DIMERIZATION OF ERYTHROSE 4-PHOSPHATE

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1. Introduction

Erythrose 4-P has been inferred to play a key role in the non-oxidative pentose phosphate pathway [1], and in the path of carbon in photosynthesis [2], despite the fact that it has never been convincingly detected and characterized in any tissue. The studies of Kornberg and Racker [3], in which synthetic erythrose 4-P was added to transaldolase and fructose 6-P, to produce the recognized products heptulose 7-P and triose 3-P, enabled the hypothetical '4 carbon fragment' [4] to be confidently assumed to be erythrose 4-P.

Erythrose 4-P has been reported in Krebs ascites tumour cells at a concentration of 0.005 nmol/g of packed cells [5] and in mammary gland [6] at a concentration of 9.2–13.8 nmol/ml of intracellular water. The transaldolase method [7], used for these estimations, leaves room to doubt that the aldo acceptor molecule for the dihydroxyacetone moiety of fructose 6-P was in fact erythrose 4-P. Ribose 5-P will react in the transaldolase catalyzed reaction (1) to give rise to glyceraldehyde 3-P as does erythrose 4-P (2).

Ribose 5-P + fructose 6-P $\rightleftharpoons$

D-*glycero* D-*altro* octulose 8-P + glyceraldehyde 3-P (1)

Erythrose 4-P + fructose 6-P $\rightleftharpoons$

altro heptulose 7-P + glyceraldehyde 3-P (2)

Tentative chromatographic evidence for the presence of erythrose 4-P in *Chlorella* has also been presented [8]. The tetrose 4-P has still to be isolated and characterized, which allows the speculation that erythrose 4-P may not normally exist free at any significant concentration in biological systems.

In this paper we report that erythrose 4-P spontaneously dimerizes in aqueous solution which confirms the earlier proposal of Sieban et al. [9], and may in part account for the previous difficulties encountered in its detection and estimation in biological systems.

2. Materials and methods

Erythrose 4-P was either prepared by lead (IV) oxidation of glucose 6-P [9] or was generated from erythrose 4-P dimethyl acetal which was obtained from Calbiochem (Australia) Pty Ltd and was estimated by the cysteine–H_2SO_4–fructose method [10].

The trimethylsilyl (TMS) derivative of erythrose 4-P was prepared by reacting 1.5 mg of the freeze dried material (freshly generated from commercial preparation) with 0.25 ml of the following mixture (1.0 ml of bis-trimethylsilyl trifluoroacetamide (BTFA); 0.5 ml of trimethylchlorosilane (TMCS) and 1.0 ml of acetonitrile) [21]. The mixture was heated at 80°C for 10 min to ensure reaction. Mass spectra

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were recorded at 70 eV on a double focussing AEI mass spectrometer (MS-902) with a source temperature of 180°C. The above solution (20 μl) was evaporated onto the pyrollite tip of the direct insertion probe of the mass spectrometer and the residue fractionally distilled from the probe tip by manipulation of the distance of the tip from the hot (180°C) source. The accurate mass measurement was carried out at a resolution of approx. 16 000 (10% valley) using Tetrameric bis-trifluoroethylphosphonitrilate as a reference compound.

3. Results and discussion

Erythrose 4-P has not been detected using a variety of analytical procedures, in numerous experimental conditions, such as in studies using buffered extracts of tissue homogenates [11–14]. The effects of a widely used buffer (Tris-HCl, pH 7.4), rat liver enzymes and a frequently used protein denaturing agent ($HClO_4$) on the concentration of erythrose 4-P were studied (table 1). Erythrose 4-P concentration, in distilled water, as determined by transaldolase assay procedure [7] did not alter over a 4 h period. If samples were added to $HClO_4$ and neutralized with $KHCO_3$ however, the concentration fell by 21% (after 5 min). The addition of Tris-HCl buffer, with or without rat liver enzyme preparation, lowered the concentration by approx. 75%. Inorganic phosphate was not found to accumulate [15] in any of the incubations over a 4 h period. Erythrose 4-P has been found to be converted to an unidentified ester by hemolyzate enzymes [16], by a reversible unimolecular reaction. In a similar study, erythrose 4-P was utilized by myocardial enzymes to produce small amounts of heptulose-P and hexose 6-P [17]. Similarly, small amounts of heptulose-P and hexose-P were found to accumulate over the 4 h period with liver enzymes (table 1), as detected by the cysteine–H_2SO_4 colorimetric method [18]. The precise nature of these phosphorylated compounds is presently being investigated, however, preliminary paper chromatographic examination of the reaction products [19] indicated that glucose 6-P, fructose 6-P and *altro* heptulose 7-P are formed from erythrose 4-P by rat liver enzymes by an unknown reaction pathway.

