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Tetrose Metabolism

1. THE PREPARATION AND DEGRADATION OF SPECIFICALLY LABELLED [^{14}C]TETROSES AND [^{14}C]TETRITOLS

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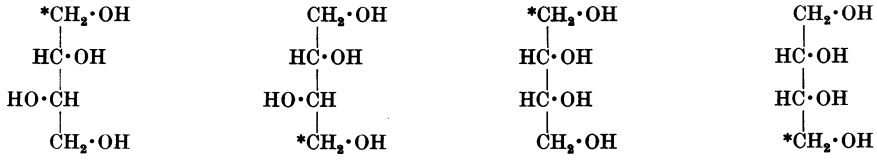
The fact that D-erythrose 4-phosphate arises in the hexose monophosphate pathway from the action of transketolase on fructose 6-phosphate, or of transaldolase on sedoheptulose 7-phosphate, has stimulated interest in tetrose metabolism, discussed in the subsequent paper (Batt, Dickens & Williamson, 1960*b*). The ease of synthesis of L-erythrulose from hydroxypyruvate in the presence of transketolase and a source of glycolaldehyde (Dickens & Williamson, 1956*a*; Dickens, 1958) provides a useful starting point for the production of a series of optically active ^{14}C-labelled tetroses and tetritols, with either specifically labelled hydroxy[^{14}C]pyruvate (Dickens & Williamson, 1958*a*) as the C_2-donor or hydroxy[^{12}C]pyruvate together with specifically labelled [^{14}C]glycolaldehyde as the acceptor aldehyde. Similarly, the aldolase-catalysed interaction of [^{14}C]formaldehyde with dihydroxyacetone phosphate (Charalampous, 1954; Peanasky & Lardy, 1958) yields a ketotetrose 4-phosphate; evidence in this paper indicates that this is the L-[4-^{14}C]erythrulose derivative, readily hydrolysable to give the free ketotetrose. L-Erythrulose is conveniently reduced by reduced diphosphopyridine nucleotide and the diphosphopyridine nucleotide-xylitol dehydrogenase of guinea-pig liver; the product with the mitochondrial enzyme is L-threitol (cf. Hollmann & Touster, 1957) and the activity of the similar enzyme purified from acetone-dried guinea-pig liver is approximately the same towards both D-xylulose and L-erythrulose (Hickman & Ashwell, 1959).

This paper describes the preparation, as a preliminary to the study of their metabolism, of various non-isotopic and specifically ^{14}C-labelled tetroses and tetritols. These include L-[1-^{14}C]- and L-[4-^{14}C]-erythrulose and D-[4-^{14}C]erythrose. In addition, preparations are given for L-threitol and *meso*erythritol (hereafter called erythritol) labelled with ^{14}C on one terminal carbon atom.

With such [^{14}C]polyols difficulties of nomenclature arise, which are discussed for the similarly labelled glycerols by Karnovsky, Hauser & Elwyn (1957). After considering possible alternatives, these authors prefer to refer to glycerol derived from D-[3-^{14}C]serine as L-[1-^{14}C]glycerol, and that from L-[3-^{14}C]serine as D-[1-^{14}C]glycerol, thus making the labelled carbon of the primary alcohol group of serine the C-1 of the polyol. It is this carbon atom of glycerol to which the phosphate group is attached in naturally occurring L-α-glycerophosphate, which is the exclusive product of the action of glycerokinase (Bublitz & Kennedy, 1954) and adenosine triphosphate upon biosynthetically prepared [1-^{14}C]glycerol. Glycerol, however, has the configuration Caa*bc, in which a* represents the isotopically labelled $CH_2 \cdot OH$ group, and glycerol is therefore an 'isotopically asymmetric' compound, in the terminology of Karnovsky *et al.* (1957), although it is important to recognize that the asymmetry towards the enzyme is independent of the isotope labelling, being an example of the Ogston (1948) phenomenon, as in the analogous case of citric acid.

The general criteria for enzymic differentiation in such instances have been well reviewed by Schwartz & Carter (1954) and by Hirschmann (1956), who point out that this type of asymmetry is dependent on a plane bisecting the molecule through b and c, resulting in opposite ends which are related as mirror images. Hirschmann proposes an extension of this in the form of a new general rule which he also applies to the case Cabc-Cabc, as illustrated by the tartaric acids: 'If tartaric acid is considered as an intermediate in an enzymic process which results in differential labelling of the carboxyl groups, the symmetrical *meso* compound can qualify as a possible intermediate, but the dys-symmetric optically active forms cannot'. (Hirschmann, 1956, p. 167.)

This appears to apply to the tetritols also. Whereas each of the two optically active forms D- and L-threitol is expected, somewhat paradoxically on this view to behave identically at both ends, *meso*erythritol should be dissimilar at the two ends of the molecule. Since the configuration of the carbon atom adjacent to the final $CH_2 \cdot OH$ group determines whether the compound is to be called



Identical L-[α-^{14}C]threitols (similarly for D-threitol)

Non-identical *meso*-[α-^{14}C]erythritols

D- or L-, this becomes obvious from consideration of the respective formulae when these are turned so as to make the labelled carbon atom C-1 (see above).

In this paper evidence supporting the identical enzymic behaviour towards the xylitol diphosphopyridine nucleotide dehydrogenase of L-[1-^{14}C]- and L-[4-^{14}C]-threitol is presented, in accordance with the above theoretical considerations. This is provided by the demonstration that when L-[4-^{14}C]-erythrulose is hydrogenated by the enzyme and reduced diphosphopyridine nucleotide, the product (which may be called either L-[1-^{14}C]threitol or L-[4-^{14}C]threitol) is capable of incorporating radioactivity into both C-1 and C-4 of an added pool of unlabelled L-erythrulose. This is evidence that both ends of the [^{14}C]threitol molecule are attacked by the enzyme, and since 80% of enzymic equilibration of C-1 and C-4 was reached there appears to be no enzymic distinction between the two terminal groups. It is suggested that this compound be designated L-[1-^{14}C(4-^{14}C)]threitol. Catalytic reduction of L-[1-^{14}C]erythrulose should similarly give L-[1-^{14}C]erythritol (i.e. D-[4-^{14}C]-erythritol), accompanied by L-[1-^{14}C]threitol; the separation of these reduction products by electrophoresis is described below.

