Estimation of the Pentose Cycle in the Perfused Cow's Udder

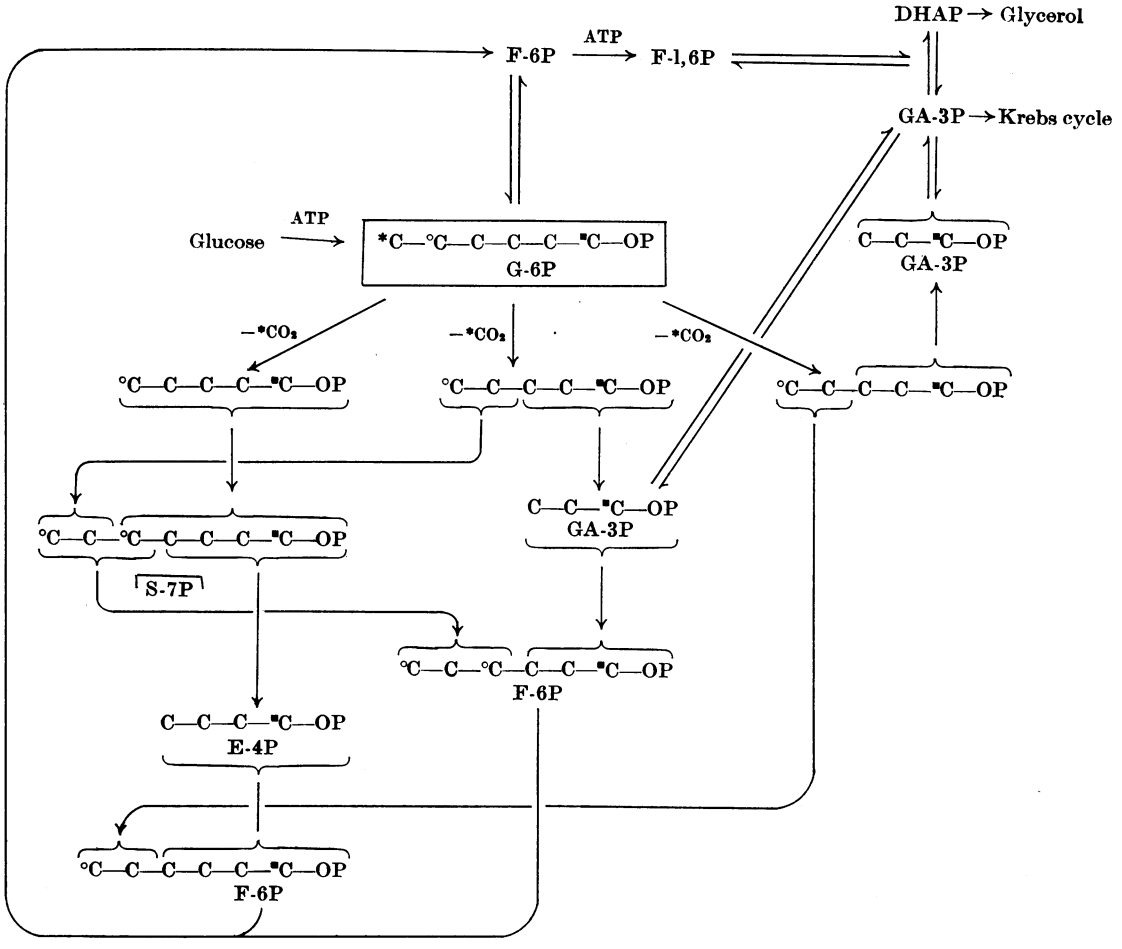
By HARLAND G. WOOD, GEORGES J. PEETERS, ROGER VERBEKE, MONIQUE LAURYSSENS AND BIRGIT JACOBSON
Department of Biochemistry, Western Reserve University, Cleveland, Ohio, U.S.A., and Veterinary School of the University of Ghent, Belgium

(*Received* 30 *December* 1964)

1. The distributions of ^{14}C have been compared in the glucose and galactose moieties of lactose obtained from cows' udders perfused with blood containing [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]-glucose. The ^{14}C of the glucose moiety was found in the same position as that of the administered glucose, but in the galactose moiety the ^{14}C from [2-^{14}C]glucose was extensively randomized into positions 1 and 3. It is concluded that the glucose moiety arose from free glucose and the galactose moiety from hexose phosphate intermediates and that the latter reflected the randomization occurring through reactions of the pentose cycle. 2. The proportion of the glucose metabolized via the pentose cycle for those cells making lactose was estimated from the distribution of ^{14}C in the galactose moiety and found to be about 23% in one experiment and 30% in another experiment. 3. The yield and distribution of ^{14}C were determined in the glycerol of fat from the tissue in experiments with [2-^{14}C]- and [6-^{14}C]-glucose. There was a greater randomization of ^{14}C in the glycerol than in C-1, C-2 and C-3 of the galactose moiety of lactose. The ratio of the yield of ^{14}C in the glycerol from [2-^{14}C]glucose to that of [6-^{14}C]glucose was very low and from this ratio it was calculated that less than 10% of the glucose was metabolized by the Embden–Meyerhof pathway and approx. 60–70% was converted into lactose. 4. [6-^{14}C]Glucose and [6-^{3}H]glucose were used to determine whether the ^{3}H at the C-6 position remained stable during its conversion into glyceride of fat from the tissue. Twenty-seven per cent of the ^{3}H was labilized during this conversion. Therefore it was not possible to use [2-^{14}C]glucose and [6-^{3}H]glucose in a single experiment to measure the relative conversion of the C-2 and C-6 positions of glucose to glycerol.

