The Pentose Phosphate Pathway in Rabbit Liver

STUDIES ON THE METABOLIC SEQUENCE AND QUANTITATIVE ROLE OF THE PENTOSE PHOSPHATE CYCLE BY USING A SYSTEM *IN SITU*

By J. F. WILLIAMS, K. G. RIENITS, P. J. SCHOFIELD AND M. G. CLARK

School of Biochemistry, University of New South Wales, Box 1 P.O., Kensington, N.S.W. 2033, Australia

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1. The reactions of the pentose phosphate cycle were investigated by the intra-portal infusion of specifically labelled [^{14}C]glucose or [^{14}C]ribose into the liver of the anaesthetized rabbit. The sugars were confined in the liver by haemostasis and metabolism was allowed to proceed for periods up to 5 min. Metabolism was assessed by measuring the rate of change of the specific radioactivity of CO_2 , the carbon atoms of glucose 6-phosphate, fructose 6-phosphate and tissue glucose. 2. The quotient oxidation of [$1\text{-}^{14}\text{C}$]glucose/oxidation of [$6\text{-}^{14}\text{C}$]glucose as measured by the incorporation into respiratory CO_2 was greater than 1.0 during most of the time-course and increased to a maximum of 3.1 but was found to decrease markedly upon application of a glucose load. 3. The estimate of the pentose phosphate cycle from C-1/C-2 ratios generally increased during the time-course, whereas the estimate of the pentose phosphate cycle from C-3/C-2 ratios varied depending on whether the ratios were measured in glucose or hexose 6-phosphates. 4. The distribution of ^{14}C in hexose 6-phosphate after the metabolism of [$1\text{-}^{14}\text{C}$]ribose showed that 65-95% of the label was in C-1 and was concluded to have been the result of a rapidly acting transketolase exchange reaction. 5. Transaldolase exchange reactions catalysed extensive transfer of ^{14}C from [$2\text{-}^{14}\text{C}$]glucose into C-5 of the hexose 6-phosphates during the entire time-course. The high concentration of label in C-4, C-5 and C-6 of the hexose 6-phosphates was not seen in tissue glucose in spite of an unchanging rate of glucose production during the time-course. 6. It is concluded that the reaction sequences catalysed by the pentose phosphate pathway enzymes do not constitute a formal metabolic cycle in intact liver, neither do they allow the definition of a fixed stoichiometry for the dissimilation of glucose.

The currently accepted scheme of the pentose phosphate cycle is a defined multienzyme pathway (Wood & Katz, 1958), which includes oxidative (reaction 1) and non-oxidative segments (reactions 2-5) (Horecker, Gibbs, Klenow & Smyrniotis, 1954; Gibbs & Horecker, 1954).

imposes the cyclic nature and the stoichiometry of the reaction sequence (reaction 6). The definition also stipulates a fixed order of sequence for the reactions catalysed by transketolase (EC. 2.2.1.1) and transaldolase (EC 2.2.1.2) and further imposes the condition that the net flow of carbon is from

The definition requires that the fructose 6-phosphate formed in reactions (3) and (4) is converted into glucose 6-phosphate by glucose phosphate isomerase (EC 5.3.1.9) and this condition

pentose phosphate to hexose 6-phosphate (Wood, Katz & Landau, 1963).

The defined mechanism of the cycle is based on the results of two experiments (Horecker *et al.*

1954; Gibbs & Horecker, 1954). These involved the incubation of [^{14}C]ribose 5-phosphate and [2,3- ^{14}C]pentose 5-phosphate with extracts of acetone-dried powders prepared from rat liver and pea root tissue. The incubations were carried out for 17h (liver) and 4h (pea root). With liver the [1- ^{14}C]ribose 5-phosphate gave rise to 1,3-labelled hexose 6-phosphate, with 74% of the ^{14}C in C-1 and 24% in C-3. The 2,3-labelled pentose 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 pattern found in the hexose 6-phosphate isolated from the pea root incubation was essentially the same as that from liver.

It was concluded that this unique distribution of ^{14}C was explained by reactions sequentially catalysed by ribose 5-phosphate isomerase (EC 5.3.1.6), ribulose 5-phosphate 3-epimerase (EC 5.1.3.1), followed by transketolase, transaldolase and transketolase (Horecker *et al.* 1954; Gibbs & Horecker, 1954).

An important consequence of the definition of the mechanism is the ordered distribution of C-2 and C-3 of substrate glucose into C-1, C-2 and C-3 of the two fructose 6-phosphate molecules formed by one turn of the cycle (reaction 7).

The two molecules of fructose 6-phosphate are distinguished by their labelling patterns because of their different paths of synthesis. The 2,3,2 pattern results from reaction (3), catalysed by transaldolase, and the 2,3,3 pattern is established in reaction (4), catalysed by transketolase.

In their theoretical treatment of the pentose phosphate cycle, Katz & Wood (1960) used the exclusive labelling patterns generated in hexose 6-phosphate by recycling of the ^{14}C of substrate glucose to quantitate the pathway. Their definition of the cycle is such that the distribution pattern is a unique property of the pathway and the degree of distribution is a direct function of the quantitative participation of the cycle in the overall glucose metabolism of tissues.

The method of Katz & Wood (1960) involves the application of [^{14}C]glucose exclusively labelled at either C-2 or C-3. After isotopic steady state (Landau, Bartsch, Katz & Wood, 1964) is established between the fructose 6-phosphate generated in the pentose phosphate cycle and the hexose 6-phosphate pool, either glucose 6-phosphate and/or fructose 6-phosphate should be isolated from tissues and the ratios of the specific radioactivity of C-1/C-2 or C-3/C-2 determined. These ratios theoretically allow an estimate of the relative contribution of the pentose phosphate cycle to total glucose metabolism (Katz & Wood, 1960; Wood *et al.* 1963; Landau & Katz, 1965).

In practice all reported estimates of the pentose phosphate cycle by using the method of Katz & Wood (1960) have involved either the isolation of the glucose units of glycogen or of glycerol from triglyceride, followed by their degradation (Bernstein, Lentz, Malm, Schambye & Wood, 1955; Stjernholm & Wood, 1958) and determination of ^{14}C ratios in C-1, C-2 and C-3. This expedient procedure followed from the assumption that after metabolism of [^{14}C]glucose the ^{14}C -labelling pattern in the glucose monomers of glycogen or in glycerol reflects the distribution of ^{14}C in glucose 6-phosphate or fructose 6-phosphate.

A characteristic feature of the development of biochemical knowledge of the pentose phosphate cycle has been the many attempts to evaluate quantitatively the cycle before a sufficiently established body of information on the cycle or pathway mechanism had been achieved. A number of schemes for the operation of the pentose phosphate cycle or pathway have been proposed [Beevers, 1956; Racker, 1956; Korkes, 1956; Kitos, Wang, Mohler, King & Cheldelin, 1958; Davies, 1961; Horecker, 1961, 1962 (p. 73)]. These proposals are distinguished from each other and from the Katz & Wood (1960) scheme by their reaction sequence and stoichiometry. The main features in favour of the model of Katz & Wood (1960) are: a reasonable consistency with much of the existing data, and an

accord with the principle of minimum hypothesis. A distinction among, and measurement of, the pathways of glucose metabolism theoretically should be easy with isotopic tracers. In practice the evaluation of experimental findings has not proved as simple as might be expected from the cursory consideration of known pathways. Sols (1961) emphasized that 'calculations of pathways' of glucose metabolism are fraught with many sources of error and such calculations should be interpreted with great caution.

The studies reported here were made to seek information as to the validity of some of the assumptions used in making the calculations on the magnitude of the pathways of glucose metabolism. The experiments were principally designed to investigate the mechanism of the pentose phosphate cycle in rabbit liver *in situ*, by kinetic studies which involved the metabolism of specifically ^{14}C -labelled glucose and ribose. From the results obtained an attempt was made to assess the quantitative extent of the pentose phosphate cycle by an application of the method of Katz & Wood (1960). Their method was rigorously followed in that hexose 6-phosphate intermediates, rather than distantly related derivatives of hexose 6-phosphate, were isolated for degradation. The reaction sequence *in situ* of the pentose phosphate cycle was investigated by measuring changes in the specific radioactivity of CO_2 and all the carbon atoms of glucose, glucose 6-phosphate and fructose 6-phosphate during the time-course experiments.

MATERIALS AND METHODS

Animals. Animals and surgical techniques for the preparation of the liver, the infusion of the relevant ^{14}C -labelled compound and the isolation and determination of the specific radioactivity of the resultant CO_2 are as previously detailed (Williams, Rienits & Clark, 1971). All animals were 40 h-starved unless otherwise stipulated.

Materials. D-[1- ^{14}C]-, D-[2- ^{14}C]- and D-[6- ^{14}C]-glucose and D-[1- ^{14}C]ribose were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All enzymes, substrates and coenzymes used were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., or Sigma Chemical Co., St Louis, Mo., U.S.A. Reagents and solvents were of A.R. grade or the highest quality commercially available (British Drug Houses Ltd., Poole, Dorset, U.K., or May and Baker Ltd., Dagenham, Essex, U.K.).

The water used in all aspects of the degradation experiments was deionized and then twice distilled in glass apparatus.

Extraction of liver. After the desired period of confinement of the ^{14}C -labelled substrate in the liver the whole organ was extirpated into liquid N_2 and crushed to a fine powder. Approx. 60 g of the frozen liver was taken and divided into three portions; 10 g was used for the isolation of CO_2 and the determination of its specific radioactivity

(Williams *et al.* 1971); 10 g was treated as described by Williams *et al.* (1971) for the determination of lactate, pyruvate, P_i and the adenine nucleotides. Approx. 40 g was accurately weighed and homogenized in ice-cold 0.6 M- HClO_4 in a large glass Potter-Elvehjem-type homogenizer. The homogenate was filtered with suction through a sintered-glass filter funnel (porosity 3) and the filtrate neutralized with 10% (w/v) KOH to pH 8.0 by glass electrode. After 1 h the precipitated KClO_4 was removed by centrifugation, and the supernatant adjusted to a final volume of 250 ml with water. This preparation maintained at pH 8.0 is hereafter called 'the extract'. All operations were conducted at 0°C .

