Pentose Cycle and Reducing Equivalents in Rat Mammary-Gland Slices

By JOSEPH KATZ and P. A. WALS

Research Institute, Cedars-Sinai Medical Center, Los Angeles, Calif. 90029, U.S.A.

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1. Slices of mammary gland of lactating rats were incubated with glucose labelled uniformly with ^{14}C and in positions 1, 2, 3 and 6, and with ^3H in all six positions. Glucose carbon atoms are incorporated into CO_2 , fatty acids, lipid glycerol, the glucose and galactose moieties of lactose, lactate, soluble amino acids and proteins. C-3 of glucose appears in fatty acids. The incorporation of ^3H into fatty acids is greatest from $[3\text{-}^3\text{H}]\text{glucose}$. ^3H from $[5\text{-}^3\text{H}]\text{glucose}$ appears, apart from in lactose, nearly all in water. 2. The specific radioactivity of the galactose moiety of lactose from $[1\text{-}^{14}\text{C}]\text{-}$ and $[6\text{-}^{14}\text{C}]\text{-glucose}$ was less, and that from $[2\text{-}^{14}\text{C}]\text{-}$ and $[3\text{-}^{14}\text{C}]\text{-glucose}$ more, than that of the glucose moiety. There was no randomization of carbon atoms in the glucose moiety, but it was extensive in galactose. 3. The pentose cycle was calculated from ^{14}C yields in CO_2 and fatty acids, and from the degradation of galactose from $[2\text{-}^{14}\text{C}]\text{glucose}$. A method for the quantitative determination of the contribution of the pentose cycle, from incorporation into fatty acids from $[3\text{-}^{14}\text{C}]\text{glucose}$, is derived. The rate of the reaction catalysed by hexose 6-phosphate isomerase was calculated from the randomization pattern in galactose. 4. Of the utilized glucose, 10–20% is converted into lactose, 20–30% is metabolized via the pentose cycle and the rest is metabolized via the Embden–Meyerhof pathway. About 10–15% of the triose phosphates and pyruvate is derived via the pentose cycle. 5. The pentose cycle is sufficient to provide 80–100% of the NADPH requirement for fatty acid synthesis. 6. The formation of reducing equivalents in the cytoplasm exceeds that required for reductive biosynthesis. About half of the cytoplasmic reducing equivalents are probably transferred into mitochondria. 7. In the Appendix a concise derivation of the randomization of C-1, C-2 and C-3 as a function of the pentose cycle is described.

Glucose metabolism in rat adipose tissue and mammary gland *in vitro* is characterized in each by high rates of lipogenesis and a high contribution of the pentose cycle (Katz, 1961). In adipose-tissue studies with ^{14}C -labelled and ^3H -labelled glucose Katz & Rognstad (1966) have established that at high rates of lipogenesis about half of the reducing equivalents for fatty acid synthesis are provided by the pentose cycle and the rest via transhydrogenation from cytoplasmic NADH. In adipose tissue nearly all the cytoplasmic reducing equivalents are consumed in reductive biosynthesis and few, if any, are transferred to mitochondria (Katz & Rognstad, 1966; Rognstad & Katz, 1966). Comparative studies on the supply and fate of reducing equivalents in other tissues are very limited. In fat tissue CO_2 , lipid and lactate are essentially the sole products, whereas in lactating mammary gland there is also extensive synthesis of lactose and proteins. The multiplicity of the metabolic pathways renders calculations of metabolic pathways and reducing equivalent balance more complex. Wood *et al.* (1965) have estimated the contribution of

the pentose cycle to glucose metabolism in the udder of the lactating cow. For rat mammary gland there is as yet no critical evaluation of the contribution of the pentose cycle and its role in providing NADPH for fatty acid synthesis.

In the present work we have used glucose labelled with ^{14}C , uniformly and in positions 1, 2, 3 and 6, and with ^3H in positions 1, 3, 4, 5 and 6. The contribution of the pentose cycle was calculated by several procedures and a balance of carbon and hydrogen and reducing equivalents was established. The supply and disposal of cytoplasmic reducing equivalents was found to differ in several respects from that in adipose tissue.

Materials and Methods

Part of the studies were done in 1965 and a part in 1970–71. Different strains of rats were used in these periods and there was some variation in methods, and some of the results are reported separately.

Animals

In 1965 rats of the Addis Slonaker strain, bred in this Institute, were used. In 1970 rats of the Wistar strain were purchased. The rats were kept on a commercial pellet diet and used 14–19 days *post partum*.

Labelled sugars

[4-³H]- and [3-³H]-Glucose, used in 1965, were synthesized in this laboratory (Rognstad *et al.*, 1965). [3-³H]-, [4-³H]- and [5-³H]-Glucose, used in 1970, were purchased from Amersham/Searle (Des Plaines, Ill., U.S.A.) and New England Nuclear Corp. (Boston, Mass., U.S.A.). [3-¹⁴C]Glucose was a gift from the National Bureau of Standards or was later purchased from Amersham/Searle. Other labelled sugars were purchased from New England Nuclear Corp. The sugars were examined by paper chromatography and degraded to establish specific labelling. In earlier work ¹⁴C-labelled sugars were degraded by fermentation with *Leuconostoc mesenteroides* (Stjernholm & Wood, 1958), followed by degradation of lactate and acetate as described by Katz *et al.* (1955). In later work ¹⁴C-labelled and ³H-labelled glucose were degraded enzymically by the method of Schmidt *et al.* (1970). By and large the ¹⁴C-labelled sugars from commercial sources were satisfactory, but this was not always so with the ³H-labelled sugars. Some samples contained 10–20% of a contaminant that co-chromatographed with glucose in butanol–acetic acid and in other commonly used chromatographic solvents. Its presence was detected during enzymic degradation because of the presence of a non-volatile non-ionic compound, containing ³H, that was not adsorbed on an anion-exchange resin after the conversion of glucose into phosphate esters. To eliminate possibilities of degradation artifacts, glucose samples labelled with ¹⁴C and ³H were mixed and degraded. With one batch of [6-¹⁴C, 5-³H]glucose, after conversion into phosphate esters was complete (by analysis), 28% of the ³H, but only 1.5% of the ¹⁴C, was eluted with water from the columns. The contaminant was non-volatile and could be a non-metabolized sugar stereoisomer. The presence of such an impurity would not be readily detected by chemical degradation. This component was removed by phosphorylation of glucose with hexokinase to glucose 6-phosphate, adsorption of the ester on Dowex 1 (acetate form) resin and elution with 1 M-ammonium acetate, followed by hydrolysis with acid phosphatase. Also, some batches contained considerable ³H not in the specified position. Our experience suggests caution in the use of ³H-labelled sugars from commercial sources.

Incubation

This was essentially as described by Katz & Kornblatt (1962). In short, slices about 0.5 mm thick

were prepared with a McIlvain–Buddle chopper and incubated in bicarbonate buffer, pH 7.2 (Krebs & Henseleit, 1932) in an atmosphere of O₂ + CO₂ (95:5). The separation of labelled fractions was essentially as described by Katz & Kornblatt (1962) and Schmidt & Katz (1969).

Water-soluble compounds

In the earlier experiments, organic acids were isolated by continuous ether extraction. In later work lactate was isolated by chromatography on Dowex columns. A portion of the medium was passed through tandem columns (1 cm × 10 cm) of Dowex 50 (H⁺ form) and Dowex 1 (acetate form). The water eluate was used for isolation of lactose. The lactate was eluted from the Dowex 1 column with 1 M-acetic acid. Amino acids were eluted from the Dowex 1 column with aq. 1 M-NH₃ soln.

The neutral fraction, containing glucose and lactose, was concentrated and then chromatographed on Whatman no. 3 paper with butan-1-ol–acetic acid–water (50:6:25, by vol.). These sugars were eluted and assayed for radioactivity. From the lactose/glucose ratio and total radioactivity in this fraction, the incorporation into lactose was calculated. The lactose was hydrolysed by boiling for 2 h in 0.5 M-H₂SO₄ and the H₂SO₄ was neutralized with BaCO₃. The supernatant was evaporated to dryness and chromatographed on paper with pyridine–ethyl acetate–water (12:5:7, by vol.), and the spots were detected by radioautography, eluted and assayed. Galactose was degraded completely by fermentation with *L. mesenteroides*, or the ¹⁴C and ³H in positions 1 and 6 of the glucose and galactose were obtained by periodate oxidation with and without prior reduction with borohydride by the method of Bloom (1962).

Degradation of glycerol

The aqueous phase after saponification of the lipids was desalted by passage through tandem columns (1 cm × 10 cm) of Dowex 50 (H⁺ form) and Dowex 1 (acetate form) and concentrated to a small volume. The glycerol was degraded without further isolation by enzymic conversion into 3-phosphoglycerate followed by degradation according to the procedure of Genovese *et al.* (1970).

Other procedures

The incorporation of ³H into water was determined as described by Katz & Wals (1970). Two-dimensional chromatography of desalted medium was as described by Katz & Kornblatt (1962). Measurements of ¹⁴C and ³H radioactivities were by liquid-scintillation counting (Katz & Wals, 1970). The efficiencies of counting were 55% in the ¹⁴C channel and 12%

in the ^3H channel, with a cross-over of about 10% from the ^{14}C channel and 1% from the ^3H channel.

Calculations

Incorporation of glucose carbon atoms into the various compounds was calculated from the concentration of glucose in the medium and the fraction of $[\text{U-}^{14}\text{C}]$ glucose recovered in each product. (Such calculations are valid only for uniformly labelled compounds.) The incorporation of other labelled atoms was expressed as specific yield; that is, total radioactive isotope recovered in products was set to 100 and incorporation into each compound expressed as a percentage. The three pathways in mammary gland are the Embden-Meyerhof pathway, the pentose cycle and lactose synthesis. The incorporation into lactose was obtained from the ^{14}C yield from $[\text{U-}^{14}\text{C}]$ glucose. Calculation of the contribution of the pentose cycle from ^{14}C yields requires suitable corrections for the presence of 'non-triose pathways' (Katz & Wood, 1960; Wood *et al.*, 1963). These pathways, which occur without cleavage of glucose to triose phosphates, are, for example, the synthesis of glycogen and galactose. The synthesis of the glucose moiety of lactose represents actually another path, since its precursor is unphosphorylated glucose in the medium, rather than hexose 6-phosphates (see the Discussion section).

