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A method for the determination of glucose synthesis in isolated bovine hepatocytes

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A simple method for determining glucose synthesis from radiolabeled precursors in isolated bovine hepatocytes using ion exchange resins is presented. This method allows processing of multiple small volume samples using suspensions of anion and cation exchange resins rather than traditional stacked column separation methods. Hepatocytes were isolated from calf liver by collagenase perfusion of the caudate lobe and were incubated with ^{14}C-labeled lactate or propionate as gluconeogenic substrates. Glucose synthesis was determined in an aliquot of cell suspension that was vortexed with a slurry of anion exchange (acetate form) resin, followed by a slurry of cation exchange resin. Newly synthesized, labeled glucose was recovered in the supernatant after centrifugation and quantitated by scintillation counting. Using this procedure, more than 98% of the unused labeled precursor was bound to the ion exchange resin and essentially 100% of a labeled glucose tracer was recovered in the supernatant. Pretreatment of hepatocyte suspensions with glucose oxidase was shown to eliminate the accumulation of radioactivity in the supernatant, thus confirming the specificity of this technique for measurement of newly synthesized glucose. This method was sensitive to changes in the rate of hepatic gluconeogenesis that resulted from changes in substrate concentration or the addition of glucagon or fatty acids to the hepatocyte incubations. (J. Nutr. Biochem. 10:205–209, 1999)

Keywords: gluconeogenesis; hepatocytes; metabolism

Introduction

Gluconeogenesis and overall hepatic glucose production are the primary sources of circulating glucose in the ruminant.[1,2] In vitro, glucose production has been determined by measuring total or net glucose release into the media or by using radiolabeled gluconeogenic precursors. Determination of net glucose is relatively simple, but it does not distinguish between glucose derived from glycogenolysis and that derived from gluconeogenesis from various precursors. Furthermore, the presence of exogenous glucose in the media can limit the ability to detect changes in rate of production. The use of labeled precursors provides a means to determine the rate of gluconeogenesis from specific carbon sources and thus eliminates some of the inaccuracies associated with enzymatic determination of total glucose production.

A major limitation in isotopic methods is the process needed to separate unused precursor from newly synthesized glucose. Unlike studies of lipogenesis, where solvent extraction can be used to separate these components, both the products and the substrates for gluconeogenesis are water soluble. Traditionally, column chromatography using "stacked" anion and cation exchange resins has been employed to separate precursors such as lactate, propionate, and alanine from glucose.[3–6] These column methods greatly limit the number of samples that can be processed and therefore are not well suited for studies with isolated cells and multiple comparisons. This article describes a modification of the traditional ion exchange column separation that uses slurries of these resins for determination of gluconeogenesis in isolated hepatocytes.

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Materials and methods

Materials

Chemicals were obtained from Sigma (St. Louis, MO USA) unless otherwise noted. Radiolabeled (^{14}C) lactate, propionate and glucose were obtained from Amersham (Arlington Heights, IL USA). Ion exchange resins were obtained from BioRad (Richmond, CA USA).

Cell preparation

The preparation of isolated hepatocytes was based on that previously used for rat hepatocytes[7] and was essentially the same as that described by Forsell et al.[8] for bovine hepatocytes. Briefly, livers were obtained from 1-month-old male Holstein calves that had been anesthetized with intravenous pentobarbital. Immediately after removal of the liver from the animal, the caudate lobe was cut from the liver and flushed with 150 to 200 mL of saline through one of the large veins using a 50-mL syringe. The cleared lobe was then flushed with 100 mL of calcium free Krebs-Henseliet buffer and transferred to the perfusion apparatus, which was similar to that described by Reese and Byard.[9] The total time from disruption of blood flow in the animal to connection with the perfusion apparatus was less than 5 minutes. The lobe was placed on a Buchner funnel and cannulated through one of the large veins on the cut surface of the tissue. The liver was flushed with an additional 600 to l,000 mL of calcium-free Krebs buffer at 38°C in a nonrecirculating perfusion to clear any remaining blood and to deplete the tissue of calcium. The perfusate was then switched (three-way valve) to Krebs buffer containing 1.2 mM $CaCl_2$ and 85 to 90 mg of collagenase (type II) per 200 mL of buffer. The collagenase containing buffer was recirculated through the liver at a flow rate of 100 to 150 mL/min for 10 to 20 minutes. Outflow from the liver was allowed to drain into the funnel and was collected in a water-jacketed reservoir (39°C). Buffers were continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide. After perfusion, the tissue was disrupted with the flat end of a plastic spatula and scraped into a beaker. The disrupted liver was shaken (90 oscillations/min) in the collagenase-containing buffer for an additional 5 to 10 minutes in a 38°C shaking water bath. Isolated cells were obtained by filtering the liver through 100-μ nylon mesh (Spectra Mesh N, Fisher Scientific, Pittsburgh, PA USA) and centrifugation at 50 × g for 2 minutes. The resulting hepatocyte pellet was resuspended and washed three times in Krebs buffer (pH 7.4). The final cell pellet was resuspended in 10 to 20 volumes of this buffer. Greater than 85% of these cells excluded trypan blue dye. Viable cell number was determined by counting an aliquot of trypan stained cells in a hemocytometer.