There is extensive evidence that a substantial part of the metabolism of glucose in the lactating mammary gland is via the pentose cycle, perhaps more than in any other tissue. Glock & McLean (1953, 1954) demonstrated a 60-fold increase in glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–NADP oxidoreductase, EC 1.1.1.49) and a 20-fold increase in 6-phosphogluconate dehydrogenase (6-phosphogluconate–NADP oxidoreductase, EC 1.1.1.44) of rat mammary gland with the onset of lactation, which dropped precipitously on its termination. Abraham, Hirsch & Chaikoff (1954), using slices of mammary gland from lactating rats, showed that the yield of $^{14}CO_2$ from [1-^{14}C]glucose was more than 10 times that from [6-^{14}C]glucose and conversely the yield of ^{14}C in lipid was twice as great from C-6 as from C-1. From the ^{14}C yields in lipids they calculated that at least 60% of the glucose was utilized via the pentose pathway. On the basis of the respiratory $^{14}CO_2$ from [1-^{14}C]- compared with [6-^{14}C]-glucose Black, Kleiber, Butterworth, Brubacher & Kaneko (1957) concluded that at least 40% of the total glucose used by the cow is metabolized via the pentose pathway and that the value is higher in specific organs such as the mammary gland. The latter was judged from the yield of ^{14}C in milk constituents including alanine and serine of the casein and glycerol of the fat. The more recent investigations of mammary gland have been reviewed by Glock & McLean (1958), Hansen & Carlson (1961) and Katz (1961).

It was pointed out by Katz & Wood (1960) and Wood, Katz & Landau (1963) that the above calculations which have been made with [1-^{14}C]- and [6-^{14}C]-glucose are not valid because no adjustment was made for the difference in the ^{14}C concentration of the hexose phosphates arising from the two labelled sugars via the pentose cycle. The fructose 6-phosphate arising from [6-^{14}C]-glucose via the cycle still contains the ^{14}C but that from [1-^{14}C]glucose does not, since it is lost as



Scheme 1. Abbreviated scheme of the pentose cycle showing the randomization of carbon atoms occurring in fructose 6-phosphate as a consequence of the pentose cycle. Note that C-1 of glucose 6-phosphate is converted into CO_2 and C-2 is randomized to C-1 and C-3 of the fructose 6-phosphate, whereas C-6 retains the same position in the fructose 6-phosphate. Note also that glyceraldehyde 3-phosphate is both produced and utilized in the pentose cycle. *, ° and ■ indicate ^{14}C from C-1, C-2 and C-6 of the glucose 6-phosphate respectively. DHAP, Dihydroxyacetone phosphate; E-4P, erythrose 4-phosphate; F-6P, fructose 6-phosphate; F-1,6P, fructose 1,6-diphosphate; GA-3P, glyceraldehyde 3-phosphate; G-6P, glucose 6-phosphate; S-7P, sedoheptulose 7-phosphate.

carbon dioxide (Scheme 1). For this reason the role of the pentose cycle was over-estimated in previous determinations (see Katz, 1961).

Katz & Wood (1960) presented a new procedure for the estimation of the pentose cycle which is based on the degree of randomization of ^{14}C which occurs in C-1, C-2 and C-3 of the fructose 6-phosphate formed from [2-^{14}C]glucose via the pentose cycle (Scheme 1). The proportions of ^{14}C in C-1, C-2 and C-3 of the hexose monophosphate or its derivative are used for the calculation. This method has the advantage that it may be used for measurement of the pentose cycle when glucose is utilized by non-triose phosphate pathways, such as lactose synthesis. In contrast the methods based on yields of ^{14}C in carbon dioxide or triose phosphate derivatives from [1-^{14}C]- and [6-^{14}C]-glucose are valid only in the absence of non-triose phosphate pathways (Wood *et al.* 1963; Landau & Katz, 1965). In addition a method of estimating pathways has been developed by Landau, Bartsch, Katz & Wood (1964) that does not require the usual assumption that there is complete equilibration of ^{14}C between the glucose 6-phosphate and fructose 6-phosphate

pools. The relative rate of the hexose phosphate-isomerase (D-glucose 6-phosphate ketol-isomerase, EC 5.3.1.9) reaction is estimated from the difference in the ^{14}C patterns of these two hexose phosphates, which in turn permits estimation of the pentose cycle. This procedure has been successfully applied by Landau & Katz (1964) to the measurement of pathways of glucose metabolism in adipose tissue of rats.

The present study is concerned with the application of the newer methods of estimating the pentose cycle in perfused udders of cows. In addition evidence is presented about the origin of the glucose and galactose moieties of lactose in this tissue.