The procedures compared here to calculate the contribution of the pentose cycle are: (a) ^{14}C yields in fatty acids from $[\text{1-}^{14}\text{C}]$ - and $[\text{6-}^{14}\text{C}]$ -glucose; (b) specific yields from $[\text{1-}^{14}\text{C}]$ - and $[\text{6-}^{14}\text{C}]$ -glucose; (c) fatty acid and glycerol yields from $[\text{1-}^{14}\text{C}]$ - and $[\text{3-}^{14}\text{C}]$ -glucose; (d) degradation of galactose from $[\text{2-}^{14}\text{C}]$ glucose; (e) degradation of glycerol from

$[\text{2-}^{14}\text{C}]$ - and $[\text{3-}^{14}\text{C}]$ -glucose. Equations based on $[\text{3-}^{14}\text{C}]$ glucose have not previously been presented. In the Appendix the equations are derived and the expressions used for the calculation in the text are summarized.

Results

^{14}C -labelled glucose

Incorporation of glucose carbon atoms. The pattern of labelling from $[\text{U-}^{14}\text{C}]$ -, $[\text{1-}^{14}\text{C}]$ -, $[\text{2-}^{14}\text{C}]$ -, $[\text{3-}^{14}\text{C}]$ - and $[\text{6-}^{14}\text{C}]$ -glucose is illustrated for a typical experiment in Table 1. About 60–70% of the utilized ^{14}C from all labels is present in CO_2 plus lipid. The rest is accounted for by lactate, lactose, soluble amino acids and the tissue residue, mainly protein. Two-dimensional chromatography revealed little, if any, other labelled organic acid other than lactate. The major labelled amino acids were alanine and glutamate, followed by serine and aspartate. In addition, another compound accounting for 1% of utilized glucose was always observed on paper chromatograms. The spot moved slowly in butanol-acetic acid, but moved in phenol approximately as fast as glutamate. In one experiment it was eluted and hydrolysed, yielding glucose and galactose. It is probably neuraminyl-lactose, described by Carubelli *et al.* (1964).

Table 2 summarizes the incorporation of glucose carbon atoms into the products, and Table 3 summarizes the distribution of individual carbon atoms (C-1, C-2, C-3 and C-6) observed in two groups of experiments. The rate of utilization of glucose was quite variable between animals, ranging for most experiments between 10 and $30\mu\text{mol/h}$ per g. About

Table 1. Utilization of ^{14}C -labelled glucose by rat mammary-gland slices

Slices (400mg) were pooled from two 15-day-old rats and incubated for 2h in 2ml of Krebs-Henseleit bicarbonate buffer containing glucose (19mm).

Labelled substrate	% of added ^{14}C in products	% recovery of ^{14}C *	Specific yield (%) in						
			CO_2	Fatty acids	Lipid glycerol	Lactate	Amino acids	Residue	Lactose
$[\text{1-}^{14}\text{C}]$ Glucose	60	102	38	25	7.0	11	3.5	3.5	12
$[\text{2-}^{14}\text{C}]$ Glucose	54	98	21	34	9.0	10	5.5	4.5	16
$[\text{3-}^{14}\text{C}]$ Glucose	55	101	44	12	9.5	11	4.0	3.5	16
$[\text{6-}^{14}\text{C}]$ Glucose	59	104	2.5	52	7.5	12	7.0	6.0	13
$[\text{U-}^{14}\text{C}]$ Glucose	59	100	31	32	8.0	7.5	5.5	4.0	12
			Yield ($\mu\text{g-atoms/2h}$ per g)†						
			87	90	22	21	16	11	34

* Includes residual glucose.

† Calculated from results for $[\text{U-}^{14}\text{C}]$ glucose.

60–70% of total carbon atoms as well as any of the individual glucose carbon atoms was recovered in all experiments in CO_2 plus lipids, but relative incorporation into CO_2 and fatty acids differed greatly from carbon atom to carbon atom. The specific yield in CO_2 ranged from 45 to 2% in the sequence $[3\text{-}^{14}\text{C}] > [1\text{-}^{14}\text{C}] > [\text{U-}^{14}\text{C}] \approx [2\text{-}^{14}\text{C}] \gg [6\text{-}^{14}\text{C}]$. The specific yield in fatty acids was nearly in a reverse order, 60% from $[6\text{-}^{14}\text{C}]$ glucose to 12% from $[3\text{-}^{14}\text{C}]$ glucose.

A great variation was observed in the glucose incorporation into lactose. Not only the total in-

corporation but the fraction incorporated into lactose varied markedly. In most experiments 10–20% of utilized glucose carbon atoms were recovered in lactose, but values as high as 27% and as low as 4% were encountered. The reason for this variability is not known. The fraction incorporated into lactate was also variable. The incorporation of the various carbon atoms into lactate as well as lactose, glycerol, soluble amino acids and protein did not vary greatly from carbon atom to carbon atom.

Table 4 compares lipogenesis *in vitro* in mammary

Table 2. *Incorporation of glucose carbon atoms by rat mammary-gland slices*

The experimental conditions were as described in Table 1. Expts. 1–5 were performed in 1965, Expts. 6–9 in 1971 (see the text). Results are given as means with the ranges in parentheses.

Expt. no.	Concn. of glucose (mM)	Yield ($\mu\text{g-atoms/3h per g}$)					
		CO_2	Fatty acids	Lipid glycerol	Lactate	Amino acids and Residue	Total
1–5	10–12	70 (36–105)	61 (23–110)	13 (6–18)	18 (12–27)	23 (11–30)	221 (120–350)
Yield ($\mu\text{g-atoms/2h per g}$)							
6–9	18–20	95 (64–111)	95 (63–118)	24 (21–31)	33 (21–45)	29 (20–40)	300 (220–345)

Table 3. *Distribution of glucose carbon atoms incorporated by rat mammary-gland slices*

The conditions were as described in Tables 1 and 2.

Expt. no.	Labelled substrate	Specific yield (%) in					
		CO_2	Fatty acids	Lipid glycerol	Lactate	Amino acids and residue	Lactose
1–5	$[\text{U-}^{14}\text{C}]$ Glucose	31 (27–36)	25 (17–34)	6 (5–9)	11 (7–17)	13 (7–17)	17 (11–27)
	$[1\text{-}^{14}\text{C}]$ Glucose	45 (37–49)	22 (16–30)	4.5 (4–6)	8 (4–12)	8 (4–13)	13 (8–24)
	$[6\text{-}^{14}\text{C}]$ Glucose	7 (3.5–9)	46 (31–57)	6 (5–8)	12 (7–17)	15 (8–20)	16 (11–26)
	$[2\text{-}^{14}\text{C}]$ Glucose	27 (25–30)	30 (18–39)	6 (5–8)	9 (6–12)	11 (6–17)	17 (10–28)
6–9	$[\text{U-}^{14}\text{C}]$ Glucose	30 (28–32)	30 (27–35)	9 (7–11)	11 (8–13)	10 (7–15)	10 (7–14)
	$[1\text{-}^{14}\text{C}]$ Glucose	40 (37–47)	24 (21–27)	8 (7–9)	11 (5–15)	7 (6–10)	10 (7–17)
	$[6\text{-}^{14}\text{C}]$ Glucose	2.5 (1.5–3)	54 (32–62)	10 (8–13)	12 (8–19)	10 (7–18)	11 (7–15)
	$[3\text{-}^{14}\text{C}]$ Glucose	45 (42–48)	14 (12–16)	12 (9–15)	10 (6–15)	8 (6–13)	11 (8–15)

Table 4. Comparison of glucose utilization by mammary-gland and liver slices of lactating rats

Slices (500mg) of mammary gland and liver from the same rat were incubated separately in 2.5 ml of Krebs-Henseleit bicarbonate buffer containing glucose (25mm). N.D., Not determined.

		% of added ^{14}C or ^3H in products					
		Mammary gland			Liver		
Rat	Labelled substrate	CO_2	Fatty acids	Lipid glycerol	CO_2	Fatty acids	Lipid glycerol
A	[U- ^{14}C]Glucose	28	30	6.0	1.3	0.14	2.4
	[1- ^{14}C]Glucose	35	19	3.8	2.8	0.17	N.D.
	[6- ^{14}C]Glucose	6.4	50	4.9	0.9	0.22	N.D.
B	[U- ^{14}C]Glucose	27	32*	4.2	1.0	0.10	1.9
	[1- ^{14}C]Glucose	33	22	4.9	2.3	0.15	1.4
	[6- ^{14}C]Glucose	3.4	52	6.4	0.6	0.13	1.2
	^3H -labelled water		0.018*			0.0004	

* Corresponds to 105 μg -atoms of C and 100 μg -atoms of H/g of tissue.

gland and livers of lactating rats. Fatty acid synthesis from glucose as well as endogenous sources, as judged by the incorporation of ^3H -labelled water, is negligible in liver slices compared with that in the gland. It appears that fatty acids of milk fat are synthesized in the gland or derived from the diet. Maximal rate of fatty acid synthesis observed by us is of the same order as that in adipose tissue of starved-re-fed rats, but much less than that *in vivo*.

The pattern of labelling from [1- ^{14}C]-, [2- ^{14}C]- and [6- ^{14}C]glucose in CO_2 and lipid is similar to that reported by others (Abraham & Chaikoff, 1959; McLean, 1964; Walters & McLean, 1968) and that seen in rat adipose tissue. [3- ^{14}C]Glucose has been, however, used rarely. The merit of this label for the measurement of the pentose cycle has been pointed out by Katz (1961). In the Embden-Meyerhof path, C-3 and C-4 of hexose 6-phosphates label the carboxyl group of pyruvate, and appear mainly in CO_2 . In the pentose cycle C-3 is introduced into C-2 and C-1 of hexose 6-phosphates, and thus labels acetyl-CoA and fatty acids. As shown in Tables 1 and 3, C-3 is the best precursor for $^{14}\text{CO}_2$, but incorporation into fatty acids is substantial, over half that from [1- ^{14}C]glucose. Abraham *et al.* (1957) and Abraham & Chaikoff (1959) reported labelling of fatty acids from [3- ^{14}C] plus [4- ^{14}C]glucose, but surprisingly they report very low recovery (half that from [1- ^{14}C]glucose) in CO_2 . We have found (J. Katz & P. A. Wals, unpublished work), as expected, that the $^{14}\text{CO}_2$ yield from [3- ^{14}C] plus [4- ^{14}C]glucose was substantially higher and fatty acid yield considerably lower than from [3- ^{14}C]glucose.

Contribution of the pentose cycle. Calculations of the contribution of the pentose cycle rest on a number of critical assumptions. One test for verification is to

compare observed specific radioactivities of hexose 6-phosphates with those predicted from theory. According to the model, and the theoretical calculations given in the Appendix, the effects would be: (a) to introduce ^{14}C from positions 1, 2 and 3 of glucose into positions 4, 5 and 6 of hexose 6-phosphates, but not to introduce any ^{14}C from positions 4, 5 and 6 of glucose into positions 1, 2 and 3 of hexose 6-phosphates; (b) to decrease markedly the specific radioactivity of hexose 6-phosphates (relative to substrate glucose) from [1- ^{14}C]glucose, but to increase that from [2- ^{14}C]glucose and more so from [3- ^{14}C]glucose; (c) to decrease slightly the specific radioactivity of hexose 6-phosphates from [6- ^{14}C]glucose.