Incubations

Cells (3–6 × $l0^6$/mL) were aliquoted into disposable plastic 7-mL vials containing labeled gluconeogenic precursors, to a final volume of 400 μL. Cells were incubated with 2 or l0 mM uniformly labeled ^{14}C lactate (100 dpm/nmole) or 2 mM 2-^{14}C propionate (200 dpm/nmole) as the gluconeogenic precursor. Glucagon (10^{-7} M) and/or palmitate [1.0 mM final concentration in 2% bovine serum albumin (BSA)] were added to certain incubations to determine the ability of these compounds to alter the rate of glucose synthesis. Vials were capped and incubated at 38°C for 0 to 3 hours. Incubations were terminated by the addition of 400 μL of ice-cold water. Cell suspensions were immediately frozen and stored for determination of label incorporation into glucose. It was determined that termination of incubations with acid ($HClO_4$ or H_2SO_4) followed by a neutralization step was not acceptable for measurement of gluconeogenesis, in that unused label was not removed, resulting in high background radioactivity. This is presumably due to the increased ionic strength that resulted from acidification and neutralization.

The method for determining glucose synthesis that was described by Brown et al.[10] also avoids use of acidification and utilizes a combination of heat and chloroform for the termination of incubations. In our experiment, the addition of cold water followed by freezing was shown to rapidly stop the metabolism of hepatocytes and prevent further synthesis of glucose (data not shown).

Gluconeogenesis assay

The method used to determine the synthesis of glucose in the present study was a modification of column chromatographic methods that have been used previously.[4–6,10] As with column separations for determination of glucose synthesis, a combination of anion and cation exchange resins was used. The primary modification was the use of these resins as a 50% (v/v) slurry in water. Prior to use, commercially available ion exchange resins were washed several times in de-ionized water. Initial development of this assay was conducted using l0-mM standard solutions of ^{14}C-labeled lactate, propionate, and glucose. The optimal separation of precursors from newly synthesized glucose was obtained with acetate as the counterion on the anion exchange resin (AG 1 × 8, 200–400 mesh) and the standard hydrogen form of the cation exchange resin (AG 50 × 8, 200–400 mesh). For hepatocyte media, a 50-μL aliquot of cells and media that was frozen and thawed was diluted with 1 mL of water in a 12 × 75 mm tube and placed on ice. A 50% slurry of anion exchange resin (500 μL, acetate counterion) was added and mixed (3 × 30 seconds) by vortex mixer. This was followed by the addition of 500 μL of a 50% slurry of cation exchange resin and mixing (3 × 30 seconds). "Stock" solutions of the ion exchange resins were kept in suspension by constant stirring on magnetic stir plates. After mixing, samples were centrifuged at l,000 × g for 15 minutes at 4°C. A 0.5- or l.0-mL aliquot of the resulting supernatant was transferred to a scintillation vial and mixed with 10 mL of aqueous scintillation cocktail for determination of unbound radioactivity.

Confirmation that the radioactivity in the supernatant represented newly synthesized glucose was made by pre-incubating the hepatocyte media samples or a 10 mM ^{14}C glucose standard with glucose oxidase. Glucose oxidase converts uncharged glucose to gluconic acid, which is bound by the anion exchange resin. A 50-μL aliquot of cell suspension was added to 100 μL of glucose oxidase (120 units, type V) in 50 mM acetate buffer (pH 5.1) or to acetate buffer alone (blank). Samples were then incubated for 90 minutes at 35°C. Following the incubation, samples were placed on ice, diluted with 900 μL of water, vortexed with anion and cation resins and further processed as described above.

