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The Fate of ^{14}C in Glucose 6-Phosphate Synthesized from [1-^{14}C]Ribose 5-Phosphate by Enzymes of Rat Liver

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1. Glucose 6-phosphate was synthesized from ribose 5-phosphate by an enzyme extract prepared from an acetone-dried powder of rat liver. Three rates of ribose 5-phosphate utilization were observed during incubation for 17h. An analysis of intermediates and products formed throughout the incubation revealed that as much as 20% of the substrate carbon could not be accounted for. 2. With [1-^{14}C]ribose 5-phosphate as substrate, the specific radioactivity of [^{14}C]glucose 6-phosphate formed was determined at 1, 2, 5 and 30min and 3, 8 and 17h. It increased rapidly to 1.9-fold the initial specific radioactivity of [1-^{14}C]ribose 5-phosphate at 3h and then decreased to a value approximately equal to that of the substrate at 6h, and finally at 17h reached a value 0.8-fold that of the initial substrate [1-^{14}C]ribose 5-phosphate. 3. The specific radioactivity of [^{14}C]ribose 5-phosphate decreased to approx. 50% of its initial value during the first 3h of the incubation and thereafter remained unchanged. 4. The distribution of ^{14}C in the six carbon atoms of [^{14}C]glucose 6-phosphate formed from [1-^{14}C]ribose 5-phosphate at 1, 2, 5 and 30min and 3, 8 and 17h was determined. The early time intervals (1–30min) were characterized by large amounts of ^{14}C in C-2 and in C-6 and with C-1 and C-3 being unlabelled. In contrast, the later time intervals (3–17h) were characterized by the appearance of ^{14}C in C-1 and C-3 and decreasing amounts of ^{14}C in C-2 and C-6. 5. It is concluded that neither the currently accepted reaction sequence for the non-oxidative pentose phosphate pathway nor the 'defined' pentose phosphate-cycle mechanism can be reconciled with the labelling patterns observed in glucose 6-phosphate formed during the initial 3h of the incubation.

The reaction scheme of the pentose phosphate pathway or cycle consists of two parts, an oxidative segment, in which glucose 6-phosphate‡ is oxidatively decarboxylated to ribulose 5-phosphate via 6-phosphogluconic acid (Horecker *et al.*, 1951), and a non-oxidative segment, in which pentose 5-phosphates are transformed to hexose 6-phosphate and triose phosphate (Horecker *et al.*, 1954; Gibbs & Horecker, 1954). Hexose 6-phosphates and triose phosphate are known to be products of ribose metabolism in extracts of a number of animal and plant tissues and micro-organisms, e.g. erythrocytes (Dische, 1938), liver (Schlenk & Waldvogel, 1946), spinach and peas (Axelrod *et al.*, 1953), *Escherichia coli* (Racker, 1948) and yeast (Sable, 1952). The nature and sequence of the reactions comprising the pentose phosphate pathway of glucose metabolism in tissues are based on the results of the experiments of Horecker *et al.* (1954) and Gibbs & Horecker (1954). The experiments involved the incubation of either [1-^{14}C]ribose 5-phosphate or [2,3-^{14}C]pentose 5-phosphate with buffer extracts of enzymes from acetone-dried powders prepared from rat liver and pea root and pea leaf tissue. The acetone-dried powder preparations of enzyme have the advantage over fresh tissue of being free of nicotinamide and adenine nucleotides. The absence of nicotinamide nucleotides prevented oxidation of the hexose 6-phosphate formed from ribose 5-phosphate by enzyme-catalysed reactions in the non-oxidative pathway. The prevention of oxidation and hence 'recycling' (Katz & Wood, 1960) blocked the subsequent randomization of ^{14}C in hexose 6-phosphate and permitted the investigation of the ^{14}C-labelling pattern of the hexose 6-phosphate products of one sequence of the reactions of the pathway (Scheme 1). The formation of hexose 6-phosphate from the triose 3-phosphate produced in the pathway was specifically prevented in the incubations involving liver enzymes by the omission of Mg^{2+} from the

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‡ All sugars and sugar phosphates mentioned in this paper have the D-configuration unless otherwise stated.

reaction mixtures, thereby inhibiting the action of hexose diphosphatase (EC 3.1.3.11) during the time course of hexose 6-phosphate formation from ribose 5-phosphate.

The incubation with liver enzymes was carried out for 17h and with pea root enzymes for 4h. With liver the [1-^{14}C]ribose 5-phosphate gave rise to [1,3-^{14}C]hexose 6-phosphate, with 74% of the ^{14}C in C-1 and 24% in C-3. The [2,3-^{14}C]pentose 5-phosphate yielded hexose 6-phosphate labelled in the following manner: 45% in C-4, 28% in C-2, 20% in C-3 and 7% in C-1. The isotope distribution in the hexose 6-phosphates formed in the incubations of pea root tissue was essentially the same as that formed in liver. These experiments have determined the detailed reaction sequences and the metabolic-pathway status of the non-oxidative pentose phosphate pathway (Scheme 1).

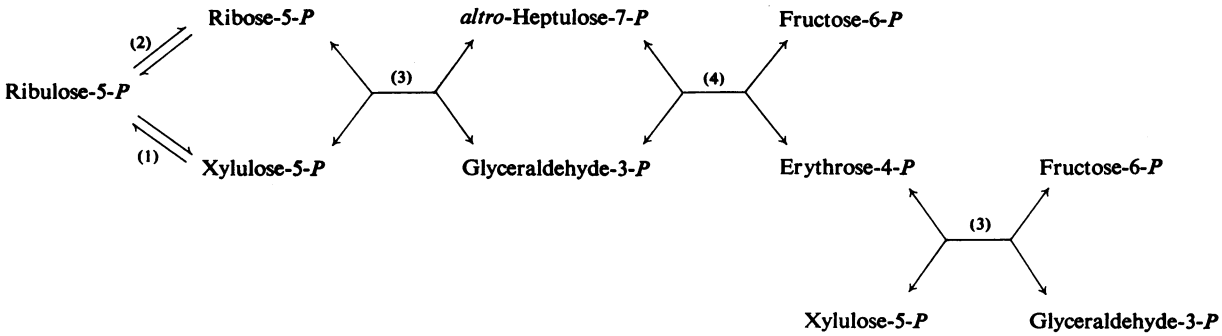
The carbon-balance studies of Glock (1952) and the mechanistic studies of Horecker *et al.* (1954) have resulted in the proposal that the stoicheiometry of the reactions of the non-oxidative segment of the pathway required 3mol of pentose 5-phosphate to form 2mol of hexose 6-phosphate and 1mol of glyceraldehyde 3-phosphate. A pentose phosphate cycle for glucose metabolism was defined by Wood & Katz (1958), such that fructose 6-phosphate formed in the reactions of the non-oxidative segment was converted into glucose 6-phosphate by glucose phosphate isomerase (EC 5.3.1.9), and the resulting stoicheiometry for one turn of the cycle was:

$$\text{Glucose-6-}P + 6\text{NADP}^+ \rightarrow 3\text{CO}_2 + \text{glyceraldehyde-3-}P + 6\text{NADPH} + 6\text{H}^+$$

There have been extensive investigations of the enzymes, intermediates and quantitative contributions of the pentose phosphate pathway to glucose metabolism in a number of animal tissues and micro-organisms [see reviews by Katz (1961), Hollman (1964) and Sable (1966)]. In liver the enzyme composition (Novello & McLean, 1968) and the identity and concentrations of intermediates (Greenbaum *et al.*, 1971) are in accord with Scheme 1. The relative contributions to $^{14}CO_2$ from the metabolism of [1-^{14}C]-, [2-^{14}C]- or [6-^{14}C]-glucose in liver slices, isolated liver cells and liver *in situ* are consistent with the operation of an active oxidative segment of the pathway. By the methods of Katz & Wood (1963), Katz *et al.* (1966) and Wood *et al.* (1963), a variety of quantitative estimates of the pathway with [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]-glucose in liver has been made. The metabolism of variously labelled glucose in liver cells (Baquer *et al.*, 1973) has been followed by measurement of the incorporation of ^{14}C into CO_2 and lipid; a constant value of 16% for the contribution of the pentose phosphate cycle to total glucose metabolism was found. For liver *in vivo*, using ^{14}C incorporation from [2-^{14}C]glucose into glucose units of glycogen, the pentose phosphate cycle estimate was 2.9%.

The distribution of ^{14}C in the glucose units of liver glycogen has always yielded low values of 3–5% for the pentose phosphate cycle [see Katz (1961) for a review of the data, and Hostetler & Landau (1967)].