The theoretical values for the relative specific radioactivities of the hexose 6-phosphates and their C-1-C-3 moieties have been calculated from the equations of the Appendix and they are plotted in Fig. 1. The difference between the specific radioactivities of the hexose 6-phosphates from [1- ^{14}C]-, [2- ^{14}C]- or [3- ^{14}C]glucose and the 'top' C-1-C-3 moiety is due to the incorporation of glyceraldehyde 3-phosphate from the triose pool into positions 4, 5 and 6 of fructose 6-phosphate. When the pentose-cycle activity is low, the glyceraldehyde 3-phosphate is derived from both the dihydroxyacetone phosphate and the glyceraldehyde 3-phosphate entities, but when the contribution of the pentose cycle is very high it is derived mainly from the glyceraldehyde 3-phosphate entity, and C-1, C-2 and C-3 are oxidized to CO_2 . This accounts for the difference between total specific radioactivity of hexose 6-phosphates and that of the C-1-C-3 moiety and explains the convex or concave shape of the curves. The curves of Fig. 1 were calculated by assuming operation of two pathways

Fig. 1. Effect of the pentose cycle on the specific radioactivities of hexose 6-phosphates from labelled glucose in rat mammary-gland slices

The relative specific radioactivities of the hexose 6-phosphates from [1- ^{14}C]-, [2- ^{14}C]-, [3- ^{14}C]- and [6- ^{14}C]-glucose (—) and the C-1-C-3 moieties (---) from [1- ^{14}C]-, [2- ^{14}C]- and [3- ^{14}C]-glucose are shown. The specific radioactivities of the C-1-C-3 of the hexose 6-phosphates from [1- ^{14}C]-, [2- ^{14}C]- and [3- ^{14}C]-glucose were calculated respectively from eqns. (A4), (A14) and (A14a) of the Appendix, and the specific radioactivities of the hexose 6-phosphates were obtained by multiplying the above values by $(1+r)$ (see the Appendix). The curve for [6- ^{14}C]-glucose was plotted from eqn. (A5a) of the Appendix, and the $H_{(6)}/H_{(1)}$ ratio (.....) from eqn. (1) of the Appendix.

(the pentose cycle and the Embden-Meyerhof pathway), complete equilibration of hexose 6-phosphates and triose phosphates and unidirectional reactions via transaldolase and transketolase, but unless departures from the assumptions are pronounced the general pattern will not be greatly changed.

It is generally accepted that the glucose moiety of lactose is derived from free glucose, and this is also supported by our findings (see below). The randomization in galactose and the ratio of galactose and glucose would then reflect the labelling pattern and

specific radioactivity of the hexose 6-phosphate pool. In Table 5 the observed galactose/glucose ratios are reported and compared with a calculated value for a 25% contribution by the pentose cycle. Table 5 also presents the $C_{(6)}/C_{(1)}$ and $C_{(2)}/C_{(5)}$ ratios in galactose from [1- ^{14}C]- and [2- ^{14}C]-glucose respectively. By and large the agreement with theory is satisfactory. The high ratio in galactose from [3- ^{14}C]-glucose is especially noteworthy.

Another test for the validity of the model is to calculate the contribution of the pentose cycle with as many methods as possible and under a variety of assumptions. In adipose tissue the agreement was found to be satisfactory (Ho & Jeanrenaud, 1967; Landau & Katz, 1965). Such a comparison for mammary gland is presented below. In Table 6 the distribution of ^{14}C in lactose from [2- ^{14}C]-glucose is reported. In the two experiments the rate of glucose uptake differed greatly, but the distribution patterns of ^{14}C were nearly identical. There was virtually no randomization in the glucose moiety, but there was ^{14}C in all six positions in galactose. The absence of randomization in the glucose entity of lactose formed in perfused cow udder from [2- ^{14}C]-glucose was reported by Wood *et al.* (1957, 1965), and our findings confirm their conclusion that the glucose entity is derived without phosphorylation from circulating glucose. Wood *et al.* (1965) found extensive randomization in C-1, C-2 and C-3 of galactose but very little ^{14}C in C-4, C-5 and C-6. The low labelling in C-4-C-6 was interpreted by Wood as due to the low triose phosphate formation via the Embden-Meyerhof path, since most of the glucose is used for lactose synthesis (see the Discussion section). The ^{14}C distribution in galactose from [2- ^{14}C]-glucose resembles that obtained with glycogen in adipose tissue (Landau *et al.*, 1964).

The contribution of the pentose cycle was calculated from both the $C_{(1)}/C_{(2)}$ and $C_{(6)}/C_{(5)}$ ratios in galactose (from Table 6) by eqns. (5) and (6) of the Appendix. In the derivation of these equations it is assumed that glucose 6-phosphate and fructose 6-phosphate are completely equilibrated. This is actually not the case and an alternative calculation, without assuming equilibration, is presented below.

Randomization in C-4, C-5 and C-6 of galactose and in glycerol. Landau *et al.* (1964) pointed out that randomizations in C-1-C-3 of glycogen represents that in glucose 6-phosphate, and the randomization in glycerol that in fructose 6-phosphate. If the equilibration of the hexose 6-phosphates were complete the pattern in glycogen and glycerol would be the same, but if the rate of the reaction catalysed by hexose 6-phosphate isomerase were insufficient for complete equilibration, the randomization from [2- ^{14}C]-glucose in glycerol would exceed that in glycogen. Landau *et al.* (1964) derived equations to calculate the rate of the hexose 6-phosphate iso-

Table 5. *Distribution of ^{14}C in the glucose and galactose moieties of lactose formed from ^{14}C -labelled glucose by mammary-gland slices of lactating rats*

Where the range (in parentheses) is given, it is from four to six experiments; otherwise all the values are reported. For the theoretical ratios, see the Appendix. Experimental conditions were those described in Tables 1–3.

Labelled substrate	^{14}C ratio in lactose		^{14}C ratio in galactose	
	Galactose/glucose	Theory for contribution of pentose cycle of 25%	$\text{C}_{(6)}/\text{C}_{(1)}$ or $\text{C}_{(2)}/\text{C}_{(5)}$	Theory for contribution of pentose cycle of 25%
[1- ^{14}C]Glucose	0.80 (0.70–0.95)	0.72	0.11 (0.09–0.12)	0.09
[6- ^{14}C]Glucose	0.89 (0.82–0.98)	0.92	0	0
[U- ^{14}C]Glucose	0.95 (0.85–1.03)	1.0		
[2- ^{14}C]Glucose	1.18 (1.0–1.35)	1.19		
[3- ^{14}C]Glucose	1.31, 1.32, 1.49	1.34		

Table 6. *Degradation of lactose sugars formed from [2- ^{14}C]glucose by rat mammary-gland slices*

The lactose from Expts. 3 and 4 of Table 2 was isolated, hydrolysed, and glucose and galactose were isolated and degraded (see the Materials and Methods section). The specific radioactivity of the carbon atom with the highest radioactivity in each sugar was set as 100, and the specific radioactivities of the other carbon atoms in each sugar were related to this carbon. 'Recovery of ^{14}C (%)' compares the mean specific radioactivity of the six carbon atoms in each sugar with the specific radioactivity obtained by combustion of the sugar to CO_2 (see the Materials and Methods section).

	Expt. 3		Expt. 4	
	Glucose utilized ($\mu\text{mol}/3\text{h}$ per g)	...	50	
	Expt. 3		Expt. 4	
	Lactose formed ($\mu\text{mol}/3\text{h}$ per g)	...	6	
Relative specific radioactivity				
	Glucose	Galactose	Glucose	Galactose
C-1	0.3	31.5	0.5	31.5
C-2	100	100	100	100
C-3	1.7	21.5	1.7	19.3
C-4	0.6	2.2	0.2	3.5
C-5		11.5		16.5
C-6		5.2		7.7
Recovery of ^{14}C (%)	87	96	95	94

merase reaction (in both directions) from the $\text{C}_{(1)}/\text{C}_{(2)}$ ratios in glycogen and $\text{C}_{(3)}/\text{C}_{(2)}$ ratio in glycerol. Landau & Bartsch (1966) found that the randomization pattern in C-4, C-5 and C-6 of glycogen from [2- ^{14}C]glucose is greater than in C-1, C-2 and C-3 of glycogen. They showed that, according to theory, the ^{14}C in C-4–C-6 of the hexose 6-phosphates and glycogen is derived from glyceraldehyde phosphate, and the randomization pattern should equal that in glycerol. They devised equations, reproduced in eqns. (11) and (12) of the Appendix, to calculate the

rate of the pentose cycle, without assuming complete equilibration of the hexose 6-phosphates, and the rate of the hexose 6-phosphate isomerase reaction. This calculation requires the determination of the ratios $\text{C}_{(1)}/\text{C}_{(2)}$ and $\text{C}_{(6)}/\text{C}_{(5)}$ in glucose 6-phosphate from [2- ^{14}C]glucose. Glycogen and galactose may be considered to represent the ^{14}C pattern in glucose 6-phosphate. Landau & Bartsch (1966) used glycogen to obtain these ratios and we have here used galactose. The ratios in galactose (Table 6) average 0.47 (5.2/11.5, 7.7/16.5), as compared with a $\text{C}_{(1)}/\text{C}_{(2)}$

Table 7. *Calculation of the contribution of the pentose cycle in rat mammary-gland slices with and without equilibration of the hexose 6-phosphates and of the rate of the hexose 6-phosphate isomerase reaction*

The results are from Table 6; calculations are from equations given in the Appendix.

Contribution of pentose cycle (%)	Expt. 3	Expt. 4
(a) Assuming complete equilibration		
From $C_{(1)}/C_{(2)}$ ratio in galactose by eqn. (5)	22.5	23
From $C_{(3)}/C_{(2)}$ ratio in galactose by eqn. (6)	27.5	28
(b) Equilibration incomplete		
From $C_{(1)}/C_{(2)}$ and $C_{(6)}/C_{(5)}$ ratio in galactose by eqn. (11)	27.5	28
Relative reaction rate of hexose 6-phosphate isomerase		
From $C_{(1)}/C_{(2)}$ and $C_{(6)}/C_{(5)}$ ratios in galactose by eqn. (12)		
Rate of hexokinase reaction is 1		
Fructose-6P $\rightarrow$ glucose-6P	3.1	
Glucose-6P $\rightarrow$ fructose-6P	3.3	
Reaction rate ($\mu\text{mol/h per g}$)		
(a) Hexokinase	6	16
(b) Glucose 6-phosphate dehydrogenase	4.8	12.5
(c) Hexose 6-phosphate isomerase		
Fructose-6P $\rightarrow$ glucose-6P	18.6	50
Glucose-6P $\rightarrow$ fructose-6P	20	53

Table 8. *Distribution of ^{14}C in lipid glycerol and lactate formed from labelled glucose in rat mammary-gland slices*

The results were obtained in Expt. 7. The specific radioactivity of the carbon atom labelled to the greatest extent was set to 100.

