RELATIONSHIP BETWEEN ISOTOPIC REVERSIBILITY AND FUTILE CYCLES IN ISOLATED RAT LIVER PARENCHYMAL CELLS

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Summary: Evidence is presented for isotopic flow between glucose and glucose-6P and between fructose-6P and fructose 1,6diP during gluconeogenesis. Inhibitors of glucokinase (glucosamine, 2-deoxyglucose) inhibit the isotopic counterflow. Transaldolase exchange is ruled out by using hexoses labeled in the top half of the molecule.

We have previously shown (1, 2) in kidney cortex segments that the pathway of gluconeogenesis is isotopically reversible; that is, during active gluconeogenesis, label from ^{14}C-glucose appears in "glycolytic" products such as lactate, CO_2 and amino acids. Many enzyme reactions in the pathway are readily reversible: all the reactions between fructose diphosphate and phosphoenolpyruvate as well as phosphohexose isomerase. Also considerable isotopic exchange can occur between phosphoenolpyruvate and pyruvate, not via a pyruvate kinase exchange reaction but as a result of cycles involving pyruvate, dicarboxylic acids and phosphoenolpyruvate (3). However the steps between glucose and glucose-6P and between fructose-6P and fructose 1,6diP are both catalyzed by different enzymes in opposite directions and the enzymes involved are essentially irreversible. Since the kidney cortex is not homogenous it is possible that the isotopic counterflow was due to real glycolysis in a small portion of different cells, rather than to isotopic exchange in the gluconeogenic cells. To reexamine the significance of isotopic reversibility in the gluconeogenic pathway we have turned to the technique of Berry and Friend (4) which produces an essentially

homogenous population of isolated liver parenchymal cells.

METHODS: Liver cells prepared according to Berry and Friend (4) were incubated in 25 ml flasks at 37° in Krebs-Henseleit (5) buffer. The incubation procedure and methods for isolation of products were those used previously for kidney cortex (6) or adipose tissue (7) segments. ^3HHO yields from [2-^{3}H] glucose were obtained by putting an aliquot of the medium through three columns: Amberlite CG-120 (H^+); Dowex 1 (acetate) and Dowex 1 (borate); the borate column retains the unused [2-^{3}H] glucose. To determine the isotopic distribution of ^{14}C in carbons 1 and 6 of glucose, the glucose was first converted to gluconate by iodine oxidation (8). The gluconate was purified on Dowex 1 (acetate) and oxidized with periodate to give C-1 as CO_2 and C-6 as formaldehyde.

RESULTS AND DISCUSSION: Isolated rat liver parenchymal cells were incubated with lactate as the major substrate together with a series of [U-^{14}C] labeled substrates added at tracer (< 0.1 μmole) levels. Cells from normally fed or fasted-refed animals were used for the experiments reported in Table 1. In these cells simultaneous gluconeogenesis and lipogenesis occurs when high concentrations of lactate are added. Net gluconeogenesis is determined by analysis of glucose and glycogen at the start and at the end of the incubation, while lipogenesis is determined by measuring the incorporation of tritium into lipids from ^3HHO (9). The results with the [U-^{14}C] compounds are similar to those previously obtained with kidney cortex segments, with the relative isotopic yields reflecting the distance (in terms of length of metabolic pathway) of the point of entry of the labeled compound into the products of the gluconeogenic or the "glycolytic" (exchange) pathway. If two substrates which enter the pathway at

Table 1. ^{14}C Yields in Glucose and Lipids in Isolated Liver Cells.

Expt. No.	[U-^{14}C] Labeled Compound	Intermediate Where ^{14}C Enters Pathway	Specific ^{14}C Yields Glucose	Lipids	Ratio of Yields [Glucose] / [Lipids]
1	Glucose	Glucose	.890	.022	40
	Galactose	Glucose-6P	.705	.060	11.8
	Mannose	Fructose-6P	.619	.074	8.2
	Glycerol	Dihydroxy-acetone-P	.497	.146	3.4
	D-Glyceralde-hyde	Glyceralde-hyde-3P	.471	.133	3.5
	Fructose	Dihydroxy-acetone-P Glyceralde-hyde-P	.550	.145	3.8
	D-glycerate	2P-Glycerate	.406	.184	2.2
	Lactate	Lactate	.168	.300	0.6
2	Glucose	Glucose	.920	.011	84
	Galactose	Glucose-6P	.741	.025	30
	Mannose	Fructose-6P	.701	.030	23
	Glycerol	Dihydroxy-acetone-P	.694	.045	15.4
	D-Glyceralde-hyde	Glyceralde-hyde-3P	.600	.040	15.0
	Fructose	Dihydroxy-acetone-P Glyceralde-hyde-3P	.680	.048	14.1
	L-Lactate	Lactate	.307	.076	4.0

