Further evidence for the classical pentose phosphate cycle in the liver

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Isolated rat hepatocytes were incubated with [3-¹⁴C]xylitol or D-[3-¹⁴C]xylulose plus xylitol or glucose at substrate concentrations. The glucose formed was isolated and degraded to give the relative specific radioactivities in each carbon atom. C-4 of glucose had the highest specific radioactivity, followed by C-3, with half to one-fifth that of C-4. Only about 1% of the total radioactivity was in C-1. The data are compared with the predictions of the classical pentose phosphate cycle [Horecker, Gibbs, Klenow & Smyrniotis (1954) *J. Biol. Chem.* **207**, 393–403], and the proposed new version of the pentose phosphate cycle in liver [Longenecker & Williams (1980) *Biochem. J.* **188**, 847–857], which they denoted as the 'L-type pentose cycle'. The Williams pathway predicts that the specific radioactivity of C-1 of glucose should be half that of C-4 (after correction for approximately equal labelling on C-3 and C-4 of hexose phosphate in the pathway involving fructose 1,6-bisphosphatase). The actual labelling in C-1 is 20–350-fold less than this. When the hepatocytes are incubated with phenazine methosulphate, to stimulate the oxidative branch of the pentose phosphate cycle, the predicted relationship between (C-2/C-3) and (C-1/C-3) ratios of specific radioactivities is nearly exactly in accord with the classical pentose phosphate cycle. Glucose and glucose 6-phosphate were isolated and degraded from an incubation of hepatocytes from starved/re-fed rats with [3-¹⁴C]xylitol. Although the patterns were of the classical type, there was more randomization of ¹⁴C into C-2 and C-1 in the glucose 6-phosphate isolated at the end of the incubation than in the glucose which was continuously produced.

Williams and co-workers (Williams & Clark, 1971; Williams *et al.*, 1971, 1978*a,b*) have been investigating possible variations in the mechanism of the pentose phosphate cycle for many years. In view of the early evidence from Williams' laboratory, we had previously re-examined whether or not the isotopic data from isolated rat liver cells agreed with the classical formulation of the pentose phosphate cycle as proposed by Horecker *et al.* (1954). Two separate studies were made in this regard. In one (Rognstad, 1976), we used hepatocytes from starved/re-fed (high-carbohydrate) rats, in which lipogenesis is high and the requirements for NADPH generation are large. Using substrates which introduced ¹⁴C on C-3 or C-2 of glucose 6-phosphate, we found distributions of ¹⁴C in glucose in good agreement with that expected from the classical pentose phosphate cycle. In another study (Rognstad & Katz, 1974), we used hepatocytes from starved rats, with substrate concentrations of [1-¹⁴C]xylitol, where the non-oxidative segment of the cycle is very active. There we found nearly perfect accord between the labelling pattern found in

glucose with that predicted by the classical pathway, in that the labelling of C-1 was nearly twice that of C-3. More recently, in a collaborative study (Cohen *et al.*, 1981), using mixed ¹⁴C- and ¹³C-labelled substrates in rat hepatocytes, the isotopic patterns in glucose were again found to be in accord with the classical pentose phosphate cycle.

However, Longenecker & Williams (1980*a,b*) have presented further experimental evidence for a non-classical mechanism of the pentose phosphate cycle, especially in the liver, which they denote as the 'L-type pentose cycle'. Indeed, earlier (Williams *et al.*, 1978*b*) statements of caution as to the tentative nature of the evidence have more recently turned to the assertion that their 'L-type pentose cycle' is 'the only reaction sequence of the pentose pathway to be found in liver tissue' (Longenecker & Williams, 1980*a*). In view of these studies, we have attempted to make yet another re-examination of the mechanism of the pentose phosphate cycle in the liver. Again, we find complete accord between our data and the classical scheme of the pentose phosphate cycle, and wide divergence from the

results predicted by the Williams scheme. [Williams (1980) has presented a recent review of his work on the pentose cycle. Critical replies (Katz, 1981; Landau, 1981; Wood, 1981), together with a rejoinder by Williams (1981), ensued.]

