The Plasma C5 Glucose/$^2$H$_2$O Ratio Does Not Provide an Accurate Assessment of Gluconeogenesis during Hyperinsulinemic Euglycemic Clamps in either Non-Diabetic or Diabetic Humans

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Abbreviations: C5 glucose: glucose labeled with deuterium on the fifth carbon; C2 glucose: glucose labeled with deuterium on the second carbon

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Objective: Measurement of plasma C2 glucose enrichment is cumbersome. Therefore the plasma C5 glucose/$^2$H$_2$O rather than the plasma C5/C2 glucose ratio commonly has been used to measure gluconeogenesis and glycogenolysis during hyperinsulinemic euglycemic clamps. The validity of this approach is unknown.

Research Design & Methods: 10 non-diabetic and 10 diabetic subjects ingested $^2$H$_2$O the evening before study. The following morning insulin was infused at a rate of 0.6 mU/kg/min and glucose was clamped at ~5.3 mmol/l for five hours. Plasma C5 glucose, C2 glucose and $^2$H$_2$O enrichments were measured hourly from two hours onward.

Results: Plasma C2 glucose and plasma $^2$H$_2$O enrichment were equal in both groups before the clamp resulting in equivalent estimates of gluconeogenesis and glycogenolysis. In contrast, plasma C2 glucose and plasma C5 glucose enrichments fell throughout the clamp whereas plasma $^2$H$_2$O enrichment remained unchanged. Since the C5 glucose concentration and hence the C5glucose/$^2$H$_2$O ratio is influenced by both gluconeogenesis and glucose clearance whereas the C5/C2 glucose ratio only is influenced by gluconeogenesis, the C5glucose/$^2$H$_2$O ratio overestimated (p<0.01) gluconeogenesis during the clamp. This resulted in biologically implausible “negative” (i.e. calculated rates of gluconeogenesis exceeding total endogenous glucose production) rates of glycogenolysis in both the non-diabetic and diabetic subjects.

Conclusions: The plasma C5 glucose/$^2$H$_2$O ratio does not provide an accurate assessment of gluconeogenesis in non-diabetic or diabetic subjects during a traditional (i.e. 2-3 hour) hyperinsulinemic euglycemic clamp. The conclusions of studies that have used this approach need to be reevaluated.
Measurement of gluconeogenesis in humans is difficult. The deuterated water method is widely used for this purpose (1-17). This method relies on the fact that the fifth carbon of glucose is labeled during gluconeogenesis whereas the second carbon of glucose is labeled with deuterium during equilibration of glucose-6-phosphate and fructose-6-phosphate (1; 2). Therefore at steady state, the ratio of plasma glucose with deuterium on the fifth carbon (C5 glucose) to plasma glucose labeled on the second carbon (C2 glucose) equals the percent of plasma glucose that is derived from gluconeogenesis (1; 2). Measurement of C2 glucose enrichment is cumbersome. Since $^2$H$_2$O and C2 glucose enrichments are equal at steady state, many investigators have used the plasma C5 glucose / $^2$H$_2$O ratio to calculate gluconeogenesis after an overnight fast (4-7; 9-12; 18). The C5 glucose/$^2$H$_2$O ratio also has been used to measure gluconeogenesis during glucose clamps (4; 6; 7; 11; 12; 18). However, the validity of this approach is uncertain. We have reported that the rate of gluconeogenesis measured during the final hour of a three hour hyperinsulinemic euglycemic clamp in lean non-diabetic subjects using the C5/C2 glucose ratio was correlated with that measured in the same subjects using the C5 glucose/$^2$H$_2$O ratio (7). However, in those as well as other glucose clamp experiments (4; 6; 7; 11; 12; 18), gluconeogenesis calculated using the C5 glucose/$^2$H$_2$O ratio commonly exceeded total endogenous glucose production. Since endogenous glucose production equals the sum of glucose derived via gluconeogenesis and glycogenolysis, this result was biologically implausible.