With [1-^{14}C]ribose as substrate, the distribution *in vivo* of ^{14}C in the glucose units of glycogen in mouse and rat liver (Hiatt, 1957, 1958) and in human blood glucose (Hiatt, 1958) was not in accord with the ordered reaction sequence of Scheme 1. The reactions of Scheme 1 (Horecker *et al.*, 1954) and the definition of the pentose phosphate cycle (Wood & Katz, 1958) have determined that the reactions of [1-^{14}C]ribose 5-phosphate in the pentose phosphate cycle should give rise to ^{14}C distributions in the hexose 6-phosphate products at steady-state, such that there are 1.8–1.3 times as much ^{14}C in C-1 as in C-3 for contributions of the cycle which range from 10 to 100% (Katz & Wood, 1960). Hiatt (1958) found that at best C-1 and C-3 were approximately equally



Scheme 1. *Reactions of the non-oxidative pentose phosphate pathway* (*Horecker et al.*, 1954)
(1) Ribulose phosphate 3-epimerase; (2) ribose phosphate isomerase; (3) transketolase; (4) transaldolase.

labelled, although for most experiments involving liver glycogen, C-3 labelling exceeded C-1 by a factor of approx. 1.3. In human blood glucose the ratio of ^{14}C in C-3 to that in C-1 was 2.

In rat liver slices with [1-^{14}C]ribose as substrate, 84% of the ^{14}C incorporated into glucose units of glycogen was equally distributed in C-1 and C-3 (Katz *et al.*, 1955). The application of the gluconeogenic substrate [1-^{14}C]xylitol to isolated liver cells prepared from starved rats and hamsters yielded ^{14}C-labelling patterns in tissue glucose which were in near-perfect accord with the proposal that the ^{14}C of xylitol was converted into glucose by a single passage through the reactions of Scheme 1 (Rognstad & Katz, 1974). The expected effects of 'recycling' on the ^{14}C labelling of glucose 6-phosphate were not found in the glucose produced from [1-^{14}C]xylitol. The proposal of Rognstad & Katz (1974) is opposed by the conclusions of Katz (1961), the data of Hiatt (1957) (using [1-^{14}C]xylose), Williams *et al.* (1971) (using [1-^{14}C]ribose) and Eisenberg *et al.* (1959) (using [1-^{14}C]glucuronic acid, a precursor of [1-^{14}C]xylose). In all of the above situations in liver, the transfer of ^{14}C from [1-^{14}C]pentose (or its precursor) was consistent with extensive activity of the transketolase exchange reaction (Clark *et al.*, 1971) rather than an unqualified operation of the pentose pathway. Sols (1961) warned that investigations, interpretations and measurements of metabolic pathways with ^{14}C are fraught with difficulty, and, for measurements of the pentose cycle, Katz & Wood (1960) state that 'the further the compound (to be isolated and degraded) is removed from hexose phosphate, the greater are the chances of distortion of the labelling pattern by reactions such as recycling via Krebs cycle, CO_2 fixations and many others'.

An investigation of the pentose cycle in rabbit liver *in situ* with [2-^{14}C]glucose and [1-^{14}C]ribose as substrates, followed by degradation of hexose 6-phosphate in accord with the Katz & Wood (1960) directive (Williams *et al.*, 1971), yielded ^{14}C-labelling patterns in hexose 6-phosphate that could not be reconciled with a pathway having the order of reaction sequences shown in Scheme 1. With [2-^{14}C]glucose, the C-2 and C-6 of glucose 6-phosphate and fructose 6-phosphate were extensively labelled. With [1-^{14}C]-ribose only C-1 of glucose, glucose 6-phosphate and fructose 6-phosphate was heavily labelled. The kinetics and degree of $^{14}CO_2$ release from [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]-glucose in rabbit liver *in situ*, together with the magnitude of the C-1/C-6 ratio were, however, all consistent with the operations of the reactions of the oxidative segment of the pentose cycle (Williams *et al.*, 1971). We reported similar inconsistencies of ^{14}C-labelling patterns in hexose 6-phosphate, but not in liver glycogen in the isolated perfused rabbit liver (Schofield *et al.*, 1970) and in regenerating rabbit liver (Gerdes *et al.*, 1970, 1974). The failure to clearly reveal, by using specifically ^{14}C-labelled glucose, the operation of the pentose cycle from the ^{14}C-labelling patterns in hexose 6-phosphates from liver, together with other data reported above (Hiatt, 1957, 1958; Katz *et al.*, 1955), has caused us to question the reaction scheme (Scheme 1) for liver tissue. In this and the following paper (Williams *et al.*, 1978) we report results of a repeat and extension of the definitive experiment which established the nature and order of the reaction sequences in Scheme 1. Particular attention has been focused on the reactions occurring at times shorter than the 17h incubation time used for liver in the original experiment (Horecker *et al.*, 1954) and these investigations have been made in a search for the reactions that resulted in the unusual ^{14}C-labelling patterns found in the hexose 6-phosphates formed in liver *in situ* after metabolism of [2-^{14}C]glucose (Williams *et al.*, 1971). In bringing into question the mechanism of the pentose pathway, it is of note that there are at least five alternative reaction schemes for the non-oxidative segment of the pentose pathway or its cycle form (Beevers, 1956; Racker, 1956; Korkes, 1956; Kitos *et al.*, 1958; Davies, 1961).

Materials and Methods

Enzymes and chemicals

Enzymes, sugar phosphates and coenzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., or from Sigma Chemical Co., St. Louis, MO, U.S.A. Erythrose 4-phosphate was prepared from glucose 6-phosphate as described by Simpson *et al.* (1966). [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]-Glucose, [1-^{14}C]ribose, sodium DL-[1-^{14}C]- and [2-^{14}C]-lactate and sodium [1-^{14}C]- and [2-^{14}C]-acetate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals and solvents were of analytical reagent grade (BDH, Poole, Dorset, U.K. or May and Baker, Dagenham, Essex, U.K.). Acetone was purified by the method of Vogel (1967) and was used for the preparation of the acetone-dried rat liver enzyme preparation. Xylulose and ribulose were prepared from xylose and ribose respectively by refluxing the sugars in dry pyridine as described by Schmidt & Treiber (1933).

1-^{14}C-labelled substrates

[1-^{14}C]Ribose 5-phosphate was prepared from [2-^{14}C]glucose by the following method. [2-^{14}C]-Glucose (3.0μmol) (specific radioactivity 33.4mCi/mmol) was added to 3.0ml of 0.4M-triethanolamine hydrochloride, pH7.6, together with 1.0ml of a solution containing 10μmol of ATP, 2.0μmol of $MgCl_2$, 12μmol of $NADP^+$, 28 units of hexokinase (EC 2.7.1.1; Boehringer; yeast enzyme; 140 units/mg of protein), 14 units of glucose 6-phosphate dehydrogenase (EC 1.1.1.49; Boehringer; yeast enzyme;

350 units /mg of protein) and 2.4 units of 6-phosphogluconate dehydrogenase (EC 1.1.1.44; Sigma type V; yeast enzyme; 50 units/mg of protein). (Ribose phosphate isomerase was not added, since it was found that there was sufficient activity of this enzyme as a contaminant in the 6-phosphogluconate dehydrogenase to effect the conversion of ribulose 5-phosphate into ribose 5-phosphate.) The formation of pentose 5-phosphate was followed spectrophotometrically by measuring the increase in A_{366} in an Eppendorf photometer. After attainment of equilibrium (3h) the solution was heated at 100°C (for 5min) and then centrifuged at 10000*g* for 10min. Non-radioactive ribose 5-phosphate (100μmol) of composition 92% ribose 5-phosphate, 6% xylulose 5-phosphate and 2% ribulose 5-phosphate and no arabinose 5-phosphate (analysed by a combination of paper and gas–liquid chromatography; P. F. Blackmore, unpublished results) was added to the supernatant and the pH adjusted to 8.0 with aq. 1M-NH_3. [1-^{14}C]Ribose 5-phosphate was isolated from the above solution by ion-exchange chromatography (Williams *et al.*, 1971). Approx. 100μmol of [1-^{14}C]ribose 5-phosphate was isolated (specific radioactivity varied between 0.52 and 0.65mCi/mmol depending on the preparation). The radiochemical purity of [1-^{14}C]ribose 5-phosphate was ascertained by (*a*) column chromatography on Dowex AG 1 (X4; borate form) (Williams *et al.*, 1971) and (*b*) paper chromatography of the dephosphorylated [^{14}C]pentose 5-phosphate. Whereas procedure (*a*) did not permit detection of other pentose 5-phosphates, such as arabinose 5-phosphate, xylulose 5-phosphate and ribulose 5-phosphate, procedure (*b*) unequivocally showed that these other pentose phosphates were absent.

Liver enzyme preparation

Adult albino rats of the Wistar strain were fed *ad libitum* on a diet of Allied Rat and Mouse KUBES (Allied Feeds, Rhodes, Sydney, Australia) and water. Adult (18-week-old) New Zealand White cross-bred rabbits were fed *ad libitum* on a diet of Allied Medicated Rabbit and Guinea Pig Pellets (Allied Foods) and water.