Calculations

The results are expressed as nanomoles of lactate or propionate converted to glucose per unit time and per million cells. This was determined using the following equation.

$$\text{Nanomoles substrate converted to glucose per } 10^6 \text{ cells} = \frac{\text{Corrected dpm in supernatant} \times \text{Total assay volume} \times \text{Total incubation volume}}{\text{Volume of supernatant used} \times \text{Specific activity of substrate (dpm/nmole)} \times \text{Cell number per vial } (\times 10^{-6})}$$

Corrected dpm refers to value obtained after subtraction of appropriate blanks. Typical volumes for total assay and incubation volumes were 2.05 mL and 800 μL (after addition of water to stop reaction), respectively. Volume of supernatant and incubation media were 1.0 mL and 50 μL, respectively.

Table 1 Anion exchange resins: Effect of counterion on binding of labeled gluconeogenic precursors and glucose

Counterion	Labeled substrate (10 mM)*	Total activity (dpm)†	Activity recovered in supernatant‡	% Retained by resin§
Chloride	Lactate	73,262 ± 958	62,710 ± 1,361	14.4
	Propionate	69,447 ± 833	65,002 ± 744	6.4
	Glucose	61,221 ± 593	67,056 ± 297	0
Formate	Lactate	12,391 ± 336	591 ± 10	95.2
	Propionate	23,626 ± 279	3,597 ± 64	84.8
	Glucose	ND	ND	ND
Acetate	Lactate	12,391 ± 336	69 ± 5	99.4
	Propionate	23,626 ± 279	461 ± 26	98.1
	Glucose	72,694 ± 439	75,035 ± 2,065	0

*Procedure as described in Materials and methods using 50 μL of labeled lactate, propionate, or glucose in Krebs-Henseliet buffer. Results are the mean ± SEM of quadruplicate assays with each substrate resin combination.
†dpm recovered in a 500-μL aliquot of samples where resin slurry was replaced with an equal volume of water (50 μL sample + 2.0 mL water).
‡dpm recovered in supernatant after vortexing and centrifuging sample in presence of ion exchange resin (50 μL sample + 1.0 mL water + 0.5 mL anion exchange resin + 0.5 mL cation exchange resin).
§% retained (or bound) = 100 − (activity recovered in supernatant/total activity × 100).
ND–not determined.

Results and discussion

The use of slurries of the ion exchange resins for the separation of newly synthesized glucose from labeled precursors and other metabolites of these precursors was demonstrated and shown to be sensitive to changes in rates of gluconeogenesis. This assay was inspired by previous work using a charcoal suspension to separate labeled acetyl CoA from newly formed acetylcarnitine as part of a carnitine assay described by Parvin and Pande.[11] This assay was developed as a means to bypass the use of stacked columns for the separation of glucose from lactate and other gluconeogenic precursors, as has previously been used.[4–6] Previously, Brown et al.[10] described the use of a combination of a column step using Sephadex, followed by use of a slurry of cation exchange resin to separate unused lactate from newly synthesized glucose in primary cultures of rat hepatocytes. The present work extends this to use of slurries of both anion and cation resins. The order in which the resin slurry was added to the samples was found to be important, in that the addition of the anion resin followed by that of the cation was essential for the separation of label. This is also the recommended order in the column method described by Mills et al.,[5] and is presumably related to the likelihood that most of the radioactivity is in the form of anions.

The influence of counterion on the anion exchange resin and its effect on the ability to retain labeled lactate or propionate and to exclude glucose in the assay is shown in *Table 1*. With chloride as the counterion, only 14% and 6% of the lactate and propionate were retained, respectively. The binding was increased to 95% and 86% for these substrates, respectively, with formate as the counterion, and was maximal (>98%) with the acetate form of the resin. The recovery of labeled glucose was always greater than 100% (103% with acetate resin), presumably due to the error in the calculations made by assuming that the volume of the resin slurries (500 μL each) was totally available in the aqueous phase. This would imply total exclusion of glucose under these conditions. From the results in *Table 1*, it is concluded that the use of a slurry of the acetate form of an anion exchange resin, followed by a cation resin, traps greater than 98% of the unused gluconeogenic precursor and enables essentially 100% of the newly synthesized glucose to be recovered in the supernatant.

