ISOTOPIC EVIDENCE FOR FUTILE CYCLES IN LIVER CELLS

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Summary: To estimate futile cycles in the metabolism of glucose in liver, rat hepatocytes were incubated with glucose labelled with tritium in position 2 and 5 and uniformly with ^{14}C. The yield in water from 2-^{3}H glucose was 1.5 times that from 5-^{3}H glucose and 2 to 3 times that of ^{14}C utilization. Lactate addition had little effect on the water yield from 2-^{3}H glucose but depressed that from 5-^{3}H glucose and utilization of ^{14}C. Our results indicate the occurrence of futile cycles glucose → glucose-6P → glucose and fructose-6P → fructose 1,6diP → fructose-6P in rat liver. An estimate of recycling at the glucose-6P level is presented.

Experiments with ^{14}C labelled compounds establish that in rat hepatocytes synthesis and glycolysis proceed simultaneously (1). Most of the steps between pyruvate and glucose are reversible and shared by the two pathways but there are three irreversible segments catalyzed respectively by glucokinase and glucose-6 phosphatase, phosphofructokinase and fructose diphosphatase, and pyruvate kinase and pyruvate carboxylase plus carboxykinase, where recycling may occur. The cycles will cause energy dissipation (ATP hydrolysis) and they have been designated as "futile cycles". Evidence for recycling at the pyruvate level in perfused liver (2) and in kidney cortex slices (3) has been provided, and we have devised a method to quantitate this cycle (3). In rat kidney cortex during gluconeogenesis from pyruvate one third to one half of the formed phosphoenolpyruvate is hydrolyzed back to pyruvate (3).

We introduce here the use of 2-^{3}H and 5-^{3}H glucose to estimate the futile cycles at the glucose-6P and fructose 1,6diP levels. Tritium from position 2 is lost irreversibly as water

in the isomerization of hexose phosphates and this label has been used to study glucose recycling in vivo (4). Tritium from position 5 is lost at the triose-P stage and we have shown that apart from glycogen or lactose this tracer appears nearly exclusively in water in rat adipocytes (5) and mammary gland slices (6). It is thus expected that water formation from 2-^{3}H glucose would be some estimate of glucose phosphorylation and that from 5-^{3}H glucose, of aldolase cleavage of fructose 1,6diP and a minimal estimate of phosphofructokinase.

METHODS. Rats of the Wistar strain 200-280 g, were used. They were either fasted overnight or meal fed from 6 to 9 a.m., a commercial diet high in sucrose (Nutritional Biochemical). Hepatocytes were prepared essentially according to Berry and Friend (7), except that hyaluronidase was omitted. The cells were washed with Krebs-Henseleit bicarbonate buffer (8) and incubated in this buffer in an atmosphere of 95% O_2 - 5% CO_2. From 0.15 to 0.25 ml packed cells were incubated in 2 ml with shaking. The experiments were terminated with perchloric acid and the flask contents were transferred into tared 15 ml centrifuge tubes with sufficient alcohol to have a final concentration of 60% alcohol, sufficient to precipitate the solubilized glycogen. The supernatant was processed as described elsewhere (1). In early work labelled water was obtained by lyophilization, but in later work the use of borate columns to adsorb glucose was found much more convenient (1). The lipids were extracted from the residue and the residual defatted dry weight obtained by reweighing the tared tubes. Activity in glycogen was obtained after KOH digestion of the residue. 1 ml of packed cells corresponds to 150 to 200 mg defatted dry residue.

RESULTS. Table 1 presents the distribution of tritium from 2-^{3}H

Table 1. Incorporation of Glucose Labelled Uniformly with ^{14}C and with Tritium in Positions 2 and 5 by Rat Hepatocytes.