Plasma C5 glucose concentrations are determined both by the rate of appearance of C5 glucose into and the rate of disappearance of C5 glucose from the plasma pool. Therefore the use of the C5 glucose/$^2$H$_2$O ratio during a clamp is only accurate when C5 glucose has achieved a new steady state. Since hyperinsulinemic clamps typically are relatively short (e.g. 2-3 hours) and the glucose pool large, we became concerned that plasma C5 glucose concentration was “artificially” elevated because the clearance was not sufficiently rapid for C5 glucose concentration to have re-achieved a steady state at a lower concentration. If so, this would be particularly problematic when the C5 glucose/plasma $^2$H$_2$O ratio was used to assess gluconeogenesis in groups in whom insulin action, and therefore glucose clearance, differed. The present experiments addressed this question by measuring both plasma C5/C2 glucose and the C5 glucose/$^2$H$_2$O ratios in diabetic and non-diabetic subjects prior to and every hour from two hours onward during a five hour hyperinsulinemic euglycemic clamp.

**Research Design and Methods**

**Subjects**

Results for this report are derived from 10 non-diabetic subjects and 10 subjects with type 2 diabetes mellitus in whom sufficient plasma was available to permit measurement of C5 glucose, C2 glucose and $^2$H$_2$O enrichment at hourly intervals during a prolonged hyperinsulinemic euglycemic clamp. Subject characteristics are given in supplemental table 1.

**Experimental Design**

Details of the experimental design have been described in detail elsewhere (19). Subjects were admitted to the Mayo Clinical Research Unit on the evening before study given a standard meal at 1700 and 1.67 grams $^2$H$_2$O/kg of body water in divided doses at 1800, 2000 and 2200. Insulin was infused in the diabetic subjects during the night to maintain glucose at ~5.5 mmol/l. A primed (fasting glucose divided by 5.5 mmol/L times 12 μCi) continuous (0.12 μCi/min) infusion
of $[3^-\text{H}]$ glucose was started at 0700; infusions of insulin (0.6 mU/kg/min), somatostatin (60 ng/kg/min), growth hormone (3 ng/kg/min) and glucagon (0.65 ng/kg/min) were started at 1000 (time 0 min). Glucose containing $[3^-\text{H}]$ glucose was infused in amounts sufficient to maintain euglycemia as previously described (20).

Analytical techniques
Plasma glucose, insulin, $[3^-\text{H}]$ glucose specific activity and enrichment of deuterium on the 2\textsuperscript{nd} and 5\textsuperscript{th} carbons of plasma glucose were measured as previously described (1; 2; 21).

Calculations
Rates are expressed in the figures and text as $\mu$mol per kg lean body mass per minute. Rates of glucose appearance, disappearance and endogenous glucose production were calculated using the steady state equations of Steele et al (22) as previously described (19). Rates of gluconeogenesis were calculated either by multiplying the plasma C5/C2 glucose ratio by endogenous glucose production or plasma C5 glucose/$^2\text{H}_2\text{O}$ ratio by the total rate of glucose appearance (2). Glycogenolysis was calculated by subtracting the rate of gluconeogenesis from endogenous glucose production. The results form the diabetic subjects have been previously been published in part elsewhere (19).

Statistical Analysis
Data in the text and figures are expressed as mean $\pm$ SEM. Student’s paired t test was used to determine whether rates calculated using the plasma C5/C2 glucose ratio differed from those calculated using the plasma C5 glucose/$^2\text{H}_2\text{O}$ ratio. A p value of less than 0.05 was considered as statistically significant.

Results
Plasma glucose and insulin concentrations (supplemental figure 1) Plasma glucose concentrations in the non-diabetic and diabetic subjects averaged 5.31 $\pm$ 0.16 and 5.41 $\pm$ 0.10 mmol/l before the clamp and did not change during the clamp.

