75	304	215
68	303	330	89	289	142
140	285	400	125	280	230

[a]Molar extinction coefficients were calculated on the assumption that the observed absorption was due to 2.5mM D-ribulose 5-phosphate.

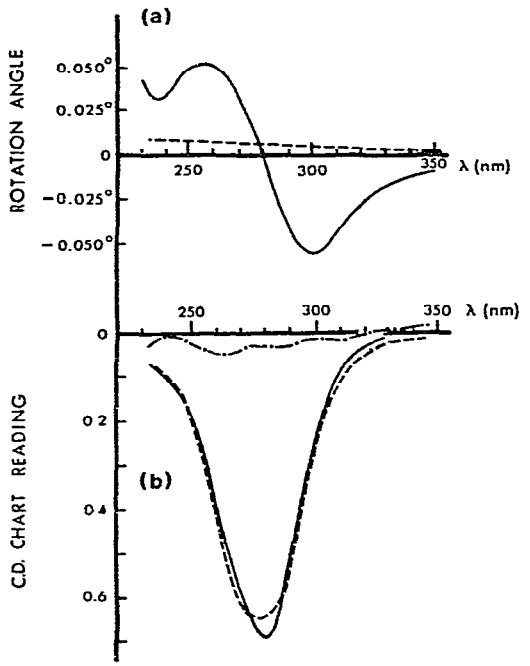

Fig. 3. O.r.d. and c.d. spectra in a 1-cm cell at 18°: (a) ———, o.r.d. spectrum of nonincubated isomerase product from 10mM D-ribose 5-phosphate in 50mM triethanolamine chloride buffer, pH 7.4; — — —, o.r.d. spectrum of 10mM D-ribose 5-phosphate in the same buffer (both spectra corrected for the base-line with buffer alone); and (b) c.d. spectra of the same mixture: ———, nonincubated; — — —, after incubation for 3 h at 37°; and —·—·—, spectrum of the buffer alone.

the absorbance at 280 nm increased from 0.18 to 0.60. A second ultrafiltrate of absorbance 0.18 was prepared 150 min after the first and kept on ice. The c.d. and o.r.d. were measured on both solutions, the absorbances of which were found to be almost unchanged at the conclusion of the measurements. The height of the peak of the incubated sample was 95% of the height of the peak of the nonincubated sample, indicating that less than 5% of the species responsible for the c.d. peak could have disappeared during the 3-h incubation at 37° (Fig. 3). Both samples were assayed enzymically for D-ribulose 5-phosphate[11,12]. Values of 2.38 ± 0.03mM (std. dev. of 4 determinations) and 2.20 ± 0.06mM (std. dev. of 4 determinations) were obtained for the nonincubated and incubated samples, respectively, so not more than 7.5% of the D-ribulose 5-phosphate had been destroyed. No difference could be detected between the two o.r.d. spectra. The o.r.d. spectrum had a negative peak at 300 nm ($[\alpha]$ −850°), a positive peak at 258 nm ($[\alpha]$ +1,030°), and an inversion of sign at 280 nm. D-Ribose 5-phosphate alone had a weak dispersion (Fig. 3). The strong negative peak [(θ) −4,420°, nonincubated sample] at 280 nm in the c.d. spectrum indicated that the compound responsible for the absorption peak at 280 nm was optically active.

Gray and Barker[5] have shown that a number of ketose phosphates that cannot exist in ring forms are present in solution as the open-chain, keto form and absorb in the 268–288 nm region with extinction coefficients ranging from 15 to 85. The initial product of the action[6] of isomerase on D-ribose 5-phosphate (ε_{280} 85) and of D-gluconate 6-phosphate dehydrogenase on D-gluconic acid 6-phosphate (ε_{290} 54–80) has an extinction coefficient within this range and, as revealed by its c.d. spectrum, is optically active. It is concluded that this initial product has the asymmetric structure usually attributed to the open-chain, keto form of D-ribulose 5-phosphate.