Experimental animals were killed by cervical dislocation. The entire liver was quickly removed (6s), sliced into small pieces (0.5g) and homogenized in acetone at −10°C by the method of Horecker (1950). The acetone-dried powder (1.7g) was extracted with 12vol. of 0.1M-Na_2HPO_4 (pH7.4) for 15s in a glass homogenizer fitted with a Teflon pestle and the extracted material centrifuged at 10000*g* for 10min. The supernatant was treated with $(NH_4)_2SO_4$ and the fraction sedimenting between 40 and 65% saturation was retained (Horecker *et al.*, 1954). The precipitate was dissolved in 0.25M-glycylglycine/KOH buffer, pH7.4, to give the 'rat liver enzyme preparation' (volume 5.3ml). The protein content of the rat liver enzyme preparation was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The maximal catalytic activities of the following enzymes were determined by the methods described by Shonk & Boxer (1964): glucokinase (EC 2.7.1.2), glucose phosphate isomerase (EC 5.3.1.9), glucose phosphomutase (EC 2.7.5.1), 6-phosphofructokinase (EC2.7.1.11), triose phosphate isomerase (EC5.3.1.1), L-glycerol 3-phosphate dehydrogenase (EC 1.1.1.8), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), phosphoglycerate kinase (EC 2.7.2.3), phosphoglyceromutase (EC 2.7.5.3), phosphopyruvate hydratase (EC 4.2.1.11), lactate dehydrogenase (EC 1.1.1.27) and hexose bisphosphatase (EC 3.1.3.11). The activity of hexokinase was determined as described by Sharma *et al.* (1963), aldolase B (EC 4.1.2.13) as described by Raijkumar *et al.* (1966), pyruvate kinase (EC 2.7.1.40) as described by Irving & Williams (1973), isocitrate dehydrogenase (NADP) (EC 1.1.1.42) as described by Ochoa (1955), aspartate aminotransferase (EC 2.6.1.1) as described by Bergmeyer & Bernt (1965*a*) and alanine aminotransferase (EC 2.6.1.2) as described by Bergmeyer & Bernt (1965*b*). The catalytic activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were determined as described by Glock & McLean (1953). The catalytic activities of transaldolase (EC2.2.1.2), ribose phosphate isomerase (EC 5.3.1.6) and ribulose phosphate 3-epimerase (EC 5.1.3.1) were determined as described by Clark *et al.* (1972*a*). Transketolase (EC 2.2.1.1) was determined by the method (*b*) of Clark *et al.* (1972*b*). The activity of ribokinase (EC 2.7.1.15) was determined by measuring the rate of incorporation of radioactivity into ribose 5-phosphate isolated from a reaction mixture containing rat liver enzyme preparation (0.1ml), triethanolamine hydrochloride buffer, pH7.6 (0.25mmol), ATP (1.7μmol), $MgCl_2$ (10μmol) and [U-^{14}C]ribose (1.0×10^6d.p.m., 0.5μmol) in a total volume of 3.0ml at 30°C. Samples (1.0ml) were removed at 0, 10 and 30min and the ribose 5-phosphate was isolated by the ion-exchange column-chromatographic procedure of Williams *et al.* (1971). NAD^+ and $NADP^+$ were determined spectrofluorimetrically (Klingenberg, 1965*a*,*b*) in an Eppendorf photometer fitted with a fluorescence attachment 1070. The combined content of NADPH and NADH was determined by the method of Klingenberg (1965*c*) modified for spectrofluorimetry. ATP, and ADP and AMP were determined by methods described by Lamprecht & Trautschold (1965) and Adam (1965) respectively.

The catalytic capacity of the liver enzyme preparation to synthesize glucose 6-phosphate and fructose 6-phosphate from ribose 5-phosphate at 34°C was determined as described by Horecker *et al.* (1954).

Formation of glucose 6-phosphate from [1-^{14}C]ribose 5-phosphate at 25°C

Glucose 6-phosphate was formed in an incubation mixture identical with that of Horecker *et al.* (1954), and which contained 36μmol of [1-^{14}C]ribose 5-phosphate (specific radioactivity 0.54mCi/mmol) and 0.25ml of the rat liver enzyme preparation (in 0.25M-glycylglycine/KOH buffer, pH7.4); distilled water was added to a final volume of 3.3ml. Toluene (0.2ml) was added to prevent microbial growth. The incubation was allowed to proceed for 17h during which time samples of 0.4ml were removed at 1, 2, 5 and 30min and 3, 8 and 17h. The pH was checked both at the commencement and at the completion of the incubation and the temperature of the incubation was maintained at 25±0.1°C. Each sample was added to 1.0ml of 0.6M-$HClO_4$ at 0°C; after 30min the precipitated protein was removed by centrifugation (10000*g* for 10min) and the supernatant adjusted to pH8.0 by the addition of saturated $KHCO_3$; the precipitated $KClO_4$ was removed by centrifugation (10000*g* for 10min). To the supernatant was added 100μmol of each of ribose 5-phosphate, glucose 6-phosphate and fructose 6-phosphate. The solution (total volume 5.0ml) was adjusted to pH8.0 with aq. 1M-NH_3, and the sugar phosphates were resolved by ion-exchange chromatography (Williams *et al.*, 1971).

Specific radioactivity and the degradation of ribose 5-phosphate

The fractions containing ribose 5-phosphate after ion-exchange chromatography were pooled, the ammonium tetraborate was removed, treated with plant acid phosphatase and deionized as previously described (Williams *et al.*, 1971). The deionized sample of ribose (85–95μmol) dissolved in 0.1ml of water was applied as a band to Whatman 3MM paper and chromatographed in butan-1-ol/acetic acid/water (2:3:5, by vol.) with authentic ribose, ribulose and xylulose as markers. The region corresponding to authentic ribose and coincident with the radioactivity, as located in a Nuclear–Chicago Actigraph III Radioactivity Scanner, was eluted from the developed chromatogram with water, concentrated to 0.4ml and rechromatographed in an identical fashion in propan-2-ol/water (4:1, v/v). The ribose was eluted from the developed chromatogram as before and the specific radioactivity of ribose (and hence of ribose 5-phosphate) was calculated from the ribose (Mejbaum, 1939) and radioactivity content of each eluted sample. The remainder of the [^{14}C]ribose in the eluate was diluted to 0.5mmol with unlabelled ribose and fermented by *Lactobacillus plantarum* strain 142-2 obtained from National Collection of Industrial Bacteria, Aberdeen, Scotland (N.C.I.B. catalogue number 8026) to lactate and acetate by the method of Bernstein (1953). The acetate and lactate formed during fermentation were separated by the method of Bernstein (1953). The acetate was purified by chromatography on Celite (Swim & Krampitz, 1954), and the lactate after ether extraction was purified by descending chromatography on Whatman 3MM paper, in butan-1-ol/acetic acid/water (12:3:5, by vol.). The chemical degradation of lactate and acetate to yield each individual carbon atom as CO_2 was conducted as previously described by Williams *et al.* (1971). Sodium DL-[1-^{14}C]- and [2-^{14}C]-lactate and sodium [1-^{14}C]- and [2-^{14}C]-acetate were also degraded to verify the specificity of the degradation procedures. The CO_2 was collected as $BaCO_3$ and the radioactivity determined.

Specific radioactivity and the degradation of glucose 6-phosphate

The fractions containing glucose 6-phosphate obtained from ion-exchange chromatography were pooled and treated in a manner identical with that described above for the ribose 5-phosphate fractions. The specific radioactivity of glucose (and hence glucose 6-phosphate) was calculated from the glucose (Washko & Rice, 1961) and radioactivity content of each sample of glucose (80–85μmol) eluted from the paper chromatograms. The glucose content was diluted to 1mmol with unlabelled glucose and fermented to yield CO_2, ethanol and lacatate by using *Leuconostoc mesenteroides* strain 39 (N.C.I.B. catalogue number 8699) (Bernstein *et al.*, 1955). The fermentation procedure and the methods for the chemical degradation of ethanol and lactate were as described by Williams *et al.* (1971).

Radioactivity measurements

Radioactivity measurements were made in an automatic scintillation counter (Nuclear–Chicago, Unilux I or Packard Tri-Carb model 2002). Analysis of the radioactivity in the fractions collected during column chromatography was achieved by placing a sample (0.01ml) into a scintillation vial with 5ml of scintillant [composed of 350ml of toluene, 350ml of 1,4-dioxan, 210ml of methanol, 73g of naphthalene, 4.45g of 2,5-diphenyloxazole and 73mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene]. Samples of $BaCO_3$ were counted as suspensions (Cluley, 1962). All radioactivity measurements (except those concerned with establishing column profiles) were conducted at least three times on duplicate samples.