Treatment of the extract. Glucose, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate were determined spectrophotometrically in a 5 ml sample of the extract by the following enzymic methods: glucose (Slein, 1963); glucose 6-phosphate and fructose 6-phosphate (Hohorst, 1963); fructose 1,6-diphosphate (Bücher & Hohorst, 1963).

An extension of the method of Khym & Zill (1952) was used for the separation of the sugars and sugar phosphates as the borate complexes. This was achieved by anion-exchange chromatography based on a linear-gradient elution with ammonium tetraborate (Lefebvre, Gonzalez & Pontis, 1964). Analytical grade resin, Dowex 1 (X4; 200–400 mesh; Cl^- form) (Bio-Rad Laboratories, Calif., U.S.A.), was converted into the borate form by equilibration with 0.8 M-potassium tetraborate for 18 h. A suspension of the resin was allowed to settle in a glass column to give a bed size $1.0\text{ cm} \times 50\text{ cm}$, and the resin washed with distilled deionized water until the effluent was free of Cl^- .

A 245 ml sample of the extract was maintained at pH 8.0, and $100\text{ }\mu\text{mol}$ each of unlabelled carrier glucose, glucose 6-phosphate, fructose 6-phosphate, ribose 5-phosphate and fructose 1,6-diphosphate was added. The mixed solution was pumped on to the column followed by 250 ml of water. Sugars and sugar phosphates were separated by elution by using a linear gradient of 0.1 M–0.4 M-ammonium tetraborate (Fig. 1). Fractions of 5.0 ml were collected at a constant flow rate of 2.5 ml/min in an LKB Radi Rac Fraction Collector type 3400. Flow was maintained by an LKB ReCyChrom Pump, type 4912. Every fourth fraction was sampled for analysis to ascertain the elution profile of the chromatogram. Ribose and ribose 5-phosphate were determined by the orcinol method (Mejbaum, 1939). Hexose and hexose phosphates were determined by a modification of the anthrone method of Graham & Smydzuk (1965); 0.5 ml was taken and added to 4.0 ml of frozen anthrone reagent [0.2% (w/v) anthrone in 98% (w/w) H_2SO_4] together with 1.5 ml of water in thick-walled glass tubes ($15\text{ cm} \times 2.5\text{ cm}$ with a glass wall thickness of 0.2 cm) which had been frozen in liquid N_2 . The tubes were heated at 100°C for exactly 5 min, cooled in ice and the extinctions determined at 625 nm. The fractions under each peak were pooled, and evaporated to dryness *in vacuo* at 45°C in a rotary film evaporator (Buchi Roto-vapor R). The residual white powder was redissolved in a minimum volume of water, the borate and ammonia were removed by three successive evaporations each with 300 ml of redistilled methanol. Individual sugar and sugar phosphate fractions free of borate were each dissolved in 5 ml of water and freeze-dried. The isolated

Fig. 1. Separation of sugars and sugar phosphates isolated from the HClO_4 extract of rabbit liver. Chromatography was carried out by using a column (50 cm $\times$ 1 cm) of Dowex 1 (X4) resin in the borate form. Sugars and sugar phosphates were eluted with a linear gradient of 0.1–0.4 M-ammonium tetraborate. Ribose (1) and ribose 5-phosphate (6) (-----) were determined by the orcinol method (E_{676}). Glucose (2), fructose (3), glucose 1-phosphate (4), fructose 6-phosphate (5), glucose 6-phosphate (7) and fructose 1,6-diphosphate (8) (—) were determined by using the colorimetric anthrone method (E_{625}). Each method is described in the Materials and Methods section.

sugars and sugar phosphates were assayed and the radioactivity was determined.

Isolation and purification of sugars for degradation. The separation of ribose, fructose, glucose 1-phosphate, fructose 6-phosphate and ribose 5-phosphate, glucose 6-phosphate as well as fructose 1,6-diphosphate was complete (Fig. 1).

The glucose fraction (approx. 300 μmol) was concentrated to 0.5 ml in a Buchi rotary evaporator and applied as a band on Whatman no. 4 paper. By using authentic glucose as a reference the sugar was isolated from possible contaminants (lactate, ribose, glycerol and fructose) by descending chromatography in butan-1-ol-acetic acid-water (12:3:5, by vol.). Glucose was located by spraying a small section of the chromatogram and the reference spot with alkaline AgNO_3 (Smith, 1958). The remainder of the glucose was eluted and concentrated to 5 ml, assayed, and the specific radioactivity determined.

The glucose 6-phosphate fraction (containing approx. 100 μmol) was concentrated to 5 ml. Then 20 mg of plant acid phosphatase (type I, Sigma Chemical Company), 2.5 ml of 0.02 M- MgCl_2 and 2.5 ml of 0.4 M-acetic acid (previously adjusted to pH 4.8 with 2 M- NaOH) were added. The mixture was incubated at 37°C for 3 h and the reaction stopped by heating at 100°C for 10 min. The supernatant was concentrated to 0.5 ml and passed through a mixed-bed resin (10 g) composed of equal parts of Dowex 50 W (H^+ form) and Dowex 1 (HCO_3^- form). The eluate was concentrated to 0.2 ml and applied as a band to Whatman no. 4 paper. Separation of free glucose was achieved as described above.

The combined fractions containing both the fructose 6-phosphate and ribose 5-phosphate were pooled and the solution was divided into two equal parts. (a) A 2.5 ml sample containing approx. 50 μmol of each sugar phos-

phate was adjusted to pH 8.0 with 1 M- NH_3 and incubated for 10 min at 25°C with 0.2 ml of glucose phosphate isomerase (10 mg of protein/ml). The glucose 6-phosphate formed from fructose 6-phosphate was converted into glucose and isolated by the paper-chromatographic method described above. (b) Ribose 5-phosphate and fructose 6-phosphate were dephosphorylated and the free sugars separated by paper chromatography as described above.

Degradation of glucose. Gunsalus & Gibbs (1952) showed that *Leuconostoc mesenteroides* stoichiometrically fermented glucose to produce CO_2 from C-1, ethanol from C-2 and C-3, and lactate from C-4, C-5 and C-6. *L. mesenteroides* was used to achieve the partial degradation of all samples of glucose.

A culture of *L. mesenteroides* strain 39 (kindly provided by Professor Martin Gibbs) was stored as an agar broth at 5°C, maintained by monthly transfer into stock agar and grown in culture (Bernstein *et al.* 1955). Crops of 12–16 l were grown in batch culture at 30°C. For satisfactory fermentations it was essential that the organisms were in a suitable stage of activity and hence the cells were subcultured while in the exponential growth phase (1–2 h after the beginning of gas evolution). To monitor the growth of the organisms, cells were cultured in sterile Erlenmeyer flasks (100 ml, 250 ml and 5 litre) fitted with a side arm (10 cm $\times$ 1.5 cm diam.). Cell density was measured by tipping the well-mixed medium into the side arm and reading the extinction in an EEL Portable Colorimeter by using an EEL ORT red filter against a blank, containing medium but no inoculum. Subcultures of 2% (v/v) inoculum were made when the mid-exponential phase was reached. Cells from the 5 litre flask in the exponential growth phase were harvested at 27000 g in a Sorvall refrigerated centrifuge fitted with a Continuous Flow System (Sorvall type KSB I) fitted to a SS34 Rotor.

The inflow rate of the culture was 120 ml/min. Cells were washed twice by resuspending in 20 vol. of 1M- NaH_2PO_4 buffer (adjusted to pH 6.0 with 2M- NaOH), and centrifuged at 27000g for 10 min. The total cell harvest was suspended in the 1M-sodium phosphate buffer, pH 6.0, to give a concentration of 1g wet wt. of cells/3 ml of suspension.

Fermentations were carried out on glucose prepared from the column-chromatographic fractions of glucose, fructose 6-phosphate and glucose 6-phosphate. The variable amounts of glucose isolated were made to 1 mmol by addition of carrier glucose before fermentation. Fermentations were carried out in double side arm Warburg flasks of 125 ml capacity; the main compartment contained the glucose solution (1 mmol in a vol. of 5 ml) together with 15 ml of 1M-sodium phosphate buffer, pH 6.0. A 3 ml sample of a suspension of *L. mesenteroides* equivalent to 1g wet wt., sufficient to ferment 1 mmol of glucose in 4 h (Gordon, 1968), was placed in the side arm. The centre well contained 2 ml of 2.5M- NaOH (CO_2 -free). A control was included in each batch of fermentations, and specific radioactivities were corrected for any dilution indicated by the yields of endogenous CO_2 ; this normally constituted 2.5–5% of that obtained from the fermentation of 1 mmol of glucose. The flasks were flushed with N_2 (CO_2 -free) and allowed to equilibrate at 30°C for 10 min, at which time the bacterial cell suspension was tipped into

the main compartment and incubation was continued for 3.5 h in a Warburg bath (150 oscillations/min). At the end of the incubation period the $\text{NaOH-Na}_2\text{CO}_3$ was quantitatively removed from the centre cell into 5 ml of 0.4M- BaCl_2 solution. The contents of the main compartment together with rinse water (10 ml) were centrifuged at 27000g for 10 min; the supernatant solution was recovered and used for the isolation of ethanol and lactic acid.

Isolation of ethanol and conversion into acetic acid. The supernatant solution from the fermentation (33 ml) was adjusted to pH 8.0 with 30% (w/v) NaOH . Ethanol was separated from lactate by distillation and was completely isolated in the distillate when 25 ml had been collected. The ethanol was oxidized to acetic acid in a stoppered flask by the addition of 10 ml of acid dichromate solution (16.7 mmol of CrO_3 in 10 ml of 4.5M- H_2SO_4). Oxidation was allowed to proceed at room temperature for 12 h. The acetic acid (0.8–0.9 mmol) was recovered by steam distillation; approx. 500 ml of distillate was collected, and the acetic acid converted into the sodium salt by titration with 0.1M- NaOH to pH 8.3. The solution of sodium acetate was concentrated to 10 ml, transferred quantitatively to the reaction vessel B (Fig. 2) and evaporated to dryness.