In connexion with the tetrose syntheses, this paper also describes the preparation by means of D-amino acid oxidase from DL-[^{14}C]serine of hydroxy[^{14}C]pyruvate and L[^{14}C]serine. The latter is a useful source of [^{14}C]glycolaldehyde, which is produced by decarboxylation with ninhydrin.

A preliminary account of this work has been presented (Batt, Dickens & Williamson, 1960*a*).

ANALYTICAL METHODS

Formaldehyde. This was determined colorimetrically by the acetylacetone method of Nash (1953) and by the chromotropic acid method (Elwyn *et al.* 1957); also gravimetrically as the dimedone derivative.

L-*Erythrulose.* Enzymic oxidation of reduced diphosphopyridine nucleotide (DPNH) by diphosphopyridine nucleotide (DPN)-xylitol dehydrogenase (Hickman & Ashwell, 1959) was followed photometrically at 340 mμ. In pure solution L-erythrulose was determined by the measurement of hydrogen uptake, in the presence of platinic oxide catalyst, in Warburg manometers.

Tetritols. The formaldehyde liberated by periodate was determined by chromotropic acid.

Formic acid. This was also formed by periodate treatment, and was first reduced to formaldehyde (method of West & Rapoport, as modified by Faulkner, 1956) and the total resulting formaldehyde determined by comparison with a pure D-threitol standard. Alternatively, formate was oxidized to CO_2 (Pirie, 1946) and collected as $BaCO_3$.

Glucose. This was determined by the method of Nelson (1944).

Glycolaldehyde. The method of Dickens & Williamson (1958*b*) was used.

Glyoxylic acid. This compound, formed in periodate oxidation, was determined colorimetrically at 520 mμ on the solution of the 2:4-dinitrophenylhydrazone in N-NaOH (Friedemann, 1957), with a sodium glyoxylate standard. The method is not specific for any particular α-oxo acid.

Serine. The serine eluted from Amberlite IR-120 (H^+ form) resin was determined as formaldehyde liberated by periodate from the CH_2·OH group.

Glycollic acid. This was determined colorimetrically with 2:7-dihydroxynaphthalene.

Measurement of radioactivity. This was at infinite thickness with correction where necessary for self-absorption on 1 cm.^{2} disks in polythene planchets. Details were identical with those given by Dickens & Williamson (1959).

Total carbon of radioactive compounds. After oxidation with persulphate and silver nitrate, the CO_2 was converted into $BaCO_3$ for counting (Katz, Abraham & Baker, 1954).

High-voltage ionophoresis (Efron, 1959). Tetritol solutions were applied to the base line either as 5 μl. spots or for preparative purposes as a band 30 cm. wide on Whatman no. 1 papers, 42 cm. × 46 cm. Saturated aqueous boric acid solution, adjusted to pH 6·0 (Rammler & MacDonald, 1958), was the liquid phase. The current (120 mA at 4000 v) was applied for 2·5 hr. Tetritols were localized on marginal strips by the benzidine–periodate spray. We are much indebted to Dr Mary Efron for kindly allowing the use of her apparatus for the electrophoresis.

Paper chromatography. Descending liquid phase was used with Whatman no. 1 paper, the excess of liquid dripping from the free end. The usual experimental period was 16 hr. Solvents normally used were: A, butan-2-one–acetic acid–aq. 4% (w/v) boric acid (9:1:1); B, ethyl acetate–pyridine–water (12:5:4). Detection was either by dipping the dried paper in aniline–phosphoric acid–acetic acid solution (cf. Dickens & Williamson, 1956*b*), or, for polyols and sugars having adjacent hydroxyls, by the periodic acid–benzidine reagent (Gordon, Thornburg & Werum, 1956). The presence of radioactive spots was detected by a thin-window Panex monitor (model 50A), and for semi-quantitative purposes counts were made on squares 2 cm. × 2 cm. cut from the paper and measured in the standard lead castle fitted with a thin end-window counter and scaler (see Measurement of radioactivity, above).

Where necessary, phosphate esters were separated in methanol–aq. NH_3 soln. (sp.gr. 0·88)–water (6:1:3; cf. Charalampous & Mueller, 1953). Spots were developed by

Table 1. *Paper chromatography of tetroses and tetritols*

Solvent	Distance moved relative to glucose (R_G)*			
	L-Erythrulose	D-Erythrose	Erythritol	D-Threitol
A. Butan-2-one–acetic acid–aq. 4% boric acid (9:1:1)	8·9	10·1	5·9	6·0
B. Ethyl acetate–pyridine–water (12:5:4)	2·25	2·00	1·56	1·44

* These values are typical but markers should always be used for identification purposes.

molybdate spray and subsequent exposure to ultraviolet light.

Some typical data are included in Table 1.

MATERIALS

Radioactive substances. These were obtained from The Radiochemical Centre, Amersham, Bucks, with the following approximate specific activities: [^{14}C]paraformaldehyde, 1 mc/m-mole; sodium [3-^{14}C]pyruvate, 1 mc/m-mole; DL-[3-^{14}C]serine, 1 mc/m-mole; L-[3-^{14}C]serine, 3 mc/m-mole; D-[1-^{14}C]glucose and D-[6-^{14}C]glucose, 2 mc/m-mole.

Other substances. Recrystallized lithium hydroxypyruvate monohydrate was prepared as described by Dickens & Williamson (1958*a*). Lithium or sodium hydroxy[3-^{14}C]-pyruvate were similarly prepared from [3-^{14}C]pyruvate; the enzymic preparation is described below. L-Erythrulose was prepared as the crystalline *o*-nitrophenylhydrazone by the action on erythritol of *Acetobacter suboxydans*, N.C.I.B. 7069, kindly supplied by the Curator, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen (cf. Whistler & Underkofler, 1938). L-Erythrulose solutions were prepared as required by refluxing the hydrazone with benzaldehyde and a little benzoic acid, followed by ether extraction of these substances from the cooled filtrate. The product was chromatographically pure and was used as a diluent for the ^{14}C-labelled erythrulose. The *cyclo*hexylammonium salt of fructose 1:6-diphosphate (McGilvery, 1953) was prepared from the barium salt.