Labelled substrate	Glycerol				Contribution of pentose cycle* (%)			
	Relative proportion of ¹⁴ C recovered			% recovery of ¹⁴ C	From C ₍₁₎ /C ₍₂₎ ratio	From C ₍₃₎ /C ₍₂₎ ratio	From C ₍₂₎ /C ₍₃₎ ratio	From C ₍₂₎ /C ₍₁₎ ratio
	C-1	C-2	C-3					
[2- ¹⁴ C]Glucose	24	100	40	94	32	33		
[3- ¹⁴ C]Glucose	100	37	13	89			27	29
[6- ¹⁴ C]Glucose	1	5	100	101				
	Lactate							
[1- ¹⁴ C]Glucose	9	14	100	100				
[2- ¹⁴ C]Glucose	12	100	24	93				
[3- ¹⁴ C]Glucose	100	20	6	97				
[6- ¹⁴ C]Glucose	3	14	100	95				
[U- ¹⁴ C]Glucose	96	100	98	104				

* Calculated from eqns. (5)–(8) of the Appendix.

ratio of 0.31. The unequal ratios indicate incomplete equilibration of glucose 6-phosphate and fructose 6-phosphate. From these two ratios the pentose cycle (without assuming equilibration of hexose 6-

phosphates) and the rate of the hexose 6-phosphate isomerase reaction were calculated and the values are reported in Table 7.

The rate of the reaction catalysed by hexose 6-

phosphate isomerase is roughly three times the rate of glucose phosphorylation (Table 7), of the same order as in adipose tissue. In Table 7 also the absolute rates (as $\mu\text{mol/h per g}$) of the reactions catalysed by hexokinase, glucose 6-phosphate dehydrogenase and hexose 6-phosphate isomerase are calculated. The fluxes of the hexokinase and glucose 6-phosphate dehydrogenase reactions are similar.

Since the randomization pattern in glycerol should in theory be that in positions 4, 5 and 6 in galactose (see above), the degradation of glycerol is thus likely to cause an overestimate of the contribution of the pentose cycle. Table 8 summarizes the results for the degradation of glycerol derived from $[2-^{14}\text{C}]$ - and $[3-^{14}\text{C}]$ -glucose. The contribution of the pentose cycle calculated from the four ratios is in close agreement, about 32%. This is considerably higher than is obtained by other methods. The pattern of ^{14}C distribution in lactate differed markedly from that in glycerol (Table 8). The pattern in lactate is difficult to interpret, but it appears to be less randomized than in glycerol. Also, the results given in Table 3 indicate that the ^{14}C yields in lactate from the differently labelled sugars do not correlate well with the yields in fatty acids. This could be explained if glycerol and lactate formation was not all in the same cells and a part of the lactate was formed in cells with an exclusive Embden-Meyerhof pathway (see the Discussion section).

Contribution of the pentose cycle from fatty acid and CO_2 yields. The calculation of the contribution of the pentose cycle from fatty acid ratios from $[1-^{14}\text{C}]$ - and $[6-^{14}\text{C}]$ -glucose is well established, and occasionally ratios from $[6-^{14}\text{C}]$ - and $[\text{U-}^{14}\text{C}]$ -glucose have been used (Flatt & Ball, 1964). [Equations for using these sugars were derived by Katz & Wood (1960), but, as discussed there, the calculation is very sensitive to experimental error.] Other fatty acid ratios can also be employed, and that with $[3-^{14}\text{C}]$ -glucose offers definite advantages. In Fig. 2 ratios of ^{14}C in the acetyl unit (fatty acids) and triose phosphates (glycerol) have been plotted from the equations given in the Appendix. The plot of the $[3-^{14}\text{C}]/[1-^{14}\text{C}]$ -glucose ratio in fatty acids as a function of the contribution of the pentose cycle is nearly linear and provides the most sensitive means for estimation of the contribution of the pentose cycle, especially when it is below 10%. With ratios from the dihydroxyacetone phosphate entity such as those from $[1-^{14}\text{C}]$ -, $[2-^{14}\text{C}]$ - and $[3-^{14}\text{C}]$ -glucose it is not necessary to assume equilibration of triose phosphates, nor to correct for non-triose pathways.

Since $[3-^{14}\text{C}]$ -glucose has been rarely used, the yields in fatty acids and glycerol from this sugar are presented (in Table 9) for each individual experiment and compared with those from $[1-^{14}\text{C}]$ -glucose. The agreement between the values for the contribution of the pentose cycle calculated from fatty acids

Fig. 2. Effect of the pentose cycle on the labelling of triose phosphates and acetyl-CoA from labelled glucose in rat mammary-gland slices

—, Ratio of yields in acetyl-CoA from $[3-^{14}\text{C}]/[1-^{14}\text{C}]$ -glucose (A), $[1-^{14}\text{C}]/[2-^{14}\text{C}]$ -glucose (B) and $[1-^{14}\text{C}]/[6-^{14}\text{C}]$ -glucose (C); ----, ratio of ^{14}C yields in triose phosphates from $[3-^{14}\text{C}]/[1-^{14}\text{C}]$ -glucose. The curves for the $[3-^{14}\text{C}]/[1-^{14}\text{C}]$ -glucose ratios were plotted from eqns. (9) and (10) of the Appendix, that for the $[1-^{14}\text{C}]/[6-^{14}\text{C}]$ -glucose ratio from eqn. (2) of the Appendix, and that for the $[1-^{14}\text{C}]/[2-^{14}\text{C}]$ -glucose ratio by dividing eqn. (A6) by eqn. (A13) of the Appendix.

from the $[3-^{14}\text{C}]/[1-^{14}\text{C}]$ -glucose and $[1-^{14}\text{C}]/[6-^{14}\text{C}]$ -glucose ratios is good. On application of the correction for non-triose pathways to the $[1-^{14}\text{C}]/[6-^{14}\text{C}]$ -glucose ratio the agreement becomes even closer, as demonstrated in Table 10. On the other hand, the values calculated from the ratio in glycerol are considerably lower, and the discrepancy is greater than can be accounted for by experimental error. The reason for the difference is not clear.

Table 10 compares the values for the contribution of the pentose cycle estimated from the specific CO_2 yield and ratios in fatty acids. The value from CO_2

Table 9. *Specific yield in fatty acids and glycerol from [3-¹⁴C]glucose and calculation of the contribution of the pentose cycle in rat mammary-gland slices*

Expt. no.	Specific yield from [3- ¹⁴ C]glucose (%)		[3- ¹⁴ C]/[1- ¹⁴ C]-Glucose ratio		Contribution of pentose cycle (%)		
	Fatty acids	Glycerol	Fatty acids	Glycerol	From [3- ¹⁴ C]/[1- ¹⁴ C]-glucose ratio		From [1- ¹⁴ C]/[6- ¹⁴ C]-glucose ratio
					Fatty* acids	Glycerol†	Fatty‡ acids
5	16	5	0.47	1.77	21	22	23
6	27	7.5	0.59	1.55	26	17	29
7	21	9	0.58	1.67	25	20	27
8	25	7	0.48	1.35	21	12	19
9	26	8	0.50	1.63	22	19	24

* Eqn. (9) of the Appendix.

† Eqn. (10) of the Appendix.

‡ Eqn. (2) of the Appendix.

Table 10. *Calculation of the contribution of the pentose cycle in rat mammary-gland slices by several methods*

Method Labelled substrate Appendix eqn. no. Expt. no.	Contribution of pentose cycle (%)			
	CO ₂	Fatty acids	Fatty acids	Degradations
	[1- ¹⁴ C]- and [6- ¹⁴ C]- Glucose	[1- ¹⁴ C]- and [6- ¹⁴ C]- Glucose	[1- ¹⁴ C]- and [3- ¹⁴ C]- Glucose	[2- ¹⁴ C]- and [3- ¹⁴ C]- Glucose
	(13) or (14)	(4)*	(9)	(11)
1	23	28		
2	26	30		
3	17	22		27
4	21	24		28
5	22	22	21	
6	19	26	26	
7	18	27	25	30†
8	15	19	21	
9	19	23	22	
Mean	20	25	23	

* Corrected for non-triose phosphate pathways.

† From eqns. (5)–(8) of the Appendix; the mean from Table 8 is likely to be an overestimate (see the text).

yields was calculated both with and without correction for incomplete equilibration of triose phosphates, but the results were nearly the same. It is apparent that estimation of the contribution of the pentose cycle from ¹⁴CO₂ yields is consistently less than that from fatty acids. Possible reasons for the discrepancy are considered in the Discussion section.

³H-labelled glucose

Specific yield. The incorporation of ³H from glucose labelled in positions 1 to 6 is illustrated for one

experiment (the same as that of Table 1) in Table 11. The means and ranges of specific yields obtained in all experiments are shown in Table 12. In the earlier experiments [3-³H]- and [4-³H]-glucose were prepared by enzymic synthesis and in later work these were commercially obtained. The major product was water, ranging from a mean yield of 82% with [5-³H]glucose to a 45% yield with [3-³H]glucose. The labelling of fatty acids was most extensive from [3-³H]glucose (35%), followed by [1-³H]glucose (25%); that from [6-³H]- or [2-³H]-glucose was about 10%, that from [4-³H]glucose was 3–4%, and only

Table 11. *Utilization of ^3H -labelled glucose by rat mammary-gland slices*

Experimental details were as given for Table 1.

Specific yield (%) in

Labelled substrate	% of ^3H utilized	Water	Fatty acids	Lipid glycerol	Lactate	Amino acids and residue	Lactose
[2- ^3H]Glucose	57	64	12	4	4	2	12
[3- ^3H]Glucose	58	44	33	6	3	2	12
[4- ^3H]Glucose	57	78	3	4	5	1	11
[5- ^3H]Glucose	53	81	1	3	2	1	13
[6- ^3H]Glucose	60	51	12	8	10	6	13

Table 12. *Incorporation of glucose hydrogen atoms by rat mammary-gland slices*

The results are given as means with the ranges in parentheses.