About 0.25 ml of liver cells (expt. 1 from fasted-refed rats; expt. 2 from normally fed rats) were incubated with 40 µmoles of L-lactate for 60 minutes in 2 ml of Krebs-Henseleit buffer with U-^{14}C labeled substrates added at tracer (< 0.1 µmole) levels. Rates of lipogenesis expressed as µatoms ^{3}H incorporated from ^{3}HHO/ml cells/hr were 120 in expt. 1 and 30 in expt. 2. Specific yields are defined as the fractional yield of the substrate metabolized in the product.

different intermediates gave identical [glucose]/[lipid] ratios, the rate of isotopic exchange between the intermediates would have to be infinite. It can be shown that a 2 fold difference in

ratios of the product yields from two different intermediates indicates a rate of isotopic exchange between the intermediates equal to the net flux in the pathway. In liver cells there is about a 3 fold difference in the [glucose]/[lipid] ratio between ^{14}C labeled glucose and galactose, and about a 1.5 to 2.5 fold difference in this ratio between mannose and glycerol or glyceraldehyde. These results indicate extensive isotopic exchange reactions between glucose and glucose-6P and between fructose-6P and the triose phosphates. Since there are rather obvious philosophical objections to having net enzyme fluxes in opposite directions at these steps, we have tried to determine whether the isotopic data represented net reactions or exchange pathways.

In regard to possible exchange reactions as causes for the isotopic reversibility, we have considered two major possibilities (a) the reported [^{14}C] glucose-[^{14}C] glucose-6P exchange catalyzed by glucose-6 phosphatase (10); and (b) the [^{14}C] fructose-6P-[^{14}C] glyceraldehyde-3P reaction catalyzed by transaldolase (11).

The main enzyme which phosphorylates glucose in the liver parenchymal cells is thought to be glucokinase, although there are somewhat conflicting reports on the role of a (low K_m) hexokinase in these cells (12,13). Glucokinase is inhibited by glucosamine and 2-deoxyglucose, competitive inhibitors of glucose with K_I's reported to be 1 mM and 16 mM respectively (14). The [^{14}C] glucose-[^{14}C] glucose-6P exchange reaction catalyzed by glucose-6 phosphatase has a K_m for glucose of 100 mM (15); the various reactions catalyzed by glucose-6 phosphatase are inhibited by a number of sugars, but only at very high concentrations with K_I's well above 0.1 M (16). Glucosamine and 2-deoxyglucose are competitive inhibitors (and substrates) for yeast hexokinase. Thus they would appear to be likely inhibitors of both of the pos-

Table 2. Effect of Glucosamine and 2-Deoxyglucose on Isotopic Counterflow from Glucose During Gluconeogenesis in Liver Cells.

Expt. No.	Glucosamine (mM)	Deoxyglucose (mM)	^{14}C Yield in CO_2 (% Added ^{14}C)	^{3}H Yield in Water (% Added ^{3}H)
1	0		1.45	24.9
	1.0		1.14	17.8
	2.0		1.02	15.7
	5.0		0.75	11.8
2		0		34.0
		10		23.6
		25		15.1
		50		11.6

Isolated liver cells (about 0.25 ml) from fasted rats were incubated for 60 minutes in 2 ml Krebs-Henseleit buffer containing 10 mM glucose and 20 mM L-lactate, together with [U-^{14}C-2-^{3}H] glucose in expt. 1 (48 hour fasted rats) and [2-^{3}H] glucose in expt. 2 (18 hour fasted rats) and the inhibitors at the concentrations shown. Glucose production was about 10 μmoles/flask in both experiments. Glucosamine and 2-deoxyglucose caused no significant changes in glucose production.

sible (gluco- and hexo-) kinases in liver parenchymal cells, and should have little effect on the exchange reaction between glucose and glucose-6P catalyzed by glucose-6 phosphatase. Table 2 shows that glucosamine and 2-deoxyglucose do inhibit the glucose → glucose-6P reaction in isolated liver cells, at concentrations which are in the region of the reported K_I's of these substrates. This indicates that the isotopic results probably do reflect a "futile cycle" (which requires ATP) between glucose and glucose-6P rather than a simple isotopic exchange reaction.

