Origins of the hydrogen bound to carbon 1 of glucose in fasting: significance in gluconeogenesis quantitation

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Origins of the hydrogen bound to carbon 1 of glucose in fasting: significance in gluconeogenesis quantitation. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E717–E723, 1999.—Healthy subjects ingested [2H]2O. [2H]enriched the hydrogen bound to carbon 1 of blood glucose 1.3 to 1.8 times more than the hydrogens bound to carbon 6. Enrichment at carbon 1 was more than at carbon 5 after 14 h, but not after 42 h, of fasting. After overnight fasting, when [2,3-3H]sucinate was infused, 34 times as much [3H]was bound to carbon 6 as to carbon 1. On [1-2H,1-3H,1-14C]galactose infusion, the ratios of [2H] to [14C] and of [3H] to [14C] in blood glucose were 30% less than in the galactose. [3H] at carbon 6 was 1% of that at carbon 1 of the glucose. Thus, although the two hydrogens bound to carbon 1 and the two bound to carbon 6 of fructose 6-phosphate (p) during gluconeogenesis are equally enriched in [3H] via pyruvate's equilibration with alanine, one of each is further enriched via hydration of fumarate that is converted to glucose. That hydrogen at carbon 1 of fructose 6-phosphate (P) is also enriched in fructose 6-P's equilibration with mannose 6-P. [2H] from [2H]2O at carbon 1 to carbon 2 of blood glucose cannot then quantitate gluconeogenesis because of 1-[2H]glucose formation during glycogenolysis. Triose-P cycling has a minimal effect on quantitation. [3H] recovery in glucose from [1-2H]galactose does not quantitate galactose conversion via UDP-glucose to glycogen.

deuterium oxide; galactose; succinate; glycogenolysis

AFTER INGESTION of [2H]2O by subjects in the fasted state, the ratio of [2H] bound to carbon 6 to carbon 2 of blood glucose provides a measure of the contribution of gluconeogenesis to glucose production (8). That is because binding at carbon 2 occurs during gluconeogenesis and glycogenolysis, whereas binding at carbon 6 occurs only during gluconeogenesis. However, the ratio gives an underestimate because 1) binding of [3H] at carbon 6 depends on the exchange of the hydrogens of pyruvate and alanine and of oxaloacetate and fumarate with those of water in their equilibrations before oxaloacetate's conversion to glucose, and 2) the conversion of glycerol to glucose does not result in binding of [3H] at carbon 6. The ratio of [2H] bound to carbon 5 to carbon 2 of blood glucose quantitates gluconeogenesis (2, 9), because binding of [2H] at carbon 5 occurs 1) in the hydration of phosphoenolpyruvate, an intermediate in the conversion of oxaloacetate to glucose and 2) in the extensive equilibration with glyceraldehyde 3-phosphate (P) of dihydroxyacetone 3-P, an intermediate in the conversion of glyceraldehyde to glucose, and 3) because no binding of [3H] occurs at carbon 5 in glycogenolysis.

If the hydrogen bound to carbon 1 of glucose were bound only via gluconeogenesis, the ratio of [2H] at carbon 1 to carbon 2 of glucose could also be used to estimate the contribution of gluconeogenesis to glucose production. The method for measuring the [2H] enrichment at carbon 1 is easier than that at carbon 5 (see METHODS). Because labeling via the oxaloacetate's equilibration with fumarate is through a hydration reaction that is extensive and stereospecific, binding of [3H] at carbon 1 could exceed that at carbon 6. However, labeling at carbon 1 could also occur via equilibration between mannose 6-P and glucose 6-P via fructose 6-P.

The aim of this study was then 1) to measure the extent of labeling of the hydrogen bound to carbon 1 compared with the hydrogens bound to carbons 2, 5, and 6 of blood glucose when subjects ingest [2H]2O in the fasted state and 2) to determine the pathways of labeling at carbon 1.