Plasma insulin concentrations in the non-diabetic and diabetic subjects averaged 31 $\pm$ 4 and 178 $\pm$ 41 pmol/l prior to the clamp and increased to 177 $\pm$ 11 and 203 $\pm$ 14 pmol/l during the clamp.

Glucose infusion rate required to maintain euglycemia and $[3^-\text{H}]$ glucose specific activity (supplemental figure 2) The glucose infusion rate required to maintain euglycemia increased in both groups during the first four hours of the clamp then plateaued thereafter. The glucose infusion rate required to maintain euglycemia during the final hour of study was higher ($p<0.01$) in the non-diabetic than diabetic subjects (45.9 $\pm$ 3.6 vs. 21.9 $\pm$ 5.6 $\mu$mol/kg/min).

Plasma $[3^-\text{H}]$ glucose specific activity remained constant in both groups during the clamp enabling accurate measurement of glucose turnover.

Glucose disappearance and endogenous glucose production (figure 1) Glucose disappearance increased in both groups during the first four hours of the clamp then remained unchanged thereafter. In contrast, endogenous glucose production was maximally suppressed in both groups within three hours. Glucose disappearance during the final hour of study was higher ($p<0.01$) in the non-diabetic than diabetic subjects whereas endogenous glucose production did not differ between groups.

Plasma $^2\text{H}_2\text{O}$, C2 glucose and C5 glucose enrichment (figure 2) Plasma $^2\text{H}_2\text{O}$ and C2 glucose enrichment did not differ in either the non-diabetic or diabetic subjects before the clamp. Plasma $^2\text{H}_2\text{O}$ enrichment remained unchanged in both groups during the clamp. In contrast, plasma
C2 glucose and C5 glucose enrichment decreased (p<0.01) in both groups during the clamp.

Rates of gluconeogenesis and glycogenolysis calculated using the plasma C5 glucose/$^2$H$_2$O and plasma C5/C2 glucose ratios (figures 3)

Rates of gluconeogenesis and glycogenolysis measured before the clamp using the plasma C5 glucose/$^2$H$_2$O ratio did not differ from those measured using the plasma C5/C2 glucose ratio in either group. In contrast, rates of gluconeogenesis measured during the clamp using the plasma C5 glucose/$^2$H$_2$O ratio were consistently greater (p<0.01) and glycogenolysis consistently lower (p<0.01) than those measured using the plasma C5/C2 glucose ratio in both the non-diabetic and diabetic subjects with the difference being most evident during the first two hours of the clamp.

Of note, rates of glycogenolysis calculated using the plasma C5 glucose/$^2$H$_2$O ratio were lower (p<0.05) than zero at 120 minutes in the non-diabetic subjects indicating that calculated rates of gluconeogenesis exceeded endogenous glucose production. Rates of glycogenolysis calculated using the plasma C5 glucose/$^2$H$_2$O ratio then converged toward zero thereafter. A similar pattern was observed in the diabetic subjects with rates of glycogenolysis calculated using the C5 glucose/$^2$H$_2$O ratio decreasing to less than zero at 120, 180 and 240 minutes then converging toward zero thereafter.

Correlations (figure 4)

Rates of gluconeogenesis measured in the non-diabetic subjects using the C5 glucose/$^2$H$_2$O ratio correlated (p<0.05) with those measured using the C5/C2 glucose ratio until 240 minutes implying that the contribution of gluconeogenesis relative to the contribution of glucose clearance to C5 glucose concentration increased with time.

Discussion

The present studies confirm that the C5 glucose/$^2$H$_2$O and the C5/C2 glucose ratios provide equivalent estimates of gluconeogenesis and glycogenolysis following an overnight fast in both non-diabetic and diabetic subjects. In contrast, use of the C5 glucose/$^2$H$_2$O ratio during an euglycemic hyperinsulinemic clamp systematically overestimates gluconeogenesis and underestimates glycogenolysis.