L-Threitol was prepared enzymically as follows. L-Erythrulose (150 μmoles) was incubated at 37° in a total volume of 20 ml. containing DPN-xylitol dehydrogenase (1 ml.), DPN (10 mg.), liver alcohol dehydrogenase (0·2 ml., C. F. Boehringer und Söhne, Mannheim, Germany), aq. 0·02 N-NH_3 soln. (5 ml.) and ethanol (1 ml.). Disappearance of erythrulose was followed by photometric estimation of DPNH oxidation (see Analytical methods section) on samples incubated with DPN-xylitol dehydrogenase. No erythrulose remained after 90 min. and the whole solution was heated to 100° for 5 min., cooled, filtered, treated with Bio-Deminrolit G and evaporated to dryness *in vacuo*. The residue in 0·5 ml. of water was chromatographed with solvent B, the single band corresponding to the threitol marker was eluted and the eluate used directly as a solution of L-threitol.

D-Threitol was kindly provided by Professor J. H. Birkinshaw (Birkinshaw, Stickings & Tessier, 1948).

Commercial preparations purchased from C. F. Boehringer und Söhne were barium fructose 1:6-diphosphate, DPN and DPNH. Bio-Deminrolit G was supplied by The Permutit Co. Ltd. (see Dickens & Williamson, 1956*b*). Since Bio-Deminrolit G is no longer obtainable, its equivalent may be prepared by stirring 1 lb. quantities of Zeo-Karb 225 (H^+ form) with 2 N-HCl (2 l.) and De-Acidite G (Cl^- form) with aq. 2 N-NH_3 soln. (2 l.), washing the resins with distilled water until free of chloride, collecting the moist resins and thoroughly mixing. The resulting mixed-bed resin removes electrolytes with less than 5% loss of sugars. We are indebted to Dr T. R. E. Kressman of The Permutit Co. Ltd. for this suggestion.

Enzyme preparations. These were made as follows: transketolase (de la Haba, Leder & Racker, 1955) was prepared from The Distillers Co. Ltd. yeast, the stages of treatment with protamine and alumina being omitted; it was free from carboxylase activity and had 10 000 Racker units of activity/ml. of final solution or 1450 units/mg. of protein; DPN- and triphosphopyridine nucleotide (TPN)-xylitol dehydrogenase were from guinea-pig liver (Hickman & Ashwell, 1959); D-amino acid oxidase was prepared from pig kidney but otherwise as described by Negelein & Brömel (1939); it was purified to the end of Stage 2 of these authors and was devoid of catalase activity.

Dialysed muscle fractions. Rat skeletal muscle (90 g.) was chilled and mechanically macerated for 1 min. in ice-cold 0·01 M-$NaHCO_3$; the extract was filtered through gauze (700 ml.) and fractionated by additions of $(NH_4)_2SO_4$. Fraction 1 (0·45 saturation) and Fraction 2 (0·45–0·60 saturation) were dissolved in 2-amino-2-hydroxymethyl-propane-1:3-diol (tris) buffer (30 ml., 0·02 M, pH 7·6) and dialysed at 2° against distilled water before use. In some experiments similar preparations from guinea-pig muscle were used. Fraction 1 consists of crude phosphohexokinase. Fraction 2 is used here as crude aldolase, or, according to Charalampous (1954), crude phosphoketotetrose aldolase. The more recent work of Peanasky & Lardy (1958), already mentioned, makes it doubtful if the latter is a separate enzyme different from ordinary aldolase, but this question does not appear finally decided as yet. Fraction 2 also served as a phosphohexose-isomerase preparation.

A third muscle enzyme used was fructokinase, prepared as described by Staub & Vestling (1951). This enzyme, which is without action on glucose, did not phosphorylate L-erythrulose, although it was highly active towards fructose and adenosine triphosphate (ATP). Consequently it can be used to distinguish or separate these two ketoses.

Commercially obtained enzymes. These included liver alcohol dehydrogenase, α-glycerophosphate dehydrogenase and aldolase (C. F. Boehringer und Söhne), yeast hexokinase and intestinal alkaline phosphatase (Sigma Chemical Co., St Louis, Mo., U.S.A.).

PREPARATIONS

Hydroxy[3-^{14}C]*pyruvate and* L-[3-^{14}C]*serine from* DL-[3-^{14}C]*serine.* DL-[3-^{14}C]Serine (11·4 μmoles, 100 μc), together with DL-[^{12}C]serine (40 μmoles),

was incubated aerobically at 37° in Warburg vessels containing O_2 and pyrophosphate buffer (0·1 ml.; 0·1 M, pH 8·5) in the presence of D-amino acid oxidase (3 ml.) until uptake of O_2 became very slow (14·1 μmoles of O_2 equivalent to 28·2 μmoles of serine). Unlabelled L-serine (200 μmoles) was added, followed by 5 ml. of 0·5 N-H_2SO_4. After removal of the protein precipitate by centrifuging the supernatant contained approximately 10 μmoles of hydroxypyruvate by enzymic analysis (Dickens & Williamson, 1958*b*). Carrier lithium hydroxypyruvate (200 μmoles) was added and the solution was passed through an Amberlite IR-120 (H^+ form) column. The combined eluate and water washings (40 ml.) were extracted continuously with ethyl ether for 70 hr. After the extract had been shaken with a little water, the ether was removed under reduced pressure and the solution of the residue in water (10 ml.) was the preparation of hydroxy-[3-^{14}C]pyruvate used for the synthesis of L-[1-^{14}C]-erythrulose. By enzymic and colorimetric analysis (Dickens & Williamson, 1958*b*) the solution typically contained 125 μmoles (7·4 μc) of hydroxypyruvate. The loss is probably largely due to the alkaline incubation conditions (cf. Dickens & Williamson, 1958*a*).

The Amberlite column, after removal of the hydroxypyruvate as described above, was eluted with 50 ml. of aq. 2 N-NH_3 soln. The eluate was evaporated to dryness *in vacuo* and the residual L-[3-^{14}C]serine (179 μmoles, 40 μc) made up to 8 ml. with water

Several such batches of hydroxypyruvate (and L-serine) were freshly prepared as required.