The phenomenon of erythrose 4-P behaving as two distinctly different compounds is observed when erythrose 4-P is being purified by ion-exchange chromatography, after its synthesis from glucose 6-P by lead (IV) oxidation (fig.1) [9]. The erythrose 4-P material from peak 2 and peak 3 have distinctly different enzymatic and chemical properties, consistent with peak 2 material being monomeric while peak 3 material is dimeric. When equimolar amounts of the material from peak 2 and peak 3 (fig.1) (determined by total phosphate analysis

Table 1
Concentration of erythrose 4-P under a variety of incubation conditions

Component	Incubation A	B	C	D
50 mM Tris-HCl, pH 7.4 (μmol)	50	50	–	–
Dialyzed 100 000 *g* supernatant of a 10% homogenate of rat liver (mg)	–	10.4	–	–
Erythrose 4-P (μmol)	10	10	10	10
Concentration of erythrose 4-P in μmol after incubation for				
5 min	2.2	2.5	7.9	9.7
4 h	2.2	2.5	8.1	9.7

Each incubation in a total vol. 3.1 ml was maintained at 30°C. Samples of 0.6 ml were removed from incubations A, B and C and added to 0.5 ml of 0.6 M-$HClO_4$ and neutralized with saturated $KHCO_3$. Samples of 0.6 ml were removed from incubation D and diluted with water to the same extent as the $HClO_4$ treated samples. Each value is the mean of duplicate estimations from two experiments.

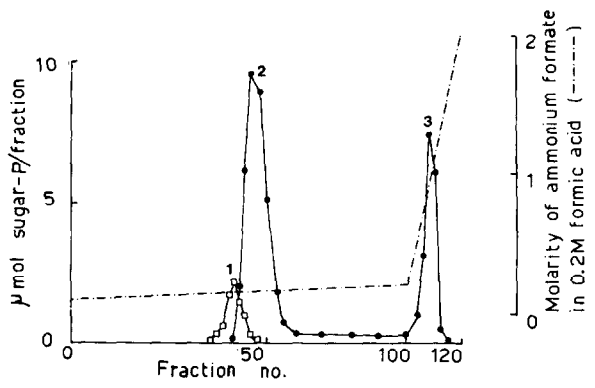


Fig.1. Chromatographic separation of the Pb (IV) (1.7 nmol) oxidation products of glucose 6-P (1.0 mmol). The preparation was applied to a column (1.5 × 3.0 cm) of Bio-Rad AG 1-X8, 200–400 mesh ion-exchange resin in the formate form with 20 ml of 0.2 M formic acid. Elution was achieved with two successive linear gradients of ammonium formate in 0.2 M formic acid. The flow rate was 1.0 ml/min and each fraction was 10 ml. The position of erythrose 4-P, in the fractions, was located by the cysteine–H_2SO_4–fructose colorimetric method [10]. Glucose 6-P was located by the cysteine–H_2SO_4 method [18]. Peak identification (1) unreacted glucose 6-P; (2) erythrose 4-P monomer and (3) erythrose 4-P dimer.

[15,20], were reacted in the transaldolase method of Racker [7], the material from peak 3 reacted more slowly than the material from peak 2, and took approx. 8 times longer to reach equilibrium. The material from peak 3 reacted with the H_2SO_4–cysteine–fructose colorimetric reagent [10], to give a molar extinction at 460 nm, which was approximately 55% of that from peak 2.