METHODS

Perfusion of half-udder and collection of milk. Cows which produced at least 10l. of milk daily were obtained from the slaughterhouse. Different cows were used for each experiment, except for Expts. 18 and 19, which were done with the two halves from a single udder. Just before slaughter 10i.u. of oxytocin (Syntocinon; Sandoz A.-G., Basle, Switzerland) was injected intravenously and the cow was milked as completely as possible. The udder was then immediately removed and bisected along the median septum, and one half was connected to the perfusion apparatus. The perfusions were for 120min. at 38° by the method of Peeters & Massart (1952) and Verbeke, Laurys-sens, Peeters & James (1959). Fresh heparin-treated cow's blood (9·5l.) was used as perfusion fluid and the labelled glucose was added to this a few minutes after beginning the perfusion. Throughout the perfusion 0·35M-sodium acetate was added to the reservoir of blood by a constant-drip device at the rate of 2·5ml./min. Milk was collected at the end of the experiment by the injection of oxytocin (10i.u.). The yield of milk varied from 85 to 200ml. The half-udder was then cooled in ice, the skin was removed and the gland was cut in slices, which were frozen.

Isolation of fractions from the milk and tissue. The procedures for the isolation of the fat and lactose were those used by Verbeke *et al.* (1959) and by Wood, Joffe, Gillespie, Hansen & Hardenbrook (1958*a*). The fat was separated from the milk by centrifugation and then the casein was precipitated from the skimmed milk by adjustment to pH4·5. The filtrate was freeze-dried and the lactose was extracted and crystallized as described by Reiss & Barry (1953). Fat from tissue was isolated by extraction of the minced tissue for several days with ethanol–ether (3:1, v/v) in a Soxhlet apparatus. The ethanol–ether was removed by distillation and the fat was taken up in light petroleum (b.p. 35–60°).

Isolation and degradation of the glycerol from fat. The fat was saponified and the tribenzoate was prepared from the resulting glycerol and recrystallized (m.p. 74–76°) as described by Wood *et al.* (1958*a*).

The glycerol was degraded with *Aerobacter aerogenes* as described by Stjernholm & Wood (1958) to obtain the specific activity of the ^{14}C of each of the three carbon atoms. Before fermentation the glycerol was separated from the saponified tribenzoate as described by Wood, Gillespie, Joffe, Hansen & Hardenbrook (1958*b*).

Isolation and degradation of glucose and galactose of the lactose. The procedure was as described by Schambye, Wood & Kleiber (1957), except that the lactose was hydrolysed with 0·1N-HCl for 2hr. at 120° and the HCl was removed with Duolite A-4 resin in the OH^- form. After separation on a cellulose column the glucose and galactose were crystallized and were then fermented with *Leuconostoc mesenteroides*. The glucose moieties from Expts. 13 and 14 were not completely degraded, but C-6 was converted with periodate into formaldehyde, which was then precipitated as the dimedone derivative.

Radioactivity measurements. The ^{14}C radioactivity was measured with a gas-phase proportional counter (Bernstein & Ballentine, 1950), after conversion into CO_2 by combustion according to Van Slyke & Folch (1940). The average micromolar specific activity of a compound was determined by multiplying the specific activity of the CO_2 from combustion by the number of carbon atoms in the compound. The recovery of ^{14}C in the degradations was calculated on the basis of the sum of the specific activities of the individual carbon atoms as compared with the average micromolar specific activity.

The ^3H radioactivity was determined with a Packard scintillation counter, as described by Harrington (1964), by using nearly maximum sensitivity for ^3H but low efficiency for ^{14}C. This total radioactivity was corrected for that part of the counts which was contributed by ^{14}C. The latter was calculated from the ^{14}C radioactivity assayed with the CO_2 gas proportional counter and the factor for the difference in efficiency of the two counters.

Labelled glucose. The different specifically labelled glucoses were obtained from the National Bureau of Standards.

RESULTS

Table 1 shows results of experiments which were done at different times and with udders from different cows. There was considerable variation in the results from udders of different cows and for this reason two experiments were set up (Table 2), with the two halves of the gland from a single cow. The right half was perfused with a mixture of [2-^{14}C]glucose and [6-^3H]glucose and the left half with [6-^{14}C]glucose and [6-^3H]glucose. It was considered that the ^3H of position 6 of glucose might be stable during the formation of glycerol, since the latter is formed directly from triose phosphate. If this were so, the conversion of C-2 and C-6 of glucose into glycerol could be determined in a single experiment by use of [2-^{14}C]glucose and [6-^3H]glucose, thus eliminating the variability between experiments. This was not possible, however, since the ^3H of [6-^3H]glucose was labilized during the conversion into glycerol, as shown by the $^{14}C/^3H$ ratio of Expt. 18, which was 1·38 instead of 1 (Table 2). Therefore only 73% of the ^3H was stable during the conversion into glycerol ($1/1{\cdot}38 = 0{\cdot}73$). Presumably the ^3H was removed by reversible conversion of the triose phosphate into phosphoenolpyruvate and its derivatives. Since the $^{14}C/^3H$ ratio in the glucose

Table 1. *Distribution of* ^{14}C *in the hexose moieties of lactose of milk and in glycerol of fat from tissue of cows' udders perfused with specifically labelled glucose*

[2-^{14}C]Glucose (0·25 mc) was used in Expt. 12 and 0·5 mc of [6-^{14}C]glucose and [1-^{14}C]glucose in Expts. 13 and 14 respectively. Other experimental details are given in the text.