Specific yield (%) in

Labelled substrate	No. of expts.	Water	Fatty acids	Glycerol	Lactate	Amino acids and residue	Lactose
[^3H]Glucose	5	50 (45-52)	25 (15-31)	5 (4-6)	4 (3-7)	3 (2-4)	13 (8-21)
[2- ^3H]Glucose	3	68 (64-74)	11 (10-13)	4.5 (4-5)	4.5 (4-5)	2 (1.5-3)	12 (4-17)
[3- ^3H]Glucose	9	45 (39-52)	35 (26-41)	5 (4-8)	3.5 (2-6)	1.5 (0.5-2.5)	10 (4-20)
[4- ^3H]Glucose	9	78 (70-89)	3 (2-4)	5 (2-6)	4 (2-7)	1 (0.5-2.5)	9 (4-20)
[5- ^3H]Glucose	3	82 (77-87)	0.5 (0.2-1)	3 (2-3)	2.5 (2-3)	1 (0.5-1)	10 (4-15)
[6- ^3H]Glucose	9	56 (46-65)	11 (8-16)	7 (4-8)	9 (5-14)	6 (4-9)	11 (4-25)

traces appeared from [5- ^3H]glucose. ^3H from all positions was incorporated into lactose roughly to the same extent as was ^{14}C . The incorporation of ^3H into glycerol, lactate and amino acids and residue was low and variable. Incorporation into lactate and amino acids appeared to be less from enzymically prepared sugars than from commercial sugars. Considerable caution must be used with the interpretation of low yields of ^3H . Some of the sugars contained 10% of the ^3H in positions other than the nominal ones. Also, such fractions as amino acids, residue and glycerol represent a group separation rather than the isolation of a specific compound. Moreover simultaneous assay of a small amount of ^3H in the presence of excess of ^{14}C is not very accurate.

$^3\text{H}/^{14}\text{C}$ ratios. A clearer picture of the fate of ^3H emerges from $^3\text{H}/^{14}\text{C}$ ratios. The ratios of major interest, for mammary gland, are summarized in

Table 13. The combination of ^3H - and ^{14}C -labelled substrates was that actually used in the experiment. The findings parallel largely those observed with rat adipose tissue. The $^3\text{H}/^{14}\text{C}$ ratio from [1- ^{14}C ,3- ^3H]glucose exceeds that in fatty acids from [1- ^{14}C ,1- ^3H]glucose. Since the hydrogen atoms from positions 1 and 3 should be incorporated to equal extents into NADPH, the difference was explained as being due to isotope discrimination (Katz *et al.*, 1965) against ^3H from position 1 but not from position 3. Strong support for this interpretation is provided by $^3\text{H}/^{14}\text{C}$ ratios greater than 1 in glycerol and galactose (Table 13). As in adipose tissue, the ^3H from position 6 appears without significant loss in triose phosphates (glycerol) but is lost extensively in the conversion into acetyl-CoA. The $^3\text{H}/^{14}\text{C}$ ratio in lactate is somewhat higher than that in adipose tissue.

The incorporation of ^3H from position 4 in fatty acids and lactate occurs by reduction via NADH. If

Table 13. $^3\text{H}/^{14}\text{C}$ ratios in some compounds formed in rat mammary-gland slices from glucose labelled with ^3H and ^{14}C

The results are given as means with the ranges in parentheses.

Labelled substrate	No. of expts.	$^3\text{H}/^{14}\text{C}$ ratio in			
		Fatty acids	Lipid glycerol	Lactate	Galactose
[1- ^{14}C ,1- ^3H]Glucose	5	1.12 (1.05–1.21)	1.13 (1.09–1.22)	0.74 (0.65–0.82)	1.21 (1.07–1.30)
[6- ^{14}C ,6- ^3H]Glucose	9	0.24 (0.18–0.29)	0.98 (0.87–1.06)	0.80 (0.67–0.92)	1.01 (0.94–1.04)
[U- ^{14}C ,3- ^3H]Glucose	5	1.56 (1.37–1.75)	0.86 (0.68–1.0)	0.11 (0.05–0.20)	0.80 (0.72–0.89)
[U- ^{14}C ,4- ^3H]Glucose	8	0.11 (0.07–0.18)	0.55 (0.37–0.64)	0.58 (0.35–0.67)	0.79 (0.69–0.94)
[1- ^{14}C ,3- ^3H]Glucose	4	1.39 (1.24–1.55)	1.02 (0.93–1.07)	0.34 (0.22–0.50)	0.95, 0.98, 1.01

the NADH were all derived by oxidation of glyceraldehyde 3-phosphate, and in the absence of exchange reactions or isotope effects, the $^3\text{H}/^{14}\text{C}$ ratio in lactate from [U- ^{14}C ,4- ^3H]glucose should be 1. The ratio in glycerol should be well above 1, since ^3H from position 4 of glucose would label position 1 of glycerol, and reductively NADH would label position 2. The observed ratios in these compounds indicate a dilution by three- to four-fold. The exchange mechanisms with protons that may dilute the specific radioactivity of the glucose hydrogen appearing in NADH have been discussed (Katz & Rognstad, 1966) and are likely to operate in mammary gland. These effects make it difficult to determine quantitatively the pathway of this hydrogen and the contribution of NADH to reductive synthesis of fatty acids (see the Discussion section).

The incorporation of ^3H from [2- ^3H]glucose resembles that in adipose tissue (Katz & Rognstad, 1969). The hydrogen from this position is, in one single transfer via hexose 6-phosphate isomerase, in part exchanged with protons. If the reversible rate of the hexose 6-phosphate isomerase reaction is much higher than the glucose flux, most of the ^3H from position 2 would appear in water. This appears to be the case in rat *in vivo* (Katz & Dunn, 1967). When the pentose cycle is active, the ^3H from position 2 would appear on C-1 of pentose 5-phosphates and subsequently on C-1 and C-3 of fructose 6-phosphate. Since in mammary gland the net influx into fructose 6-phosphate via the pentose cycle and Embden-Meyerhof pathway are of the same order (see Table 7), and the rate of the hexose 6-phosphate isomerase reaction is relatively low, substantial amounts of ^3H would appear on C-1 and C-3, and label subsequently fatty acids and other products.

The metabolism of [5- ^3H]glucose by rat adipose tissue has been described by Katz & Wals (1971). Some 97–98% of the metabolized ^3H appeared in water and the rest in glycerol. In mammary gland this ^3H was also retained in lactose. [5- ^3H]Glucose appears to be a valuable tool for use in following glucose catabolism. The yield in water provides a sensitive means for determining rates of glucose utilization too small to measure by other means.

The $^3\text{H}/^{14}\text{C}$ ratio in the glucose moiety of lactose was essentially that of substrate glucose. From Table 13 it is apparent that ^3H on C-3 and C-6 of galactose is handled as is the corresponding carbon atom. The ^3H in positions 4 and 5 is lost relative to the loss of ^{14}C with [U- ^{14}C]glucose. This is expected, because of the exchange between C-4–C-6 of hexose 6-phosphates and triose phosphates (see above).

Incorporation of ^3H from ^3H -labelled water into lipids. In a few experiments ^3H -labelled water was used as a substrate and incorporation into fatty acids and glycerol was determined. The results are summarized in Table 14. About 1 μg -atom of ^3H appears/ μg -atom of C incorporated from glucose into either fatty acids or glycerol.

Reducing equivalents

Supply of NADPH for fatty acid synthesis. In rat adipose tissue, when lipogenesis is high, about half of the NADPH is supplied by the pentose cycle and about half by transhydrogenation from NADH. We have attempted here to assess the roles of these pathways in mammary gland. The fatty acids formed in rat mammary-gland slices from glucose or acetate range in chain length from C_8 to C_{14} (see above). The mean chain length is C_{10} to C_{12} , requiring 1.60–

Table 14. *Incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ and ^3H from ^3H -labelled water into lipids by rat mammary-gland slices*

Experimental conditions were as given for Expts. 6–9 in Table 2.

Expt. no.	Incorporation of ^{14}C ($\mu\text{g-atoms}/2\text{h}$ per g) into		Incorporation of ^3H ($\mu\text{g-atoms}/2\text{h}$ per g) into		$^3\text{H}/^{14}\text{C}$ ratio in	
	Fatty acids	Glycerol	Fatty acids	Glycerol	Fatty acids	Glycerol
11	118	31	106	30	0.90	0.97
12	91	23	95	26	1.06	0.95
13	32	6.5	37	6.7	1.15	1.03
14	82	19	79	23	0.96	1.20
Mean					1.02	1.04

Table 15. *Contribution of the NADPH formed via the pentose cycle to reductive synthesis of fatty acids in rat mammary-gland slices*

Expt. no.	Fatty acids formed ($\mu\text{mol}/2\text{h}$ per g)	NADPH required ($\mu\text{mol}/3\text{h}$ per g)	NADPH formed ($\mu\text{mol}/3\text{h}$ per g)		% of requirement from pentose cycle	
			Minimal*	Maximal*	Minimal*	Maximal*
1	22	38	37	45	97	119
2	55	92	83	96	90	104
3	12	20	19	24	95	126
4	47	79	61	69	77	88
5	15	25	32	32	128	128
		($\mu\text{mol}/2\text{h}$ per g)	(NADPH formed in $\mu\text{mol}/2\text{h}$ per g)			
6	54	90	73	86	81	95
7	31	52	44	53	85	102
8	45	76	50	57	66	75
9	47	79	61	67	77	85

* The minimal NADPH production was calculated from the contribution of the pentose cycle on the basis of CO_2 yields, the maximal from that on the basis of fatty acid yields given in Table 10.

1.67 μmol of NADPH for reduction/ μmol of acetyl-CoA. Assuming the higher value, the amount of fatty acids formed and the NADPH requirement for reduction are tabulated in Table 15. Since there is a consistent difference of about 20% in the estimation of the contribution of the pentose cycle from either $^{14}\text{CO}_2$ yields or from fatty acid ratios, we have computed a minimal and a maximal formation of NADPH.

In eight out of nine experiments the formation of NADPH is sufficient to supply no less than 80% of the reducing equivalent requirement, and it is likely that all of it can be supplied by the pentose cycle. Another method for the calculation of NADPH

yields is possible. Since the dilutions of C-1 and of ^3H in position 3 via recycling are the same, the label in NADP^3H from $[\text{3-}^3\text{H}]\text{glucose}$ should equal the $^{14}\text{CO}_2$ specific yield derived via the pentose cycle from C-1 of glucose. The contribution of the Embden-Meyerhof pathway to CO_2 formation from C-1 of glucose is very low and can be readily corrected for (see Table 16). If all the NADP^3H were used exclusively for fatty acid reduction, the specific yields in fatty acids from $[\text{3-}^3\text{H}]\text{glucose}$ and $^{14}\text{CO}_2$ yields from C-1 via the pentose cycle should be nearly the same. Those yields are tabulated in Table 16, and it is seen that the formation of NADP^3H in all experiments exceeds the recovery in fatty acids. The

Table 16. Recovery in fatty acids of ^3H from NADP^3H formed in the pentose cycle in rat mammary-gland slices

Expt. no.	Specific yield in fatty acids from $[3\text{-}^3\text{H}]\text{glucose}$ (%)	Specific yield in $^{14}\text{CO}_2$ via the pentose cycle from $[1\text{-}^{14}\text{C}]\text{glucose}$ (%)	^3H incorporated from NADP^3H into fatty acids (%)
1	38	42	91
2	41	46	89
3	26	32	81
4	38	42	90
5	33	44	75
6	37	40	92
7	30	37	81
8	32	36	89
9	34	40	85
Mean			86

results of Table 16 suggest that about 15% of NADP^3H serves functions other than fatty acid reduction.