Conversion of L-[3-^{14}C]*serine into* [2-^{14}C]*glycolaldehyde*. The solution of L-[3-^{14}C]serine (8 ml., 179 μmoles, 40 μc), prepared as described above, was mixed with 100 mg. of citrate [mixture of 19 g. of citric acid and 2 g. of trisodium citrate, well ground together (cf. Greenberg & Rothstein, 1957)] and 100 mg. of ninhydrin contained in a special 50 ml. conical absorption flask closed by a rubber seal (cf. Dickens & Williamson, 1959). The centre well contained 2 ml. of 2 N-NaOH (CO_2-free). The flask was evacuated and heated inside a protective container for 1·5 hr. at 100°, when the naphtharesorcinol test (Dickens & Williamson, 1958*b*) showed a 45% yield of glycolaldehyde. (The yield of CO_2 estimated as $BaCO_3$ was nearly quantitative and had only 1% of the total radioactivity.) The solution was deionized by Bio-Deminrolit G and concentrated to 5 ml.; 38·8% recovery of ^{14}C was found both by periodate and persulphate oxidations (see Analytical Methods section), indicating that all ^{14}C is in the CH_2·OH group.

L-[1-^{14}C]*Erythrulose and* L-[4-^{14}C]*erythrulose: synthesis by transketolase*. For the preparation of the 1-^{14}C-labelled compound, [3-^{14}C]hydroxypyruvate (96 μmoles, 5·9 μc; in 8 ml. of water) and [^{12}C]-glycolaldehyde (100 μmoles) were incubated at 37° in $N_2 + CO_2$ (95:5, v/v) with transketolase (1·75 ml.), phosphate buffer (2·5 ml.; 0·5 M, pH 6·2), thiamine pyrophosphate (2 mg.) and magnesium chloride (100 μmoles); total volume was 13·25 ml. A portion (2·65 ml.) was measured into a separate Warburg vessel and the CO_2 evolution followed manometrically. The whole was deionized with Bio-Deminrolit G, filtered, the filtrate was evaporated to dryness *in vacuo* and the residue of [1-^{14}C]erythrulose was redissolved in 5 ml. of water (4·5 μc, 66 μmoles of erythrulose).

Exactly the same procedure was used with [2-^{14}C]glycolaldehyde (87 μmoles, 15 μc; in 5 ml. of water), lithium hydroxy[^{12}C]pyruvate (100 μmoles), transketolase and other additions as described above. Evolution of CO_2 was 75 μmoles and after deionization the residue of L-[4-^{14}C]erythrulose was made up to 5 ml. of aqueous solution (12·3 μc in 75 μmoles).

Conversion of [^{14}C]*paraformaldehyde into* [^{14}C]-*formaldehyde*. [^{14}C]Paraformaldehyde (1·00 mg. = 28 μc) was weighed with addition of unlabelled paraformaldehyde (8·85 mg.) and 15 ml. of water into a flask with efficient reflux; the mixture was gently boiled under a fume hood for 2 hr. to effect complete solution and depolymerization. After distillation with small additions of water, 21 ml. of distillate contained 293 μmoles of formaldehyde (acetylacetone-analysis method) or 91% of the calculated yield.

Enzymic synthesis of [1-^{14}C]*fructose* 1:6-*diphosphate*. D-[1-^{14}C]Glucose (5·0 mg., 12·5 μc) was phosphorylated in a total volume of 6·8 ml. containing $NaHCO_3$ (0·6 ml., 0·15 M), $MgCl_2$ (0·2 ml., 0·1 M), sodium adenosine triphosphate (Sigma Chemical Co.; 0·6 ml., 0·1 M) and yeast hexokinase (Sigma Chemical Co.; 4 mg.). The reaction course was followed manometrically [at 37°, gas phase $N_2 + CO_2$ (95:5, v/v)]. When gas evolution was virtually complete, 1·0 ml. of N-HCl was added, the mixture was cooled to 0°, neutralized and the slight precipitate removed by centrifuging. After concentration *in vacuo* to 2·0 ml. the solution (now containing [1-^{14}C]glucose 6-phosphate and some [1-^{14}C]fructose 6-phosphate) was reincubated in a Warburg vessel under $N_2 + CO_2$ (95:5, v/v) at 37° with the following additions: $NaHCO_3$ (0·3 ml., 0·15 M), $MgCl_2$ (0·1 ml., 0·1 M), phosphohexose isomerase (0·05 ml. of dialysed muscle fraction 2, see Materials section), phosphohexokinase (0·10 ml. of dialysed muscle fraction I, see Materials section), sodium adenosine triphosphate (0·4 ml., 0·1 M). After 60 min. at 37°, 0·5 ml. of N-HCl and water (6 ml.) were added, and the mixture was neutralized and centrifuged from insoluble matter. The supernatant (10·0 ml.) was assayed for fructose 1:6-

diphosphate and triose phosphate by observation of the fall of the absorption at 340 mμ due to added DPNH (0·1 ml. of solution was taken for analysis: consecutive additions of α-glycerophosphate dehydrogenase, and then aldolase, in the spectrophotometer cuvettes were made as usual). The total volume (10·0 ml.) contained 13·7 μmoles of triose phosphate and a further 28·0 μmoles of triose phosphate were released on addition of aldolase, equivalent to 14·0 μmoles of fructose 1:6-diphosphate present. The glucose taken (5 mg.) was equivalent to 56 μmoles of triose phosphate; combined recovery was 75%.

Aldolase synthesis of [1-^{14}C]*erythrulose.* The above-mentioned solution (9 ml.) was used directly for the preparation of erythrulose 1-phosphate, since in the presence of aldolase and triose phosphate isomerase the entire material is available in the form of dihydroxyacetone phosphate for aldolase condensation with formaldehyde. For this purpose, unlabelled fructose 1:6-diphosphate (0·4 ml., 0·1 M), unlabelled formaldehyde (1·0 ml., 133 μmoles), water (6 ml.) and muscle fraction 2 (2·0 ml., see Materials section) were added and the solution was incubated at 37°. Samples (0·2 ml.) were removed at 0, 30 and 60 min. for estimation of formaldehyde and, after 60 min., the mixture was cooled to 0° and 4 ml. of perchloric acid was added, the precipitated protein was removed, the supernatant neutralized to pH 7·5 with 20% (w/v) KOH, and after it had been kept for several hours at 0° the $KClO_4$ was removed and the filtrate made to 30 ml. with water.

This solution containing erythrulose 1-phosphate was incubated with alkaline phosphatase (50 mg.) at 37° for 3 hr., then acidified with N-HCl (1 ml.) and the solution treated with Bio-Deminrolit G until neutral. The filtrate and water washings from the resin were concentrated *in vacuo* to 10 ml., containing [1-^{14}C]erythrulose (total radioactivity by persulphate oxidation 5·0 μc; radioactivity counted as the erythrulose phenylosazone 5·2 μc; mean 41% overall recovery of 1-^{14}C of the glucose taken).