Materials and methods

Hepatocytes were prepared as described (Katz *et al.*, 1978) from rats deprived of food for 24 h or from rats starved for 2 days, then re-fed overnight with a high-carbohydrate diet. Incubations were performed in glass 25 ml Erlenmeyer flasks at 38°C in a rotary shaker set at 125 rev./min. The medium was Krebs–Henseleit (1932) buffer, with O₂/CO₂ (19:1) in the gas phase. The incubations were terminated by injection of 0.5 ml of 20% (v/v) HClO₄. The neutral fraction from ion-exchange column treatment of the medium (Katz *et al.*, 1978) was dried and chromatographed on Whatman 3MM paper with phenol/water (4:1, v/v) as solvent, to separate glucose from residual xylitol or xylulose. The glucose was degraded by slight modifications (Rognstad, 1980) of the procedure of Schmidt *et al.* (1970). In the experiment with hepatocytes from starved/re-fed rats, glucose 6-phosphate was isolated by high-pressure liquid chromatography and purified as described by Katz *et al.* (1978).

D-[3-¹⁴C]Xylulose was synthesized by overnight incubation of dihydroxy[1-¹⁴C]acetone 3-phosphate (25 µmol) with excess (500 µmol) of glycolaldehyde plus muscle aldolase (100 EC units) to form D-[3-¹⁴C]xylulose 1-phosphate which was isolated on Dowex 1 (acetate), eluted with 4 M formic acid, dried and enzymically dephosphorylated. The D-[3-¹⁴C]xylulose was purified by paper chromatography in phenol/water (4:1, v/v) and butan-1-ol/acetic acid/water (4:1:2, by vol.). [3-¹⁴C]Xylitol was prepared by incubating D-[3-¹⁴C]xylulose with polyol dehydrogenase plus an excess of NADH. [3-¹⁴C]Xylitol was purified as described (Rognstad & Katz, 1974).

Results and discussion

On examination of the proposed 'L-type pentose cycle' mechanism (see Fig. 2 of Longenecker & Williams, 1980a), the most striking difference between it and the classical pathway is the predicted re-distribution of carbon from C-4 to C-1 of glucose 6-phosphate in a single turn of the Williams cycle. No such redistribution occurs in the classical pathway. Carbon on C-4 of glucose 6-phosphate will be converted into C-1 of the triose phosphates via the pentose phosphate cycle (or via phosphofructokinase), and re-formation of fructose 6-phosphate by fructose biphosphatase will cause labelling on both C-3 and C-4 by virtue of prior partial isotopic equilibration of the triose phosphates. The labelling pattern would be expected to be superimposed on the

operation of either type of pentose phosphate cycle. In the classical Horecker *et al.* (1954) pathway, carbon on C-3 of glucose 6-phosphate is redistributed into C-2 and, less extensively, into C-1 of glucose 6-phosphate on further recycling. One can show (Rognstad, 1976) that the classical scheme predicts that the (C-1/C-3) ratio in glucose 6-phosphate should be the square of the (C-2/C-3) ratio, when label is originally introduced on C-3 of glucose 6-phosphate. (This derivation neglects the reversibility of the transaldolase and transketolase reactions.) Thus if the (C-2/C-3) ratio is 0.10, the predicted (C-1/C-3) ratio is (0.10)², or only 0.01.

In order to examine the redistribution of carbon from C-4 of glucose 6-phosphate, we have used not a C-4-labelled hexose, but rather C-3-labelled pentose 5-phosphate precursors, namely [3-¹⁴C]xylitol or D-[3-¹⁴C]xylulose. All versions of the pentose phosphate cycle accept that the oxidative sequence proceeds from glucose 6-phosphate to 6-phosphogluconate to ribulose 5-phosphate, and in this sequence a C-4-labelled glucose 6-phosphate becomes a C-3 labelled pentose 5-phosphate. The subsequent redistribution of carbon from C-3-labelled pentose 5-phosphate in the non-oxidative segment is the same as would occur from C-4 of glucose 6-phosphate in the complete pentose cycle.