Isolation of lactic acid. The residue from the ethanol distillation (8 ml) was diluted to 40 ml with water, adjusted to pH 3.0 with 5M- H_2SO_4 and the lactic acid was isolated by a 24 h liquid-liquid extraction by using 300 ml

Fig. 2. Combustion train for the degradation of lactic acid, acetic acid, methylamine and glucose. The apparatus was continuously flushed with N_2 which had first been passed through an alkaline sparge system (A) to remove any contaminant CO_2 . CO_2 which resulted from the oxidation of the above compounds in the reaction vessel (B) was swept through two acid-permanganate scrubbers (C) [containing 15 ml of 5% (w/v) KMnO_4 and 5 ml of 1M- H_2SO_4] to remove any traces of SO_2 or SO_3 . The CO_2 -traps (E) contained a solution of 0.1M- $\text{Ba}(\text{OH})_2$ which had been previously filtered to remove insoluble matter. The U-tube (D) was packed with glass wool to prevent the transfer of vapour droplets. Temperature of the reaction vessel was maintained by a thermostated water bath (F). Further details are given in the Materials and Methods section.

of freshly distilled diethyl ether. The ether was evaporated from the receiver flask and the 20 ml of aqueous lactic acid residue steam distilled (1 vol.) to remove volatile acids. The lactic acid was determined by titration with 0.1M-NaOH to pH 8.3, and evaporated to a volume of 5 ml.

Degradation of lactic and acetic acids. Chemical degradations of lactic acid and acetic acid were carried out in the combustion train (Fig. 2).

Decarboxylation of lactic acid. Lactic acid was decarboxylated by the method of Katz, Abraham & Chaikoff (1955) to give CO₂ equivalent to C-4 of glucose. The reaction mixture was composed of 5 ml of sodium lactate solution (0.8–1.0 mmol), 1 ml of 2.5M-H₂SO₄ and 10 ml of 5% (w/v) KMnO₄ (freshly prepared). The sodium lactate and H₂SO₄ were added to the reaction vessel B (Fig. 2) and N₂ was swept through the system to remove contaminant CO₂. The CO₂ traps were then connected, KMnO₄ was added, and the reaction mixture maintained at 90°C for 30 min. The CO₂ released was trapped as BaCO₃. Acetate (0.7–0.9 mmol) (equivalent to C-5 and C-6 of glucose) was recovered by steam distillation of the permanganate solution; 30 vol. of distillate was collected and the acetic acid was titrated with 0.1M-NaOH to pH 8.3. The sodium acetate solution was evaporated to dryness.

Decarboxylation of acetic acid. The decarboxylation of acetic acid was carried out by the Schmidt oxidation (Phares, 1951). In this reaction, acetate (C-5 and C-6 of glucose) from the decarboxylation of lactate, and acetate (C-2 and C-3 of glucose) from the oxidation of ethanol, yield CO₂ from the C-5 and C-3 respectively of the original glucose molecule. The reaction mixture consisted of sodium acetate (0.7–0.9 mmol), 1 ml of 18M-H₂SO₄ and 100 mg of NaN₃. The H₂SO₄ was added to the reaction vessel B (Fig. 2) containing the sodium acetate. The reaction vessel was flushed with N₂ to remove CO₂ (BaCO₃ formed during this step was discarded) and then frozen by immersion in liquid N₂. Sodium azide was added, the flask was reconnected to the combustion train and the temperature of the reaction mixture raised to 50°C. After the initial surge of gas had subsided the temperature of the reaction mixture was further raised to 70°C and maintained at this temperature for 30 min. ¹⁴CO₂ was collected as Ba¹⁴CO₃. The residual solution contained C-2 or C-6 of the original glucose, as methylamine.

Oxidation of methylamine. On conclusion of the Schmidt reaction, the contents of the reaction vessel (B) were transferred to a 150 ml round-bottom flask and the solution (15 ml) was made alkaline with 30% (w/v) NaOH. The methylamine was recovered by distillation of two-thirds of the volume into 20 ml of 0.05M-H₂SO₄. The methylamine hydrogen sulphate (0.2–0.5 mmol) was concentrated to a volume of 1 ml in a Buchi rotary film evaporator and then placed in the reaction vessel B (Fig. 2). Total oxidation of methylamine carbon was effected by wet combustion with 40 ml of Van Slyke-Folch reagent (Van Slyke & Folch, 1940).

Degradation of [¹⁴C]glucose standards. The specificity of the fermentation and the accuracy of the subsequent chemical steps of the degradation reactions were verified by including duplicate samples (1 mmol) of [¹⁴C]glucose standard as controls. The glucose standard was prepared by mixing equal amounts of [1-¹⁴C]glucose, [2-¹⁴C]glucose and [6-¹⁴C]glucose; the specific radioactivity of each ¹⁴C-

labelled species of glucose was determined by the wet-combustion method of Van Slyke & Folch (1940) and these are shown, together with the degradation results, in Table 1.

Radioactivity. All radioactivity measurements were made by the gas-flow counting technique. The radioactivity of CO₂ derived from individual carbon atom oxidation or total molecule oxidation was counted as Ba¹⁴CO₃. All counts were corrected to infinite thinness. A small volume (usually 0.1 ml) of the aqueous sample to be counted was pipetted on to a copper-corrugated planchet, dispersed with the aid of methanol (0.5 ml), dried and the radioactivity counted. Two Nuclear-Chicago (model D47/C110B) gas-flow counters were used and were operationally maintained as follows. Each month a standard ¹⁴C source of medium count rate (approx. 165 c.p.m.) was repetitively counted (10000 counts) for 48 h.

Typical satisfactory data were mean and standard deviation 165 ± 1.69 (*n* = 41). Of the 41 sample counts 27 were within ± 1 count of the mean and 34 of the 41 were within ± 2 counts. A daily application of χ² test for the proper functioning of an ionization counter was also made to ensure that each machine was 'in control' (Chase, 1960).

RESULTS

Specific radioactivity of CO₂ from specifically ¹⁴C-labelled sugars. The results in Fig. 3 show the changes with time in the specific radioactivity of ¹⁴CO₂ resulting from the metabolism of [1-¹⁴C]glucose, [2-¹⁴C]glucose and [6-¹⁴C]glucose by the infused rabbit liver. Table 2 summarizes the details of the data including the specific radioactivity of the glucose isolated from the liver at the same time as the CO₂ samples were taken.

The data show only a gradual increase in the specific radioactivity of CO₂ during the initial minute of metabolism of [1-¹⁴C]glucose; thereafter the rate rapidly increased, reaching a maximum specific radioactivity at 3 min and subsequently decreased in experiments of 4 and 5 min duration. The rate of change of the relative specific radio-

Table 1. *Degradation of specifically labelled D-[¹⁴C]-glucose standards*

Results show the distribution of ¹⁴C in the individual carbon atoms of a glucose standard preparation. The results are expressed as specific radioactivities of the individual carbon atoms and include standard deviation; the number of determinations made are given in parentheses.

Carbon atom	Expected	Found
1	30.00	30.90 ± 1.50 (5)
2	25.00	25.10 ± 3.50 (5)
3	0	0.15 ± 0.03 (5)
4	0	1.35 ± 0.06 (5)
5	0	0.12 ± 0.03 (5)
6	25.00	22.30 ± 3.26 (5)

Fig. 3. Changes with time of the specific radioactivity of CO₂ resulting from the metabolism of [¹⁴C]ribose and [¹⁴C]glucose. A 1.0 ml sample of 0.7 mm-ribose containing 10 μCi of [¹⁴C]ribose or 1.0 ml of 0.33 mm-glucose containing 10 μCi of the specifically labelled [¹⁴C]glucose was infused into the liver of a 40 h-starved rabbit. Metabolism of the ¹⁴C-labelled sugars (■, [¹⁻¹⁴C]ribose; □, [¹⁻¹⁴C]glucose; ●, [²⁻¹⁴C]glucose; ○, [⁶⁻¹⁴C]glucose) was confined to the liver by haemostasis and allowed to proceed for intervals ranging from 0.5 to 5.0 min. CO₂ was isolated from the liver at the indicated times and the specific radioactivities were determined. The values are the means of at least three individual experiments.

activity of ¹⁴CO₂ (the relative specific radioactivity of ¹⁴CO₂ is the ratio of the specific radioactivity of CO₂ to the specific radioactivity of glucose, both sampled from the tissue at the same instant) confirmed that there was a maximum yield of ¹⁴CO₂, from [¹⁻¹⁴C]glucose, at 3 min.

The specific radioactivity of ¹⁴CO₂ resulting from the metabolism of [⁶⁻¹⁴C]glucose showed a rapid increase during the first minute and exceeded the values reported with [¹⁻¹⁴C]glucose. The increase thereafter (1–4 min) was approximately linear. The C-1/C-6 ratio (yield of ¹⁴CO₂ from [¹⁻¹⁴C]glucose relative to the yield of ¹⁴CO₂ from [⁶⁻¹⁴C]glucose) was greater than 1.0 in all experiments of more than 1 min duration and the ratio reached a maximum of 3.1 after 3 min when the optimum specific radioactivity of ¹⁴CO₂ from [¹⁻¹⁴C]glucose was attained. Further, as shown in Fig. 4, increased blood glucose concentrations were found to decrease ¹⁴CO₂ formation by reactions involving the oxidation of

Table 2. Metabolism of ¹⁴C-labelled glucose to CO₂ by rabbit liver in situ

The values are the means ± s.d. with numbers in parentheses; other values are the means of duplicate determinations. The relative molar specific radioactivity (RMSA) is the specific radioactivity of CO₂/specific radioactivity of glucose ratio. A 1.0 ml sample of 0.33 mm-glucose containing 10 μCi of specifically ¹⁴C-labelled glucose was infused intraportally into the liver of the 40 h-starved anaesthetized rabbit. Metabolism of glucose was confined to the liver by haemostasis and allowed to proceed for periods of 0.5–5.0 min. For other details see the Materials and Methods section.