Analytical procedures

Analytical procedures for the determination of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, glucose 6-phosphate and fructose 6-phosphate and glucose 1-phosphate were as described

by Bücher & Hohorst (1965), Hohorst (1965) and Bergmeyer & Klotzsch (1965) respectively. Ribose 5-phosphate was determined by a procedure involving reactions (1)–(5), where reactions (1), (2) and (3) are catalysed by ribose phosphate isomerase, ribulose phosphate 3-epimerase and transketolase respectively.

$$\text{Ribose-5-}P \rightleftarrows \text{ribulose-5-}P \quad (1)$$

$$\text{Ribulose-5-}P \rightleftarrows \text{xylulose-5-}P \quad (2)$$

$$\text{Xylulose-5-}P + \text{erythrose-4-}P \rightleftarrows \text{fructose-6-}P + \text{glyceraldehyde-3-}P \quad (3)$$

$$\text{Fructose-6-}P \rightleftarrows \text{glucose-6-}P \quad (4)$$

$$\text{Glucose-6-}P + \text{NADP}^+ \rightarrow \text{6-}P\text{-gluconolactone} + \text{NADPH} + \text{H}^+ \quad (5)$$

The reaction mixture for the determination of ribose 5-phosphate contained 1.0ml of 0.4M-triethanolamine hydrochloride buffer, pH7.6, sample (0.01–0.1ml), 0.025ml of 0.3M-$MgCl_2$, 0.025ml of 1.05M-thiamin pyrophosphate, 0.05ml of 5mM-$NADP^+$ and 5 units of each of glucose 6-phosphate dehydrogenase and glucose phosphate isomerase. After the change in A_{340} due to the presence of glucose 6-phosphate and fructose 6-phosphate in the sample had ceased, 0.05ml of 0.5M-erythrose 4-phosphate, 0.2 unit of ribulose phosphate 3-epimerase and 0.05 unit of transketolase were added. When there was no further change in absorbance, the reaction for the determination of the ribose 5-phosphate was commenced by the addition of 2 units of ribose phosphate isomerase. The method thus described was found to be specific for ribose 5-phosphate, provided the value of sedoheptulose 7-phosphate was subtracted from the final answer, since sedoheptulose 7-phosphate will react with erythrose 4-phosphate and transketolase to form fructose 6-phosphate and ribose 5-phosphate. This method was used in preference to other methods [for example, Racker (1965*c*) and Mejbaum (1939)], which were found not to be specific for ribose 5-phosphate.

Xylulose 5-phosphate was determined by a method involving reactions (6), (7) and (8), where reaction (6) was catalysed by transketolase.

$$\text{Xylulose-5-}P + \text{erythrose-4-}P \rightleftarrows \text{fructose-6-}P + \text{glyceraldehyde-3-}P \quad (6)$$

$$\text{Glyceraldehyde-3-}P \rightleftarrows \text{dihydroxyacetone-}P \quad (7)$$

$$\text{Dihydroxyacetone-}P + \text{NADH} + \text{H}^+ \rightarrow \text{L-glycerol-3-}P + \text{NAD}^+ \quad (8)$$

The reaction mixture for the determination of xylulose 5-phosphate contained 0.05ml of sample, 1.0ml of 0.4M-triethanolamine hydrochloride buffer, pH7.6, 0.05ml of 2mM-NADH, 0.5 unit of each of triose phosphate isomerase and glycerol phosphate dehydrogenase, 0.01ml of 5mM-erythrose 4-phosphate, 0.025ml of 0.3M-$MgCl_2$ and 0.025ml of 1.05mM-thiamin pyrophosphate. After the change in A_{340} due to the presence of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in the sample had ceased, 0.05 unit of transketolase was added to start the reaction. The change in A_{340} was recorded and reached equilibrium after 20–30min at 30°C. Ribulose 5-phosphate was determined in the same cuvette by measuring the further decrease in absorbance after the addition of 0.2 unit of ribulose phosphate 3-epimerase (reactions 2, 6, 7 and 8).

The method of Racker (1965*d*) for the determination of erythrose 4-phosphate is not specific, since transaldolase catalyses reaction (9) as well as reaction (10) and thereby measures both ribose 5-phosphate and erythrose 4-phosphate. The following alternative method for the measurement of erythrose 4-phosphate was used (reactions 11, 4 and 5).

$$\text{Fructose-6-}P + \text{ribose-5-}P \rightleftarrows \text{octulose-8-}P + \text{glyceraldehyde-3-}P \quad (9)$$

$$\text{Fructose-6-}P + \text{erythrose-4-}P \rightleftarrows \textit{altro}\text{-heptulose-7-}P + \text{glyceraldehyde-3-}P \quad (10)$$

$$\text{Fructose} + \text{erythrose-4-}P \rightleftarrows \text{fructose-6-}P + \text{erythrose} \quad (11)$$

The reaction mixture was composed of 1.0ml of 0.4M-triethanolamine hydrochloride buffer, pH7.6, 0.25ml of sample, 0.025ml of 0.3M-$MgCl_2$, 0.025ml of 1.05mM-thiamin pyrophosphate, 0.05ml of 5mM-$NADP^+$ and 0.5 unit of each of glucose 6-phosphate dehydrogenase and glucose phosphate isomerase. After the change in A_{340} due to the presence of glucose 6-phosphate and fructose 6-phosphate in the sample had ceased, 0.05ml of 1.0M-fructose and 0.05 unit of transketolase were added to start the reaction. With a standard solution of erythrose 4-phosphate, the equilibrium was reached in 20–30min. By using an Eppendorf photometer with a log–linear converter and scale expander allowing a full-scale deflection of 0–0.03 absorbance unit on a Ricken Denshi SP-J3 recorder, the sensitivity of the method permitted reproducible measurements of as little as 3nmol of erythrose 4-phosphate per ml of sample.

Total heptulose 7-phosphate was determined by the method described by Racker (1965*a*) for sedoheptulose 7-phosphate. This method did not discriminate between *altro*-2-heptulose 7-phosphate and *manno*-2-heptulose 7-phosphate (Williams *et al.*, 1978).

Fructose 1,6-bisphosphate was determined in the sample that had been assayed for glucose 6-phosphate and fructose 6-phosphate content (Hohorst, 1965) by the addition of 0.2 unit of hexose bisphosphatase (Racker, 1965*b*).

Results

The substrate, [1-^{14}C]ribose 5-phosphate

Duplicate samples of the prepared [1-^{14}C]ribose 5-phosphate were degraded together with a sample of the [^{14}C]ribose 5-phosphate which remained in the reaction mixture after 17h incubation of [1-^{14}C]ribose 5-phosphate with the rat liver enzyme preparation.

In order to guarantee the efficiency of the microbiological and chemical degradations, a [1-^{14}C]-ribose reference standard accompanied the degradations. The results of the degradations (Table 1) confirmed that the position of ^{14}C was in C-1 of all samples examined. The percentage distribution of ^{14}C in [1-^{14}C]ribose 5-phosphate sampled at zero time was 94.8 ± 4.2 ($n = 4$), and the data in Table 1 also show that the ^{14}C was not randomized in the [1-^{14}C]ribose 5-phosphate remaining in the reaction mixture after 17h incubation with the rat liver enzyme preparation.

The liver enzyme preparation

All liver enzyme preparations, whether extracted from acetone-dried powders of the livers of male or female rats or rabbits, were found to have the same catalytic capacity to synthesize hexose 6-phosphate from ribose 5-phosphate (0.19 ± 0.02 μmol/h per mg of protein at 34°C) as that reported by Horecker *et al.* (1954) for rat liver.

The data of Table 2 show the maximal catalytic activities of some of the enzymes of rat and rabbit liver enzyme preparations. Except for pyruvate kinase and phosphoglyceromutase only minor variations in the activities of the enzymes examined were detected, irrespective of the source of liver enzyme preparation. Both pyruvate kinase and phosphoglyceromutase were approximately 2-fold more active in the preparations derived from rat liver. Ribokinase was absent from all preparations, and hexose bisphosphatase could not be detected without the addition of Mg^{2+}. As reported previously (Horecker *et al.*, 1954) hexose bisphosphatase was stimulated by the addition of $MgCl_2$. A final concentration of 20mM-$MgCl_2$ produced some activity (Table 2), but the maximum activity (0.22 unit/ml) was only achieved at 150–200mM-$MgCl_2$. A comparison of the data of Table 2 with the data of Novello & McLean (1968) and Shonk & Boxer (1964) indicated that the liver enzyme preparation contained higher proportions of pentose phosphate-pathway enzymes than did crude liver extracts [for example the ratio of the activity of glucose 6-phosphate dehydrogenase (or transketolase) to pyruvate kinase was increased approximately 5–8-fold]. None of the following could be detected in the rat liver enzyme preparation: NAD^+, $NADP^+$, NADH, NADPH, ATP, ADP and AMP. In order to determine the effect of the prolonged incubation (17h at 25°C) on the rat liver enzyme preparation the catalytic activities of seven of the constitutive enzymes, as well as the capacity to synthesize hexose 6-phosphate from ribose 5-phosphate at 34°C, were examined (Table 3). Apart from a 22% increase in the activity of 6-phosphogluconate dehydrogenase, there was no significant change in the activity of the enzymes examined. More importantly the catalytic capacity of the rat liver enzyme preparation to synthesize hexose 6-phosphate from ribose 5-phosphate at the higher temperature of 34°C did not change and the catalytic activity of hexose bisphosphatase remained undetectable throughout the incubation.