	Sp. activity (counts/min./μmole)		
	[2-^{14}C]Glucose (Expt. 12)	[6-^{14}C]Glucose (Expt. 13)	[1-^{14}C]Glucose (Expt. 14)
Lactose of milk	1200	1710	
Glucose moiety	759	1320	228
Galactose moiety	424	382	54·5
Glycerol of fat from tissue	32·2	232	

Position of ^{14}C		Specific activities (on the basis of 100 for the reference carbon)*					
Hexose	Glycerol	Glucose	Galactose	Glycerol	Galactose	Glycerol	Galactose
C-1	C-3	0·4	30·6	48	18·8	100[5]	100[6]
C-2	C-2	100[1]	100[2]	100[3]	2·4	10·5	3·8
C-3	C-1	0·7	18·8	37	1·8	7·1	8·1
C-4		0·002	0·7		0·8		0·6
C-5		0·01	4·9		0·7		0·6
C-6		0·10	0·7		100[4]		4·3

* Specific activities of the carbon atoms assigned a value of 100 were (in counts/min./μmole): 1 = 717, 2 = 264, 3 = 15·5, 4 = 292, 5 = 182·7, 6 = 47·6. The recovery of ^{14}C in the degradation products was: Expt. 12, glucose moiety, 96%, galactose moiety, 97%, and glycerol, 101%; Expt. 13, galactose moiety, 95%, and glycerol, 103% (C-6 of the glucose moiety contained 1250 counts/min./μmole); Expt. 14, galactose moiety, 103% (C-6 of the glucose moiety contained 1·29 counts/min./μmole). There was some dilution (~ 10%) during the glycerol fermentation because of formation of endogenous lactate by the bacteria (Stjernholm & Wood, 1958). The specific activity for the reference carbon and the recovery of ^{14}C in the degradation of glycerol are on the basis of the lactate recovered from the fermentations.

Table 2. *Distribution of* ^{14}C *and* ^{3}H *in the hexose moieties of the lactose of milk and in the glycerol of the fat from the tissue and from the milk obtained by perfusion of a cow's udder with* ^{14}C- *and* ^{3}H-*labelled glucose*

Expt. 18 was with the left half of the udder (3·15 kg.). [6-^{14}C]Glucose (0·25 mc) and [6-^{3}H]glucose (2·5 mc) were added to the 9·5 l. of blood used for the perfusion. The yield of milk was 113 ml. Expt. 19 was with the right half of the same udder (2·90 kg.). [2-^{14}C]Glucose (0·25 mc) and [6-^{3}H]glucose (2·5 mc) were added to the 9·5 l. of blood for the perfusion. The yield of milk was 86 ml. In each case the blood contained approx. 5 g. of glucose at the onset of perfusion and 1 g. at the conclusion. Perfusion was for 120 min. at 38°, as described in the text. The cow had been producing 14 l. of milk daily.

	[6-^{14}C]- and [6-^{3}H]-Glucose (Expt. 18)			[2-^{14}C]- and [6-^{3}H]-Glucose (Expt. 19)			
	^{14}C (counts/min./μmole) (*A*)	^{3}H (counts/min./μmole) (*B*)*	Ratio $^{14}C/^{3}H$ (*A*/*B*)	^{14}C (counts/min./μmole) (*C*)	^{3}H (counts/min./μmole) (*D*)*	Ratio $^{14}C/^{3}H$ (*C*/*D*)	$\frac{^{14}C \text{ from 2-}^{14}C}{^{14}C \text{ from 6-}^{14}C}$ (*C*/*A*)
Lactose of milk	1411	1191	1·18	1328	1130	1·17	0·94
Glucose moiety	934	916	1·02	941	924	1·02	1·01
Galactose moiety	347	304	1·14	407	319	1·28	1·17
Fat of tissue: glycerol	208	151	1·38	22·1	150	0·15	0·11
Fat of milk: glycerol	1·56	1·05	1·48	0·35	1·44	0·24	0·22

* Both of the original glucose mixtures gave 2·33 times the counts/min./μmole for ^{3}H radioactivity as for ^{14}C radioactivity. The tritium values have been adjusted for this difference by dividing the ^{3}H counts/min./μmole by 2·33 to make the ^{14}C and ^{3}H values equivalent.

Table 3. *Distribution of ^{14}C in the hexose moieties of lactose and the glycerol of fat obtained from a cow's udder perfused with ^{14}C- and ^{3}H-labelled glucose*

Experimental details are given in the legend to Table 2 and in the text. Values are specific activities of ^{14}C on the basis of 100 for the reference carbon.*

Position of ^{14}C		[6-^{14}C]- and [6-^{3}H]-Glucose (Expt. 18)			[2-^{14}C]- and [6-^{3}H]-Glucose (Expt. 19)		
Hexose	Glycerol	Glucose	Galactose	Glycerol	Glucose	Galactose	Glycerol
C-1	C-3	0·2	9·8	100[3]	0·4	37·0	44·7
C-2	C-2	0·8	2·1	8·2	100[4]	100[5]	100[6]
C-3	C-1	0·3	1·2	3·4	1·2	24·6	47·7
C-4		0·2	0·7		0·3	0·6	
C-5		0·1	0·4		0·6	1·1	
C-6		100[1]	100[2]		1·2	1·1	

* Specific activities of the carbon atoms assigned a value of 100 were (in counts/min./μmole): 1 = 904, 2 = 281, 3 = 160, 4 = 880, 5 = 241·5, 6 = 9·95. The recovery of ^{14}C in the products of degradation was 98, 95, 98, 95, 98 and 95% respectively in these experiments and was calculated as described in Table 1.

moiety of lactose was 1·02 in both Expts. 18 and 19 very little labilization of the ^{3}H occurred during the conversion of the [6-^{3}H]glucose into the glucose moiety.