If ^3H from NADP^3H via the pentose cycle should be the sole source of reductive hydrogen, the $^3\text{H}/^{14}\text{C}$ ratios from $[3\text{-}^3\text{H}]\text{glucose}$ and ^{14}C -labelled sugars can be calculated. The combination used in Expts. 1–5 was $[3\text{-}^3\text{H}]$ - and $[\text{U-}^{14}\text{C}]\text{-glucose}$ and in Expts. 6–9 it was $[3\text{-}^3\text{H}]$ - and $[1\text{-}^{14}\text{C}]\text{-glucose}$. The expressions for the $^3\text{H}/^{14}\text{C}$ ratios in fatty acids would be: For the $[3\text{-}^3\text{H}]/[\text{U-}^{14}\text{C}]\text{-glucose}$ ratio:

$$\frac{3 \times 1.67}{2(1 + 2P)}$$

For the $[3\text{-}^3\text{H}]/[1\text{-}^{14}\text{C}]\text{-glucose}$ ratio:

$$\frac{1.67(2 - P)}{2(1 - P)}$$

where P represents the contribution of the pentose cycle. For a 20–25% contribution of the pentose cycle, these ratios would then range from 1.67 to 1.78 for the $[3\text{-}^3\text{H}]/[\text{U-}^{14}\text{C}]\text{-glucose}$ ratio and from 1.79 to 1.95 for the $[3\text{-}^3\text{H}]/[1\text{-}^{14}\text{C}]\text{-glucose}$ ratio. The mean observed ratios were 1.56 and 1.39, suggesting that the NAD^3H from $[3\text{-}^3\text{H}]\text{glucose}$ contributed 70–90% of the reducing equivalents required for the synthesis of fatty acids. The lower end of this range appeared more likely. In rat adipose tissue the $[3\text{-}^3\text{H}]/[\text{U-}^{14}\text{C}]\text{-glucose}$ ratio was about 1, indicating a considerably lower contribution of the pentose cycle to NADPH formation. The mean $^3\text{H}/^{14}\text{C}$ ratio in fatty acids from $[\text{U-}^{14}\text{C}, 4\text{-}^3\text{H}]\text{glucose}$ was 0.11, as compared with 1.56 from that with $[\text{U-}^{14}\text{C}, 3\text{-}^3\text{H}]\text{-glucose}$ (Table 13). Assuming a dilution factor 4 for the ^3H from position 4, this would correspond to a contribution of 20–25% of NADH hydrogen to fatty acid reduction.

Thus an estimate consistent with the data is that the pentose cycle contributes about three-quarters of the cell NADPH , with not more than one-quarter being derived from NADH through transhydrogenation, catalysed presumably, as in adipose tissue, by malate dehydrogenase and ‘malic’ enzyme (decarboxylating) (Rognstad, 1969).

Reducing-equivalent balance. The determination of reducing equivalent balance is complicated by the incorporation of ^{14}C into amino acids and protein. We have assumed that the labelling in amino acids represents transamination. This does not affect the reducing-equivalent balance and the ^{14}C yield in amino acids is thus equivalent to pyruvate accumulation.

In mammary gland cytoplasmic NADPH is generated via the pentose cycle and NADH by the oxidation of glyceraldehyde 3-phosphate. The reduced nicotinamide nucleotides are consumed in the synthesis of lipid and lactate. From the ^{14}C yields and the contribution of the pentose cycle (the averages of Table 15 being used) we have established an approximate balance. The calculations in Table 17 are presented for four experiments (out of nine), and are representative of the ranges obtained. The estimates should be considered to be rough approximations, but several features may be reliably inferred. In all experiments the yield of NADPH was roughly the same as that of NADH . The reduced nicotinamide nucleotides were always in considerable excess over that needed for cytoplasmic biosynthesis. The excess ranged from 30 to 50% (Table 17). Some of this may be used in reactions not taken into account, but clearly a major fraction is transferred into mitochondria. If, as is generally accepted, citrate acts as a carrier for cytoplasmic acetyl-CoA, an amount of oxaloacetate equal to that of the acetyl-CoA incorporated into fatty acids would be formed via

Table 17. *Reducing equivalent balance from glucose in rat mammary-gland slices*

Expt. no. ...	1	2	3	4
	Metabolic rates			
	(μmol/3 h per g)		(μmol/2 h per g)	
Glucose utilized	28.5	59	58	37
Glucose phosphorylated*	26	53.5	55	35
Acetyl-CoA converted into fatty acids	22	55	54	31
Acetyl-CoA oxidized	4	1	2	3
	Contribution of pathways (%)			
Pentose cycle	26	28	24	23
Galactose	9	11	6	5
Embden-Meyerhof pathway	65	61	70	72
Reducing equivalents formed and used in cytoplasm				
	(μmol/3 h per g)		(μmol/2 h per g)	
NADPH formed	39	92	78	49
NADH formed	36	76	80	51
Total reducing equivalents formed	75	168	158	100
Reducing equivalents used for fatty acids	38	92	90	52
Reducing equivalents used for glycerol and lactate	7	15	22	18
Total reducing equivalents used	45	107	112	70
Excess of reducing equivalents in cytoplasm	30	61	46	30
Reducing equivalents produced in mitochondria and transferred into mitochondria from cytoplasm				
	(μmol/3 h per g)		(μmol/2 h per g)	
Pyruvate dehydrogenase	26	56	56	34
Tricarboxylic acid cycle	16	4	8	9
Transferred from cytoplasm	30	61	46	30
Total	72	121	110	73

* The differences between the values in the two top rows represent the glucose moiety of lactose.

citrate cleavage. This would be reduced to malate, which in part could be utilized in the generation of NADPH. Most of it could be transferred to mitochondria and oxidized to oxaloacetate. Such a cycle would conserve oxaloacetate and ATP for citrate formation.

In most experiments, such as Expts. 2, 6 and 7 of Table 17, the formation of oxaloacetate is roughly similar to the excess of reduced nicotinamide nucleotides. In two experiments, illustrated by Expt. 1 of Table 17, the excess was considerably higher than the oxaloacetate formed by citrate cleavage. It is not certain whether this is due to experimental error or whether another mechanism for hydrogen transport may operate.

Discussion

Pathways

Under our conditions 10–20% of glucose is converted into lactose, 20–30% is oxidized via the pentose cycle and about 60% is converted into triose phosphates via the Embden-Meyerhof pathway. Thus 80–85% of the triose phosphates is formed via the Embden-Meyerhof pathway. About 30% of the glucose carbon atoms appears as CO₂. This is formed to approximately equal extent by oxidation via the pentose cycle and by pyruvate decarboxylation. The contribution of the tricarboxylic acid cycle is small and it accounts for less than 10% of the CO₂. The contribution of the pentose cycle in mammary gland

is similar to that in epididymal fat of starved-re-fed rats. The rates of fatty acid synthesis *in vitro* in the two tissues are of the same order, but the fraction of glucose carbon atoms converted into fatty acids is 45–50% in adipose tissue, as compared with a maximum of 35% in mammary gland. Therefore the NADPH yield via the pentose cycle is sufficient to supply most of the reducing-equivalent requirement for lipogenesis in mammary gland.

Recycling via the pentose cycle affects, in a characteristic fashion, the labelling of the products with ^{14}C and ^3H . Results reported here with both isotopic labels strongly support the operation of a model of glucose metabolism developed by us and studied extensively with adipose tissue. Mathematical analysis based on this model permits a rational interpretation of many of the findings obtained by the use of isotopic labels, such as yields from various labels, the relative specific radioactivities of galactose and the randomization of carbon atoms in this sugar. Our results confirm that glucose is derived from free glucose without phosphorylation and that galactose is derived from the hexose 6-phosphate pool. Specific radioactivities of galactose derived from [1- ^{14}C]-, [2- ^{14}C]-, [3- ^{14}C]- and [6- ^{14}C]-glucose differ markedly, and this is satisfactorily explained by theory. Further support for the validity of the model is provided by the degradation of galactose and glycerol and the incorporation of ^3H .

In general, ^3H in glucose is incorporated into water more extensively than is ^{14}C into CO_2 . ^3H in its original position is retained to a large extent in lactose and on C-3 of glycerol and lactate. ^3H from position 5 is released completely as water in catabolism, which suggests the use of this label as an irreversible tracer for glucose catabolism and turnover. ^3H from positions 1, 2, 3 and 4 labels the hydrogen of reduced nicotinamide nucleotides and reduced products, but the amount varies greatly. The labelling resembles closely that observed in the fat pad. The major findings and their interpretation are: (1) the ^3H yield in fatty acids from position 3 of glucose exceeds considerably that from position 1, and $^3\text{H}/^{14}\text{C}$ ratios from [1- ^{14}C , 1- ^3H]glucose exceed 1 in glycerol and galactose (this is due to the operation of isotope effects against ^3H transfer from [1- ^3H]glucose to NADPH, but not from [3- ^3H]glucose); (2) the ^3H yields from [4- ^3H]glucose in lactate, glycerol and fatty acids are much less than would appear if the major product from this label were NAD^3H (this is accounted for by the occurrence of exchange reactions and dilution of the specific radioactivity of the ^3H appearing on C-1 of glyceraldehyde 3-phosphate); (3) the relatively large retention of ^3H from position 2 in fatty acids and other products is explained by the extensive formation of [1- ^3H]pentose phosphates, with subsequent formation of hexose 6-phosphates labelled in positions 1 and 3. For a detailed discussion

readers are referred to the studies with adipose tissue (Katz & Rognstad, 1966, 1969; Rognstad & Katz, 1969).

Pentose cycle

The contribution of the pentose cycle was estimated from incorporation of ^{14}C into CO_2 and fatty acids and randomization in galactose. The total range of values is 20–30%. Although this constitutes a satisfactory agreement for many purposes, there appeared to be consistent differences between the values obtained by some methods. The contribution of the pentose cycle calculated from CO_2 yields was significantly less than that calculated from fatty acid yields (Table 10). The values calculated from galactose randomization may be higher, but the data are too limited for a conclusion to be reached. The differences may in part reflect the approximations in the theory.