Aldolase synthesis of L-[4-^{14}C]*erythrulose.* The preparation was similar to the one mentioned above, starting from unlabelled fructose 1:6-diphosphate (*cyclo*hexylammonium salt, 120 μmoles), [^{14}C]formaldehyde (126 μmoles, 11 μc), tris buffer (3 ml.; 0·2 M, pH 7·6), $MgCl_2$ (300 μmoles), muscle fraction 2 (5·0 ml.) in a total volume of 31·2 ml. The solution was incubated at 37°. In 3 hr. 70% of the formaldehyde had disappeared and the reaction rate had fallen sharply. Isolation of the product as described above gave 5 ml. of solution containing 80 μmoles (6·75 μc) of [4-^{14}C]erythrulose, i.e. 60% yield based on the amount of [^{14}C]formaldehyde taken. Chromatography in solvent A (see Analytical Methods section) and development by aniline phosphoric acid spray showed only one main spot corresponding to the erythrulose marker and a faint secondary spot corresponding to dihydroxyacetone.

Catalytic hydrogenation of the L-[^{14}C]*erythruloses.* Portions (0·8 ml.) of the above-mentioned erythrulose solutions were transferred to the two side bulbs of Warburg vessels, of which the main part contained platinic oxide monohydrate (5 mg., Johnson, Matthey and Co. Ltd.) in 2 ml. of water. After complete saturation of the catalyst with hydrogen by shaking at 37° in a stream of H_2 (30 min.) the taps were closed and the substrate was tipped in, the manometric readings being continued until hydrogenation was complete. The values observed with pure non-isotopic L-erythrulose agreed excellently with those calculated for 1 mole of H_2/mole of ketotetrose. The filtrate and washings were combined ('mixed tetritols').

Composition of the mixed tetritols. The reduction products from [4-^{14}C]erythrulose (and also from the pure unlabelled compound) were subjected to high-voltage ionophoresis in a water-cooled apparatus as detailed in the Analytical methods section. In the pH 6 borate medium clear separation occurred of two bands only, one moving 2 cm. towards the cathode (? electro-osmosis), the other 11 cm. towards the anode. From the behaviour of authentic non-isotopic markers, these were identified as erythritol (2 cm.) and threitol (11 cm.) respectively. The latter is evidently the L-isomer when derived from reduction of L-erythrulose; synthetic D-threitol was used for identification of this band. The two bands were separately eluted with water, the volumes of the eluates adjusted to 2·0 ml. and persulphate oxidations were made on 1·0 ml. portions, to which were added 100 μmoles of non-isotopic carrier erythritol. From the radioactivities of the resulting $BaCO_3$ it was calculated that the 'mixed tetritols' contained 72% of erythritol and 28% of L-threitol.

Enzymic preparation of L-[1-^{14}C]*threitol.* This substance was prepared from both L-[1-^{14}C]erythrulose and L-[4-^{14}C]erythrulose by the following method. The labelled erythruloses (200 μmoles, 5–10 ml., 5·0 μc) were incubated with enzyme and DPNH as described for the preparation of non-isotopic L-threitol (see Materials section). The isotopic yield averaged 50% in the final chromatographically pure products from either starting material.

Preparation of D-[4-^{14}C]*erythrose.* Perlin & Brice (1956) have described a convenient preparation of non-isotopic D-erythrose from D-glucose which we have applied to D-[6-^{14}C]glucose on a micro scale. To a solution of D-[6-^{14}C]glucose (200 μmoles, 10 μc) in 3 drops of water, 3 ml. of acetic acid and

200 mg. of lead tetra-acetate (L. Light and Co. Ltd.) were added with shaking at room temperature for 10 min. A solution of oxalic acid dihydrate (55 mg.) in a little acetic acid was added and, after 20 min., the precipitate of lead salt was removed and washed with 10 ml. of acetic acid. To the residue, after removal *in vacuo* of the solvent, 0·5N-H_2SO_4 (20 ml.) was added, followed by hydrolysis at 45° for 18 hr. The pH was then brought to 5·0 by addition of saturated $Ba(OH)_2$, the solution and washings of the $BaSO_4$ precipitate were combined and shaken with Bio-Deminrolit G (2 g.) for 30 min. The filtered solution was concentrated *in vacuo* to 2 ml. and then chromatographed on paper (solvent B) and chromatographed similarly again to separate from a faint adjacent band. Markers of authentic D-erythrose were prepared from crystalline 2:4-ethylidene D-erythrose made as described by Rappoport & Hassid (1951) for the preparation of the L-isomer. The eluted D-[4-^{14}C]-erythrose (70 μmoles) was made to 5 ml. with water. After addition of carrier erythrulose to 0·1 ml. samples, persulphate oxidation gave 3·5 μc of total $^{14}CO_2$, counted as $BaCO_3$, and periodate degradation of the phenylosazone (see erythrulose degradation, described below) showed that 0·8% of the total radioactivity was in C-1 + C-2 + C-3 and 99·2% of the ^{14}C was in C-4 (counted as formaldehyde dimedone).

DEGRADATIONS OF ^{14}C-LABELLED COMPOUNDS

Action of periodic acid on L-*erythrulose.* L-Erythrulose (120 μmoles) solution was measured into an absorption flask with 2N-NaOH as CO_2 absorbent in the centre well. After the addition of periodic acid (British Drug Houses Ltd., final concentration 0·3M) the stoppered flask was kept at room temperature for 50 min., when the $BaCO_3$ prepared from the alkali weighed 16·6 mg. (84 μmoles). Formic acid, formaldehyde and glycollic acid were also determined (see Analytical methods section). The reaction mixture was neutralized with 0·15M-$Ba(OH)_2$, the supernatant and washings of the precipitate were adjusted to pH 8·0 and the formaldehyde was distilled into 30 ml. of saturated dimedone solution: yield of crystalline dimedone derivative was 54·4 mg. The distillation residual solution was acidified with 1 ml. of 5N-H_2SO_4 and steam-distilled, and the distillate after neutralization was evaporated to small volume; the sodium formate was then oxidized with mercuric chloride–sodium acetate reagent to CO_2 (24·4 mg. as $BaCO_3$, 123 μmoles).

The residual solution from the steam-distillation was added to 15 ml. of 0·1N-ceric sulphate in an absorption flask having 2N-NaOH in the centre well. The evacuated stoppered flask was heated at 100° for 1 hr.; CO_2 was evolved (weighed as $BaCO_3$, 7·3 mg., 37 μmoles). No glyoxylic acid was detected in the periodate reaction mixture.