To verify that the radioactivity recovered in the supernatant was glucose, hepatocyte media samples were preincubated with glucose oxidase prior to the addition of the ion exchange resins. Using a l0-mM labeled glucose (300 dpm/nmole) standard, it was determined that a 90-minute incubation at 37°C, with 120 units of glucose oxidase, converted greater than 90% of the glucose to charged species, which no longer appeared in the supernatant after addition of the ion exchange resins. After 30 and 60 minutes of incubation, 70% and 85%, respectively, of the glucose was metabolized under these conditions. Using 60 units of enzyme for 90 minutes resulted in slightly greater amounts of radioactivity remaining in the supernatant (90% metabolized with 60 units versus 94% with 120 units). In *Table 2*, the results of incubating a series of hepatocyte media samples with glucose oxidase are shown. The samples are from a single preparation of cells that were incubated for 1, 2, or 3 hours with and without glucagon (10^{-7} M). Aliquots of the frozen and thawed cell suspensions were incubated with glucose oxidase in acetate buffer or in buffer alone prior to the addition of ion exchange resins. The results indicate that in the 1-hour incubation, 70% of the radioactivity can be accounted for by glucose. This increases to 90% in the 3-hour incubations. In this particular assay, 94% of the glucose standard was metabolized in the 90-minute incubation with glucose oxidase. The correction for the amount of radioactivity not specifically accounted for as glucose and the conversion of dpm to nanomoles of lactate converted per million cells indicates an increased rate of synthesis with time. Also shown in *Table 2* is the effect of glucagon on the rate of glucose formation. ln this cell preparation, glucagon stimulated gluconeogenesis 48%, 82%, and 66% over that in controls after 1, 2, and 3 hours of incubation, respectively.

The average rates of lactate conversion for ten cell

Table 2 Gluconeogenesis assay validation: Effect of glucose oxidase on recovery of radioactivity from hepatocyte incubations*

Incubation time (min)	Glucagon (10^{-7}M)	Activity recovered in supernatant glucose oxidase preincubation† (dpm/50 μL supernatant)‡ −	+
60	−§	46 + 2	15 + 5
	+	64 + 6	18 + 2
120	−	150 + 17	34 + 6
	+	242 + 7	31 + 4
180	−	215 + 24	23 + 7
	+	363 + 14	44 + 7
10 mM ^{14}C glucose standard‖		56,520 + 1,479	3,558 + 40

*Hepatocytes incubated in Krebs-Henseliet buffer with 10 mM lactate (40 dpm/nmole) for 1 to 3 hours in a total volume of 400 μL. Incubations were stopped by adding 400 μL of ice-cold water and immediate freezing cells and media. The results represent the mean +SEM for quadruplicate incubations at each time point.
†A 50 μL aliquot of cell suspension was pre-incubated with glucose oxidase in 0.05 M acetate buffer or buffer alone for 90 minutes at 37°C prior to addition of ion exchange resins.
‡Incubations of hepatobytes in control buffer without glucagon.
§dpm in 500 μL aliquot of a 2.05 mL total volume (using 50 μL of smaple). Values were corrected for blank incubations (lactate incubated 3 hours without hepatocytes).
‖Incubation of glucose standard with glucose oxidase eliminated 94% of the activity.

preparations are shown in *Table 3*. As in *Table 2*, there was an increase in the rate of lactate conversion during the second hour of incubation over that observed during the first hour (0–60 minutes, 45.6 nmol/10^6 cells; 60–120 minutes, 76.9 nmo1/10^6 cells). Presumably, this is related to a lag in the use of labeled substrate and equilibration of metabolite pools, rather than to a change in the rate of total glucose production. The average glucagon response for the ten cell preparations was a 34% increase in the rate of lactate conversion.

To further demonstrate the metabolic integrity of the cells and the sensitivity of the gluconeogenesis assay, the effect of glucagon and free fatty acid on the rates of glucose synthesis from 2 or 10 mM lactate or 2 mM propionate were determined and are shown in *Table 4*. The rate of gluconeogenesis with 2 mM lactate was approximately 20% of that with 10 mM lactate under basal conditions. The rate of glucose synthesis with 2 mM propionate was similar to that

Table 3 Gluconeogenesis from lactate in isolated bovine hepatocytes

Incubation conditions	nmol lactate converted to glucose/10^6 cells
1 hour	45.6 ± 5.7
2 hour	122.5 ± 10.9
2 hour + 10^{-7} glucagon	164.4 ± 16.1*