When erythrose 4-P was chromatographed in the GW_3 solvent [19], two urea/HCl reacting areas [19] were produced, the major component having an R_{PO_4} = 0.48, while the other had an R_{PO_4} = 0.78 (approx. 10% of the total material applied to the chromatogram). Elution of the compound with R_{PO_4} = 0.48, followed by rechromatography in the same solvent, resulted in the compound remaining in the same area of the chromatogram as before (i.e., R_{PO_4} = 0.48), while rechromatography of the material with R_{PO_4} = 0.78, resulted in the bulk of the material now appearing in the R_{PO_4} = 0.48 region, with a barely detectable amount in the R_{PO_4} = 0.78 area. Moses and Calvin [8], after examining the ^{14}C metabolites formed in *Chlorella* after 3 min $^{14}CO_2$ fixation, proposed that possibly 'two forms of erythrose

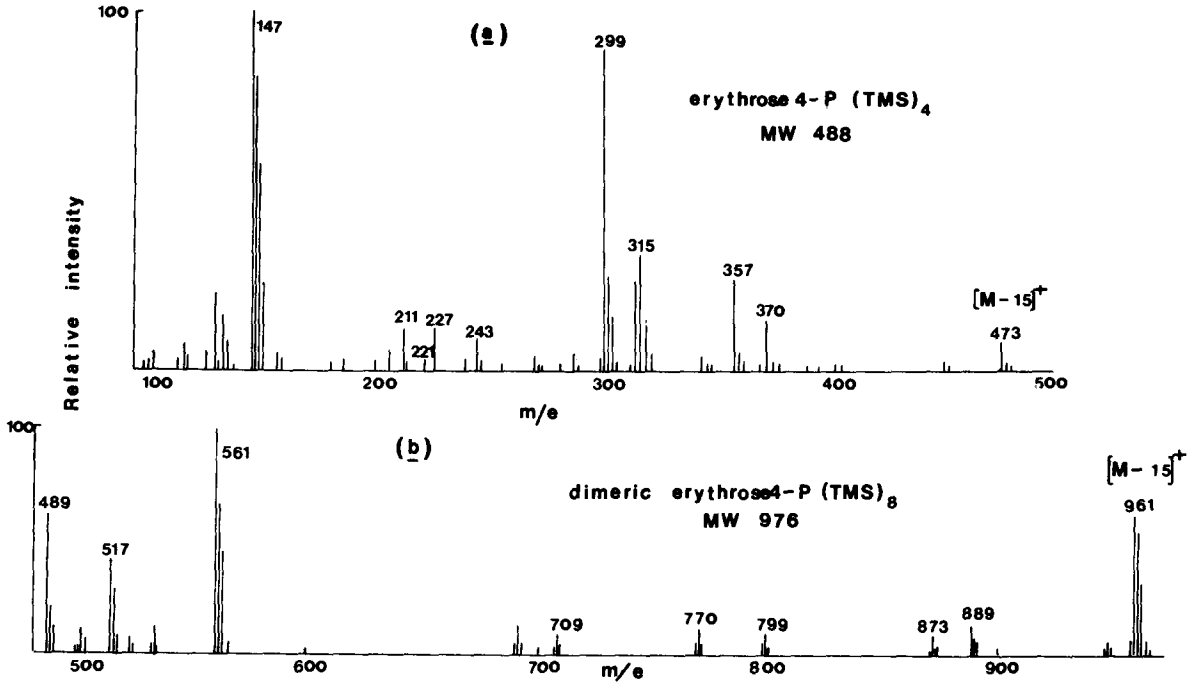
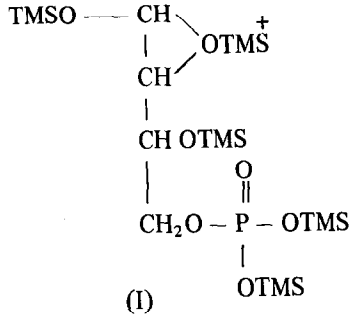


Fig.2. A portion of the mass spectra of the TMS derivatives of the monomeric and dimeric forms of erythrose 4-P.