Two possible routes of oxidation (*A* and *B*) are consistent with these findings. A third route (*C*) can be excluded by the absence of glyoxylic acid formation:

Erythrulose carbon atom no.	C-1	C-2	C-3	C-4
A	$CH_2 \cdot OH$	CO_2H	$H \cdot CO_2H$	$H \cdot CHO$
B	$H \cdot CHO$	CO_2	CHO	$CH_2 \cdot OH$
			↓	↓
			$H \cdot CO_2H$	$H \cdot CHO$
C	$H \cdot CHO$	CO_2H	CHO	$H \cdot CHO$

Reeves (1941) has shown that glycolaldehyde (the intermediate presumed to be formed from C-3 and C-4 in *B*) would be converted quantitatively into formic acid and formaldehyde. (Glycolaldehyde may similarly result from C-3 and C-4 in route *A* as an intermediate.) Pathway *B* is assumed proportional to the observed CO_2 evolution (70%) and pathway *A* to the glycollic acid formation (30%). On this basis the yields in μmoles/100 μmoles of erythrulose (recovery of total C of erythrulose 97·5%) were: CO_2, 70; glycollic acid, 30; $H \cdot CHO$, 156; $H \cdot CO_2H$, 104 (Calc.: CO_2, 70; glycollic acid, 30; $H \cdot CHO$, 170; $H \cdot CO_2H$, 100).

Although the above results are typical, individual variations in separate periodate oxidations occurred, suggesting that the extent of the two pathways is rather variable according to small experimental variations. Periodate oxidation of erythritol, on the other hand, gave consistent results; e.g. 100 μmoles of erythritol gave 176 μmoles of formaldehyde dimedone, and 182 μmoles of $BaCO_3$ from the mercuric acetate oxidation of formic acid. This corresponds respectively to 88% of formaldehyde recovered from C-1 and C-4 and 91% of formic acid from C-2 and C-3.

Degradation of erythrulose phenylosazone. In view of the variability of results with the free ketotetrose, the osazone was first prepared and then degraded by periodate oxidation. For example, a mixture of erythrulose (200 μmoles), sodium acetate trihydrate (1 g.) and phenylhydrazine hydrochloride (0·5 g.) in 3·5 ml. of water was heated at 100° for 30 min. The filtrate, together with 6·5 ml. of water washings, was cooled and the crystals of phenylosazone were collected (95%

yield). For recrystallization it was dissolved in a little hot ethanol, water was added to the filtered solution to slight turbidity and the solution was cooled.

Periodate oxidation of the erythrulose phenylosazone was as follows: the osazone (50 mg.) was dissolved in 24 ml. of warm 66% (v/v) ethanol. To the cooled solution, 0·2 ml. of N-$NaHCO_3$ and 0·8 ml of N-periodic acid were added. Rapid precipitation occurred of the bisphenylhydrazone of mesoxalaldehyde, identified by mixed m.p. with the same product derived from periodic acid oxidation of glucosazone. After 15 min. at room temperature, this was filtered on to weighed filter-paper disks (31·2 mg., 67% of calc.). To the filtrate and washings, saturated $Ba(OH)_2$ was added to pH 7–8 in order to remove excess of periodate. The supernatant and washings from this precipitate were combined and the formaldehyde was distilled into saturated dimedone solution (30 ml.). Yield of formaldehyde dimedone was 22·3 mg., or 46% of calc. for C-4 of the original erythrulose. This method is useful for determining the localization of radioactivity of C-1 + C-2 + C-3 (in the osazone) and C-4 (in the formaldehyde).

Localization of ^{14}C in preparations of labelled erythrulose and tetritols. Table 2 shows the results of degradation experiments carried out by the methods already described.

In the enzymic preparations an average of 92% of the total radioactivity appears in the expected carbon atoms of [1-^{14}C]- and [4-^{14}C]-erythrulose respectively. The extent of the randomization between these two positions, amounting variously from 2·6 to 12·8%, differs in the different preparations. Where transketolase is used, this enzyme is known to cleave L-erythrulose in the presence of an acceptor aldehyde, and a radioactive enzyme–glycolaldehyde complex has recently been shown to be formed when only [generally labelled (G)-^{14}C]-erythrulose or [G-^{14}C]fructose 6-phosphate is the substrate (Datta & Racker, 1959). Such a complex could effect a partial exchange reaction with unlabelled glycolaldehyde and thus introduce some labelling into the other C_2 moiety of erythrulose. In addition, some hydroxy[3-^{14}C]pyruvate will undergo spontaneous decarboxylation during incubation with transketolase and glycolaldehyde, yielding some [2-^{14}C]glycolaldehyde which can itself act as an acceptor aldehyde for hydroxy[3-^{14}C]pyruvate, yielding some [1:4-$^{14}C_2$]erythrulose. In the aldolase preparations also reported in Table 2, specificity of labelling was highest (97·4%) in the [4-^{14}C]erythrulose, as is to be expected since here H·^{14}CHO is the direct source of radioactivity and the dihydroxyacetone phosphate with which it combines is unlabelled. On the other hand the preparation of [1-^{14}C]erythrulose, by the similar reaction with unlabelled formaldehyde, involved a rather complicated series of steps starting with [1-^{14}C]glucose and resulting finally in aldolase cleavage of [1-^{14}C]fructose 1:6-diphosphate. Possibly at some stage of this preparation radioactive carbon was transferred from C-1 of the original hexose to formaldehyde, though such a reaction is not known to occur. Whether this is the true explanation or not, the labelling of C-4 of the erythrulose was appreciable (12·8%) when this method of synthesis was used.

In the degradation of the ketotetroses shown in Table 2, radioactivity at C-1, C-2 and C-3 is combined. However, after reduction to the tetritols the degradations, also in Table 2, show that C-1 or C-4 respectively has nearly all the total activity, only about 3–4% being present in C-2 and C-3 combined.