Table 1 presents the complete ¹⁴C distribution pattern in the glucose formed from either D-[3-¹⁴C]xylulose or [3-¹⁴C]xylitol. D-[3-¹⁴C]Xylulose was used, at a tracer concentration, in experiments in which either 10 mM-glucose or 10 mM-xylitol was the major substrate for hepatocytes isolated from starved rats. [3-¹⁴C]Xylitol was used together with substrate concentrations of xylitol in the other experiments. Phenazine methosulphate was also added as shown to stimulate the oxidative segment of the pentose cycle, since phenazine methosulphate reacts directly with NADPH to form reduced phenazine methosulphate which in turn reacts directly with O₂ to regenerate oxidized phenazine methosulphate (and H₂O₂). The use of xylitol as a substrate ensures a very active non-oxidative pentose pathway, since xylitol is a very good glucogenic substrate in the liver, with the stoichiometry considered to be

3 xylitol → 3 xylulose → 2 glucose + 1 triose phosphate.

The triose phosphate can either also be converted into glucose, or in part can be catabolized to lactate and CO₂.

As seen in Table 1, C-4 of glucose is the predominant carbon atom labelled from the 3-¹⁴C-labelled substrates. There is also considerable labelling in C-3 of glucose as a consequence of the pathway involving [1-¹⁴C]triose phosphate and [3,4-¹⁴C]fructose 1,6-bisphosphate. Although this pathway is of importance, the predominant path-

Table 1. Yields in major products and ^{14}C distributions in glucose formed from D-[3- ^{14}C]xylulose or [3- ^{14}C]xylitol in rat hepatocytes

Hepatocytes (36–47 mg dry wt.) from starved rats were incubated for 30–60 min in 5 ml of Krebs–Henseleit (1932) buffer with the labelled and unlabelled substrates shown. The glucose formed was isolated and degraded to give the relative specific radioactivities in each carbon atom, expressed with that of C-3 set equal to 100. Abbreviation used: PMS, phenazine methosulphate.

Expt. no. ...	608				617				626
Flask no. ...	1	2	5	6	1	2	3	4	5
Main substrate (10 mM) ...	Glucose	Glucose	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol	Xylitol
Labelled substrate ...		D-[3- ^{14}C]Xylulose				[3- ^{14}C]Xylitol			[3- ^{14}C]Xylitol
[PMS] (μM) ...	0	50	0	5	0	10	25	50	0
Glucose carbon	Relative specific radioactivities								
C-1	4.6	15.1	1.5	6.3	1.9	4.2	5.2	5.5	0.9
C-2	18.4	37.0	7.3	21.1	9.2	18.2	20.6	22.0	6.8
C-3	100	100	100	100	100	100	100	100	100
C-4	193	521	382	446	465	375	357	408	419
C-5	2.5	2.6	3.8	2.7	2.6	4.1	4.6	5.8	0.9
C-6	0.8	2.1	1.9	2.2	1.3	2.0	1.8	2.1	0.6
Product	Specific yields in products (% of total utilization)								
Glucose	84	43	95	73	95	72	70	71	97
Lactate	3	11	4	18	5	14	19	18	2
CO_2	13	46	1	9	0.5	14	11	11	0.5

way is the non-oxidative segment of the pentose cycle, and, in the classical version of this pathway, the label is transferred from C-3 of pentose 5-phosphate exclusively to C-4 of hexose phosphate. In the Williams version, the 'L-type pentose cycle', C-3 of the pentose phosphates is redistributed into hexose phosphate, with two-thirds of the carbon on C-4 and one-third on C-1. As shown in Table 1, the extent of labelling of C-1 of glucose in all cases is small, and it is clear that the distribution is far from that predicted by the Williams scheme. Assuming for simplicity complete equilibration of the triose phosphates, one can subtract the labelling on C-3 of glucose from that on C-4 to get the C-4 labelling owing to the non-oxidative pentose pathway alone. By the Williams scheme, the labelling on C-1 should be half of this value. However, the experimental C-1 labelling in some cases is two orders of magnitude lower than the predicted value, and in all cases the disagreement is very large. Invoking further cycling in the complete pentose cycle would not lessen the wide discrepancy between the data and the predicted result of the 'L-type pentose cycle', since, although C-1 is lost in the oxidative sequence, in each cycle C-4 of hexose phosphate is redistributed again two-thirds back to C-4 and one-third to C-1.