The last column of Table 2 shows the ratios of the ^{14}C from [2-^{14}C]glucose and [6-^{14}C]glucose respectively incorporated into the products. The ratio was 1·01 for the glucose moiety of lactose and somewhat more than 1·0 for the galactose. The ratio for the lactose was 0·94 whereas on the basis of the glucose and galactose it should have been somewhat greater than 1·0. Perhaps the lactose value is in error since the sum of the glucose plus galactose specific activities for Expt. 18 is 1281 but the observed value for lactose was 1411. The same ^{14}C ratios for the glycerol of both tissue and milk were very low. This finding is considered in the Discussion section. The ^{14}C value of the glycerol from milk was so low that it is subject to considerable error.

The penultimate column of Table 2 presents the $^{14}C/^{3}H$ ratio of Expt. 19. If there had been no labilization of ^{3}H and Expts. 18 and 19 were exact duplicates then this ratio and the ratio of the last column should be identical. The ratios are the same for the glucose moiety and they also are fairly close for galactose and glycerol. Therefore Expts. 18 and 19 were reasonably good duplicates. This is also indicated by the close agreement of the specific activities of the corresponding hexose moieties in the two experiments and the ^{3}H values for the glycerol.

The distributions of ^{14}C in the hexose moieties of the lactose and in the glycerol from the fat of the tissue are shown in Tables 1 and 3. It can be seen that the glucose moieties retained ^{14}C almost exclusively in the position which was labelled in the administered substrate whereas there was considerable randomization of ^{14}C in the galactose moiety. There was extensive randomization in the glycerol from [2-^{14}C]glucose and also some in that from [6-^{14}C]glucose.

DISCUSSION

Estimation of the pentose cycle from the distribution of ^{14}C in a hexose requires the assumption that this distribution accurately reflects that of the glucose 6-phosphate. Similarly if glycerol is to be used for estimation of the rate of the hexose phosphate-isomerase reaction (Landau *et al.* 1964), its ^{14}C distribution must reflect that of C-1, C-2 and C-3 of the fructose 6-phosphate. The validity of these assumptions will be considered first and then the actual calculations.

Free glucose as the precursor of the glucose moiety of lactose. The glucose portion of lactose arises from free (unesterified) glucose and consequently does not reflect the changes of ^{14}C patterns that occur during the interconversions of the hexose phosphate esters. Evidence for this mechanism was obtained by Wood, Schambye & Peeters (1957), Wood, Siu & Schambye (1957) and Wood *et al.* (1958*a*) and the results of Tables 1 and 3 are in complete accord. The glucose moiety has the same ^{14}C pattern as the administered glucose whereas the ^{14}C was extensively randomized in the galactose. In addition the specific activity of the galactose moiety was less than that of the glucose moiety, apparently because the ^{14}C was subject to dilution in the metabolic pools of hexose phosphate esters, whereas that of the glucose moiety was not.

The greater incorporation of ^{14}C into the galactose from [2-^{14}C]glucose than from [6-^{14}C]glucose (ratio 1·17, Table 2) can be explained by the

observations of Katz & Wood (1960). They showed that the total ^{14}C in the hexose phosphates derived from [2-^{14}C]glucose is increased by the pentose cycle whereas that from [6-^{14}C]glucose remains unaltered by the cycle. The 1·01 ratio for the glucose moiety is in accord with these conclusions since the free glucose is not subject to these changes.

Final evidence of the role of free glucose comes from the work of Watkins & Hassid (1962) and Babad & Hassid (1964). These authors observed synthesis of lactose from UDP-galactose and free glucose with particulate fractions from guinea-pig and bovine mammary glands and recently with soluble enzymes obtained from milk.

Galactose as a derivative of glucose 6-phosphate. The galactose unit of the lactose is believed to arise from glucose 6-phosphate via glucose 1-phosphate, UDP-glucose and UDP-galactose. Its ^{14}C pattern therefore should reflect that of the glucose 6-phosphate which occurs as an intermediate of the Embden–Meyerhof pathway and pentose cycle. The evidence on this point is ambiguous, however, and requires consideration. Hansen, Wood, Peeters, Jacobson & Wilken (1962) isolated glucose 6-phosphate, UDP-glucose, UDP-galactose and lactose from the tissue of a cow's udder perfused for 12min. with blood containing a mixture of labelled [1,3-^{14}C]glycerol and [2-^{14}C]glucose. The ^{14}C patterns in the galactose of the lactose and of the UDP-glucose were quite similar and were in accord with a precursor–product relationship, but the ^{14}C pattern of the glucose 6-phosphate differed from that of the galactose and had a much lower specific activity than the nucleotide hexoses. This discrepancy was explained by proposing that the glucose 6-phosphate isolated from the tissue had at least two origins. One part, which turned over slowly, acquired relatively little ^{14}C and was from the non-secretory tissue of the gland, and the second part, which was the precursor of the galactose, was from the secretory cells and had high activity. Accordingly measurements based on the ^{14}C-distribution patterns in the galactose of lactose would reflect the ^{14}C pattern of the glucose 6-phosphate of those cells involved in the secretion of lactose and therefore the estimated pentose cycle would apply only to the secretory cells. Nevertheless such estimates must reflect a major part of the glucose metabolized by the udder since the formation of lactose and casein represents a major part of the metabolism of glucose *in vivo* by the cow (Kleiber *et al.* 1955).