In the presence of $^2$H$_2$O, the fifth carbon of glucose is labeled with deuterium during gluconeogenesis (1; 2). The second carbon of glucose is labeled with deuterium during equilibration of glucose-6 phosphate and fructose-6 phosphate (1; 2). At equilibrium, the enrichment of deuterium on the second carbon of glucose equals that of plasma water. Since plasma glucose derived from either gluconeogenesis or glycogenolysis passes though the glucose-6-phosphate pool, at steady state either the C5 glucose/$^2$H$_2$O or the C5/C2 glucose ratio equals the proportion of plasma glucose derived from gluconeogenesis (1; 2). The situation changes during a hyperinsulinemic euglycemic clamp when the rate of release of C5 glucose decreases due to suppression of glucose production and the rate of clearance of C5 glucose increases due to stimulation of glucose uptake. As is evident from figure 2, C5 glucose enrichment fell throughout the five hours of the study in the diabetic subjects indicating that equilibrium never was reached. While C5 glucose enrichment also fell during the clamp in the non-diabetic subjects it appeared to approach steady state during the final hour of the study. Therefore, plasma C5 glucose concentrations
during the first portion of the clamp were higher than those that would be present when a new steady state eventually was achieved. In contrast, since C5 glucose and C2 glucose are cleared in parallel, the C5/C2 glucose ratio only is influenced by the rates of release of C5 glucose and C2 glucose enabling assessment of gluconeogenesis.

Since plasma C5 glucose enrichment is diluted by both endogenously produced and exogenously infused glucose, gluconeogenesis is calculated by multiplying the C5 glucose/\(^2\text{H}_2\text{O}\) ratio by total rate of glucose appearance. Use of this approach resulted in rates of gluconeogenesis that not only were greater than those calculated using the C5/C2 glucose ratio but also that were greater than endogenous glucose production. This yielded biologically implausible (i.e. negative) rates of glycogenolysis. A similar pattern has been observed in previous studies that have used the C5 glucose/\(^2\text{H}_2\text{O}\) ratio to calculate gluconeogenesis during a hyperinsulinemic euglycemic clamp. Use of the C5 glucose/\(^2\text{H}_2\text{O}\) ratio during a glucose clamp overestimates gluconeogenesis and underestimates glycogenolysis. In contrast, since the C5/C2 glucose ratio only is influenced by gluconeogenesis, estimates obtained using this ratio will not be altered by changes in glucose clearance. We therefore recommend using the C5/C2 glucose ratio to assess gluconeogenesis during a hyperinsulinemic clamp. We also recommend performing the clamp for a sufficient duration (e.g. at least three hours) so that the effects of insulin on gluconeogenesis have time to become readily evident.

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Glucose disappearance and endogenous glucose production. An insulin infusion was started at time zero.
Plasma enrichment of $^2$H$_2$O, C2 glucose and C5 glucose observed in the non-diabetic and diabetic subjects. An insulin infusion was started at time zero.
Rates of gluconeogenesis and glycogenolysis measured in the non-diabetic (figure 3a) and diabetic (figure 3b) subjects using the plasma C5 glucose/C2 glucose or plasma C5 glucose/$^2$H$_2$O ratios. An insulin infusion was started at time zero.
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Manuscript Figure 3b
Correlation of Gluconeogenesis Calculated with C5/C2 glucose or C5 glucose/$^2$H$_2$O

**Non-Diabetic**

- $r = 0.78$, $p = 0.008$
- $r = 0.66$, $p = 0.037$
- $r = 0.79$, $p = 0.006$
- $r = 0.77$, $p = 0.009$

**Diabetic**

- $r = 0.03$, $p = 0.930$
- $r = 0.49$, $p = 0.160$
- $r = 0.68$, $p = 0.035$
- $r = 0.85$, $p = 0.002$

120 Min, 180 Min, 240 Min, 300 Min

Manuscript Figure 4

Correlation between the rates of gluconeogenesis measured using C5 glucose/$^2$H$_2$O ratio and the C5 glucose/C2 glucose ratio.