After manipulations involving precipitation, freezing, or simply keeping the solution, compounds were produced having extinction coefficients higher than 85. These compounds, however, still appeared to retain their asymmetry, as there was little or no accompanying loss of material assayable with the enantiomer-specific D-ribulose 5-phosphate 3-epimerase (Ref. 13 and above-described results). The compound prepared from D-gluconic acid phosphate and then purified is already different in structure from that, originally formed in solution, having ε_{290} 54–80. However, it can be observed to undergo a series of spontaneous rearrangements or associations in solution, similar to, if not identical with, those of the product of the isomerase reaction in phosphate buffer, namely, a bathochromic shift to a peak at 305–307 nm, followed by a hypsochromic shift.

EXPERIMENTAL

Materials and methods. — The sodium salts of D-gluconic acid 6-phosphate and D-ribose 5-phosphate, tetrasodium D-*erythro*-pentulose 1,5-bisphosphate, D-gluconate 6-phosphate dehydrogenase (Type VI, from yeast, 30 units/mg), D-ribose 5-phosphate ketol-isomerase (Type I, from spinach, 75 units/mg), and lactate dehydrogenase

(Type III, from beef heart, 670 units/mg) were obtained from Sigma Chemical Co., St. Louis, Mo. 63178. Except where stated, all spectra were measured with a Beckman DB double-beam spectrophotometer connected to a Photovolt 43 linear-log recorder. Optical rotatory and circular dichroic spectra were measured on a Jasco o.r.d./c.d. instrument at 18° (Japan Spectroscopic Co. Ltd., Tokyo). Ultrafiltration was performed in a 10-ml cell through a Diaflo PM-30 membrane (Amicon Corp., Lexington, Mass. 02173). The absence of D-ribose 5-phosphate ketol-isomerase in the ultrafiltrate was verified by placing a portion of the solution in a spectrophotometer cell at 37° and adding D-ribose 5-phosphate. The absence of any increase in absorbance at 290 nm showed that all the isomerase had been removed[6].

Preparation of D-*ribulose* (D-erythro-*pentulose*) *5-phosphate.* — D-Ribulose 5-phosphate was prepared enzymically from D-gluconic acid 6-phosphate [4,14]. Full activity of the D-gluconate 6-phosphate dehydrogenase required the presence of both 2mM ethylenediamine tetraacetate and 2mM dithiothreitol in the reaction mixture. Only lactate dehydrogenase from beef heart was found to be sufficiently free of D-ribulose 5-phosphate 3-epimerase (EC 5.1.3.1) to be suitable for the regeneration of NADP in the preparation. The product was precipitated with barium ion, treated with charcoal to remove NADP, and isolated as the barium salt of the hydrazone which was stored in a desiccator at −20°. D-Ribulose 5-phosphate was regenerated by adding Dowex 50W (H^+, 100–200 mesh) ion-exchange resin, adjusting the pH to 2 with dilute sulphuric acid and incubating at 37° for 10 min with frequent stirring to allow complete hydrolysis of the hydrazone and fixation of barium and hydrazine on the resin. The mixture was filtered, the filtrate cooled in ice, the pH adjusted to 6.2 with sodium hydroxide, and the frozen solution stored at −20°. Determinations of hydrazine[15] showed that 99% of it was removed by this procedure. Paper chromatography in three different solvent systems[16] showed only a single spot reacting with 3,4-dinitrobenzoic acid[17] and the absence of D-ribose 5-phosphate. Enzymic assay[11,12] showed that the D-ribulose 5-phosphate contained less than 10% of D-xylulose 5-phosphate and negligible amounts of pyruvate.

Preparation of D-*xylulose* (D-threo-*pentulose*) *5-phosphate.* — This compound was prepared by the condensation of D-glyceraldehyde 3-phosphate and hydroxypyruvic (3-hydroxy-2-oxopropanoic) acid in the presence of transketolase[18]. Enzymic assays[12,18] showed that it contained less than 10% each of D-ribulose 5-phosphate and hydroxypyruvic acid. The content of D-glyceraldehyde 3-phosphate was less than 5% of the amount of D-xylulose 5-phosphate, but an approximately equimolecular amount of L-glyceraldehyde phosphate would have been present since the DL-form of the triose phosphate was used in its preparation. However, as neither glyceraldehyde phosphate nor hydroxypyruvic acid absorbs in the range 230–360 nm, there would have been no interference with the measurement of the spectrum of D-xylulose 5-phosphate.

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