Transaldolase catalyzes an exchange reaction between carbons 4, 5 and 6 of fructose-6P and glyceraldehyde-3P. Also the oxida-

Table 3. ^{14}C Yields and Distribution in Glucose from [1-^{14}C] Galactose in Liver Cells.

Expt. No.	Dietary Conditions	Lactate Conc (mM)	^{14}C Yields % Added ^{14}C Glucose	CO_2	Lipids	% Relative Distribution of ^{14}C in Glucose C-1	C-6
1	Fasted Refed	20	53.7	20.0	0.6		
2	Fasted Refed	25	52.0	8.2	0.6		
3	Fasted Refed	25	52.2	18.0	1.0		
4	Fasted Refed	20	53.1	30.2	3.1		
5	Fasted Refed	25	41.0	25.2	1.3	85	15
6	Fasted Refed	20	48.0	29.3	4.5	89	11
7	Fasted	10	56.0	3.5	n.d.	94	6
8a	Fasted	20	47.8	3.4	n.d.	94	6
8b	Fasted	80	52.3	3.2	n.d.	93	7

About 0.25 ml of liver cells were incubated in 2 ml of Krebs-Henseleit buffer for 60 minutes with [1-^{14}C] galactose added at a tracer level and lactate as the main substrate. The rates of gluconeogenesis averaged about 15 μmoles/ml cells/hour for the fasted-refed cells, and about 40 μmoles/ml cells/hour for the fasted cells.

tive pentose cycle will transfer ^{14}C from the bottom half of the hexose phosphates to glyceraldehyde-3P. These reactions thus conceivably could account for some or all of the labelling of glycolytic products from compounds which enter the pathway at or above fructose-6P. To avoid these possible pathways it is necessary to use compounds labeled in the top half of the hexose. Incubation of liver cells with [1-^{14}C] galactose also produces labelling of glycolytic products (Table 3), in experiments where the net metabolic flow was in the direction of glucose formation. The only established pathway which would cause this labelling

involves phosphofructokinase activity. Again there is apparently another futile cycle between fructose-6P and fructose 1,6diP.

While the procedure of Berry and Friend is reported to produce essentially a pure parenchymal cell population (4), it is possible that a small proportion of other, strictly glycolytic, cells (Kupfer, bile duct, etc.) is also present. Another possibility is that there is a certain proportion of normal appearing, but somewhat damaged, parenchymal cells which may require glycolysis to furnish extra energy to exist or repair damaged structures. Thus the problem may remain as to whether there are opposing fluxes in separate cells, rather than in the same cells, in which case futile cycles might not exist. To attempt to determine if futile cycling exists in the gluconeogenic parenchymal cells, we have again used [1-^{14}C] galactose as the labeled substrate, but now we have determined the distribution of ^{14}C in carbons 6 and 1 of the product of the gluconeogenic cells, glucose (Table 3, experiments 5 through 8). Phosphofructokinase (and rapid triose-P isomerase) activity would cause labelling of C-3 of both triose phosphates, which then will label C-6 and C-1 of glucose in the gluconeogenic pathway (the direction of net flux). To further rule out the possibility that the [1-^{14}C] galactose might be converted to [3-^{14}C] lactate in a glycolytic cell, and then be converted to [1, 6-^{14}C] glucose in the parenchymal cells, we have used (experiment 8b, Table 3) a large lactate trap to mask such a pathway. The large lactate pool had essentially no effect on the labelling of C-6 of glucose from [1-^{14}C] galactose, again suggesting that there is phosphofructokinase activity during gluconeogenesis in the parenchymal cells. From the ^{14}C distribution in glucose and steady state models one can make a rough estimation of the rate of phosphofructokinase activity in these experiments; the values obtained are of the same

order of magnitude as the net fluxes in the opposite direction (details to be presented elsewhere). The rate of futile cycling at the glucose-glucose-6P step has been estimated using specifically tritiated glucoses (17).

There appears to be substantial evidence for "futile cycles" in gluconeogenic cells, and if the isotopic data which produces the evidence is caused by some artifact or exchange systems, it is not by systems which are presently known.

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