Carbon-balance studies on the metabolism of ribose 5-phosphate by the rat liver enzyme preparation

Fig. 1 shows the concentration–time relationships for all of the intermediates currently assigned to the non-oxidative pentose phosphate pathway, together with glucose 1-phosphate, during the 17h incubation of ribose 5-phosphate with the rat liver enzyme preparation. The results of Fig. 1 were used to calculate the carbon-balance data of Table 4 where each value is expressed as a percentage of the

Table 1. *Distribution of ^{14}C in ribose 5-phosphate isolated from the incubation mixture at 0 and 17h*

The incubation mixture at 25°C contained 36 μmol of [1-^{14}C]ribose 5-phosphate (0.65 μCi/μmol), 0.25 ml of rat liver enzyme preparation (equivalent to 7.3 mg of protein) and water to give a total volume of 3.3 ml at pH 7.4. At intervals during the incubation samples were removed and treated as described in the text for the isolation and purification of ribose 5-phosphate. Each sample of ribose 5-phosphate was converted into ribose and degraded as described in the Materials and Methods section. The radioactivity in each carbon atom is expressed as a percentage of the total radioactivity contained in all five carbon atoms. The percentage recovery represents the percentage of radioactivity in the pentose or pentose phosphate degraded by *L. plantarum* to that measured by total combustion (Van Slyke & Folch, 1940). The percentage distribution obtained on degradation of standard [1-^{14}C]ribose was: C-1, 92.6; C-2, 0.1; C-3, 5.5; C-4, 0.2; C-5, 1.6. Each value is the mean from duplicate degradations of samples from two experiments.

Carbon atom	Time (h) ...	Distribution of ^{14}C in [^{14}C]ribose 5-phosphate (%) 0	17
1		94.8	97.0
2		0.9	3.0
3		1.4	0.0
4		1.4	0.0
5		1.5	0.0
Recovery (%)		92.6	93.8

Table 2. *Maximal catalytic activities of some of the enzymes of rat and rabbit liver enzyme preparations*
The enzyme preparations were made from acetone-dried powders of rat and rabbit liver as described in the Materials and Methods section. Enzyme activities are expressed as μmol/min per ml of the enzyme preparation. Protein was determined by the method of Lowry *et al.* (1951). The values are means ± S.D. for the numbers of determinations shown in parentheses for individual enzyme preparations.

	Catalytic activity of enzymes					
	Rat			Rabbit		
Protein (mg/ml of enzyme preparation) ...	29.2	±0.8	(8)	37.7	±1.6	(8)
Hexokinase	Nil		(4)	Nil		(4)
Glucokinase	0.047	±0.013	(7)	0.048	±0.010	(7)
Glucose 6-phosphate dehydrogenase	0.49	±0.09	(8)	0.68	±0.10	(8)
6-Phosphogluconate dehydrogenase	1.34	±0.18	(8)	0.85	±0.09	(8)
Glucose phosphate isomerase	10.1	±0.5	(8)	9.6	±0.4	(8)
Glucose phosphomutase	1.50	±0.32	(5)	1.99	±0.35	(5)
Phosphofructokinase	0.13	±0.02	(5)	0.14	±0.02	(5)
Aldolase B	1.61	±0.07	(8)	2.01	±0.09	(8)
Triose phosphate isomerase	33.4	±0.2	(8)	41.8	±0.2	(8)
L-Glycerol 3-phosphate dehydrogenase	16.3	±3.0	(5)	15.2	±3.7	(5)
Glyceraldehyde 3-phosphate dehydrogenase	0.13	±0.05	(4)	0.09	±0.03	(4)
Phosphoglycerate kinase	3.24	±0.05	(3)	3.04	±0.04	(3)
Phosphoglyceromutase	0.85	±0.12	(3)	0.34	±0.09	(3)
Phosphopyruvate hydratase	4.0	±0.6	(3)	7.3	±0.8	(3)
Pyruvate kinase	0.94	±0.11	(4)	0.42	±0.10	(4)
Lactate dehydrogenase	37.5	±2.0	(3)	49.4	±2.6	(3)
Isocitrate dehydrogenase (NADP)	8.52	±0.91	(3)	12.22	±1.02	(3)
Aspartate aminotransferase	15.2	±2.1	(3)	13.4	±2.3	(3)
Alanine aminotransferase	4.3	±1.3	(3)	3.1	±1.1	(3)
Transketolase	0.37	±0.16	(8)	0.50	±0.08	(8)
Transaldolase	0.46	±0.07	(8)	0.56	±0.08	(8)
Ribokinase	Nil		(6)	Nil		(6)
Ribose phosphate isomerase	0.37	±0.04	(8)	0.42	±0.03	(8)
Ribulose phosphate 3-epimerase	0.60	±0.05	(8)	0.55	±0.05	(8)
Hexose bisphosphatase	Nil		(8)	Nil		(8)
Hexose bisphosphatase (+20 mM-$MgCl_2$)	0.12	±0.02	(5)	0.09	±0.01	(5)

Table 3. *Effect of prolonged incubation on some of the catalytic properties of the rat liver enzyme preparation*
Each incubation was constructed to simulate the incubation conditions described in Table 1 and was composed of rat liver enzyme preparation (0.1 ml, 29.2 mg of protein/ml, containing glycylglycine/KOH buffer, pH 7.4, 0.0248 mmol) and 1.22 ml of water at 25°C. The reaction mixtures were incubated for the times shown at 25°C and then cooled to 0°C. The catalytic activities of seven enzymes of the rat liver enzyme preparation were immediately determined on samples from each reaction mixture. The catalytic capacity of the liver enzyme preparation to synthesize hexose 6-phosphate from ribose 5-phosphate at 34°C (Horecker *et al.*, 1954) was measured with a 0.5 ml sample of the incubation mixture as described in the Materials and Methods section. Each value is the mean of duplicate determinations from four individual incubations.

	Catalytic activity (μmol/min per ml of enzyme preparation)					
Time (h) ...	0	3	6	9	12	17
Glucose 6-phosphate dehydrogenase	0.49	0.37	0.44	0.53	0.43	0.39
6-Phosphogluconate dehydrogenase	1.34	1.36	1.91	2.31	1.55	1.64
Glucose phosphate isomerase	10.1	10.4	10.1	9.7	10.2	10.1
Hexose bisphosphatase	Nil	Nil	Nil	Nil	Nil	Nil
Aldolase B	1.61	1.68	1.71	1.79	1.84	1.84
Transketolase	0.37	0.41	0.39	0.38	0.37	0.37
Transaldolase	0.46	0.47	0.47	0.43	0.46	0.48
Hexose 6-phosphate formed from ribose 5-phosphate (μmol/h per mg of protein at 34°C)	0.188	0.192	0.188	0.192	0.192	0.192

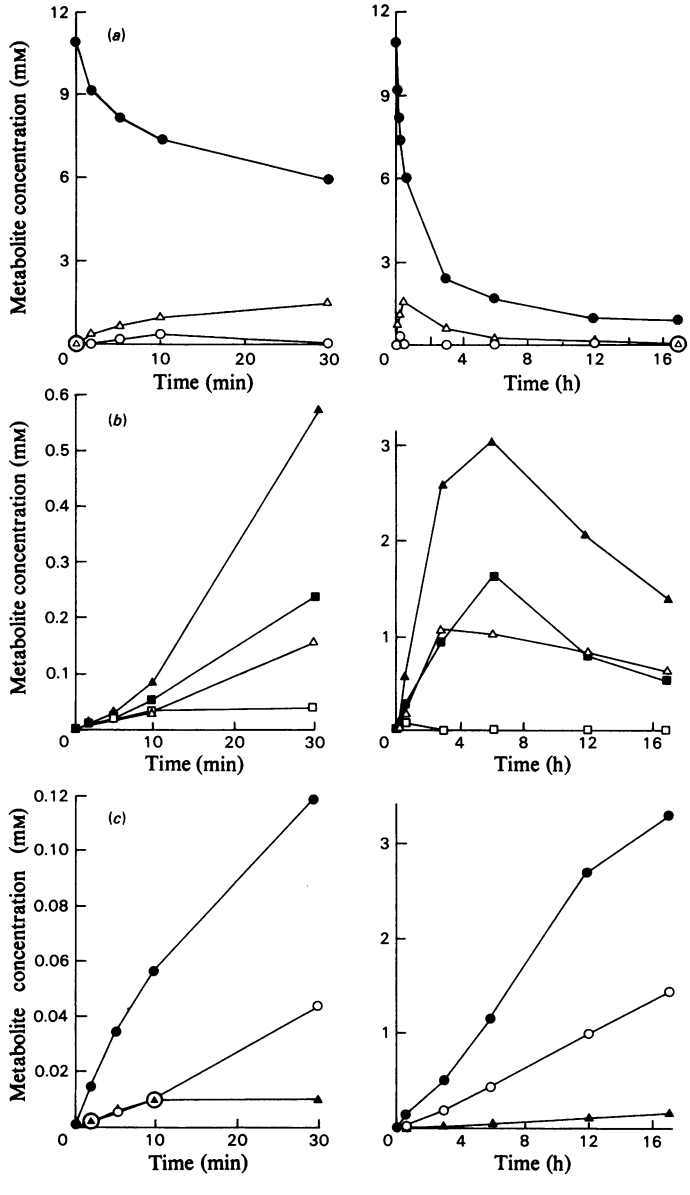


Fig. 1. *Carbon-balance studies for the formation of hexose phosphate from ribose 5-phosphate as catalysed by the rat liver enzyme preparation*