Table 2. *Degradations of erythrulose and tetritols*

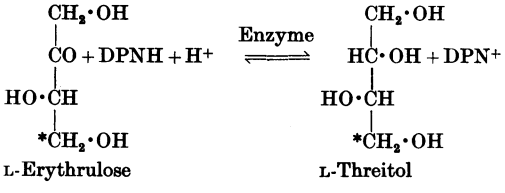
Substance degraded and method of preparation	Percentage of total ^{14}C	
	C-1 + C-2 + C-3*	C-4†
L-[1-^{14}C]Erythrulose		
Transketolase (prep. 1)	91·7	8·3
Transketolase (prep. 2)	90·0	10·0
Aldolase (prep. 3)	87·2	12·8
L-[4-^{14}C]Erythrulose		
Transketolase (prep. 4)	6·6	93·4
Transketolase (prep. 5)	11·0	89·0
Aldolase (prep. 6)	2·6	97·4
D-[4-^{14}C]Erythrose (chemical)	0·8	99·2
	C-1 + C-4‡	C-2 + C-3§
Tetritols from [1-^{14}C]erythrulose (prep. 1)‖	97·2	2·8
Tetritols from [4-^{14}C]erythrulose (prep. 4)‖	95·7	4·3

* As the osazone of mesoxalic semialdehyde.
† As formaldehyde from the osazone.
‡ As formaldehyde liberated by periodate.
§ As formic acid liberated by periodate.
‖ These tetritols were produced by catalytic hydrogenation (see text).

The labelling of substrates described in this paper is therefore sufficiently specific for their use in metabolic studies. These are described in the subsequent paper (Batt *et al.* 1960*b*).

TESTS WITH ENZYME SYSTEMS

Proof of isotopic symmetry of [1-^{14}C]- *and* [4-^{14}C]-L-*threitol.* The principle of this experiment is the study of distribution of ^{14}C between C-1 and C-4 of L-erythrulose when a pool of unlabelled L-erythrulose is incubated with DPN, DPN-xylitol dehydrogenase and L-[^{14}C]threitol:



L-Erythrulose L-Threitol

The terminal group of the threitol is that bearing ^{14}C. If in the reaction from right to left the enzyme does not distinguish between the two CH·OH groups of threitol, ^{14}C should be found after incubation in both C-1 and C-4 of the erythrulose pool. Preliminary studies showed that this equilibrium lies far to the right, as in the DPNH-catalysed D-xylulose–xylitol transformation studies by Hollmann & Touster (1957). For example, with 8 μmoles of L-threitol in the presence of 0·8 μmole of DPN$^+$ the DPNH formation was equivalent to 0·3% of the L-threitol added at pH 8·0. In a similar system with 10 μmoles of L-erythrulose added, only 0·15% of the L-threitol was oxidized as judged by DPNH formation.

To test ^{14}C incorporation the following system (total volume, 1·7 ml.) was maintained at room temperature for 2 hr.: L-[1-^{14}C]threitol, prepared enzymically from L-[4-^{14}C]erythrulose, 1·65 μmoles, 0·24 μC; unlabelled L-erythrulose, 5 μmoles; DPN-xylitol (D-xylulose) dehydrogenase 0·2 ml.; tris buffer (0·3 ml.; 0·5M, pH 8·0); $MgCl_2$ (0·3 ml., 0·1M) and DPN$^+$ (0·8 μmole). After 2 hr. reaction time, carrier L-erythrulose (150 μmoles), sodium acetate trihydrate (1 g.) and phenylhydrazine hydrochloride (0·5 g.) were added, with warming to 80° for 30 min. The osazone was collected after keeping at 0° overnight, washed, dried, counted, recrystallized and recounted. No fall of specific activity occurred on recrystallization and the total recovery of ^{14}C was 0·047 μC (20%). Oxidation with periodate of the recrystallized osazone (see Analytical methods section) showed that 42% of the total radioactivity appeared in C-1 + C-2 + C-3 and 58% in C-4 of the isolated tetrosazone. The original L-[4-^{14}C]erythrulose from which the L-[^{14}C]threitol was prepared had only 2·0% of its ^{14}C in C-1 + C-2 + C-3 and 98·0% of the total radioactivity in C-4. Hence, during the incubation with enzyme, 80% of complete exchange of isotopic labelling between C-1 and C-4 may be concluded. As discussed at the beginning of this paper, this behaviour is consistent with enzymic identity of the terminal halves of the L-threitol molecule, expected on theoretical grounds.

Substrate specificity of the triphosphopyridine nucleotide-xylitol (L-*xylulose*) *dehydrogenase.* This enzyme from mitochondrial fraction of guinea-pig liver was found by Hollmann & Touster (1957) to attack only xylitol and L-xylulose in the presence of TPN$^+$ or TPNH respectively, whereas the DPN-linked polyol dehydrogenase also described by these authors attacked a wide range of polyols (see also Hollmann, 1959). The fact that the DPN-xylitol (D-xylulose) enzyme also actively dehydrogenates L-threitol, which has close structural similarity to xylitol, led us to test the reactivity of the TPN-xylitol (L-xylulose) enzyme (prepared as described by Hickman & Ashwell, 1959) towards various substrates, including D-threitol (see Fig. 1). The results are shown in Table 3.

Hence D-threitol specifically requires TPN$^+$ and reacts quite vigorously with the TPN-xylitol (L-xylulose) dehydrogenase of guinea-pig liver. The expected dehydrogenation product is D-erythrulose, but this material was not available to us to test the reverse reaction. However, a specimen of

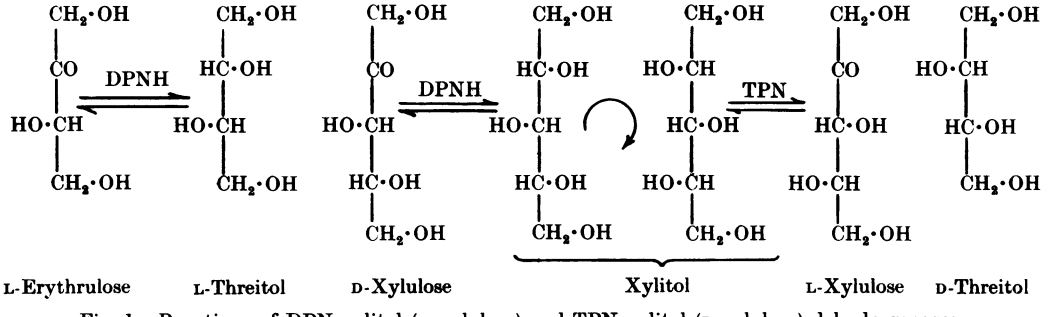


Fig. 1. Reactions of DPN-xylitol (D-xylulose) and TPN-xylitol (L-xylulose) dehydrogenases.