Distribution of ^{14}C in glycerol. The calculations from the ^{14}C patterns of the glycerol involve the ratios of ^{14}C in C-1 and C-2 and also C-3 and C-2, and therefore simple dilution by unlabelled compounds does not affect the results. Randomization of ^{14}C by reversible conversion of the triose phosphates into Krebs-cycle intermediates would cause an error, however, since the ^{14}C would no longer reflect the distribution in positions C-1, C-2 and C-3 of the fructose 6-phosphate. [6-^{14}C]-Glucose may be used to test for this randomization since in the absence of randomization the ^{14}C should be confined to C-3 of glycerol. Landau & Katz (1964) found this to be true for adipose tissue and therefore were able to use the ^{14}C patterns of glycerol for their calculations. In our case the glycerol from [6-^{14}C]glucose of Expts. 13 and 18 did contain considerable ^{14}C in C-2 and C-1 and there was more in C-2 than in C-1, as would be expected if there were reversible conversion of [3-^{14}C]triose phosphate into a C_4 symmetrical dicarboxylic acid. These reactions are also indicated by the labilization of ^{3}H from [6-^{3}H]glucose during its conversion into glycerol. Therefore the greater randomization of ^{14}C of [2-^{14}C]glucose in the glycerol than in positions C-1, C-2 and C-3 of the galactose probably results from randomization involving the C_4 dicarboxylic acids, and not from incomplete equilibration of ^{14}C by the hexose phosphate-isomerase reaction.

It was necessary in these experiments to use glycerol from the fat of the tissue for the degradations since the fat of the milk acquired very little ^{14}C during a 2hr. perfusion. The glycerol of the fat from the milk of Expt. 13 contained enough ^{14}C to permit degradation and the distribution found was: C-1, 2; C-2, 2; C-3, 100; this showed that there was very little randomization of ^{14}C. Therefore if the glycerol from milk labelled by [2-^{14}C]-glucose could have been degraded it would have provided values for calculation of the rate of the hexose phosphate-isomerase reaction.

A number of investigators have observed that glycerol of the milk fat acquires ^{14}C at a later time than does the lactose. Popják, Glascock & Folley (1952) suggested that the time-sequence is a consequence of a precursor–product relationship in which glycerol is produced from glucose. The glucose (as reflected in the lactose) thus becomes labelled first, and subsequently the glycerol. When similar observations were made by Wood *et al.* (1958*a*) they suggested that the difference in time of appearance of ^{14}C in the components of milk might arise because these compounds are secreted by different types of cells, fat by an apocrine type and the lactose and casein by a merocrine type of secretion. Folley & McNaught (1961) have reaffirmed the view that the time-sequence is the result of a precursor–product sequence; but their argument does not seem convincing because the times involved in the labelling are in hours, whereas immediate compounds of tissues become labelled in a few seconds or minutes, as observed by Hansen *et al.* (1962) for the UDP-hexoses.

Estimation of the metabolism by the pentose cycle. If complete equilibration by the hexose phosphate-isomerase reaction is assumed, the pentose cycle (PC) may be estimated by use of the following equations, which correspond to equations 6 and 7 of Wood *et al.* (1963):

$$\frac{\text{Sp. activity of C-1 of hexose 6-P}}{\text{Sp. activity of C-2 of hexose 6-P}} = \frac{2\,PC}{1+PC} \quad (1)$$

and

$$\frac{\text{Sp. activity of C-3 of hexose 6-P}}{\text{Sp. activity of C-2 of hexose 6-P}} = \frac{PC}{1+PC} \quad (2)$$

PC of these equations is the fraction of glucose metabolized by the pentose cycle and a value of 0·3 is equivalent to 30% metabolism by this pathway. From the C-1/C-2 and C-3/C-2 ratios of ^{14}C of the galactose moiety of lactose of Expt. 12, PC is 0·221 by eqn. (1) and 0·235 by eqn. (2); the corresponding values for Expt. 19 are 0·294 and 0·326. Thus within a given experiment there was close agreement between the values obtained by the two equations. Evidently the distribution patterns were not seriously distorted by the randomizations which can occur via reversible transketolase and transaldolase exchange reactions as discussed by Wood & Katz (1958). If the equilibration of ^{14}C by the hexose phosphate-isomerase reaction was not complete the present estimated value of the pentose cycle would be lower than the true value since the glucose 6-phosphate derivative would not reflect the total randomization that had occurred in the fructose 6-phosphate.

It can be shown (Wood *et al.* 1963; Landau *et al.* 1964) that there is very little error in the determination of the pentose cycle with [2-^{14}C]glucose when the rate of reversal of the hexose phosphate-isomerase reaction from fructose 6-phosphate to glucose 6-phosphate is about five times that of the utilization of glucose. Hexose phosphate isomerase is very active in most tissues and it thus seems likely that the determined values may be close to the true values.