The incubation mixture at 25°C contained 108 μmol of ribose 5-phosphate, 0.75ml of rat liver enzyme preparation (equivalent to 21.9mg of protein) and water to give a total volume of 9.90ml at pH7.4. Samples of 1ml were removed from the incubation mixture at the times shown into 2.0ml of ice-cold 0.6M-$HClO_4$. The denatured protein was removed by centrifugation (5000*g* for 10min) and the supernatant solution adjusted to pH6.5 with saturated $KHCO_3$. After removal of the precipitate of $KClO_4$ by centrifugation (10000*g* for 10min) the final volume was adjusted to 3.3ml and samples were removed for the analysis of the intermediates by the methods described in the text. (*a*) Ribose 5-phosphate (●), xylulose 5-phosphate (△), ribulose 5-phosphate (○); (*b*) heptulose 7-phosphate (▲), glyceraldehyde 3-phosphate (□), dihydroxyacetone phosphate (■), fructose 1,6-bisphosphate (△); (*c*) glucose 6-phosphate (●), fructose 6-phosphate (○), glucose 1-phosphate (▲). Each value is the mean of duplicate determinations from four individual incubations.

Table 4. *Carbon-balance studies for the rat liver enzyme-catalysed conversion of ribose 5-phosphate to glucose 6-phosphate*
Data of Fig. 1 were used to calculate the total amount of carbon contained in each of the identifiable metabolites at the times shown. Each value is expressed as a percentage of the total carbon contributed by the substrate ribose 5-phosphate at the commencement of the incubation.

Time ...	0	10min	30min	3h	6h	12h	17h
Metabolite							
Ribose 5-phosphate	100	68.2	55.0	22.9	16.5	9.3	7.3
Xylulose 5-phosphate	0	8.9	13.3	5.6	1.9	0.8	0
Ribulose 5-phosphate	0	2.8	0	0	0	0	0
Heptulose 7-phosphate	0	1.2	7.4	33.1	38.9	26.1	18.3
Glyceraldehyde 3-phosphate	0	0.2	0.2	0	0	0	0
Dihydroxyacetone phosphate	0	0.3	1.3	5.3	9.2	4.0	3.0
Fructose 1,6-bisphosphate	0	0.3	1.7	12.0	11.4	8.3	7.4
Erythrose 4-phosphate	0	0	0	0	0	0	0
Glucose 1-phosphate	0	0.1	0.1	0.3	0.5	1.1	1.8
Fructose 6-phosphate	0	0.1	0.5	1.8	4.7	11.0	16.0
Glucose 6-phosphate	0	0.6	1.3	6.0	12.7	29.2	36.2
Total	100	82.7	80.8	87.0	95.8	89.8	90.2
Carbon unaccounted for (%)	0	17.3	19.2	13.0	4.2	10.2	9.8

total carbon contributed by the substrate ribose 5-phosphate at the commencement of the incubation. After 17h at 25°C with the rat liver enzyme preparation 92.5% of the total carbon as ribose 5-phosphate was utilized to produce at least nine intermediates (Fig. 1 and Table 4). Glucose 6-phosphate and fructose 6-phosphate accounted for 52.2% of the substrate carbon after 17h incubation. This latter result compared favourably with those of Horecker *et al.* (1954), but differed greatly from the finding of Glock (1952), who reported values of 70–75% for the conversion of ribose 5-phosphate into hexose 6-phosphate when catalysed by enzymes of rat liver *in vitro*. Fig. 1(*a*) shows that there were at least three widely divergent rates of ribose 5-phosphate utilization. During the initial 10min, the maximum rate of ribose 5-phosphate utilization was 158nmol/min per mg of protein which changed in the interval from 10 to 30min to 32.9nmol/min per mg of protein, then remained unchanged at 2.39nmol/min per mg of protein for the remaining 16.5h. The rates of formation of total hexose phosphate were fairly uniform throughout the incubation at 2.9nmol/min per mg of protein during the 10–30min interval and 2.14nmol/min per mg of protein during the final 16.5h.

Of the intermediates determined, erythrose 4-phosphate could not be detected at any time interval, even though the method used allowed the accurate measurement of quantities as low as 3nmol/ml of sample. Ribulose 5-phosphate and glyceraldehyde 3-phosphate were only detected during the initial 30min of the incubation. The data of Table 4 also allowed calculation of the percentage of total carbon unaccounted for after measurement of the concentrations of the commonly accepted intermediates of the pentose pathway. The carbon deficit increased from zero to a maximum of 19.2% at 30min then declined to 4.2% at 6h. In the final 11h of the incubation there was a constant carbon deficit of approx. 10%. P_i was not formed during the 17h incubation (Taussky & Schorr, 1953).

Specific radioactivity of glucose 6-phosphate formed from [1-^{14}C]ribose 5-phosphate

The rate of change of the specific radioactivity of [^{14}C]glucose 6-phosphate during the 17h incubation is shown in Fig. 2. After 1min it exceeded that of its precursor [1-^{14}C]ribose 5-phosphate by a factor of 1.4, which approximates to the theoretically expected value of 1.5. An excessive incorporation of ^{14}C into glucose 6-phosphate continued during the initial 3h of the incubation to reach a maximum value which was 1.9-fold higher than the initial specific radioactivity of [1-^{14}C]ribose 5-phosphate. After 8h incubation the specific radioactivity of glucose 6-phosphate declined to a value (0.95×10^6 d.p.m./μmol) that did not vary for the remainder of the incubation.

The specific radioactivity of the substrate ribose 5-phosphate decreased from 1.15×10^6 d.p.m./μmol at zero time to 0.5×10^6 d.p.m./μmol at 3h, then remained essentially constant for the remainder of the incubation.

This fall in the specific radioactivity of [1-^{14}C]-ribose 5-phosphate during the incubation was an unexpected finding and opposed the mechanistic predictions of the Horecker *et al.* (1954) reaction scheme for the pentose phosphate pathway.

^{14}C distribution in hexose 6-phosphate formed from [1-^{14}C]ribose 5-phosphate

Distribution of ^{14}C in each carbon atom of the samples of glucose 6-phosphate formed from

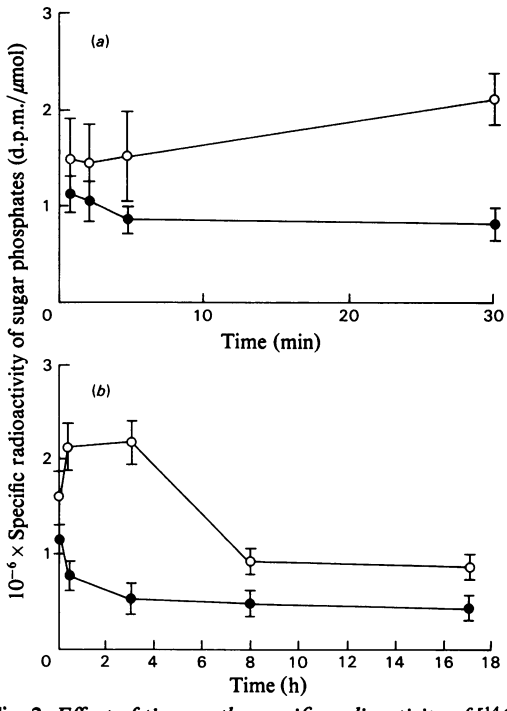


Fig. 2. *Effect of time on the specific radioactivity of* [^{14}C]*-glucose 6-phosphate and* [^{14}C]*ribose 5-phosphate isolated from the incubation of* [1-^{14}C]*ribose 5-phosphate with the rat liver enzyme preparation*

Details for the composition of the incubation mixture at 25°C and the treatment of samples are given in Table 1 and the text. The specific radioactivity of the [1-^{14}C]ribose 5-phosphate was 0.52μCi/μmol. To each neutralized sample (pH 7.0) was added 100μmol of unlabelled ribose 5-phosphate and glucose 6-phosphate and the solution subjected to the column chromatographic procedure for the separation of sugar phosphates as described by Williams *et al.* (1971). [^{14}C]Glucose 6-phosphate and [^{14}C]ribose 5-phosphate were isolated at the time intervals indicated, purified as glucose and ribose respectively and the specific radioactivity of each was determined as described in the Materials and Methods section. Each value is the mean of duplicate determinations from four experiments. The error bars represent ± S.E.M. ○, Glucose 6-phosphate; ●, ribose 5-phosphate.