On the other hand, the ^{14}C distribution pattern is in rather good accord with the classical pentose cycle mechanism. As mentioned above, upon com-

plete cycling ^{14}C originally on C-3 of glucose phosphate will be redistributed into C-2 and C-1. Although the complete pentose cycle flux is fairly low in hepatocytes from starved rats, it can be increased by the addition of phenazine methosulphate (Rognstad, 1976). As previously stressed (Rognstad & Katz, 1974; Rognstad, 1976), the mechanism of a pathway can best be studied when the flux through it is high, since interference from side reactions or exchange reactions will be less. Thus the most useful data, in regard to the complete pentose cycle, in our experiments will be those obtained with phenazine methosulphate. As mentioned above, one can derive that the C-1/C-3 ratio of specific activities should equal the C-2/C-3 ratio squared, using the model of the classical pentose phosphate cycle, assuming back reactions of transaldolase and transketolase to be negligible. The experimental C-1/C-3 values in Table 1 are all higher than the values calculated from $(\text{C-2/C-3})^2$, but at least in the cases involving phenazine methosulphate, the average error is less than 25%. Probably the error can be largely accounted for by experimental errors in the degradation procedure. Thus, degradation of an authentic [3- ^{14}C]glucose standard yielded about 0.5% of total activity on C-1. The commercial [3- ^{14}C]glucose had been chemically synthesized, with no possibility of labelling occurring on C-1, and the 0.5% represents the error

Table 2. Distribution of ^{14}C in glucose and glucose 6-phosphate formed in hepatocytes from starved/re-fed rats incubated with $[3\text{-}^{14}\text{C}]\text{xylitol}$

Hepatocytes (28 mg defatted dry wt.) from rats starved for 2 days and re-fed with a high-carbohydrate diet overnight were incubated in 5 ml of Krebs–Henseleit buffer with 5 mM- $[3\text{-}^{14}\text{C}]\text{xylitol}$ plus 10 mM-dihydroxyacetone, for 90 min at 38°C . The glucose and glucose 6-phosphate were isolated and degraded. The results are expressed as relative specific radioactivities with C-3 set equal to 100.

Glucose carbon	Relative specific radioactivity	
	Glucose	Glucose 6-phosphate
C-1	4.9	8.2
C-2	14.4	29.5
C-3	100	100
C-4	328	472
C-5	1.6	14.6
C-6	4.3	10.8

in the degradation procedure. Likewise, degradations of $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]\text{glucose}$ standards by our procedure yield up to 1% 'crossover' into other carbon atoms in the top half of glucose. With the modified (Rognstad, 1980) procedure, however, there is very little crossover ($<0.2\%$) between the top (C-1, 2, 3) and bottom (C-4, 5, 6) halves of glucose. The degree of labelling on C-6 in Table 1 likewise probably can be attributed largely to degradative error, with crossover from the C-4 position. Most of the C-6 specific radioactivities are less than 0.5% of those on C-4. The labelling on C-5, however, may include a real contribution from fructose 6-phosphate $\rightleftharpoons$ fructose 1,6-bisphosphate cycling, which can cause redistribution of ^{14}C from C-2 to C-5.

Williams and co-workers (Williams *et al.*, 1978a; Longenecker & Williams, 1980a) have cautioned against the use of products that are metabolically distant from the pentose phosphate cycle, rather than the hexose phosphates, for isolation and degradation. To the extent that the products really are metabolically distant from the glucose 6-phosphate of the liver parenchymal cell, we would concur. However, glucose, the direct product of glucose 6-phosphate metabolism in the liver, cannot be considered metabolically distant. The same is true for glycogen, with the exception of experiments with substrates such as $[^{14}\text{C}]\text{galactose}$, which enter into the pathway in the middle of the sequence glucose 6-phosphate $\rightleftharpoons$ glycogen. Under conditions of gluconeogenesis, glucose may even be a better substrate for degradation than is glucose 6-phosphate (when the ^{14}C -labelled substrate is not glucose itself). To the degree that one may have cellular heterogeneity in the particular preparation used (other cell types, or damaged cells, in hepatocyte preparations, or the

