ON THE USE OF [^{3}H, ^{14}C]LABELLED GLUCOSE IN THE STUDY OF THE SO-CALLED "FUTILE CYCLES" IN LIVER AND MUSCLE

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Received April 10,1974

SUMMARY : High speed supernatants of liver and muscle extracts, filtered on a column of Sephadex G-25, were incubated with glucose 6-phosphate labelled with tritium in position 2,3,5 or 6, and uniformly with ^{14}C. With liver filtrates, there was a preferential loss of tritium in positions 2 and 5, relative to ^{14}C. With muscle filtrates, only the tritium in position 2 was lost. These results indicate that hydrogen exchange in position 5 of fructose 6-phosphate can occur in the liver without conversion to fructose diphosphate by phosphofructokinase, being catalyzed by transaldolase and triose phosphate isomerase. A study of the half-life of [3-^{3}H]glucose in the blood of normal fed mice gave no evidence in favor of the operation of a futile cycle between fructose 6-phosphate and fructose diphosphate in the liver *in vivo*.

Katz and Dunn (1) have reported that the half-life of [2-^{3}H] glucose in normal rats is 1.5 times shorter than that of [U-^{14}C]glucose. This phenomenon has also been observed in human subjects, but not in patients deficient in glucose 6-phosphatase (2, 3). A selective loss of tritium relative to carbon was also observed in mice with [4-^{3}H]glucose, although less pronounced than with [2-^{3}H]glucose (4). These data indicate that glucose is recycled in the body by a sequence of reactions leading to glucose 6-phosphate and in which tritium atoms in positions 2 and 4 are lost, at least in part, either by direct metabolic conversion or by exchange reaction. The loss of tritium in position 2 most likely occurs at the level of hexose 6-phosphate isomerase (1) whereas that in position 4 has been tentatively explained by exchange reactions involving triose phosphate formation from the lower half of fructose 6-phosphate by transaldolase (4). The Cori cycle is also responsible for a part of the tritium depletion and can be evaluated by the loss of tritium in position 6 of glucose (5).

Recently, Clark *et al.* (6) observed a selective loss of tritium relative to carbon in isolated liver cells incubated with

[2-^{3}H] or [5-^{3}H]glucose. The use of this cellular preparation has the great advantage of eliminating the participation of the Cori cycle. The loss of tritium in position 5 was about 60 % of that in position 2 and was believed to occur at the level of the triose phosphates, after conversion of glucose to fructose diphosphate. The authors came to the conclusion that "futile" cycles occur between glucose and glucose 6-phosphate and between fructose 6-phosphate and fructose diphosphate. Another group of workers also concluded from investigations with [5-^{3}H]glucose that there exists a recycling between fructose 6-phosphate and fructose diphosphate in the flight muscle of *Bombus affinis* when exposed to low temperature (7), in the skeletal muscle of susceptible pigs intoxicated with halothane (8), and in rat liver, *in vivo* (9).

In the present paper it is shown that the loss of tritium from position 5 of fructose 6-phosphate in the liver is explained by the operation of transaldolase and triose phosphate isomerase. The observation of Clark *et al.* (6) is therefore in direct support of a recycling of glucose up to the level of glucose 6-phosphate although not to the level of fructose diphosphate.

MATERIAL AND METHODS

[^{3}H] and [^{14}C]glucose, purchased from the Radiochemical Centre, Amersham, was converted to glucose 6-phosphate by incubation with ATP-Mg and hexokinase (4). Erythrose 4-phosphate and purified yeast transaldolase were purchased from Boehringer G.m.b.H., and other reagents from Sigma Chemical Company. Liver and muscle from fed mice were homogenized with 4 vol. 0.05 M glycylglycine buffer, pH 7.1, and centrifuged for 30 min at 150,000 x *g* (liver) or for 15 min at 10,000 x *g* (muscle). A part of the supernatant was filtered through a Sephadex G-25 column equilibrated with the same buffer. All these manipulations were performed in the cold. Filtrated and non-filtrated preparations were incubated with doubly labelled glucose 6-phosphate as indicated in Table I. At the end of the incubation, glucose 6-phosphate was isolated and counted as previously described (4). Hexose 6-phosphate isomerase (10) and transaldolase (11) activities were assayed at 37°C in the filtered preparations. The methods used for measuring the decay of doubly labelled glucose in mice have been reported (4).