[1-^{14}C]ribose 5-phosphate at incubation times ranging from 1 min to 17 h is shown in Table 5. Both C-1 and C-3 of glucose 6-phosphate contained small amounts of ^{14}C during the first 30 min of the incubation; however, in the samples isolated after 3, 8 and 17 h incubation the incorporation of ^{14}C steadily increased to reach a maximum by 17 h of 57.2 and 25.1% in C-1 and C-3 respectively. C-4 contained 10.1% of the label at 1 min which thereafter decreased to 2.7% in the sample analysed at 17 h. The percentage of label in C-5 was variable, but did not exceed 5.9% during the entire incubation. The most important features of the results of Table 5 were the appearance of label and the degree of label incorporated into C-2 and C-6 of glucose 6-phosphate sampled at 1 and 2 min. In the samples degraded at 5 min, 30 min, 3 h, 8 h and 17 h, C-2 remained labelled (approx. 13%), whereas the label in C-6 increased from 46.4% at 2 min to reach a maximum of 76.2% at 30 min, and thereafter steadily decreased to 3.4% at 17 h.

The effect of incubation time on the radioactivity content of each of the carbon atoms of glucose 6-phosphate formed from [1-^{14}C]ribose 5-phosphate

Table 5. *Distribution of* ^{14}C *in glucose 6-phosphate formed from* [1-^{14}C]*ribose 5-phosphate as catalysed by the rat liver enzyme preparation*

Details for the composition of the incubation mixture at 25°C are given in Table 1. Glucose 6-phosphate was isolated at the time intervals indicated, purified as glucose and degraded according to the procedure detailed in the text. The specific radioactivity of each carbon atom is expressed as a percentage of the total, obtained by the addition of the specific radioactivities of the six carbon atoms. The percentage recovery of label was determined by expressing the total label recovered (determined by the addition of the six individual carbon atom specific radioactivities) as a fraction of the specific radioactivity obtained from the complete oxidation of the glucose molecule by the method of Van Slyke & Folch (1940). Degradation data for the substrate [1-^{14}C]ribose 5-phosphate are given in Table 1.

Carbon atom / Sample time ...	1 min	2 min	5 min	30 min	3 h	8 h	17 h
1	1.0	0.5	1.1	2.0	28.2	40.6	57.2
2	45.0	44.0	16.1	12.9	20.9	13.4	10.2
3	1.7	0.1	2.2	1.9	9.8	16.3	25.1
4	10.1	3.1	4.9	6.9	4.5	4.6	2.7
5	0.9	5.9	0.9	0.1	1.9	3.7	1.4
6	41.3	46.4	74.8	76.2	34.7	21.4	3.4
Recovery (%)	98.4	100.3	107.7	97.9	98.9	98.2	98.6

Table 6. *Effect of time on the radioactivity content of each carbon atom of [^{14}C]glucose 6-phosphate formed from [1-^{14}C]ribose 5-phosphate by the rat liver enzyme preparation*

The total radioactivity of each carbon atom of the degraded glucose 6-phosphate was calculated from the specific radioactivity (Fig. 2) and concentration of glucose 6-phosphate in the incubation mixture at that time interval (Fig. 1), and the percentage of radioactivity contained by the relevant carbon atom (Table 5). The percentage of original label was determined by the ratio, total radioactivity in the six carbon atoms of glucose 6-phosphate/total radioactivity in C-1 of the substrate, [1-^{14}C]ribose 5-phosphate, at the commencement of the incubation.

		$10^{-3}\times$ Radioactivity (d.p.m.)						
Carbon atom	Time ...	1 min	2 min	5 min	30 min	3 h	8 h	17 h
1		0.4	0.4	1.6	14.9	1038.0	2130.0	5542.0
2		19.0	35.9	24.0	96.7	768.0	702.0	991.0
3		0.7	0.1	3.3	14.2	361.0	855.0	2430.0
4		4.3	2.6	7.3	51.8	165.0	241.0	262.0
5		0.3	4.8	1.3	0.7	69.8	194.0	135.5
6		17.4	37.8	111.6	572.0	1276.0	1122.0	330.0
$10^{-3}\times$ Total radioactivity in glucose 6-phosphate (d.p.m.)		42.1	81.6	149.1	750.3	3677.8	5244.0	9690.5
Percentage of original label		0.08	0.16	0.29	1.44	7.08	10.09	18.65

is shown in Table 6. All carbon atoms except C-6 showed an increase in radioactivity as the time of incubation increased to 17h. The radioactivity content of C-6 increased to a maximum of 1.27×10^6 d.p.m. at 3h and then decreased to 0.33×10^6 d.p.m. at 17h.

Discussion

The results of Horecker *et al.* (1954) and Gibbs & Horecker (1954) have provided the main experimental evidence for the introduction and acceptance of the current scheme for the reactions and the reaction sequence of the non-oxidative pentose phosphate pathway of glucose metabolism (Scheme 1). The ordered sequence of reactions proposed for the enzymic conversion of pentose 5-phosphate into hexose 6-phosphate was inferred from data gathered with an experimental system in which the hexose 6-phosphate accumulated and was unable to recycle back through the reactions of the oxidative pentose phosphate pathway. It was reasoned that the obstruction of a recycling process by the omission of $NADP^+$ would prevent the redistribution of ^{14}C isotope in hexose 6-phosphate (Wood & Katz, 1958) and effect a 'freezing' of the ^{14}C distribution pattern in the hexose 6-phosphate formed from pentose 5-phosphate. It was an assumption of the experiments that the reaction pathway would be revealed by the nature and degree of ^{14}C distribution in the hexose 6-phosphate product. Under the coenzyme-limited conditions the reaction scheme proposed in Scheme 1 gave rise to the synthesis of two different isotopically labelled species of hexose 6-phosphate from [1-^{14}C]ribose 5-phosphate, namely [1,3-^{14}C]fructose 6-phosphate and [1-^{14}C]fructose 6-phosphate. The theoretical stoicheiometry further stipulated that each ^{14}C-labelled species of fructose 6-phosphate was formed in equal quantities and that the theoretical percentage distribution of ^{14}C in fructose 6-phosphate at any time was 66.6% in C-1, 0% in C-2, 33.3% in C-3, 0% in C-4, 0% in C-5 and 0% in C-6.

The reaction mechanism shown in Scheme 1 does not permit the labelling of any other carbon atom of hexose 6-phosphate and predicts that the molar specific radioactivity of hexose 6-phosphate will be 1.5 times the molar specific radioactivity of the ribose 5-phosphate and will remain constant at this value throughout the incubation. In contrast, the results of Figs. 1 and 2 and Tables 5 and 6 indicate that there is more than one reaction rate and possible pathway for the conversion of pentose 5-phosphate into hexose 6-phosphate by reactions catalysed by rat liver enzymes. At least three different rates of ribose 5-phosphate utilization were observed, although the rates of synthesis of glucose 6-phosphate, fructose 6-phosphate and glucose 1-phosphate were approximately linear (Figs. 1*a* and 1*c*). The combined rate of formation of all three hexose phosphates was only one-sixtieth of the initial rate of ribose 5-phosphate utilization. There have been numerous reports of various rates of ribose 5-phosphate utilization during the time course of its incubation with enzymes prepared from many tissues: erythrocyte haemolysates (Dische, 1951); higher plants (Axelrod *et al.*, 1953); liver (Glock, 1952); acetone-dried powder enzyme preparations of muscle, erythrocytes and Krebs II carcinoma ascites cells (Dickens & Williamson, 1956). During the 17h time course of this study the relative amounts of ribose 5-phosphate, xylulose 5-phosphate and ribulose 5-phosphate (Table 4) were not maintained at

equilibrium (Tabachnik *et al.*, 1958), which would prevail if the activities of ribose phosphate isomerase and ribulose phosphate 3-epimerase were alone controlling the concentrations of these intermediates.

It is useful to contrast the markedly different rates of ribose 5-phosphate utilization with the patterns of changing concentration and accumulation of intermediates of ribose 5-phosphate and hexose 6-phosphate metabolism together with the varying patterns of ^{14}C distribution into each of the carbon atoms of glucose 6-phosphate formed during the incubation. During the first 3h of the incubation, the rapid rate of ribose 5-phosphate utilization (Fig. 1*a*) coincided with (i) the relatively rapid accumulation of heptulose 7-phosphate, fructose 1,6-bisphosphate and dihydroxyacetone phosphate, all of which began to decrease in concentration after 6h (Fig. 1*b*), (ii) the heavy labelling of C-2 and C-6 of glucose 6-phosphate formed during the initial 3h and an almost total absence of ^{14}C from C-1 and C-3, and (iii) the rapid increase in the specific radioactivity of glucose 6-phosphate to 1.9 times that of the [1-^{14}C]ribose 5-phosphate substrate after 3h.