known degree of cellular heterogeneity in a whole liver perfusion), glucose represents the product of the functioning liver parenchymal cell, whereas the glucose 6-phosphate pools in all the types of cells may possibly become labelled by the particular ^{14}C -labelled substrate used. In a reasonably homogeneous hepatocyte preparation, assuming no great changes in metabolism during the period investigated (metabolic steady state), one would expect a reasonable correspondence between the ^{14}C distributions in glucose and glucose 6-phosphate. In lengthy experiments, metabolic steady state may not hold, and there could be wide divergences between the glucose 6-phosphate pattern obtained at the end of the experiment, and representing metabolic fluxes at this instant in time, and the glucose pattern, which represents metabolic pathways over the whole experimental period. We have only limited data on comparison of ^{14}C distributions in glucose 6-phosphate and glucose. In Table 2 are shown the relative specific radioactivities of the six carbon atoms of glucose and glucose 6-phosphate from an incubation of $[3\text{-}^{14}\text{C}]\text{xylitol}$ with hepatocytes from starved/re-fed rats. The glucose 6-phosphate was isolated at the end of the rather long (90 min) incubation. The probable reason for the increased randomization of ^{14}C into C-2 and C-1 of the glucose 6-phosphate is that, in these experiments in which no glucose was initially added, glycogenolysis is rapid at the start and gradually slows down as glucose accumulates in the medium. The increased randomization in the glucose 6-phosphate at 90 min thus probably does not reflect increased pentose-cycle flux, but an essentially constant pentose cycle, with a decrease in the glycogenolytic flux through glucose 6-phosphate. The reason for the considerably greater labelling in C-5 and C-6 in the glucose 6-phosphate is not known. It could reflect increased cycling between ^{14}C -labelled lactate, accumulated during the long incubation, and triose phosphate. Although there are quantitative differences in the ^{14}C distribution patterns, again neither the glucose nor the glucose 6-phosphate pattern is remotely close to a pattern predicted by the Williams scheme. Also, H. R. Williams & Landau (1972) have shown similar ^{14}C distributions in glucose, glucose 6-phosphate and glycogen in liver with $[6\text{-}^{14}\text{C}]\text{fructose}$ as the labelled substrate.

Williams (1981) has also pointed out that non-equilibration of glucose 6-phosphate and fructose 6-phosphate could affect isotopic distribution patterns, and under certain conditions this may be true. However, with xylitol as the gluconeogenic substrate (in the absence of phenazine methosulphate) in hepatocytes from starved rats, lack of complete isotopic equilibration of the hexose phosphates would be of little importance. As shown in Table 1, under those conditions, 95% or more of the

[^{14}C]xylitol used is converted into glucose (other significant products being CO_2 and lactate), and thus essentially all the ^{14}C entering either fructose 6-phosphate or glucose 6-phosphate is converted into the same product, glucose. Even under conditions when a major flux to lactate may also occur, as in the experiments with phenazine methosulphate, lack of hexose 6-phosphate equilibration would only function to increase the discrepancy between our experimental data and the predicted output from the Williams model, since in his scheme the redistribution from C-4 into C-1 occurs in glucose 6-phosphate and not fructose 6-phosphate.

There are also recent reports of non-isotopic data which conflict with the Williams scheme. Thus Wood & Gascon (1980) found that arabinose 5-phosphate, a key intermediate in the 'L-type pentose cycle', was not metabolized by rat liver homogenate.

The isotopic data obtained previously by us (Rognstad & Katz, 1974; Rognstad, 1976), by H. R. Williams & Landau (1972), and in the current study provide strong evidence for the operation of the classical pentose phosphate cycle in liver. Our present results show that the ^{14}C randomization is greatly divergent from the pattern predicted by the scheme of Williams and co-workers (Longenecker & Williams, 1980a,b; Williams, 1980).

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