Time in liver (min)	[¹⁻¹⁴ C]Glucose			[²⁻¹⁴ C]Glucose			[⁶⁻¹⁴ C]Glucose		
	10 ⁻³ × Sp. radio-activity of CO ₂ (c.p.m./mmol)	10 ⁻⁵ × Sp. radio-activity of glucose (c.p.m./mmol)	10 ³ × RMSA	10 ⁻³ × Sp. radio-activity of CO ₂ (c.p.m./mmol)	10 ⁻⁵ × Sp. radio-activity of glucose (c.p.m./mmol)	10 ³ × RMSA	10 ⁻³ × Sp. radio-activity of CO ₂ (c.p.m./mmol)	10 ⁻⁵ × Sp. radio-activity of glucose (c.p.m./mmol)	10 ³ × RMSA
0.5	2.82 ± 0.22 (3)	2.56 ± 0.62 (3)	11.0	0.18 ± 0.02 (3)	6.35 ± 0.31 (3)	0.28	2.29	5.75	3.98
1.0	13.20	—	—	0.53 ± 0.47 (3)	9.37 ± 4.83 (3)	0.57	4.57 ± 0.91 (3)	9.15 ± 3.40 (3)	5.00
1.5	13.66 ± 5.15 (3)	5.27 ± 1.76 (3)	25.9	0.72 ± 0.37 (3)	7.84 ± 4.00 (3)	0.92	—	—	—
2.0	29.22 ± 0.32 (4)	6.30 ± 0.81 (3)	46.4	2.37 ± 0.31 (4)	4.32 ± 1.80 (4)	0.55	7.62 ± 0.62 (3)	7.8 ± 1.20 (3)	9.76
3.0	23.95 ± 1.56 (3)	6.17	38.8	23.61 ± 0.40 (3)	15.90	14.84	9.35	7.28	12.85
4.0	20.24 ± 3.40 (13)	13.94 ± 5.80 (12)	14.5	3.74 ± 1.20 (4)	39.99 ± 10.40 (4)	0.93	11.29 ± 0.89 (3)	3.65 ± 1.20 (3)	30.93
5.0	—	—	—	—	—	—	11.30 ± 2.90 (10)	7.67 ± 4.20 (8)	14.70

C-1 of glucose, but not to affect the rate of $^{14}\text{CO}_2$ formation from C-6 of glucose in experiments of 5 min duration.

The initial (first 3 min) rate of increase in the specific radioactivity of $^{14}\text{CO}_2$ resulting from the metabolism of $[2\text{-}^{14}\text{C}]\text{glucose}$ was slow when compared with the rates of $^{14}\text{CO}_2$ formation from the metabolism of $[1\text{-}^{14}\text{C}]$ - and $[6\text{-}^{14}\text{C}]\text{glucose}$. There was a rapid increase in the specific radioactivity of CO_2 after 3 min followed by an equally rapid decrease.

An approximate estimate of the percentage of ^{14}C of the glucose fraction which gave rise to CO_2 can be made from the relative specific radioactivity. The values varied with both the position of the label in the glucose and with the holding time.

By using $[1\text{-}^{14}\text{C}]\text{glucose}$ as substrate 1.1–4.6% of the label was incorporated into CO_2 during 5 min, whereas CO_2 arising from $[6\text{-}^{14}\text{C}]\text{glucose}$ and $[2\text{-}^{14}\text{C}]\text{glucose}$ contained 0.4–3% and 0.03–1.5% of the label respectively. These estimates were based on the assumption that the position of ^{14}C in the glucose was not randomized during the period of metabolism.

The values obtained for the metabolism of $[2\text{-}^{14}\text{C}]\text{glucose}$ to $^{14}\text{CO}_2$ were variable during the initial 3 min period (Table 2) and the rate of change

of the relative specific radioactivity following the metabolism of $[2\text{-}^{14}\text{C}]\text{glucose}$ was erratic. Values calculated from the results with $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$ generally showed a good correlation between the specific radioactivity of $^{14}\text{CO}_2$ and the relative specific radioactivity. Irrespective of the position of ^{14}C in the infused glucose, the specific radioactivity of the glucose isolated from the liver varied more than the specific radioactivity of CO_2 at all time-intervals. The results in Table 3 show that the changing pattern with time of radioactivity in C-1 of glucose 6-phosphate, resulting from the metabolism of $[2\text{-}^{14}\text{C}]\text{glucose}$, is very similar to that for the incorporation of label into CO_2 (Table 2).

Distribution of ^{14}C in glucose, glucose 6-phosphate and fructose 6-phosphate resulting from the metabolism of $[2\text{-}^{14}\text{C}]\text{glucose}$. The accepted process for introducing ^{14}C from $[2\text{-}^{14}\text{C}]\text{glucose}$ into C-1 and C-3 of hexose 6-phosphate is the pentose phosphate cycle (Wood & Katz, 1958; Katz & Wood, 1960). In the liver from the starved animal there is extensive glucose synthesis by gluconeogenesis (Utter, 1963) and the immediate precursors of this glucose are glucose 6-phosphate and fructose 6-phosphate (Utter, 1963; Krebs, 1964).

It is a condition of the pentose phosphate cycle as defined by Wood & Katz (1958) and Katz & Wood (1960) that the fructose 6-phosphate formed in the cycle will completely equilibrate with the total pool of cytoplasmic hexose 6-phosphate. Such equilibration should therefore include the hexose 6-phosphates of the final common pathway of gluconeogenesis. For this reason it was assumed that the distribution of ^{14}C from $[2\text{-}^{14}\text{C}]\text{glucose}$ into C-1 and C-3 of tissue glucose would provide an effective method for investigating and possibly measuring the contribution of the pentose phosphate cycle.

The results in Table 4 show that the concentrations of the metabolites were relatively constant for

Fig. 4. Effect of increasing blood glucose concentration on the relative specific radioactivity of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{glucose}$ ($\square$) and $[6\text{-}^{14}\text{C}]\text{glucose}$ ($\bullet$). Adult female rabbits, starved for 40 h, were anaesthetized and the hepatoportal system was surgically exposed as described by Williams *et al.* (1971). The blood glucose concentration was increased by injecting various amounts of glucose through the right ear vein. The blood glucose concentration (as determined from analysis of blood taken from the left ear vein) settled at a steady concentration after 8 min. $[1\text{-}^{14}\text{C}]\text{Glucose}$ or $[6\text{-}^{14}\text{C}]\text{glucose}$ ($10\mu\text{Ci}$) was then infused via the hepatic portal vein as described by Williams *et al.* (1971) and metabolism was confined to the liver by haemostasis and allowed to proceed for 5 min. The glucose and CO_2 from the liver were then isolated, and specific radioactivities determined (Williams *et al.* 1971).

Table 3. Incorporation of radioactivity into C-1 of glucose 6-phosphate after metabolism of $[2\text{-}^{14}\text{C}]\text{glucose}$

Data on the concentration, specific radioactivity and % distribution of label into C-1 of glucose 6-phosphate are given in Tables 5 and 6. These data and the mass of the liver were used to calculate the total radioactivity in C-1 of glucose 6-phosphate.

Time (min)	Mass of liver (g)	Total radioactivity in C-1 of glucose 6-phosphate (c.p.m./kg wet wt. of liver)
1	71	8018
2	68	46580
3	83	48046
4	80	78091
5	70	41644

Table 4. Concentration of CO₂, glucose and hexose phosphates in the liver of a starved rabbit during various intervals of the holding time

Female rabbits (3.0–3.5 kg, and 40 h-starved) were anaesthetized (15 min) and the livers surgically exposed. An ischaemic condition was imposed on the liver by clamping shut the outflow of blood from the hepatic veins for the times shown. A 1.0 ml sample of 0.33 M-glucose was infused into the liver intraportally at the rate of 4.0 ml/min for 15 s before closing the hepatic veins. The results are mean values $\pm$ s.d. for five animals. For other experimental details see the Materials and Methods section.

Time in liver (min)	Concn. of metabolite				
	(mmol/kg wet wt. of liver)		(μ mol/kg wet wt. of liver)		
	CO ₂	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate
1.0	8.30 $\pm$ 0.35	20.9 $\pm$ 4.2	241 $\pm$ 40	65.5 $\pm$ 7.3	42.0 $\pm$ 21.2
2.0	8.12 $\pm$ 0.38	21.5 $\pm$ 6.1	267 $\pm$ 28	50.1 $\pm$ 6.5	25.3 $\pm$ 13.8
3.0	8.74 $\pm$ 0.29	19.0 $\pm$ 4.8	310 $\pm$ 42	60.0 $\pm$ 6.9	41.4 $\pm$ 23.5
4.0	7.99 $\pm$ 0.35	17.6 $\pm$ 5.8	300 $\pm$ 47	64.2 $\pm$ 5.9	55.3 $\pm$ 37.4
5.0	8.80 $\pm$ 0.30	18.3 $\pm$ 6.0	280 $\pm$ 68	49.5 $\pm$ 6.8	57.0 $\pm$ 39.0

each class of compound over the 5 min holding time. There was a 1.5-fold variation in the concentration of both glucose 6-phosphate and fructose 6-phosphate and a twofold variation in the concentrations of fructose 1,6-diphosphate. However, in general the results obtained were similar to those reported for the starved rat (Muntz & Vanko, 1962; Muntz, 1968; Gumaa & McLean, 1968; Gumaa, Greenslade & McLean, 1968).