The lower rate of ribose 5-phosphate utilization occurred after 3h of incubation when the labelling pattern of glucose 6-phosphate was characterized by increasing radioactivity in C-1 and C-3 and decreasing radioactivity in C-2 and C-6; however, C-2 still retained a significant percentage of radioactivity even at 17h. This latter pattern of ^{14}C distribution in C-1 and C-3 may be related to the reaction sequence for the pentose phosphate pathway (Scheme 1), although the theoretically predicted ratio of the specific radioactivity of C-1/specific radioactivity of C-3 (C-1/C-3 ratio) of 2 was not found. Horecker *et al.* (1954) reported C-1/C-3 to be 3.08, and the results of Table 5 gave values of 2.88, 2.49 and 2.28 for C-1/C-3 at 3h, 8h and 17h respectively; this last value was closer to the theoretical predictions of the reaction sequences of Scheme 1. The slower phase of ribose 5-phosphate utilization coincided with: (*a*) a decrease in the concentrations of heptulose 7-phosphate, fructose 1,6-bisphosphate and dihydroxyacetone phosphate in the incubation mixture; (*b*) a steady decrease in the specific radioactivity of glucose 6-phosphate formed during the interval from 3 to 8h, after which time the specific radioactivity remained constant and equal to the initial value of the specific radioactivity of the [1-^{14}C]ribose 5-phosphate substrate; (*c*) a decrease in the radioactivity of C-6 of glucose 6-phosphate (Table 6) by approx. 71% during the last 9h of the incubation; (*d*) a constancy of the specific radioactivity of ribose 5-phosphate after the initial decrease that occurred during the first 3h. As there was no change in the ^{14}C-labelling pattern of the [1-^{14}C]ribose 5-phosphate sampled from the incubation mixture after 17h (Table 1), it is established that residual ribose 5-phosphate has not participated in reactions which resulted in any ^{14}C randomization of its carbon atoms during a 17h incubation.

As the possibility remained that the distribution patterns in glucose 6-phosphate (Table 5) were due to a set of unique properties of the rat liver enzyme preparation, a comparison was made between rat and rabbit liver enzyme preparations, prepared in an identical fashion. This was considered important because investigations by us of the operation of the pentose phosphate cycle were all conducted using rabbit liver (e.g. Schofield *et al.*, 1970; Williams *et al.*, 1971; Gerdes *et al.*, 1974) and all of these studies failed to show that the reaction sequence of the pentose phosphate pathway (Scheme 1) or a cyclic scheme of these reactions was being followed. A representative series of enzymes (Table 2) was examined in both preparations and, except for pyruvate kinase and phosphoglyceromutase, only minor variations in the activities of the enzymes examined between preparations from either rat or rabbit liver were found, and furthermore, the activities of the enzymes usually associated with the reactions of the non-oxidative pentose phosphate pathway were sufficiently high to account for the synthesis of hexose 6-phosphate from pentose 5-phosphate. This was considered to be good evidence that the rat or rabbit liver enzyme preparations were most suitable systems to carry out mechanistic studies *in vitro* on the non-oxidative pentose phosphate pathway.

In order to investigate the possibility that the different rates of ribose 5-phosphate utilization and low rates of hexose 6-phosphate formation together with the two different types of isotope-distribution patterns in glucose 6-phosphate (namely labelling of C-2 and C-6 during the first 3h and labelling of C-1 and C-3 during the last 14h) were due to changes in enzyme activity during the incubation, the stability of the rat liver enzyme preparation was examined (Table 3). A representative series of enzymes, in particular the group-transferring enzymes transketolase, transaldolase and aldolase (Table 3), from the rat liver enzyme preparation was shown to remain constant throughout the incubation, together with the ability of the enzyme preparation to catalyse the formation of hexose 6-phosphate from pentose 5-phosphate at a constant rate (0.188–0.192μmol/h per mg of protein at 34°C) during the different time intervals of the 17h incubation.

The distribution of ^{14}C in C-1 and C-3 of glucose 6-phosphate may be attributed to, and is consistent with, the ordered reaction sequence catalysed by transketolase, transaldolase and transketolase (Horecker *et al.*, 1954), whereas the distribution of ^{14}C into C-2 and C-6 cannot be reconciled with the operation of any of the recognized pathways of pentose phosphate metabolism in liver tissue. An

exception is the following series of reactions which theoretically may occur *in vivo* but are prevented in the enzyme preparation used in the present work or in the experiment of Horecker *et al.* (1954).

$$[1\text{-}^{14}C]\text{Ribose-5-}P \dashrightarrow [1,3\text{-}^{14}C]\text{fructose-6-}P \quad (12)$$

$$[1,3\text{-}^{14}C]\text{Fructose-6-}P + \text{ATP} \rightarrow [1,3\text{-}^{14}C]\text{fructose-1,6-}P_2 + \text{ADP} \quad (13)$$

$$[1,3\text{-}^{14}C]\text{Fructose-1,6-}P_2 \rightleftarrows [1,3\text{-}^{14}C]\text{dihydroxyacetone-}P + \text{glyceraldehyde-3-}P \quad (14)$$

$$[1,3\text{-}^{14}C]\text{Dihydroxyacetone-}P \rightleftarrows [1,3\text{-}^{14}C]\text{glyceraldehyde-3-}P \quad (15)$$

$$[1,3\text{-}^{14}C]\text{Fructose-6-}P + [1,3\text{-}^{14}C]\text{glyceraldehyde-3-}P \rightleftarrows [1,3,4,6\text{-}^{14}C]\text{fructose-6-}P + \text{glyceraldehyde-3-}P \quad (16)$$

or

$$[1,3\text{-}^{14}C]\text{Dihydroxyacetone-}P + [1,3\text{-}^{14}C]\text{glyceraldehyde-3-}P \rightleftarrows [1,3,4,6\text{-}^{14}C]\text{fructose-1,6-}P_2 \quad (17)$$

$$[1,3,4,6\text{-}^{14}C]\text{Fructose-1,6-}P_2 \rightleftarrows [1,3,4,6\text{-}^{14}C]\text{fructose-6-}P + P_i \quad (18)$$

$$[1,3,4,6\text{-}^{14}C]\text{Fructose-6-}P \dashrightarrow {}^{14}CO_2 + [2,3,5\text{-}^{14}C]\text{xylulose-5-}P \quad (19)$$

$$[2,3,5\text{-}^{14}C]\text{Xylulose-5-}P + [1,3,4,6\text{-}^{14}C]\text{fructose-6-}P \rightleftarrows [1,3,5\text{-}^{14}C]\text{xylulose-5-}P + [2,3,4,6\text{-}^{14}C]\text{fructose-6-}P \quad (20)$$

The above reactions are catalysed by the following enzymes: (12) the sequential action of transketolase, transaldolase and transketolase; (13) fructose 6-phosphate kinase; (14 and 17) aldolase; (15) triose phosphate isomerase; (16) transaldolase exchange reaction (Williams *et al.*, 1971); (18) hexose bisphosphatase; (19) the sequential action of glucose phosphate isomerase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and ribulose phosphate 3-epimerase; (20) transketolase exchange reaction (Clark *et al.*, 1971).

The operation of the above series of reactions will theoretically relocate ^{14}C from C-1 of pentose 5-phosphate to C-1 and C-3 of hexose 6-phosphate as well as C-2 and C-6 by the operation of several reactions of glycolysis and transketolase and transaldolase exchange reactions. This series of reactions is prevented from occurring in the rat liver enzyme preparation both by the absence of the nucleotides ATP and $NADP^+$ and of the appropriate concentration of Mg^{2+} which is necessary for the activation of hexose bisphosphatase (Horecker *et al.*, 1954).

It is a theoretical condition of both the experimental system used by Horecker *et al.* (1954) for the conversion of $[1\text{-}^{14}C]$pentose 5-phosphate into hexose 6-phosphate and the conventional reaction scheme of the pentose phosphate pathway (Scheme 1) that there is no redistribution of ^{14}C isotope in the molecule (Table 1) and that the specific radioactivity of $[1\text{-}^{14}C]$ribose 5-phosphate should not change during the time course of the reaction. The data of Table 2 show that there is an early and significant fall in specific radioactivity of ribose 5-phosphate. It will be shown that this unexpected but significant fall in specific radioactivity is a necessary condition of the proposed new reaction scheme for the pentose phosphate pathway which is presented in the following paper (Williams *et al.*, 1978).

The results in Table 4 not only demonstrate different rates of pentose 5-phosphate utilization and hexose 6-phosphate production, but indicate that a significant proportion of substrate carbon is not accounted for (maximum 20% of carbon unaccounted at 30min) by the analysis of the intermediates and products of the pathway. Since free inorganic phosphate was not formed during the incubation, this indicates that phosphorylated intermediates are being formed and that they may also play a role in relocating ^{14}C from C-1 of pentose 5-phosphate to C-2 and C-6 of hexose 6-phosphate. In the following paper (Williams *et al.*, 1978) a new reaction scheme for the non-oxidative pentose phosphate pathway is proposed, which is consistent with all of the data obtained *in vitro* presented here and which will also account for the results obtained for the metabolism of glucose and ribose in rabbit liver *in situ* (Williams *et al.*, 1971).

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