Values are the mean ± SEM of the average of duplicate incubations from 10 hepatocyte preparations with 10 mM (U-^{14}C) lactate as the substrate.
*Glucagon effect significant at $p < 0.05$ by pooled t-test, or $p < 0.0001$ by paired t-test (within animal).

observed with 10 mM lactate. Under basal conditions, the addition of glucagon resulted in 25 to 48% increases in the rate of gluconeogenesis depending on the substrate. Responsiveness of hepatocyte suspensions to glucagon has been shown to be dependent on the degree of receptor damage in the collagenase procedure. The relative response seen here was similar to that reported in suspensions of ovine hepatocytes[12] or in cultured bovine cells.[13] The addition of l.0 mM palmitate in 2% albumin to the incubations resulted in a fourfold increase in lactate conversion to glucose at the 2 mM concentration. At l0 mM lactate or 2 mM propionate, the addition of palmitate/BSA resulted in a 2.5-fold increased rate of substrate conversion. These results are as expected due to the sparing effect of fatty acids on lactate or propionate utilization for oxidation,[14–16] as well as to a stimulation of gluconeogenesis by reducing equivalents generated in fatty acid oxidation.[17] Glucagon and palmitate had additive effects.[14,18] The relative stimulation of glucose synthesis by glucagon with 2 mM lactate was similar in the presence (+27%) or absence (+25%) of palmitate. However, in incubations with l0 mM lactate or 2 mM propionate, where the rates of gluconeogenesis were greater, the response to glucagon was decreased in the presence of palmitate. The decreased response under these conditions most likely is related to the maximal capacity for the cells to respond to metabolic and hormonal stimuli.

Direct comparison of the rates of glucose production in the present studies with those previously reported for hepatocytes prepared from ruminant liver is complicated by differences in source of tissue (age of animals; bovine vs. ovine or caprine) and expression of results (usually per gram of tissue rather than per cell). Many of these previous studies have reported total net glucose synthesis using the glucose oxidase method[8,14,19] rather than specific rates from a gluconeogenic precursor. However, if it is assumed that the values reported for lamb hepatocytes by Clark et al.[14] regarding the number of cells per gram of tissue (6×10^8 cells/g liver) and the wet:dry weight ratio (3.7) are applicable to the bovine, then the present results can be converted to correspond to previous methods of expression. The value of 122.5 nmol of lactate converted to glucose/(10^6 cells/2 hr) (*Table 3*) is equivalent to 30.6 nanomoles of glucose/(10^6 cells/hr). This would correspond to a rate of 1.13 μmoles glucose/(min/g liver) on a dry weight. basis, which is similar to the rate of glucose (net) synthesis reported by Forsell et al.[8] for bovine cells and to the rate of propionate conversion to glucose in caprine hepatocytes reported by Aiello and Armentano.[15]

Thus, the method described above represents a rapid and reliable means to determine rates of gluconeogenesis in isolated hepatocytes. Rates determined using this procedure are similar to those previously reported for hepatocytes using column separation techniques and were shown to be sensitive to substrate and hormonal manipulations of the cells. Although a direct comparison to column procedure was not conducted, it is likely that the use of slurries of ion exchange resins saves considerable time over that associated with columns and is more adaptable to processing large numbers of samples simultaneously.

Table 4 Effect of substrate and presence of glucagon and palmitate on gluconeogenesis in isolated heoatpcytes

	Media			
	Basal		Basal + 1.0 mM Palmitate/2% BSA	
Substrate[1]	Glucagon (0)	Glucagon (10^{-7} M)	Glucagon (0)	Glucagon (10^{-7} M)
2 mM lactate	18.0 ± 1.7[a]	22.5 ± 3.1[a]	83.2 ± 3.9[b]	105.1 ± 6.5[c]
10 mM lactate	79.2 ± 4.5[a]	117.5 ± 9.0[b]	215.5 ± 20.9[c]	250.6 ± 27.3[c]
2 mM propionate	94.8 ± 5.7[a]	129.2 ± 1.8[b]	217.2 ± 1.8[c]	239.6 ± 5.7[d]

Results are mean of quadruplicate incubations (±SEM) from a representative preparation of hepatocytes.
[1]Substrate (nmol substrate converted to glucose/10^4 cells/2 hrs).
[a,b,c,d]Means within a row without a common superscript differ ($P < 0.05$).

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