RESULTS

Table I shows the loss of tritium relative to ^{14}C that occurred when doubly labelled glucose 6-phosphate was incubated with

Table I. *Loss of ^{3}H relative to ^{14}C from doubly labelled glucose 6-phosphate in vitro.* 0.5 ml of tissue preparation was incubated at pH 7.1 and 37 °C with 5 µmoles of doubly labelled glucose 6-phosphate (G-6-P) in a final volume of 1 ml. The duration of the incubation varied from 20 to 60 min and the amount of tissue from 20 to 100 mg per test, according to the velocity of tritium loss. ATP-Mg : 5 mM ATP, 5 mM Mg Acetate; E4P : 0.5 mM erythrose 4-phosphate; TA : purified yeast transaldolase, 3 U/g of tissue; "filtrated" and "not filtrated", refer to tissue preparations that were, or not, filtered through a Sephadex G-25 column. The values shown are means ± SEM, with the number of determinations in parentheses. All values below 0.1 µmole/min per g are not significantly different from zero. It has been checked that no loss of tritium occurred in the presence of transaldolase and erythrose 4-phosphate alone.

Labelling of G-6-P	Tissue	Additions	G-6-P deprived of ^{3}H (µmoles/min per g of tissue) "filtrated"	"not filtrated"
2-^{3}H,U-^{14}C	Liver	None	5.10 ± 0.27 (5)	6.55 ± 0.10 (4)
"	Muscle	None	9.16 ± 1.12 (4)	9.90 ± 0.71 (4)
3-^{3}H,U-^{14}C	Liver	None	0.07 ± 0.05 (6)	0.05 ± 0.02 (6)
"	Liver	ATP-Mg	1.01 ± 0.12 (3)	1.01 ± 0.26 (3)
"	Muscle	None	0.03 ± 0.02 (6)	0.63* ± 0.26 (7)
"	Muscle	ATP-Mg	2.08 ± 0.43 (3)	2.20 ± 0.61 (3)
5-^{3}H,U-^{14}C	Liver	None	2.14 ± 0.15 (7)	3.11 ± 0.31 (6)
"	Liver	E4P	3.11 ± 0.03 (3)	3.24 ± 0.47 (3)
"	Muscle	None	0.06 ± 0.03 (7)	1.04* ± 0.30 (6)
"	Muscle	E4P+TA	3.96 ± 0.55 (3)	3.98 ± 0.55 (3)
6-^{3}H,U-^{14}C	Liver	None	0.05 ± 0.01 (4)	0.09 ± 0.02 (4)
"	Muscle	None	0.01 ± 0.01 (4)	0.04 ± 0.01 (4)

*Values not significantly different from each other.

liver or muscle extracts filtered or not on Sephadex G-25. A loss of tritium in position 2 occurred with both types of preparation and was not influenced by gel filtration. It was 1.5 to 1.8-fold more rapid in muscle than in liver.

Tritium in position 3 was stable in filtered preparations of both liver and muscle and in non-filtered liver extracts but not in non-filtered muscle extracts. A loss of tritium in position 3 occurred in all preparations upon addition of ATP and magnesium, being then about twice faster in muscle than in liver.

Tritium in position 5 was unstable in filtered or non-filtered liver extract. The rate of exchange was increased in the filtrate by the addition of erythrose 4-phosphate; it varied from 40 to 60 % of

that in position 2. No loss of tritium in position 5 occurred in filtered muscle extract unless transaldolase and erythrose 4-phosphate were added. In the non-filtered muscle preparation, the loss in position 5 was comparable to that in position 3.

Tritium in position 6 was stable in filtered and non-filtered liver and muscle extracts.

Table II shows that the activity of hexose 6-phosphate isomerase was 1.8 times as high in muscle as in liver, whereas that of transaldolase was 40 times higher in liver than in muscle.

Table II. *Activities of hexose 6-phosphate isomerase and transaldolase in liver and muscle from normally fed mice.* Activities are expressed in μmoles of substrate converted in one min per 1 g of tissue at 37 °C. Values given are means ± SEM, with the number of experiments in parentheses.