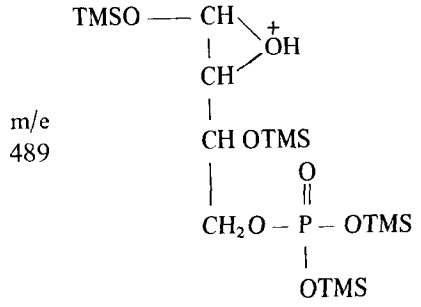
phosphate were present in the cell extract'. This proposal was made since two chromatographically different ^{14}C-phosphorylated compounds were observed which after dephosphorylation cochromatographed with authentic erythrose. These two observations would be consistent with there being monomeric and dimeric forms of erythrose 4-P.

To verify the proposal that erythrose 4-P is capable of dimerizing in aqueous solution, the TMS derivative of 'erythrose 4-P' was prepared and the mass spectra recorded. The first fraction to be volatilized showed a mass spectrum consistent with that of the monomeric form of tetra-TMS-erythrose 4-P, with the $[M\text{-}CH_3]^+$ ion at m/e 473 (fig.2a) and typical fragmentation and rearrangement ions at m/e 357, 315, 299, 243, 227 and 211 [21] as expected. The molecular ion M^+ (m/e 488) was absent from the spectrum.

The mass spectrum of the next and major fraction to be volatilized showed the most intense high mass ion at m/e 961, which corresponds to the $[M\text{-}CH_3]^+$ ion of the proposed octa-TMS-erythrose 4-P dimer (mol. wt. 976) (fig.2b). An accurate mass measurement performed on this ion confirmed its composition as $C_{31}H_{79}O_{14}P_2Si_8$ (measured mass 961.3080; calculated for $C_{31}H_{79}O_{14}P_2Si_8$; 961.3099). Ions were also observed at m/e 889, 799 and 709, which we believe to arise by the successive losses of CH_3, TMSOH and a further TMSOH from an incompletely silylated hepta-TMS-erythrose 4-P dimer of mol. wt. 904. The fragmentation ions with m/e of 561 and 489 possibly represent the following two rearrangement ions (I) and (II) formed by bond cleavage accompanied by migration of a TMS group and H atoms respectively. Once again as with the monomeric tetra-TMS erythrose 4-P, and in keeping with previous studies of TMS derivatives of sugar-Ps [21], no M^+ ion was observed.

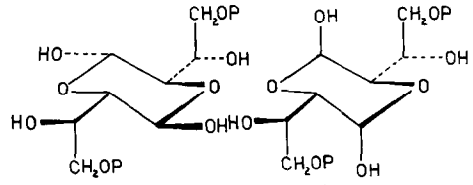


(I)



(II)

The structure of the proposed cyclic *bis* hemiacetal of erythrose 4-P could possibly be that represented by (III) or (IV) with III being the more thermodynamically favoured configuration.



(III) (IV)

Both isomers have a two-fold axis of symmetry in which the assymetric ring carbons are internally compensated. This would help to explain the apparent anomalous finding that erythrose 4-P is not optically active [22], despite the presence of chiral centres. Other hydroxycarbonyl compounds which are not able to form internal hemiacetals, are also capable of dimerizing by forming cyclic *bis* hemiacetals, e.g. ribulose 5-P [23], glycolaldehyde [24], glyceraldehyde and 1,3-dihydroxyacetone [25].

Another phenomenon to be considered in explanation of the apparent utilization of erythrose 4-P in Tris buffer (table 1) is that of Schiff base formation between the aldehyde of erythrose 4-P and amine of Tris or carbinolamine formation as has been suggested by Chirgwin et al. [26]. Although the very tight, specific molecular mechanism for the binding of 'erythrose 4-P' to phosphoglucose isomerase is unknown [26], the existence of a dimeric form of erythrose 4-P may in part explain this mechanism and account for the unusually tight binding of erythrose 4-P dimer to a site separate from the catalytic site. 4-Phosphoerythronate, which cannot dimerize,

is a poor inhibitor [26]. Further support for the existence of dimer III or IV is being sought by n.m.r. spectroscopic studies of erythrose 4-phosphate.

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