The results in Table 5 show the concentration and specific radioactivity of metabolites in experiments involving the infusion of [2-¹⁴C]glucose with holding times ranging from 1 to 5 min. There was an increase in the specific radioactivity of all intermediates, as well as glucose (the precursor) and CO₂, in experiments of 1–3 min duration. The specific radioactivity of fructose 6-phosphate and fructose 1,6-diphosphate reached maximum values at 5.0 min. The specific radioactivity of CO₂ and tissue glucose reached their maximum value at 4 min and then declined. The relative specific radioactivity of fructose 6-phosphate reached a higher value than the other compounds examined. The specific radioactivity of fructose 6-phosphate was 8–18 times that of glucose 6-phosphate and the specific radioactivity of glucose 6-phosphate ranged from 17 to 92% of that of glucose. The low relative yield of ¹⁴C in CO₂ has already received comment.

The results in Table 6 show the distribution of ¹⁴C in all six carbon atoms of glucose, glucose 6-phosphate and fructose 6-phosphate after 1–5 min metabolism of [2-¹⁴C]glucose. The results show that the contribution of the pentose phosphate cycle, calculated from the distribution of ¹⁴C into C-1 and C-3 of the intermediates, was negligibly small in the early time-course experiments. There appeared to be a gradual increase with increasing time of metab-

olism but the values were rather variable. The low estimates of the pentose phosphate cycle reported here confirm the values *in vivo* (less than 3%) reported for rat liver by Hostetler & Landau (1967).

It is a condition of the pentose phosphate cycle definition that C-3 should possess half of the concentration of ¹⁴C of C-1. It was noted that the varying pattern of ¹⁴C concentration in C-3 of glucose and the hexose phosphates fluctuated widely between samples, and the ordered prediction of the theoretical pentose phosphate cycle for isotope distribution in C-1 and C-3 was not realized.

The other major feature of the results of Table 6 was the high concentration of ¹⁴C in C-5 and C-6 of the phosphorylated intermediates. In the 1 and 2 min experiments C-6 of both glucose 6-phosphate and fructose 6-phosphate contained 30–40% of the total label, whereas C-5 of fructose 6-phosphate increased from 23 to 63% in the 3–5 min experiments. C-6 of glucose 6-phosphate during 3–5 min contained 9–41% of the label.

In these experiments the labelling patterns in the six carbon atoms of glucose did not reflect the ¹⁴C patterns of the hexose phosphates. The ¹⁴C in glucose at all time-intervals was still predominantly in its original position. There was some randomization of label into C-1 and C-3 of glucose. The total concentration of ¹⁴C in C-5 and C-6 of glucose ranged from 1 to 8%, whereas the same carbon atoms in the hexose phosphates contained 18 to 80% of the label.

Pentose phosphate cycle in the liver of the fed rabbit. The low and equivocal estimates of the pentose phosphate cycle reported in Table 6 raised the question as to whether the nutritional state of the experimental animal (40 h-starved) was responsible

Table 5. *Concentration, specific radioactivity and relative specific radioactivity of CO₂, glucose and hexose phosphates after metabolism of [2-¹⁴C]glucose in starved rabbit liver*

Results are the means of duplicate determinations on single animals. The general experimental conditions were as described for Table 4. A 1.0 ml sample of 0.33 mM-glucose containing 10 μ Ci of [2-¹⁴C]glucose was infused into the liver intraportally at the rate of 4.0 ml/min for 15 s before closing the hepatic veins. For other experimental details see the Materials and Methods section.

Time in liver (min)	Concn. of metabolite				
	(mmol/kg wet wt. of liver)		(μ mol/kg wet wt. of liver)		
	CO ₂	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate
1.0	8.50	24.0	239	62.5	34.1
2.0	8.00	18.7	274	41.9	20.5
3.0	8.68	19.1	318	62.2	45.8
4.0	8.20	15.1	306	59.3	133.8
5.0	8.90	12.7	260	43.0	61.2
	$10^{-3} \times$ Sp. radioactivity (c.p.m./mmol)				
	CO ₂	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate
1.0	1.10	1490	610	4840	10
2.0	1.14	1580	850	7470	2430
3.0	3.43	5670	5210	28750	2090
4.0	23.04	26520	4400	—	1050
5.0	3.25	12260	2810	46300	2810
	Relative specific radioactivity				
	CO ₂	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate
1.0	0.00074	1.0	0.41	3.25	0.01
2.0	0.00072	1.0	0.54	4.73	1.54
3.0	0.00061	1.0	0.92	5.07	0.37
4.0	0.00087	1.0	0.17	—	0.04
5.0	0.00026	1.0	0.23	3.78	0.23

for the failure to demonstrate a significant and quantitatively consistent estimate of the cycle in liver. For this reason estimates of the pentose phosphate cycle were made in the livers of fed rabbits. The animals were either fed *ad libitum* (regimen A) or starved for 40 h and then allowed unlimited access to food for 24 h (regimen B).

The results in Table 7 indicate that glucose 6-phosphate and fructose 6-phosphate concentrations altered with the nutritional state. However, the variation in glucose 6-phosphate concentration was within the range of values reported for liver (Muntz & Vanko, 1962; Muntz, 1968; Gumaa & McLean, 1968; Gumaa *et al.* 1968). The glucose concentration and the fructose 6-phosphate concentration in the animal on regimen A were both lower by a factor of three in rabbit liver than the values reported for liver from rats on a similar feeding regimen (Muntz & Vanko, 1962).

Compared with the values from starved animals (Table 5) the animal on regimen A showed greatly

enhanced specific radioactivities of the hexose phosphates and CO₂. However, compared with animals which were 40 h-starved, animals on both feeding regimens brought about the distribution of even smaller quantities of radioactivity from [2-¹⁴C]glucose into C-1 and C-3 of glucose and the hexose 6-phosphates. The concentration of ¹⁴C in C-2 of glucose and hexose 6-phosphates was assigned a value of 100 and used to calculate the relative concentration of ¹⁴C in C-1 and C-3. The contribution of the pentose phosphate cycle calculated from C-1/C-2 ratios was negligible. These findings are in agreement with the results of Marks & Feigelson (1957) for fed rats as calculated by Katz (1961).

'Pulse' experiment with [2-¹⁴C]glucose. The results in Table 6 are complex and reflect the interactions of the many processes that operate on these central metabolites. The results could raise some doubt as to whether sufficient time had been allowed in these experiments to observe the operation of the pentose phosphate cycle.

Table 6. *Distribution of ¹⁴C in glucose, glucose 6-phosphate and fructose 6-phosphate isolated from livers after metabolism of [2-¹⁴C]glucose*

Data on the concentration, specific radioactivity and relative specific radioactivity of the compounds used in these experiments are given in Table 5. The percentage recovery represents the percentage of activity in the hexose or hexose phosphate degraded by *L. mesenteroides* compared with that measured by total combustion. The specific radioactivity of the hexose and hexose 6-phosphate degraded are recorded as well as the percentage contribution of the pentose phosphate cycle to total glucose catabolism. The % pentose phosphate cycle values were calculated from formulae given in the Discussion section and involve the relative radioactivities of C-1, C-2 and C-3. The percentage distribution obtained on degradation of the [2-¹⁴C]glucose employed in these experiments was C-1, 0; C-2, 99.0; C-3, 0.6; C-4, 0.2; C-5, 0.2; C-6, 0; with an 85% recovery. The general experimental conditions were as described for Table 2. Details of the procedures are given in the Materials and Methods section.

Holding time in liver...	1.0min						2.0min						3.0min						4.0min						5.0min					
	Glucose 6- phosphate			Fructose 6- phosphate			Glucose			Fructose 6- phosphate			Glucose			Fructose 6- phosphate			Glucose			Fructose 6- phosphate			Glucose			Fructose 6- phosphate		
	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in	Metabolite	Glucose	radioactivity in
C-1	0.3	96.3	91	5.5	44.0	36.3	1.7	1.0	20.0	2.9	2.8	2.9	0.7	4.3	5.8	0.4	10.8	5.7	2.8	78.4	76.4	38.7	0	1.6	0	10.8	5.7	2.8	78.4	
C-2	0.5	96.3	91	3.3	44.0	36.3	5.3	95.2	27.8	38.1	91.3	63.5	54.0	88.3	67.0	84.7	76.4	36.5	15.2	0	0	0	0	0	76.4	36.5	15.2	0	0	
C-3	0.7	96.3	91	6.2	44.0	36.3	5.4	1.9	13.6	7.0	0.6	3.4	12.1	0.9	3.1	0	1.6	0	0	0	0	0	0	0	1.6	0	0	0	0	
C-4	0.7	96.3	91	6.2	44.0	36.3	5.4	1.9	13.6	7.0	0.6	3.4	12.1	0.9	3.1	0	1.6	0	0	0	0	0	0	0	1.6	0	0	0	0	
C-5	1.4	96.3	91	6.7	44.0	36.3	18.5	0.2	0	6.3	5.0	0	3.8	0.9	3.1	3.6	3.3	5.6	4.2	0	0	0	0	3.3	5.6	4.2	0	0		
C-6	0.8	96.3	91	34.3	44.0	36.3	32.8	1.2	32.3	43.6	3.6	8.3	22.8	0.7	7.6	48.9	7.2	11.4	62.7	0	0	0	0	7.2	11.4	62.7	0	0		
Recovery (%)	91	96.3	91	89	44.0	36.3	78	105	98	80	87	104	91	90.1	81	74.5	68	78	101.5	0	0	0	0	68	78	101.5	0	0		
10 ⁻³ × Sp. radio- activity of material degraded (c.p.m./mmol)	38.2	96.3	91	18	44.0	36.3	47.0	9.7	6.9	20.5	95	56.2	69.5	1770	34.7	26.9	600	14.2	84.8	0	0	0	0	600	14.2	84.8	0	0		
% Pentose phosphate cycle	0.1	96.3	91	7.1	44.0	36.3	2.4	0.5	—	4.1	1.6	1.9	0.6	2.5	4.7	0.5	8.3	9.2	11.2	0	0	0	0	8.3	9.2	11.2	0	0		
C-1/C-2	0.5	96.3	91	8.1	44.0	36.3	17.1	2.1	95.8	22.4	0.7	5.6	28.9	6.4	—	—	2.1	—	—	0	0	0	0	2.1	—	—	0	0		
C-3/C-2	0.5	96.3	91	8.1	44.0	36.3	17.1	2.1	95.8	22.4	0.7	5.6	28.9	6.4	—	—	2.1	—	—	0	0	0	0	2.1	—	—	0	0		

Table 7. Concentration, specific radioactivity and relative distribution of ^{14}C in C-1, C-2 and C-3 of glucose and hexose phosphates from the livers of fed rabbits after the metabolism of $[2\text{-}^{14}\text{C}]\text{glucose}$

Animals were either fed *ad libitum* (regimen A), or starved for 40 h and then allowed access to food for 24 h (regimen B). The general experimental conditions were as described in Table 6. Details of the experimental procedures are given in the Materials and Methods section. Results are the means of duplicate determinations.