Tissue	Hexose 6-phosphate isomerase	Transaldolase
Liver	104 ± 8 (4)	3.71 ± 0.4 (4)
Muscle	177 ± 12 (4)	0.095 ± 0.002 (4)

We have also compared the half-life of glucose labelled with ^{3}H in various positions to that of [^{14}C]glucose in the blood of mice *in vivo*. Fig. 1 shows that the loss of tritium in position 3 was similar to that in position 6, which is taken as a measure of the Cori cycle (5). The amount of tritium lost from position 5 in excess of that from position 6, was about 40 % of that from position 2.

DISCUSSION

The loss of tritium in position 2 of glucose 6-phosphate

An exchange of the hydrogen in position 2 of glucose 6-phosphate with the protons of the medium is known to be catalyzed by hexose 6-phosphate isomerase (12). The isomerization of hexose phosphates is considered to occur by an intramolecular hydrogen transfer with variable rates of hydrogen exchange. The ratio of exchange to transfer increases with temperature, and also depends on the source of enzyme. In this study, the ratio of muscle to liver activities was around 1.8

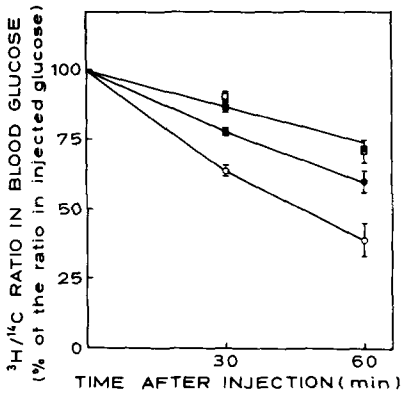

Fig.1 - *The $^3H/^{14}C$ ratio of blood glucose after injection of [2-^3H,U-^{14}C]glucose (○), [3-^3H,U-^{14}C]glucose (■), [5-^3H,U-^{14}C] glucose (●) and [6-^3H,U-^{14}C]glucose (□).* Trace amounts of [^{3}H] glucose (200 µC/kg of body weight) together with [^{14}C]glucose (80 µC/kg of body weight) were injected in the tail vein of mice. Values shown are means of at least 4 animals; vertical bars represent ± SEM. The values obtained with [3-^{3}H]glucose are not statistically different from the ones with [6-^{3}H]glucose.

for the exchange reaction (Table I) as for the overall conversion (Table II). It is also known (12) that, due to an important isotopic effect, the absolute values of tritium exchange (Table I) or transfer are several fold lower than the rate of conversion of non-labelled substrate (Table II).

The absolute values shown in Table I, reaching 6 to 7 micromoles of glucose 6-phosphate deprived of tritium per g of liver in one min are markedly higher than the usual flow of metabolites in the liver (1 to 2 µmoles/min per g). However, these values have been obtained in the presence of a saturating amount of glucose 6-phosphate (5 mM), whereas the concentration of this ester in the liver (0.1 to 0.2 mM) is well below the Km of hexose 6-phosphate isomerase (0.7 mM according to ref. 10).

The loss of tritium in positions 3 and 5 of glucose 6-phosphate

In a liver extract that has been deprived of ATP and of free magnesium by filtration on a Sephadex column, phosphorylation of fructose 6-phosphate and dephosphorylation of fructose diphosphate does not occur. Even in an unfiltered extract, these reactions require the addition of magnesium to be detectable. Thus the loss of tritium in position 5 of glucose 6-phosphate that occurs in these preparations cannot be attributed to a futile recycling between fructose 6-phosphate and triose

phosphates. The absence of tritium loss from position 3 of glucose 6-phosphate is another proof that such a recycling does not occur under these conditions.