A more likely reason for the divergence may be cellular non-homogeneity of the gland. Divergence in the results of calculations of the contribution of the pentose cycle would be likely if synthesis of lactose and fatty acids occurred in different cells. Our findings indicate that these compounds, but probably not lactate, are formed in one cell type. The gland contains about 25% by weight of connective tissue (Rees & Eversole, 1964), and it is likely that glucose is also metabolized by this tissue. A common end product of glucose metabolism is lactate, and it appears likely that some of the lactate originates in connective tissue, where the pentose cycle does not occur. This is consistent with the findings that the yields in lactate from glucose labelled in various positions is fairly uniform and does not parallel the yields in fatty acids. The utilization of glucose by adventitious tissue would cause an underestimate when the contribution of the pentose cycle was calculated from specific CO_2 yields. On the other hand, estimation based on fatty acid yields depends on ratios and would not be affected. The calculation based on the labelling of glycerol formed from [1- ^{14}C]- and [6- ^{14}C]-glucose is not reliable, owing to incomplete equilibration of triose phosphates, but this does not affect the calculation based on the labelling of glycerol formed from [1- ^{14}C]- and [3- ^{14}C]-glucose. However, values obtained from the latter ratio are unreasonably low. We cannot offer any explanation for this divergence.

Calculations of the contribution of the pentose cycle from the [1- ^{14}C]/[6- ^{14}C]-glucose and [1- ^{14}C]/[3- ^{14}C]-glucose ratios of the labelling in fatty acids were found to be in close agreement. [3- ^{14}C]Glucose provides a very sensitive means for the estimation of the pentose cycle (Fig. 2). But for its limited availability and high cost its use would be the method of choice for the evaluation of the contribution of the pentose cycle in tissues that form fatty acids. As

shown in Figs. 1 and 2, the specific radioactivity of triose phosphates or the acetyl group formed from [2-¹⁴C]glucose is not greatly altered by the pentose cycle, and yields from this sugar are not a sensitive index of this pathway.

Comparative studies of the pentose cycle in mammary gland tissues of different species should be of interest. The early work in rats and cows has been reviewed by Katz (1961). It is likely that in ruminants glucose is used mainly for production of lactose and NADPH via the pentose cycle, with very little incorporation into the acids of milk fat, which are mainly derived from acetate formed in the rumen. Wood *et al.* (1965) estimated the contribution of the pentose cycle to glucose metabolism in perfused cow udder to be about 30%, but only 10–20% being metabolized via the Embden–Meyerhof pathway. In rat tissue *in vitro*, with glucose as sole substrate, the Embden–Meyerhof pathway is, on the other hand, the major path.

There have been several studies of the pentose cycle in rat mammary gland. McLean (1958, 1964) and Walters & McLean (1968) reported [1-¹⁴C]-/[6-¹⁴C]-glucose ratios of the labelling in fatty acids similar to those in the present work. Abraham & Chaikoff (1959) reported that the ratios decreased to about 0.4 in the presence of insulin, corresponding to a contribution of the pentose cycle of 33%, but Walters & McLean (1968) as well as ourselves (J. Katz & P. A. Wals, unpublished work) did not observe an effect of insulin on the ratio. Unfortunately, in such studies total glucose utilization has been rarely determined. This vitiates calculations of the contribution of the pentose cycle from ¹⁴CO₂ yields. Meaningful comparison of yields from substrates labelled in various positions is not possible unless total utilization of the label is the same. Examination of the published data reveals that often utilization varied by a factor 2 or more, and such data must be viewed with care.

In most of that work, contribution of the pentose cycle to triose phosphate formation is estimated to be very high (50–70%), as compared with the now established values of about 25%. The operation of the pentose cycle and the effects of recycling have not been appreciated even in recent work (Smith & Glascock, 1969). The erroneous values have unfortunately persisted and are quoted extensively in numerous reviews and advanced textbooks.

Reducing equivalents in lipogenesis

NADPH can be formed in the cytoplasm by the dehydrogenases of the pentose cycle, by 'malic' enzyme (decarboxylating) and by isocitrate dehydrogenase. It appears that the last enzyme plays little or no role in lipogenesis from glucose in either mammary gland or adipose tissue of the rat. In

adipose tissue the pentose cycle and the pyruvate–malate cycle (Rognstad, 1969) contribute equally to the NADPH required for fatty acid reduction. In mammary gland, on the other hand, the results given in Tables 15 and 16 indicate that most of the NADPH is provided for by the pentose cycle. The results given in Table 17 indicate that most of the NADPH is provided for by the pentose cycle and that most of the oxaloacetate formed by citrate cleavage is reduced to malate, which is transferred to mitochondria. Thus 'malic' enzyme plays a relatively small role in lipogenesis in mammary gland, at least if glucose is the substrate. Enzyme activity profiles indicate that the activity of 'malic' enzyme varies greatly in tissues of various species. It is absent from mammary gland of the lactating rabbit (Gul & Dils, 1969) and is low in adipose tissue of the cow (Bauman *et al.*, 1970).

A noteworthy difference between rat adipose tissue and mammary gland is in the fate of the cytoplasmic reducing equivalents. A striking aspect in adipose tissue is that practically all the reducing equivalents formed in the cytoplasm from glucose is consumed there in biosynthesis, and there is virtually no net transport into mitochondria. On the other hand, in mammary gland up to one-half of the cytoplasmic reducing equivalents is likely to be transported into mitochondria.

It should be stressed that, *in vivo*, fatty acid synthesis is not likely to occur from glucose alone, and that other substrates such as lactate and acetate may be important. Comparative studies of reducing-equivalent balance in lipogenesis with several substrates and in different animals would be of value. In rat mammary gland, as in adipose tissue, fatty acid synthesis from lactate is increased over 10-fold by phenazine methosulphate (Katz & Wals, 1970). It has been suggested that transport and disposal of reducing equivalents may be rate-limiting for lipogenesis in adipose tissue (Katz & Wals, 1971). Further studies are required to test this concept in the mammary gland.

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APPENDIX

Analysis of the pentose cycle has evolved from that based on a simple model (Katz & Wood, 1960) to a rather complex one (Katz & Rognstad, 1967). It appears that the simpler models, assuming complete equilibration of hexose 6-phosphates and triose phosphates and unidirectional flux by transketolase and transaldolase, are satisfactory approximations in many systems. Parts of the early analysis require revision, but the basic equations are unaltered. Rognstad & Katz (1969) have summarized the analysis for $[1-^{14}\text{C}]$ - and $[6-^{14}\text{C}]$ -glucose. We now extend that to $[2-^{14}\text{C}]$ - and $[3-^{14}\text{C}]$ -glucose, and indicate some effects of non-triose phosphate pathways.

The model of glucose metabolism is that shown in Scheme 1 of Rognstad & Katz (1969). The symbol P is used, instead of the former pc , to represent the fraction of the glucose metabolized via the pentose cycle; NTP represents the fraction metabolized via non-triose pathways. In the absence of non-triose pathways the fraction of glucose forming fructose 1,6-diphosphate is $(1-P)$, and in the presence of these pathways it is $(1-P-NTP)$. The fraction of glucose oxidized to phosphogluconate and pentose 5-phosphate is $3P$, of which two-thirds is reconverted into hexose 6-phosphates. In this process a fraction

(mol/mol of glucose) $2P$ of triose phosphates is formed, but one-half of this is incorporated into C-4, C-5 and C-6 of hexose 6-phosphates. Hence the total inflow into the triose phosphate pool is $2(1-P)$ via the Embden-Meyerhof pathway, and $2P$ via the pentose cycle. The outflow is $1P$ into hexose 6-phosphates and $2-P$ as glycerol and pyruvate. In the presence of non-triose pathways the formation of triose phosphates via the Embden-Meyerhof pathway is $2(1-P-NTP)$, and the outflow from the triose phosphate pool to glycerol and pyruvate is $(2-P-2NTP)$ mol of triose phosphate/mol of utilized glucose.

$[1-^{14}\text{C}]$ - and $[6-^{14}\text{C}]$ -Glucose

These label C-1 and C-6 of hexose 6-phosphates and C-3 of triose phosphates. Equations for the inflow and outflow of ^{14}C at steady state into these carbon atoms can be written from the considerations above. To avoid multiplicity of symbols, the rate of glucose inflow and the specific radioactivity of glucose are both set to unity. The equations of state and intermediate expressions are numbered with the letter A as a prefix. The final (working) expressions used to calculate the contribution of the pentose

cycle and other parameters or to construct Figs. 1 and 2 of the main paper are numbered separately. $C_{(1)}$ – $C_{(6)}$ refer to the carbon atoms of hexose 6-phosphate (Hex-6P) and $Tr_{(1)}$, $Tr_{(2)}$, $Tr_{(3)}$ refer to the carbon atoms of triose phosphates (Tri-P).

This leads to eqn. (4):

$$\frac{\text{Tri-P from } [1-^{14}\text{C}]\text{Glc}}{\text{Tri-P from } [6-^{14}\text{C}]\text{Glc}} = \frac{(1-P-NTP)(1+P)}{(1+2P)(1+P-NTP)} \quad (4)$$

Eqns. (3) and (4) were used in the main paper to

Labelled carbon	Inflow	Outflow	Eqn.
C-1 of Hex-6P ($C_{(1)}$)		$[1] = C_{(1)}(1+2P)$	(A1)
C-6 of Hex-6P ($C_{(6)}$)		$[1] + PTr_{(3)} = C_{(6)}(1+P)$	(A2)
C-3 of Tri-P ($Tr_{(3)}$)	$(1+P)C_{(6)} + (1-P)C_{(1)} = 2Tr_{(3)}$		(A3)

[Eqns. (A1)–(A3) appear also in the paper by Rognstad & Katz (1969), with an unfortunate error in eqn. 3. The incorrect term $Tr_3(2-PC)$ appears there instead of $2Tr_3$. Eqns. (5), (6) and (8)–(10) of that paper are also wrong, but the error cancels out and the final expressions reported are correct.]

If the substrate is $[1-^{14}\text{C}]\text{glucose}$ the inflow 1 (shown in square brackets) appears in eqn. (1), but not in eqn. (2). If the label is $[6-^{14}\text{C}]\text{glucose}$, 1 appears only in eqn. (2), and not in eqn. (1). Solving the two sets of simultaneous equations we obtain for the relative specific radioactivities:

solve for the contribution of the pentose cycle from the fatty acid ratios from $[1-^{14}\text{C}]\text{-}$ and $[6-^{14}\text{C}]\text{-glucose}$. The incorporation into galactose constitutes a non-triose pathway, but that into the glucose moiety of lactose represents another pathway, since this is derived from unphosphorylated glucose. The contribution of the pentose cycle was calculated from eqn. (4) by using the yield in galactose (approximately half that in lactose) for estimating the contribution of non-triose pathways. The incorporation into the glucose moiety of lactose was computed separately from the ^{14}C yield.