Rat No.	Diet	Lactate	Glucose in Medium Initial (μmoles/2 ml)	Glucose in Medium Final (μmoles/2 ml)	Label in Glucose	Isotope Recovered In: CO_2 (% Added Tracer)	Soluble Products (% Added Tracer)	Lipid Plus Glycogen (% Added Tracer)	Water (% Added Tracer)	Total (% Added Tracer)	Isotope* Recovery
1	Starved	-	9.5	10	2-^3H	-	0.1	0.1	21	21	98
					5-^3H	-	0.3	0.2	13	13	96
					U-^{14}C	3.7	1.5	0.5	-	5.7	97
		+	9.5	21	2-^3H	-	0.1	0.5	21	22	100
					5-^3H	-	0.4	0.8	6.7	7.9	97
					U-^{14}C	0.56	1.0	1.0	-	2.6	99
4	Meal Fed	-	29	43	2-^3H	-	0.0	0.3	24	24	95
					5-^3H	-	0.2	0.1	15	15	96
					U-^{14}C	4.6	4.0	1.0	-	9.5	98
		+	29	49	2-^3H	-	0.0	0.2	19	19	94
					5-^3H	-	0.3	0.2	6.9	7.4	101
					U-^{14}C	1.3	0.8	0.3	-	2.4	99

*Includes the activity in recovered glucose.

Experiment 1, 28 mg (defatted dry weight) of cells incubated in 2 ml for 90 minutes.
Experiment 2, 38 mg indubated in 2 ml for 60 minutes. Lactate when present 25 mM.

and 5-^{3}H glucose and ^{14}C from uniformly labelled glucose incubated with and without lactate. In hepatocytes from fasted rats there was usually a small increase in medium glucose from endogenous precursors, and a marked increase in glucose in the presence of lactate. In meal fed rats there was considerable glycogenolysis, and the production of glucose was somewhat increased by the addition of lactate. A more extensive study of glucose metabolism and lipogenesis by hepatocytes will be presented elsewhere (Clark and Katz, in preparation). In no case, up to glucose concentration of 50 mM did we observe glucose uptake from the medium. There was however utilization of added isotope. The ^{14}C labelled products, at medium glucose concentrations of up to 20 mM, were CO_2 and soluble compounds, the major one being lactate. At these concentrations the incorporation into glycogen and lipid was very low. The cells however incorporate glucose into glycogen at higher glucose concentrations, and the hepatocytes of meal fed rats have a high capacity of lipogenesis (Clark and Katz, in preparation). In the presence of lactate fatty acid synthesis as measured by the incorporation of tritium from labelled water was 400 to 600 µatoms hydrogen per gram dry weight per hour, but glucose carbon contributed little to this synthesis, especially at glucose concentrations below 10 mM. Similar findings have been reported with perfused liver (9).

As shown in table 2, under these conditions nearly the sole product from the tritium labelled glucose was water. In the absence of lactate the yield in water from position was 1.5 to 2 times that from position 5 and about 3 times the incorporation of ^{14}C. The addition of lactate had hardly any effect on the water yield from 2-^{3}H glucose but depressed that from 5-^{3}H glucose, and especially the utilization of ^{14}C (table 1).

Table 2. Yields of Water from 2-^{3}H and 5-^{3}H Glucose and Recovery of ^{14}C from Uniformly Labelled Glucose in Products by Rat Hepatocytes.

Rat No.	Diet	Period of Incubation Hours	Lactate	^{3}H in Water Utilized from Glucose 2-^{3}H % Added Isotope	5-^{3}H	^{14}C	Glucose[2] Formation µmoles/100 mg	Apparent[3] Glucose Phosphorylation	Apparent[4] Glycolysis	Apparent[5] Glucose-6 Phosphatase
1	Starved	1.5	-	21	13	5.7	+ 3.5	7.8	1.9	11
			+	21	6.7	2.6	+41	21	0.8	62
2	Starved	2.0	-	27	21	3.8	+ 3.0	6.0	0.9	9
			+	31	11	2.1	+32	10	0.7	42
3	Starved	0.5	-	7.1	4.5	3.2	0	1.7	0.8	1.7
		0.5	+	7.0	2.0	1.2	+ 7.5	1.9	0.3	9.4
		1.0	+	13	3.4	2.5	+22	4.5	0.8	27
4	Meal Fed	1.0	-	19	11	6.7	+49	9.5	3.2	59
		1.0	+	17	6.4	2.1	+56	9.2	1.1	66
4[1]	Meal Fed	1.0	-	24	15	9.5	+37	23	9.0	60
		1.0	+	19	6.9	2.4	+52	19	2.5	71
5	Meal Fed	2.0	-	29	17	10	+24	18	6.5	42
		2.0	+	25	10	4.1	+64	20	3.4	84
6[1]	Meal Fed	1.0	-	25	17	9.0	+28	8.5	3.8	37
		1.0	+	22	10	3.5	+46	9.5	1.8	56

(1) Glucose 15 mM (see table 1).
(2) Determined by analysis of the medium.
(3) Average glucose concentration multiplied by % yield in water from 2-^{3}H glucose and divided by tissue weight (defatted dry weight).
(4) Calculated as (3), using the ^{14}C yield.
(5) Sum of glucose formation and apparent phosphorylation.