Ratio of the yields of ^{14}C in the glycerol and estimation of the Embden–Meyerhof and non-triose phosphate pathways. Katz & Wood (1960) derived the equation shown below for estimation of pathways from the yields of ^{14}C in a triose phosphate derivative:

$$\frac{^{14}C \text{ in triose P derivative from [1-}^{14}C\text{]glucose}}{^{14}C \text{ in triose P derivative from [6-}^{14}C\text{]glucose}} = \frac{(EM)Q}{EM+PC} \quad (3)$$

EM and PC are the fractions of the glucose metabolized by Embden–Meyerhof pathway and pentose cycle respectively and Q is a factor to correct for the difference in the ^{14}C content of the hexose 6-phosphate pool arising from [1-^{14}C]glucose as compared with that from [6-^{14}C]glucose. Eqn. (3) is not strictly accurate when there is a non-triose phosphate pathway. Although there is net formation of glyceraldehyde 3-phosphate in the pentose cycle there also is utilization of triose phosphate in the cycle, since 2 mol. of triose phosphate is produced while only 1 mol. is being utilized (Scheme 1). This utilization of glyceraldehyde 3-phosphate was not considered in the derivation of eqn. (3) and therefore it is not correct for all circumstances. The equation was derived on the basis that triose phosphate is utilized only to form derivatives such as glycerol, fatty acids etc., but when there is a non-triose phosphate pathway the triose phosphate utilized in the pentose cycle can be converted into non-triose phosphate derivatives such as lactose. It is adequate, however, if there are no non-triose phosphate pathways because under these circumstances the net flow of ^{14}C is entirely to carbon dioxide and triose phosphate derivatives, as was assumed for the derivation. Nevertheless eqn. (3) can be used to provide an approximation of the Embden–Meyerhof pathway even in the presence of non-triose phosphate pathways when the value of the pentose cycle is known. In the present experiments the ^{14}C was determined in triose phosphate derivatives from [2-^{14}C]- and [6-^{14}C]-glucose instead of [1-^{14}C]- and [6-^{14}C]-glucose. It has been shown by Katz & Wood (1960) that when the pentose cycle is 30% the hexose 6-phosphates have a higher ^{14}C content from [2-^{14}C]glucose than from [6-^{14}C]glucose, being 1·10 times as high. Therefore, when [2-^{14}C]glucose is used instead of [1-^{14}C]glucose, 1·10 is substituted for Q in eqn. (3). The ratio of ^{14}C in glycerol of fat from [2-^{14}C]-glucose to that from [6-^{14}C]glucose was 0·22 in the milk and 0·11 in the tissue (see Table 2). Therefore $0{\cdot}22 = EM(1{\cdot}10)/(EM+0{\cdot}3)$, from which $EM = 0{\cdot}075$. Similarly, from the ratio 0·11 of the glycerol of tissue, EM is found to be 0·03. The non-triose phosphate pathways are calculated by difference and $1-EM-PC = 1-0{\cdot}08-0{\cdot}30 = 0{\cdot}62$. Accordingly in Expt. 19 it is calculated that 30% of the glucose was utilized via the pentose cycle, 8% via the Embden–Meyerhof pathway and 62% for synthesis of lactose by non-triose phosphate pathways.

The view that there was very little metabolism of glucose by the Embden–Meyerhof pathway finds support from another source. The Embden–Meyerhof pathway would convert C-1, C-2 and C-3 of the hexose into triose phosphate. The ^{14}C then would be incorporated in C-4, C-5 and C-6 of the fructose 6-phosphate because of utilization of glyceraldehyde 3-phosphate in the pentose cycle as

illustrated in Scheme 1. Thus ^{14}C from [2-^{14}C]glucose would have been introduced into C-5 of the galactose moiety. In Expt. 19 there was very little ^{14}C incorporated into the C-5 position and therefore little use of the Embden–Meyerhof pathway. In Expt. 12 there may have been more use of the Embden–Meyerhof pathway since there was some incorporation of ^{14}C into C-5 of galactose. In contrast with these results, Landau & Katz (1964) found in rat adipose tissue that a substantial amount of ^{14}C from [2-^{14}C]glucose was introduced into C-5 of the glucose unit of glycogen. They calculated that 23% of the glucose was metabolized by the pentose cycle and most of the remainder via the Embden–Meyerhof pathway. The metabolism of glucose by ruminants probably differs from that of non-ruminants (Folley & McNaught, 1961). In ruminants acetate is formed in the rumen and is reduced to fats with NADPH arising from the pentose cycle of the udder, whereas in non-ruminants this source of acetate is not available and a major part must be produced in the gland by the Embden–Meyerhof pathway. Consequently the latter pathway is prominent in the gland of non-ruminants (J. Katz & R. Rognstad, unpublished work with rat mammary gland).

There was ^{14}C from [6-^{14}C]glucose in C-1 of galactose in both Expts. 13 and 18. This can occur by conversion of [6-^{14}C]glucose into glyceraldehyde 3-phosphate via the pentose cycle and the triose phosphate can then enter the hexose via synthesis through the Embden–Meyerhof pathway. Apparently there was synthesis by the Embden–Meyerhof pathway although there was little breakdown of glucose by this pathway.

Comparison of experiments in vivo *and* in vitro. The present experiments are in accord with experiments *in vivo* by others which likewise indicate that a large part of the glucose is converted into lactose. For example, Kleiber *et al.* (1955) found that 50% of the ^{14}C administered intravenously as [U-^{14}C]glucose was recovered in lactose of milk from cows.