Feeding regimen	Time in liver (min)	Concn. of metabolite					
		(mmol/kg wet wt. of liver)			($\mu\text{mol/kg wet wt. of liver}$)		
		CO ₂	Glucose	Fructose 6-phosphate	Glucose 6-phosphate	Fructose 6-phosphate	
A	5.0	7.09	4.20		401	20	
B	5.0	8.26	14.30		273	102	
$10^{-3} \times \text{Sp. radioactivity (c.p.m./mmol)}$							
A	5.0	CO ₂	Glucose	Fructose 6-phosphate	Glucose 6-phosphate	Fructose 6-phosphate	
B	5.0	25.60	4750		15400	67300	
		13.17	2710		527	1223	
Feeding regimen...							
Metabolite...							
Relative sp. radioactivity		Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Glucose	Glucose 6-phosphate	Fructose 6-phosphate
C-1	0		0.15	0.10	0	0.10	0
C-2	100	100	100	100	100	100	100
C-3	0	0	0	—	0	0	0
% Pentose phosphate cycle							
C-1/C-2	0	0.07		0.05	0	0.05	0

To investigate the above question the following experiment, designated 'pulse', was performed. $[2\text{-}^{14}\text{C}]\text{Glucose}$ ($10\text{ }\mu\text{Ci}$) was infused into the hepatic portal vein of the anaesthetized animal in the usual manner and held in the liver for 5 min. The portal circulation was then restored for 15 s, during which time a further $10\text{ }\mu\text{Ci}$ of $[2\text{-}^{14}\text{C}]\text{glucose}$ was injected and the hepatic circulation again arrested for a further 5 min. This operation was repeated once more. The result of this procedure was to allow an accumulated $30\text{ }\mu\text{Ci}$ of $[2\text{-}^{14}\text{C}]\text{glucose}$ to undergo metabolism for a total period of 15 min.

The concentrations of lactate, pyruvate, P_i and adenine nucleotides in rabbit liver at the termination of the 'pulse' experiment are given in Table 8. The values for the $[\text{lactate}]/[\text{pyruvate}]$, $[\text{ATP}]/[\text{AMP}]/[\text{ADP}]^2$ and $[\text{free NAD}^+]/[\text{free NADH}]$ ratios are all similar to those obtained for rabbit liver before the introduction of ischaemic conditions (Williams *et al.* 1971) and the tissue concentration of lactate had increased only slightly.

The results in Table 9, when compared with the maximum values reported in Table 5, show that the 'pulse' procedure achieved a doubling of the specific radioactivity of tissue glucose, only a slight increase in the specific radioactivities of glucose 6-phosphate and fructose 6-phosphate, and a 20-fold increase in the specific radioactivity of fructose 1,6-diphosphate. Owing to the increased specific radioactivity of glucose, the relative specific radioactivity values of the hexose 6-phosphates were approximately four times lower than the averaged values of Table 5. The specific radioactivity of $^{14}\text{CO}_2$ was consistently low by using the 'pulse' experimental technique.

The patterns of isotope distribution in glucose 6-phosphate and fructose 6-phosphate (Table 9) were similar to the results in Table 6. The highest concentration of ^{14}C was again found in C-4, C-5 and C-6 of these hexose phosphates. By using glucose 6-phosphate, the estimate of the pentose phosphate cycle was 53% (C-1/C-2) and 25.4% (C-3/C-2). The estimate from fructose 6-phosphate was 7.5% (C-1/C-2) and 14.7% (C-3/C-2).

The unexpected but consistently observed pattern of isotope distribution into C-4, C-5 and C-6 as shown in Tables 6 and 9 was also found by Gordon (1968) in similar studies on the metabolism of $[2\text{-}^{14}\text{C}]\text{glucose}$ in the isolated perfused rabbit liver, and by Clark (1967) in studies on the metabolism of various specifically ^{14}C -labelled trioses in rabbit liver *in situ*.

Metabolism of $[1\text{-}^{14}\text{C}]\text{ribose}$. The reaction sequence of the pentose phosphate cycle is based on the results of experiments where $[1\text{-}^{14}\text{C}]\text{ribose}$ 5-phosphate was metabolized by enzymes extracted from rat liver and pea roots (Horecker *et al.* 1954; Gibbs & Horecker, 1954). Most of the more recent

Table 8. Concentration of metabolites and reactant ratios of the lactate dehydrogenase system and the adenine nucleotide system in the 'pulse' experiment on rabbit liver *in situ*

Concn. of metabolite ($\mu\text{mol/g wet wt. of liver}$)					$\frac{[\text{Lactate}]}{[\text{Pyruvate}]}$ ratio	$\frac{10^3 \times [\text{ATP}]}{[\text{ADP}][\text{HPO}_4^{2-}]}$	$\frac{[\text{ATP}][\text{AMP}]}{[\text{ADP}]^2}$	$\frac{[\text{free NAD}^+]}{[\text{free NADH}]}$
Lactate	Pyruvate	ATP	ADP	AMP				
6.80 ± 1.38	0.238 ± 0.076	0.29 ± 0.12	0.60 ± 0.15	1.55 ± 0.38	7.33 ± 0.10	110	1.25	315

$[2\text{-}^{14}\text{C}]\text{Glucose}$ ($10\text{ }\mu\text{Ci}$) was infused into the hepatic portal vein of the anaesthetized animal and confined to the liver for 5 min by haemostasis. The portal circulation was then restored for 15 s during which time a further $10\text{ }\mu\text{Ci}$ of $[2\text{-}^{14}\text{C}]\text{glucose}$ was infused and the hepatic circulation again arrested for a further 5 min. This operation was repeated one further time. The result of this procedure was to allow an accumulated $30\text{ }\mu\text{Ci}$ of $[2\text{-}^{14}\text{C}]\text{glucose}$ to undergo metabolism for a total period of 15 min. All other experimental details have been described (Williams *et al.* 1971). The results are mean values ($\pm$ s.d.) for five animals. The method of calculating the $[\text{free NAD}^+]/[\text{free NADH}]$ ratio was described by Williamson, Lund & Krebs (1967).

Table 9. 'Pulse' experiment

The concentration, specific radioactivity and relative specific radioactivity of CO₂, glucose, glucose 6-phosphate and fructose 6-phosphate and the distribution of ¹⁴C in glucose 6-phosphate and fructose 6-phosphate isolated from the liver after the intraportal infusion of 3.0 ml of 0.33 mM-glucose containing 30 μ Ci of [2-¹⁴C]glucose. Metabolism was allowed to proceed for a total period of 15 min. For details of the procedure see Table 8. For other details see Table 6. Except where indicated results are the means of duplicate determinations.

Concn. of metabolite (μ mol/kg wet wt. of liver)				
CO ₂	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate
8050	11380	319	79.2	42.8
$10^{-6} \times$ Specific radioactivity (c.p.m./mmol)				
CO ₂	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate
0.00505 ± 0.00028 (4)	49.2	6.25	49.0	57.8
Relative specific radioactivity				
CO ₂	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate
0.000103	1.0	0.12	0.99	1.18
Metabolite...		Glucose 6-phosphate	Fructose 6-phosphate	
Percentage of radioactivity in				
C-1		6.9	2.6	
C-2		13.3	19.7	
C-3		2.7	2.5	
C-4		17.4	3.8	
C-5		2.5	10.9	
C-6		57.2	60.5	
Recovery (%)		92.0	101.5	
$10^{-3} \times$ Sp. radioactivity of material degraded (c.p.m./mmol)		86.8	106.2	
% Pentose phosphate cycle				
C-1/C-2		53.3	7.6	
C-3/C-2		25.4	14.7	

studies on the pentose phosphate cycle have involved the application of [2-¹⁴C]glucose (Landau & Katz, 1964; Green & Landau, 1965; Hostetler, Cooperstein, Landau & Lazarow, 1966; Hostetler & Landau, 1967) which can be converted into [1-¹⁴C]-ribose 5-phosphate after decarboxylation in the oxidative segment of the cycle. Metabolism of [2-¹⁴C]glucose in the present studies has only provided equivocal evidence on both the role and the reaction sequence of the pentose phosphate cycle in rabbit liver metabolism. It was therefore decided to study the metabolism of [1-¹⁴C]ribose in the rabbit liver *in situ*.

The results in Table 10 summarize the results on the concentration, radioactivity and the distribution of ¹⁴C into CO₂ and the carbon atoms of glucose, glucose 6-phosphate and fructose 6-phosphate following the metabolism of [1-¹⁴C]ribose. The results show that with the exception of glucose the concentrations of the isolated products were within

the range of values reported for experiments with [2-¹⁴C]glucose (Table 5).

Two features distinguish these results from those obtained with ¹⁴C-labelled glucose: (a) specific radioactivity of the CO₂ was approximately three times greater than the maximum values found for the oxidation of [¹⁴C]glucose (Table 2) and (b) 65–95% of the label in glucose and the hexose phosphates was in C-1. C-3 was significantly labelled (10%) only in glucose 6-phosphate.