Table 3. *Changes in extinction with different substrates*

The enzyme (0·1 ml.) was tested with D-threitol, xylitol, erythritol or L-threitol (20 μmoles), TPN^+ or DPN^+ (0·8 μmole), in a total volume of 3·0 ml. containing tris buffer (30 μmoles), pH 8, and $MgCl_2$ (30 μmoles). Changes in extinction at 340 mμ after 20 min. at room temperature are recorded; the observations with TPN refer to the TPN-xylitol (L-xylulose) enzyme and in those with DPN the DPN-xylitol (D-xylulose) dehydrogenase was substituted under otherwise identical conditions. L-Threitol was enzymically prepared and the observation of reduction with both enzymes needs to be confirmed on chemically pure material.

Substrate	Increase in E	
	With DPN	With TPN
D-Threitol	0·009	0·120
Xylitol	0·380	0·254
Erythritol	0·004	0·003
L-Threitol	0·083	0·046

DL-erythrulose was prepared in poor yield from erythritol by the Fenton reaction (Neuberg, 1902), and purified by chromatography. The band running identically with pure L-erythrulose was eluted. The concentrated aqueous eluate reacted equally well with TPNH plus the TPN-xylitol (L-xylulose) dehydrogenase or with DPNH plus the DPN-xylitol (D-xylulose) dehydrogenase. It therefore contained, as expected, both isomers of erythrulose and the total content of DL-substance was determined in this way.

Since the TPN enzyme reduced this DL-erythrulose but not L-erythrulose, it may be concluded that D-erythrulose is a substrate for this enzyme, in addition to D-xylulose, for which according to Hollmann (1959) this enzyme is '*absolut specifisch*'.

Enzymic test of optical species of the erythrulose synthesized by aldolase. Erythrulose 1-phosphate was synthesized enzymically from dihydroxyacetone phosphate and formaldehyde, as described above: the free ketotetrose was liberated by phosphatase. Portions of the solution (1 μmole) added to the DPN-xylitol (D-xylulose) enzyme system (3 ml.) caused rapid oxidation of DPNH; authentic L-erythrulose behaved similarly. On the other hand, neither sample of ketotetrose caused any TPNH oxidation with the TPN-xylitol (L-xylulose)-dehydrogenase system. It may be concluded that the erythrulose synthesized by the aldolase reaction is present as L-erythrulose 1-phosphate. The optical species was not determined in the original preparation by this method (Charalampous & Mueller, 1953).

SUMMARY

1. The following preparations are described: (*a*) hydroxy[3-^{14}C]pyruvate and L-[3-^{14}C]serine from DL-[3-^{14}C]serine by D-amino acid oxidase; (*b*) [2-^{14}C]glycolaldehyde from [3-^{14}C]serine by decarboxylation with ninhydrin; (*c*) L-[1-^{14}C]-erythrulose and L-[4-^{14}C]erythrulose from the action of transketolase on the appropriately ^{14}C-labelled hydroxypyruvate and glycolaldehyde; (*d*) L-[4-^{14}C]erythrulose by the action of crude muscle aldolase on a mixture of H·^{14}CHO and dihydroxyacetone phosphate, followed by phosphatase hydrolysis of the erythrulose 1-phosphate so formed (it is shown enzymically that this compound is the L-isomer of erythrulose); (*e*) L-[1-^{14}C]-erythrulose similarly, by aldolase action on unlabelled formaldehyde and [1-^{14}C]dihydroxyacetone phosphate, prepared enzymically from D-[1-^{14}C]glucose.

2. L-[1-^{14}C]Threitol was prepared enzymically from either L-[1-^{14}C]erythrulose or L-[4-^{14}C]erythrulose, by means of reduced triphosphopyridine nucleotide and the 'diphosphopyridine nucleotide-xylitol (D-xylulose) dehydrogenase' of guinea-pig liver. The isotopic identity of L-[1-^{14}C]threitol and L-[4-^{14}C]threitol is experimentally proved, in confirmation of theoretical prediction. The correspondingly labelled erythritols are expected to be different compounds, but this remains to be investigated.

3. The preparation of D-[4-^{14}C]erythrose by periodate oxidation of D-[6-^{14}C]glucose is described.

4. By hydrogenation of the above-mentioned tetroses corresponding tetritols were prepared.

5. The position of isotopic labelling in the tetroses and tetritols has been determined by a study of the products of periodate oxidation of the free tetritols or of the phenylosazone of erythrulose (or erythrose).

6. The substrate specificity of the 'triphosphopyridine nucleotide-xylitol (L-xylulose) dehydrogenase' of guinea-pig liver has been shown to include D-threitol, as well as xylitol, for which substrate this enzyme was previously regarded as completely specific.

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Tetrose Metabolism

2. THE UTILIZATION OF TETROSES AND TETRITOLS BY RAT TISSUES

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Very little definite information is available about the metabolism of either tetroses or tetritols in the whole animal or in tissue preparations. This is surprising since there is now a considerable body of evidence that tetroses or their phosphates are capable of a variety of enzymic transformations. Thus the aldotetrose D-erythrose was first implicated in the pentose phosphate pathway of carbohydrate metabolism as a probable product, in the form of D-erythrose 4-phosphate, of the action of transaldolase on sedoheptulose 7-phosphate (Horecker & Smyrniotis, 1954; Horecker, Gibbs, Klenow & Smyrniotis, 1954; Horecker & Smyrniotis 1955; Horecker, Smyrniotis, Hiatt & Marks, 1955):

Sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate $\rightleftharpoons$ D-fructose 6-phosphate + D-erythrose 4-phosphate.

The reversibility of this reaction was considered probable; Racker, de la Haba & Leder (1954) were able to show that fructose 6-phosphate was itself split by transketolase yielding 'active glycolaldehyde' and again, presumably, D-erythrose 4-phosphate. The same product, together with dihydroxyacetone phosphate, was formed by the reversible cleavage by aldolase of sedoheptulose 1:7-diphosphate (Horecker *et al.* 1955; Smyrniotis & Horecker, 1956). Finally, Ballou, Fischer & MacDonald (1955) synthesized chemically D-erythrose 4-phosphate and showed that, with aldolase and dihydroxyacetone phosphate, sedoheptulose 1:7-diphosphate was formed. Kornberg & Racker (1955) showed that the synthetic tetrose ester behaved in the same