Labelled carbon	$[1-^{14}\text{C}]\text{Glucose}$	Eqn.	$[6-^{14}\text{C}]\text{-Glucose}$	Eqn.
C-1 of Hex-6P ($C_{(1)}$)	$\frac{1}{1+2P}$	(A4)	0	(A4a)
C-6 of Hex-6P ($C_{(6)}$)	$\frac{P(1-P)}{(1+2P)(2-P)(1+P)}$	(A5)	$\frac{2}{(2-P)(1+P)}$	(A5a)
C-3 of Tri-P ($Tr_{(3)}$)	$\frac{1-P}{(1+2P)(2-P)}$	(A6)	$\frac{1}{(2-P)}$	(A6a)

From the above we obtain:

$$\frac{(A5)}{(A4)} = \frac{\text{C-6 of Hex-6P from } [1-^{14}\text{C}]\text{Glc}}{\text{C-1 of Hex-6P from } [1-^{14}\text{C}]\text{Glc}} = \frac{P(1-P)}{(2-P)(1+P)} \quad (1)$$

$$\frac{(A6)}{(A6a)} = \frac{\text{Tri-P from } [1-^{14}\text{C}]\text{Glc}}{\text{Tri-P from } [6-^{14}\text{C}]\text{Glc}} = \frac{1-P}{1+2P} = \gamma \quad (2)$$

Tri-P is used in eqn. (2) rather than $Tr_{(3)}$, since C-1 and C-2 of the triose are unlabelled.

Designating $(A6)/(A6a)$ by γ we obtain:

$$P = \frac{1-\gamma}{1+2\gamma} \quad (3)$$

If non-triose pathways are present eqns. (A1) and (A2) are unaltered, but instead of eqn. (A3) eqn. (A3') must be substituted:

Labelling from $[2-^{14}\text{C}]\text{-}$ and $[3-^{14}\text{C}]\text{-glucose}$

These carbon atoms are randomized in positions 1, 2, 3 of hexose 6-phosphates. The labelled triose phosphates would also be incorporated, as with $[1-^{14}\text{C}]\text{glucose}$, as a unit into C-4–C-6 of hexose 6-phosphates. Hence all six carbon atoms of hexose 6-phosphates and all the carbon atoms of triose phosphates and acetyl-CoA are labelled. If equilibration of triose phosphates and hexose 6-phosphates is

$$\text{C-3 of Tri-P } (Tr_{(3)}) \quad (1+P-NTP)C_{(6)} + (1-P-NTP)C_{(1)} = 2Tr_{(3)}(1-NTP) \quad (A3')$$

complete the ratio $C_{(4)}+C_{(5)}+C_{(6)}/C_{(1)}+C_{(2)}+C_{(3)}$ is that given by eqn. (1), and also:

$$C_{(2)}/C_{(1)} = C_{(5)}/C_{(6)}$$

and

$$C_{(3)}/C_{(2)} = C_{(4)}/C_{(5)}$$

so that solutions for only $C_{(1)}$, $C_{(2)}$ and $C_{(3)}$ are derived below.

To obtain the total relative specific radioactivity of the hexose 6-phosphates, the radioactivity in C-4-C-6 must be added to that in C-1-C-3. The ratio between C-4-C-6 and C-1-C-3 is $r = P(1-P)/(2-P)(1+P)$ (eqn. 1). Hence the radioactivity of the hexose 6-phosphates is that in C-1-C-3 multiplied by the factor $(1+r)$.

The inflow via the pentose cycle into C-1 of hexose 6-phosphates is $2PC_{(2)}$, into C-2 of hexose 6-phosphates is $2PC_{(3)}$ and into C-3 of hexose 6-phosphates it is $[(C_{(2)}+C_{(3)})/2]2P$. The outflow is $(1+2P)$, and non-triose pathways have no effect on inflow or outflow. The equations are the same for $[2-^{14}\text{C}]$ - and $[3-^{14}\text{C}]$ -glucose except that inflow from substrate occurs either into C-2 or into C-3, with the term 1 (in square brackets) appearing in either eqn. A8) or eqn. (A9):

Carbon of hexose 6-phosphates	Inflow	Outflow	Eqn.
$C_{(1)}$	$2PC_{(2)} = C_{(1)}(1+2P)$		(A7)
$C_{(2)}$	$[1]+2PC_{(3)} = C_{(2)}(1+2P)$		(A8)
$C_{(3)}$	$[1]+(C_{(2)}+C_{(3)})P = C_{(3)}(1+2P)$		(A9)

Solving the two sets of simultaneous equations, we obtain for the relative specific radioactivities:

Carbon of hexose 6-phosphates	$[2-^{14}\text{C}]$ Glucose	Eqn.	$[3-^{14}\text{C}]$ Glucose	Eqn.
$C_{(1)}$	$\frac{2P(1+P)}{(1+3P)(1+2P)}$	(A10)	$\frac{4P^2}{(1+2P)(1+3P)}$	(A10a)
$C_{(2)}$	$\frac{1+P}{1+3P}$	(A11)	$\frac{2P}{1+3P}$	(A11a)
$C_{(3)}$	$\frac{P}{1+3P}$	(A12)	$\frac{1+2P}{1+3P}$	(A12a)
$C_{(1)}+C_{(2)}$	$\frac{1+5P+4P^2}{(1+3P)(1+2P)}$	(A13)	$\frac{2P+8P^2}{(1+3P)(1+2P)}$	(A13a)
$C_{(1)}+C_{(2)}+C_{(3)}$	$\frac{1+6P+6P^2}{(1+3P)(1+2P)}$	(A14)	$\frac{1+6P+12P^2}{(1+3P)(1+2P)}$	(A14a)

From these we obtain the ratios:

	$[2-^{14}\text{C}]$ Glucose		$[3-^{14}\text{C}]$ Glucose	
$C_{(1)}/C_{(2)}$	$\frac{2P}{(1+2P)}$	(5)	$\frac{2P}{(1+2P)}$	(7)
$C_{(1)}/C_{(3)}$	$\frac{2(1+P)}{(1+2P)}$	(6)	$\frac{4P^2}{(1+2P)^2}$	(8)

Eqns. (5)–(8) have been used in the main paper to calculate the contribution of the pentose cycle from the degradation of galactose and glycerol.

The ratio of specific radioactivities of triose phosphates from $[1-^{14}\text{C}]$ -, $[2-^{14}\text{C}]$ - or $[3-^{14}\text{C}]$ -glucose are the same as the ratios of the C-1, C-2 and C-3 moieties of hexose 6-phosphates. This holds even if equilibration of triose phosphates by triose phosphate isomerase is incomplete or in the presence of non-triose pathways. Also, the ratios in acetyl-CoA are those of the C-1+C-2 moieties of hexose 6-phosphates from these three labels. To obtain the ratios of specific radioactivities from $[3-^{14}\text{C}]$ glucose to that of $[1-^{14}\text{C}]$ glucose in acetyl-CoA or triose phosphates, eqns. (A13a) and (A14a) are divided by eqn. (A4) to obtain eqns. (9) and (10):

$$\frac{\text{Ac-CoA from } [3-^{14}\text{C}]\text{Glc}}{\text{Ac-CoA from } [1-^{14}\text{C}]\text{Glc}} = \frac{8P^2+2P}{1+3P} \quad (9)$$

$$\frac{\text{Tri-P from } [3-^{14}\text{C}]\text{Glc}}{\text{Tri-P from } [1-^{14}\text{C}]\text{Glc}} = \frac{12P^2+6P+1}{1+3P} \quad (10)$$

Eqns. (9) and (10) are plotted in Fig. 2 of the main paper. Other ratios may be obtained at will. To derive ratios from yields from $[2-^{14}\text{C}]$ - or $[3-^{14}\text{C}]$ -

glucose with that from [6-¹⁴C]glucose requires calculation of the specific radioactivity of triose phosphates from [2-¹⁴C]- or [3-¹⁴C]-glucose. Calculation from ratios with [6-¹⁴C]glucose has the disadvantage of being affected by incomplete equilibration of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate and it also requires a correction for non-triose pathways.

Incomplete equilibration of hexose 6-phosphates

The ratios $C_{(1)}/C_{(2)}$ and $C_{(6)}/C_{(5)}$ in hexose 6-phosphates would be unequal if equilibration of glucose 6-phosphate and fructose 6-phosphate is incomplete (Landau & Bartsch, 1966). These two ratios are used to calculate the contribution of the pentose cycle and the relative rate of the hexose 6-phosphate isomerase reaction in the manner described by Landau & Bartsch (1966). Experimentally these ratios may be obtained from compounds considered to be derivations of glucose 6-phosphate, as the $C_{(1)}/C_{(2)}$ and $C_{(6)}/C_{(5)}$ in glycogen in galactose. Instead of the $C_{(6)}/C_{(5)}$ ratio in glycogen in galactose, the $C_{(3)}/C_{(2)}$ ratio in glycerol may be employed (Landau & Bartsch, 1966). The expressions appear to be cumbersome, but are numerically simple to use, as in Table 7 in the main paper:

$$P = \frac{(2 + C_{(1)}/C_{(2)}) (C_{(6)}/C_{(5)})}{(2 - 3C_{(1)}/C_{(2)}) (2 - C_{(1)}/C_{(2)}) + C_{(6)}/C_{(5)} (6 - 5C_{(1)}/C_{(2)})} \quad (11)$$

and

$$E = \frac{C_{(1)}/C_{(2)} (2 - C_{(1)}/C_{(2)} + 2C_{(6)}/C_{(5)})}{(C_{(6)}/C_{(5)} - C_{(1)}/C_{(2)}) (2 - C_{(1)}/C_{(2)})} \quad (12)$$

where E is the relative rate of the hexose 6-phosphate isomerase reaction in the direction fructose 6-phosphate $\rightarrow$ glucose 6-phosphate and $E - (1 - 3P)$ is that

in the reverse direction. [If equilibration is complete, $E = \infty$, $C_{(1)}/C_{(2)} = C_{(6)}/C_{(5)}$ and eqn. (11) reduces to eqn. (5).]

Calculation of the contribution of the pentose cycle from specific CO₂ yields

The expressions given by Rognstad & Katz (1969) are reproduced here. $G1$ and $G6$ are the specific yields in CO₂ from [1-¹⁴C]- and [6-¹⁴C]-glucose and γ the ratio of fatty acids from these sugars. Eqn. (13) assumes complete equilibration of triose phosphates, and eqn. (14) contains a correction for incomplete equilibration (in mammary gland, with the low ¹⁴CO₂ yields from [6-¹⁴C]glucose, the correction is insignificant):

$$P = \frac{G1 - G6}{3 - (2G1 - G6)} \quad (13)$$

$$P = \frac{G1 - G6}{3 - (2G1 + \gamma G6)} \quad (14)$$

Corrections for non-triose pathways are not significant, but, since the glucose moiety of lactose is not derived from hexose 6-phosphates and is not included in the three pathways, the specific CO₂ yields were divided by $1/(1 - NTP/2)$, where NTP is the yield in lactose.

References

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