The rate of production of ¹⁴CO₂ from [1-¹⁴C]ribose is shown in Fig. 3 together with similar results for variously ¹⁴C-labelled glucose. Both the rate and extent of ¹⁴C incorporation into CO₂ after [1-¹⁴C]-ribose oxidation were appreciably higher than with ¹⁴C-labelled glucose.

DISCUSSION

Rates of ¹⁴CO₂ formation in situ. The finding that the C-1/C-6 ratios were greater than one (except in

Table 10. *Concentration, specific radioactivity and distribution of ^{14}C in glucose and hexose 6-phosphates isolated from liver after metabolism of $[1-^{14}\text{C}]\text{ribose}$*

A 1.0 ml sample of 0.7 mM-ribose containing 10 μCi of $[1-^{14}\text{C}]\text{ribose}$ was infused intraportally into rabbit liver *in situ* and confined by haemostasis. Metabolism was allowed to proceed for 5 min. Animals were starved 40 h before the experiment. Details of the experimental conditions and procedures are given in the Materials and Methods section. Results are the means of duplicate determinations on single animals. For other details see Table 6.

Concn. of metabolite ($\mu\text{mol/kg}$ wet wt. of liver)				
CO_2	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6-diphosphate
7820	8930	295	56.8	43.3
$10^{-6} \times \text{Specific radioactivity (c.p.m./mmol)}$				
CO_2	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	
0.110	12.0	4.08	32.1	
Metabolite...	Glucose	Glucose 6-phosphate	Fructose 6-phosphate	
Percentage of radioactivity				
C-1	92.0	64.8	80.5	
C-2	1.9	7.2	2.9	
C-3	0.8	10.0	1.5	
C-4	0.2	4.4	3.9	
C-5	2.3	6.0	0	
C-6	2.8	7.6	11.2	
Recovery (%)	93.0	83.2	101.2	
$10^{-3} \times \text{Sp. radioactivity of material degraded (c.p.m./mmol)}$	565.0	40.90	157.0	

experiments of less than 1 min duration) indicated the presence of an oxidative segment of the pentose phosphate pathway of glucose oxidation in rabbit liver *in situ*. Although the results allow a measure of the relative rate at which C-1 and C-6 of substrate glucose were metabolized to CO_2 , they do not allow a quantitative measure of the contribution of the pentose phosphate cycle to glucose metabolism. The composition of the pentose phosphate cycle CO_2 will depend on the degree of recycling of glucose 6-phosphate. If the glucose is metabolized wholly by the pentose phosphate cycle, the CO_2 will theoretically be derived equally from C-1, C-2 and C-3 (Katz & Wood, 1960). If on the other hand the contribution of the cycle is small, then nearly all of the CO_2 from the cycle will come from C-1 of glucose.

In the Embden-Meyerhof pathway C-1 and C-6 of glucose become C-3 of triose phosphate, but there is no direct or simple relationship between the triose phosphate yield in carbohydrate metabolism and the CO_2 derived therefrom. Triose phosphate can be converted into several intermediary products such as serine, glycerol and pyruvate. Of the acetyl carbon atoms that enter the tricarboxylic acid cycle, only a fraction will be liberated as CO_2 even during experiments of reasonably long duration (Felts, Chaikoff & Osborne, 1951; Abraham, Hirsch & Chaikoff, 1954). There is, at present, no un-

ambiguous method of evaluating the CO_2 yield from the oxidation of C-3 of triose phosphate.

Katz & Wood (1960) derived the following expression relating the C-1/C-6 ratios with the pathways of glucose metabolism.

$$\frac{\text{C-1}}{\text{C-6}} = \frac{Q (\% \text{ EM} \times \text{N} + \% \text{ PC} \times 3)}{\text{N} (\% \text{ EM} + \% \text{ PC})}$$

The equation contains three independent variables: EM (Embden-Meyerhof pathway); PC (pentose phosphate cycle); N (the oxidative yield of CO_2 from C-3 of triose phosphate); and Q [the dilution factor, which is dependent on PC (Wood & Katz, 1958)]. The expression is complex and in appraising the equation Katz & Wood (1960) state that even if carbohydrate metabolism were simplified to include only the Embden-Meyerhof pathway and the pentose phosphate cycle, and if recycling in the pentose phosphate cycle did not occur, $^{14}\text{CO}_2$ ratios *per se* would still not provide useful quantitative information on the participation of pathways, since changes in N affects the ratios much more extensively than does recycling in the pentose phosphate cycle.

The metabolism of $[2-^{14}\text{C}]\text{glucose}$ is believed to yield $^{14}\text{CO}_2$ in three different ways (Muntz & Murphy, 1957): (i) phosphogluconate oxidation, from C-1 of glucose 6-phosphate produced during

cyclic operations of the pentose cycle; (ii) glycolysis and subsequent oxidation of the carbonyl group of acetyl-CoA in the tricarboxylic acid cycle (original C-2 of glucose); (iii) from the pentose phosphate cycle and glycolysis, then from the carboxyl group of pyruvate (C-3 of glucose 6-phosphate).

All of the above processes were very slow in comparison with the rate of $^{14}\text{CO}_2$ formation from $[1\text{-}^{14}\text{C}]\text{glucose}$ in experiments of up to 3.0 min duration (Table 2 and Fig. 3). The delay in the significant ^{14}C enrichment of C-1 of glucose 6-phosphate (Table 3) may account for the initial lag in the appearance of $^{14}\text{CO}_2$ from $[2\text{-}^{14}\text{C}]\text{glucose}$ during the early time-course of the experiments.

The decline in the value of the specific radioactivity of $^{14}\text{CO}_2$ at 4 and 5 min in all experiments involving $[1\text{-}^{14}\text{C}]\text{glucose}$ (Table 2) may be attributed to the effect of the increasing blood glucose concentration (Fig. 4).

The results in Table 2 show that there were only small variations in the individual values of the specific radioactivities of CO_2 . These results are in striking contrast with the wide range of values for glucose. The results in Tables 2 and 3 are consistent with the view that the specific radioactivity of $^{14}\text{CO}_2$ may be more closely related to the total radioactivity in C-1 of glucose 6-phosphate, than to the specific radioactivity of glucose.

That the rates of $[^{14}\text{C}]\text{glucose}$ oxidations were slow in contrast with the rapid oxidation of $[1\text{-}^{14}\text{C}]\text{-ribose}$ (Fig. 3) was not surprising, since glucose is poorly utilized by liver (Muntz, 1968), most probably because of the slow rate of phosphorylation. The ribokinase (EC 2.7.1.15) of rabbit liver was found to be at least one hundred times more active than the hexokinase (EC 2.7.1.1) and glucokinase

The high concentration of label in C-6 of the hexose 6-phosphates may also be shown to result from a transaldolase exchange reaction. This would necessitate prior transfer of ^{14}C from C-2 of substrate glucose into C-1 of hexose 6-phosphate followed by cleavage to triose phosphates in the Embden-Meyerhof pathway, interconversion of the two trioses by triose phosphate isomerase (EC 5.3.1.1) and the transaldolase exchange of the resulting ^{14}C -labelled glyceraldehyde 3-phosphate with either fructose 6-phosphate or sedoheptulose 7-phosphate (Scheme 1). In both cases the transaldolase exchange reaction would effect incorporation of C-1, C-2 and C-3 of substrate glucose into C-6, C-5 and C-4 of fructose 6-phosphate. Although the reactions catalysed by transaldolase are freely reversible, the mechanism of the enzyme (Horecker, 1962, p. 82) will not allow the reverse exchange of C-6, C-5 and C-4 of fructose 6-phosphate to return to C-1, C-2 and C-3 of fructose 6-phosphate.

The transfer of label from $[2\text{-}^{14}\text{C}]\text{glucose}$ into C-1 of hexose 6-phosphate before its exchange into C-6 of hexose 6-phosphate may also be shown to result from an exchange reaction. The results with $[1\text{-}^{14}\text{C}]\text{ribose}$ (Table 10) show the almost exclusive transfer of label into C-1 of glucose and the hexose 6-phosphates and are consistent with the operation of the following three reactions: (i) rapid phosphorylation of $[1\text{-}^{14}\text{C}]\text{ribose}$ by ribokinase (reaction 8) (Agranoff & Brady, 1956); (ii) isomerization and epimerization of pentose phosphates (reactions 9 and 10) (Seegmiller & Horecker, 1952; Ashwell & Hickman, 1955); (iii) a transketolase exchange reaction involving erythrose 4-phosphate and ketopentose phosphate (reaction 11) (de la Haba, Leder & Racker, 1955).

(EC 2.7.1.2) was absent from starved rabbit liver. The hexokinase activity was only 25% of that of rat liver (J. F. Williams, unpublished work). Ballard & Oliver (1964, 1965) have also reported the absence of glucokinase in adult sheep liver.

Transaldolase and transketolase exchange reactions. A surprising feature of the results in Table 6 was the appearance within 1 min of some 40–50% of the total label of the hexose phosphates in C-5 and C-6 rather than in the predicted C-1, C-2 and C-3. The extensive labelling of C-5 in fructose 6-phosphate was indicative of the operation of an active transaldolase exchange reaction (Ljungdahl, Wood, Racker & Couri, 1961). The transaldolase exchange reaction is illustrated in Scheme 1.

The results of $^{14}\text{CO}_2$ production from ribose and glucose (Fig. 3) suggest that the sequence of reactions which involved ribose (reactions 8 to 11) resulted in the more rapid formation of $[1\text{-}^{14}\text{C}]\text{-hexose 6-phosphate}$ (Table 10) and thus labelling of CO_2 (Table 3), more so than any of the reactions involving the oxidation of $[1\text{-}^{14}\text{C}]\text{-}$, $[2\text{-}^{14}\text{C}]\text{-}$ or $[6\text{-}^{14}\text{C}]\text{-glucose}$. This enhancement in the rate and degree of incorporation of label into CO_2 can probably be traced to the high activity of ribokinase in reaction (8).