Our observations are adequately explained by the activity of transaldolase, through which the lower half of fructose 6-phosphate is converted to glyceraldehyde phosphate whereas the upper half is unchanged. The velocity of the tritium loss from position 5 (Table I) in liver extracts corresponds to the activity of transaldolase (Table II). Muscle tissue has been reported not to contain transaldolase (13) and we have detected only a small amount of activity (Table II); accordingly, no loss of tritium in position 5 of glucose 6-phosphate occurred in the presence of a muscle filtrate deprived of ATP, unless transaldolase and erythrose 4-phosphate were added. Exchange reaction through transaldolase requires the presence of a catalytic amount of erythrose 4-phosphate. This amount could be present in a liver Sephadex filtrate, being possibly bound to transaldolase itself. It could also be formed by the combined action of transaldolase and transketolase on fructose 6-phosphate, as proposed by Pontremoli *et al.* (14). The reaction was speeded up by the addition of erythrose 4-phosphate and was also faster in a liver extract than in the filtrate. However, since erythrose 4-phosphate is a potent inhibitor of hexose 6-phosphate isomerase (15), experiments with this tetrose phosphate are not unambiguous. The rate of tritium loss from position 5 was between 40 and 60 % of that from position 2, in liver preparations.

A loss of tritium in position 3 occurred in non-filtered muscle extracts and also in liver or muscle filtrates enriched with ATP and magnesium, indicating that, under these conditions, a recycling between fructose 6-phosphate and fructose diphosphate occurred *in vitro*. A loss of tritium from position 3 of glucose 6-phosphate relative to ^{14}C can also occur *in vivo* during recycling through the oxidative pentose phosphate pathway.

The use of doubly labelled substrates in the study of the so-called futile cycles in liver and muscle

From the above considerations, it appears that in both liver and muscle, a loss of tritium in position 3 of glucose (liver only), glucose 6-phosphate or glycogen relative to ^{14}C can be due to the operation of the oxidative pentose phosphate cycle (liver only) or to recycling of metabolites through triose phosphates (liver and muscle); this can occur either through a so-called futile recycling between fructose 6-phosphate and fructose diphosphate or, in the liver, via the Cori cycle. Since in intact fed mice, the loss of tritium from position 3 of blood

glucose was approximately the same as that from position 6, it could be entirely explained by the Cori cycle. We have therefore no evidence for a fructose 6-phosphate:fructose diphosphate recycling in the liver. This conclusion is in agreement with the absence of specific randomization of ^{14}C between carbons 1 and 6 of liver glycogen formed from [1-^{14}C]glucose (16, 17) or from [1-^{14}C]galactose (18). In contrast, a recycling between glucose and glucose 6-phosphate in the liver was evidenced by the markedly more rapid loss of tritium from positions 2, 4 and 5 than from position 6 of glucose. The loss of tritium from position 5 was, indeed, about 40 to 60 % of that from position 2, *in vivo* (Fig. 1) and in the isolated liver cells (6), as it was from glucose 6-phosphate in a liver extract (Table I); we have seen that in this case, it was adequately explained by the action of transaldolase on fructose 6-phosphate. The loss of tritium from position 2 *in vivo*, in excess of that from position 6, indicates that about 35 % of the body glucose (about 85 µmoles in a normally fed 20 g mouse) has lost its tritium through glucose:glucose 6-phosphate recycling in 60 min. Since the liver of such a mouse weighs 1 g and since the $^{3}H/^{14}C$ ratio in glucose 6-phosphate is approximately 50 % of that in glucose (2), one can calculated a rate of recycling equal to 0.35 x 2 x 85/60 = about 1 µmole/min per g of liver.

In the muscle, due to the virtual absence of transaldolase, the loss of tritium in positions 4 or 5 of glucose 6-phosphate or glycogen have the same significance as that in position 3. This has allowed the demonstration that recycling between fructose 6-phosphate and fructose diphosphate occurs extensively in the flight muscle of *Bombus affinis* maintained at 5 °C (7) and in the skeletal muscle of susceptible pigs intoxicated with halothane(8). Cycling also seems to occur, to a small extent, in resting muscles of normal pigs (8) and in the isolated rat diaphragm, since tritium was lost during the conversion of [4-^{3}H,U-^{14}C] glucose into glycogen in that preparation (4). It may be recalled that 5 % of the ^{14}C present in glycogen formed from [1-^{14}C]glucose by the isolated diaphragm was specifically recovered in position 6 (17).

ACKNOWLEDGMENT

This work was supported by the *Fonds de la Recherche Scientifique Médicale* and by NIH Grant AM 9235. L. Hue is *Chargé de Recherches du FNRS*.

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