There also is evidence from experiments *in vivo* that there is a substantial pentose cycle. When [1-^{14}C]acetate or $NaH^{14}CO_3$ was injected intravenously into cows (Schambye *et al.* 1957) the glucose moiety was labelled predominantly in C-3 and C-4, and presumably arose via blood glucose which was synthesized in the liver. In the galactose moiety there was considerable ^{14}C in C-1 and C-2 in addition to that in C-3 and C-4. By using the C-2/C-3 and C-1/C-3 ^{14}C ratios, and equations corresponding to (1) and (2), Katz (1961) estimated the pentose cycle to be 30 and 40% for the experiment with [1-^{14}C]acetate and 45 and 47% with $NaH^{14}CO_3$. The values are probably too high or the gland itself because the calculations did not take into account the small amount of randomization of ^{14}C which had occurred previously in the liver as was reflected in the glucose moiety. Other data were considered by Katz (1961) but the data cited above are probably the most reliable since the values calculated from the two equations were in better agreement than those from the other experiments.

Thanks are due to Dr Rune Stjernholm and Dr Bernard R. Landau for their assistance in the determination of the ^{3}H radioactivity and to Mr Sam Zito for measurement of the ^{14}C radioactivity. This investigation was supported by Contract AT-30-1(1320) from the Atomic Energy Commission and by the Belgian IRSIA Foundation.

REFERENCES

Abraham, S., Hirsch, P. F. & Chaikoff, I. L. (1954). *J. biol. Chem.* **224**, 955.

Babad, H. & Hassid, W. Z. (1964). *J. biol. Chem.* **239**, PC946.

Bernstein, W. & Ballentine, R. (1950). *Rev. sci. Instrum.* **21**, 158.

Black, A. L., Kleiber, M., Butterworth, E. M., Brubacher, G. B. & Kaneko, V. I. (1957). *J. biol. Chem.* **227**, 537.

Folley, S. J. & McNaught, M. L. (1961). In *Milk: The Mammary Gland and its Secretion*, vol. 1, p. 441. Ed. by Kon, S. K. & Cowie, A. T. New York and London: Academic Press Inc.

Glock, G. E. & McLean, P. (1953). *Biochim. biophys. Acta*, **12**, 590.

Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.

Glock, G. E. & McLean, P. (1958). *Proc. Roy. Soc.* B, **149**, 354.

Hansen, R. G. & Carlson, D. M. (1961). In *Milk: The Mammary Gland and its Secretion*, vol. 1, p. 371. Ed. by Kon, S. K. & Cowie, A. T. New York and London: Academic Press Inc.

Hansen, R. G., Wood, H. G., Peeters, G. J., Jacobson, B. & Wilken, J. (1962). *J. biol. Chem.* **237**, 1034.

Harrington, H. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 59.

Katz, J. (1961). In *Radioactive Isotopes in Physiology, Diagnostics and Therapy*, vol. 1, p. 705. Ed. by Schweigt, H. & Turba, F. Berlin, Göttingen and Heidelberg: Springer-Verlag.

Katz, J. & Wood, H. G. (1960). *J. biol. Chem.* **235**, 2165.

Kleiber, M., Black, A., Brown, M. A., Baxter, C. F., Luick, J. R. & Stadtman, F. H. (1955). *Biochim. biophys. Acta*, **17**, 252.

Landau, B. R., Bartsch, G. E., Katz, J. & Wood, H. G. (1964). *J. biol. Chem.* **239**, 686.

Landau, B. & Katz, J. (1964). *J. biol. Chem.* **239**, 697.

Landau, B. R. & Katz, J. (1965). In *Handbook of Physiology*, section 5, p. 253. Ed. by Renold, A. E. & Cahill, D.S., jun. Washington, D.C.: American Physiological Society.

Peeters, G. & Massart, L. (1952). *Arch. int. Pharmacodyn.* **91**, 380.

Popják, G., Glascock, R. F. & Folley, S. J. (1952). *Biochem. J.* **52**, 472.

Reiss, O. K. & Barry, J. M. (1953). *Biochem. J.* **55**, 783.
Schambye, P., Wood, H. G. & Kleiber, M. (1957). *J. biol. Chem.* **226**, 1011.
Stjernholm, R. & Wood, H. G. (1958). *Arch. Biochem. Biophys.* **78**, 28.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Verbeke, R., Lauryssens, M., Peeters, G. & James, A. T. (1959). *Biochem. J.* **73**, 24.
Watkins, W. M. & Hassid, W. Z. (1962). *J. biol. Chem.* **237**, 1432.
Wood, H. G., Gillespie, R., Joffe, S., Hansen, R. G. & Hardenbrook, H. (1958*b*). *J. biol. Chem.* **233**, 1271.
Wood, H. G., Joffe, S., Gillespie, R., Hansen, R. G. & Hardenbrook, H. (1958*a*). *J. biol. Chem.* **233**, 1264.
Wood, H. G. & Katz, J. (1958). *J. biol. Chem.* **233**, 1279.
Wood, H. G., Katz, J. & Landau, B. R. (1963). *Biochem. Z.* **338**, 809.
Wood, H. G., Schambye, P. & Peeters, G. J. (1957). *J. biol. Chem.* **226**, 1023.
Wood, H. G., Siu, P. & Schambye, P. (1957). *Arch. Biochem. Biophys.* **69**, 390.