The results in Table 10 are not a unique report of the nearly exclusive ^{14}C -labelling of C-1 of hexose following the metabolism in liver of $[1\text{-}^{14}\text{C}]\text{pentose}$. Hiatt (1957) found a similar distribution of label in

the glucose units of liver glycogen following the metabolism of [1- ^{14}C]xylose by the mouse *in vivo*. The results in Table 10 are contrary to the predicted fate of [1- ^{14}C]ribose in liver (Horecker *et al.* 1954) and therefore raise doubt as to the status of the defined pentose phosphate cycle and its mechanism in rabbit liver.

Since reactions (9) and (10) are freely reversible it is possible to introduce label into C-3 of hexose 6-phosphate by reactions (9), (10) and (11) (Horecker & Smyrniotis, 1953; Srere, Cooper, Klybas & Racker, 1955).

Evidence for the limited operation of reaction (12) may be seen from the zero concentration of ^{14}C in C-3 of glucose 6-phosphate and fructose 6-phosphate in experiments of 4 and 5 min duration (Table 6) and in the 'pulse' experiment (Table 9) where there is less than 3% labelling. C-3 of both hexose phosphates, however, is significantly labelled (3–13%) in experiments of 1–3 min duration. The ^{14}C of tissue bicarbonate can be introduced into C-3 and C-4 of hexose 6-phosphate and hexose by reactions in gluconeogenesis (Utter, 1963). Some of the label of C-3 and C-4 of the hexose intermediates in Tables 6, 9 and 10 may arise from these reactions, since glucose production continues unabated in the liver during the 'holding time' of all experiments (Williams *et al.* 1971), although the source of the C_3 unit is not known.

The sequence of reactions (8) to (9) even if allowed to proceed to equilibrium in a closed system (Horecker *et al.* 1954; Gibbs & Horecker, 1954) cannot yield the same labelling pattern in hexose 6-phosphate as that dictated by the theoretical reaction scheme for the pentose phosphate cycle, i.e. 67% of ^{14}C in C-1 and 33% of ^{14}C in C-3. It is not suggested that the transaldolase and other transketolase reactions do not achieve some net synthesis. Indeed, in the absence of any other known

reaction for the formation of erythrose 4-phosphate in animal tissues, and because of its presence in low concentration in liver (Gumaa & McLean, 1968) and its suggested high turnover in tissues (Horecker, 1962, p. 88) it is necessary that transaldolase should catalyse the formation of some erythrose 4-phosphate (reaction 13).

However, the results (Tables 6, 9 and 10) suggest that the exchange reactions of transaldolase and transketolase are so extensive, relative to the activity of the remaining reaction possibilities of these enzymes, that a contribution of the defined pen-

tose phosphate cycle cannot be verified in liver tissue.

In the investigations of the pentose phosphate cycle in the liver of the fed rabbit (Table 7), the animals were maintained on two different feeding regimens. These were used because Tepperman & Tepperman (1963) reported significant differences in the activity of the enzymes of glucose metabolism when animals were maintained on different diets. The lower specific radioactivities of CO_2 , glucose and the hexose phosphates in the livers of the animals on the fed-starved-fed cycle (regimen B) may have occurred because the 24 h feeding period after starvation was insufficient to restore the concentrations or activities of key enzymes. However, the increased incorporation of label reported for the animals fed *ad libitum* was a desired aim of the experiment although the results of the incorporation failed to show any significant contribution of the pentose phosphate cycle.

Difference in labelling patterns of glucose and hexose 6-phosphates. The ^{14}C -labelling patterns in tissue glucose would be expected to result from the mixing of at least two glucose pools, the [2- ^{14}C]glucose substrate and the glucose formed by gluconeogenesis (Williams *et al.* 1971) (Scheme 1). The results shown in Table 5 indicate that, even

though there is a constant rate of glucose formation, the specific radioactivity of cellular glucose increases with time. This increase in specific radioactivity must presumably arise because of a steady influx of the substrate $[2-^{14}\text{C}]\text{glucose}$. Further, calculations can be made from the results in Table 5 which show that the rate of glucose phosphorylation in this experimental system does not exceed one-fifth the rate of glucose formation (this value is based on the specific radioactivities of glucose and glucose 6-phosphate at 1 and 3 min and on the assumption of pre-steady-state conditions). Since gluconeogenesis is derived from unlabelled precursors and the cellular concentration of glucose is 50-fold that of glucose 6-phosphate, it is unlikely that the labelling patterns observed in glucose 6-phosphate and fructose 6-phosphate will significantly influence the labelling pattern of glucose over the time-interval of these experiments.

Difference in ^{14}C labelling patterns of hexose 6-phosphate during time-course experiments. The striking and unexpected difference in the radioisotopic composition and specific radioactivities of glucose 6-phosphate and fructose 6-phosphate (Tables 5, 7, 9 and 10) would imply that very limited metabolic and isotopic equilibria exist between the pools of these two hexose 6-phosphates. The disparity in labelling patterns is apparent in spite of the presence of an active glucose phosphate isomerase in liver (Gumaa *et al.* 1968). The higher concentration of label in C-4, C-5 and C-6 of all fructose 6-phosphate arising from the metabolism of $[2-^{14}\text{C}]\text{glucose}$ clearly shows the influence of the direct participation of fructose 6-phosphate rather than glucose 6-phosphate in the transaldolase exchange reaction (Scheme 1). On the other hand the concentration of ^{14}C in the C-1 of glucose 6-phosphate is always greater than that of C-1 of fructose 6-phosphate. This suggests that the C-1 of glucose 6-phosphate is able to retain some of the effects of the transketolase exchange reaction which is responsible for the ultimate transfer of label from $[2-^{14}\text{C}]\text{glucose}$ to C-1 of hexose 6-phosphate (reactions 1, 9, 10, 11 and 5 and Scheme 1).

The results may imply that there are two compartments for fructose 6-phosphate in liver (Scheme 1). In one compartment the distribution of label in fructose 6-phosphate and glucose 6-phosphate is a consequence of the metabolism of glucose in glycolysis and gluconeogenesis. In the other compartment the labelling pattern of fructose 6-phosphate is a consequence of its exchange reactions with transketolase and transaldolase in the pentose phosphate pathway. The effect of the transketolase and transaldolase exchange reactions on the labelling patterns of fructose 6-phosphate and glucose 6-phosphate will tend to oppose each other. The transketolase exchange reaction will direct the

transfer of label from C-2 of glucose 6-phosphate to C-1 of fructose 6-phosphate, whereas the transaldolase exchange reaction will relocate C-1, C-2 and C-3 to C-6, C-5 and C-4 of fructose 6-phosphate (Scheme 1). It is probable that the radioisotopic composition of the sampled fructose 6-phosphate reflects the mixing of the two separate compartments during extraction. The results imply that each of the compartments may be confined within the cell to a particular path or reaction in glucose metabolism and that there may be limited contact and only partial equilibration of these hexose 6-phosphates by glucose phosphate isomerase. The anomaly of calculating an increasing estimate of the pentose phosphate cycle from C-1/C-2 ratios would appear to result from the interaction of the effects of the above events during the 5 min interval of the experiments. The important issue is that the processes by which ^{14}C is distributed into hexose 6-phosphates cannot be reconciled with the defined reaction sequences in the pentose phosphate cycle.

The estimates of the pentose phosphate cycle in rabbit liver have been calculated by using equations (A) and (B) (Katz & Wood, 1960).

$$\text{C-1/C-2} = \frac{2\text{PC}}{1 + 2\text{PC}} \quad (\text{A})$$

$$\text{C-3/C-2} = \frac{\text{PC}}{1 + \text{PC}} \quad (\text{B})$$

More sophisticated equations have been derived (Landau *et al.* 1964; Katz, Landau & Bartsch, 1966; Landau & Bartsch, 1966) which theoretically correct for effects that may qualify the cycle estimate, i.e. incomplete equilibrations by glucose phosphate isomerase and triose phosphate isomerase and transaldolase exchange reactions. However, equations (A) and (B) are implicit in all other equations since all are derived for the model of the pentose phosphate cycle based on the definition of Wood & Katz (1958).

The experiments reported here were originally carried out with a view to measuring the quantitative role of the pentose phosphate cycle in liver *in situ*. To this end the experimental results were applied to equations (A) and (B). However, the widely divergent estimates of the cycle indicate that not only were the necessary conditions for the application of the equations not fulfilled (Katz & Wood, 1960) but, even more important, the failure to measure the cycle was due to the fact that processes other than the reactions of the pentose phosphate cycle were primarily responsible for the transfer of C-2 of glucose into C-1, C-2 and C-3 of the hexose 6-phosphates. This situation is completely contrary to the current mechanism for the pentose cycle (Katz & Wood, 1960; Katz, 1961; Wood *et al.* 1963).

Rat liver has been shown to be a rich source of the enzymes of the pentose phosphate pathway (Novello & McLean, 1968). The results of our experiments suggest that both glucose and ribose are metabolized in rabbit liver by reactions involving these enzymes. The results also indicate that the contribution of the defined pentose phosphate cycle to glucose metabolism is very small in rabbit liver.

The nature and extent of the exchange reactions make it most unlikely that the reaction sequences of the pentose phosphate pathway enzymes comprise a metabolic cycle or will allow the definition of a fixed stoichiometry for the dissimilation of glucose.

In contrast with the conclusions drawn earlier (Horecker *et al.* 1954), Horecker [1961, 1962 (p. 73), 1965] has more recently emphasized that the reactions of the pentose phosphate pathway are not of a cyclic nature but rather are composed of two parallel reaction sequences for the formation of pentose phosphate from hexose phosphate. One of these sequences comprises the oxidative segment of the pentose phosphate pathway (reaction 1) whereas the other involves the anaerobic synthesis of pentose phosphate from fructose 6-phosphate (Bonsignore, Pontremoli, Mangiarotti, de Flora & Mangiarotti, 1962). The results reported here more closely support the concept of Horecker for the reactions in the pathway than any of the other schemes that have been proposed (Beever, 1956; Racker, 1956; Korkes, 1956; Kitos *et al.* 1958; Davies, 1961).

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