From 25 to 40 mg cells (defatted dry weight) incubated in 2 ml buffer. Glucose concentration, except when noted otherwise, 5 mM, lactate when present 25 mM.

Table 2 presents additional data on the utilization of these glucoses. The results are presented as % of added tracer. Since there is no change or more often an increase in the glucose calculations of glycolysis by dividing the ^{14}C yield by glucose specific activity have little meaning. Such calculations have been frequently used and in table 2 we have for comparison calculated the apparent rate of glycolysis. Mean specific activity was used for the calculation.

Our results establish clearly the operation of futile cycles at the glucose-6P and fructose-1,6diP levels for the system as a whole. If the hepatocytes were heterogeneous, with solely glucose synthesis or glycolysis proceeding in separate cells, equal amounts of glycolytic products should be formed, and the yield in water from 2-^{3}H and 5-^{3}H glucose should have been the same. Thus it is likely that the two opposite reactions proceed simultaneously in each cell.

Possibilities that the detritiation is due to exchange reactions, without any net metabolic flux have been considered by us (1), and while such reactions are difficult to disprove, they appear unlikely. The rate of phosphorylation has been obtained by multiplying the fraction of added tritium recovered in water by the mean concentration of medium glucose (table 2). This calculation appears to be valid whether phosphorylation occurs everywhere or in a metabolic compartment. Since the rate of glucose production is known the rate of glucose-6 phosphatase can be readily obtained as the sum of net glucose synthesis and of phosphorylation, and the rates are reported in table 2. The rate of phosphorylation at physiological glucose concentrations was from 15 to 25% the rate of glucose-6P phosphatase.

Calculation of the apparent rate of phosphofructokinase is

more complex and will be dealt elsewhere. It requires some simplifying assumptions, knowledge of the flux from and to glycogen and analysis based on a metabolic model. However, without attempting to quantitate, it is clear that if it is assumed that there is no metabolic compartmentation of metabolites the rate of phosphofructokinase must be several times higher than that of glucose phosphorylation. Since the rate of phosphatase is from 3 to 5 times that of phosphorylation most of the 5-^{3}H hexose-6P will be cycled back to glucose. To obtain the detritiation of 5-^{3}H observed here a rate of phosphofructokinase several times that of glucokinase is required. A rate of phosphofructokinase of the same order as that of the phosphatases would however appear at variance with current concepts of the regulation of this enzyme.

DISCUSSION. If the activity of the enzymes in a futile cycle are high, regulation is required to assure net metabolic flow and restrict energy dissipation. Some investigators have considered metabolic control to operate in analogy with electric networks, as a flip flop circuit, with the enzymes either being turned on or off (10). Scrutton and Utter (11) and Newsholme and Underwood (12) have considered the operation of futile cycles in glucose metabolism. The latter (12) have proposed a role for such a cycle in the fine control of metabolism. Another possible regulatory function could be balancing ATP production and utilization, by regenerating ADP. Other functions are possible, or the futile cycles may be simply an imperfection, a leak, of the regulatory mechanism. At present the experimental data are so limited as to render speculation on a physiological role premature. The application of tritium labelled substrates provides a useful tool for the study of some of these systems.

In the interpretation of these results the problem of compartmentation is of major interest. There have been suggestions that compartmentation of metabolic pathways and separation of intermediates into isolated pools occurs in mammalian cells and microorganisms (13). Review of this work is beyond the scope of this paper (see reference 13). In our opinion the evidence for such compartmentation is not convincing. To account for our data by this approach a complex system of several compartments must be assumed and at present there is little to support such a system.

While this work was in preparation, two papers have appeared on the application of U-^{14}C, 5-^{3}H glucose to the estimation of the fructose diphosphatase-phosphofructokinase cycle (14, 15).

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