METHODS

Subjects

Healthy men and women participated in one of three protocols. They had been on their usual weight-maintaining diet, which by history contained 200 g of carbohydrate daily. They ate a dinner of 12–14 kcal/kg body weight between 1700 and 1800 in the first protocol and between 1900 and 2000 in the second and third protocols. The dinner was composed of 48% carbohydrate, 19% protein, and 33% fat. They then began fasting. The subjects were informed of the purpose and possible risks involved in the study, and each gave informed written consent. The study was approved by the Institutional Review Committees at the Karolinska Hospital and University Hospitals of Cleveland.

Labeled Materials

D-[1-3H]galactose (5.7 Ci/mmol) and D-[1-14C]galactose (56 mCi/mmol) were purchased from Moravak Biochemical (Brea, CA). [2,3-3H]sucinic acid (37.5 Ci/mmol) was purchased from Sigma Chemical (St. Louis, MO). Radiochemical purity of the galactose was reported to be 98% or more. That was confirmed by subjecting samples to HPLC with an HPX-87P column (Bio-Rad Laboratories, Hercules, CA) with water at 80°C as solvent. Purity of the succinate was reported to be >95%. That was shown by HPLC with an HPX-87H column with 0.01 N H2SO4 at 65°C as solvent. D-[1-14C]galactose and D-[1-3H]glucose, each reported to be 98% atom excess in [3H], were purchased from Isotec (Miamisburg, OH). The labeled...
galactose and the succinate, undiluted, were dissolved in isotonic saline. The solutions were shown to be sterile and pyrogen free. \(^2\H_2\O\), 99.9% \(^{3}H\), was also purchased from isotope.

\(^2\H_2\O\) Administration

This portion of the study was previously reported (9), except for the enrichment of the hydrogen bound to carbon 1 of blood glucose. Two men and three women, 24–39 yr old, with body mass indexes between 20.7 and 25.3 kg/m\(^2\) (subjects TA, CS, HH, JC, and EL in Ref. 9) fasted for 42 h. Five hours after beginning the fast, each subject ingested 2.5 ml of \(^2\H_2\O\)/kg body water and then again 4 h later. Body water was calculated at 60% of body weight in the men and 50% in the women. They were allowed to drink water ad libitum enriched to 60% of body weight in the men and 50% in the woman. They were allowed to drink water ad libitum enriched to 0.5% \(^{3}H\)\(^2\H_2\O\) beginning 5 h into the fast. At 14, 22, and 42 h, blood was drawn (20 ml) from an antecubital vein.

Analyses. Glucose was isolated from blood, and the enrichments of the hydrogens at its carbons 2, 5, and 6, previously reported (9), and of the hydrogen at its carbon 1 were determined as described in the next protocol.

Calculations. The ratio of the enrichments of the hydrogen at carbon 1 to carbon 6, carbon 1 to carbon 5, as well as at carbon 5 to carbon 2, were calculated.

Succinate with \(^{3}H\)\(^2\H_2\O\) Administration

Three men and one woman, 24–37 yr old, with body mass indexes between 22.2 and 26.6 kg/m\(^2\), fasted for 15 h. Beginning at 11 h into the fast, each was infused continuously via an antecubital vein in one arm with 50 \(\mu\)Ci/h of the \(2,3-{\(^{3}H\})\)succinate in the saline at a rate of 10 ml/h. Each subject ingested 1.25 ml \(^{2}\H_2\O\)/kg body water at 11 h and then again at 45-min intervals three more times for a total of 5 ml \(^{2}\H_2\O\)/kg body water. Body water was again calculated to be 60% of body weight in the men and 50% in the woman. Subjects were allowed to drink water ad libitum enriched to 0.5% \(^{3}H\)\(^2\H_2\O\) beginning 11 h into the fast. At 14 h and 15 h, blood was collected (60 ml), each time from an antecubital vein of the other arm for isolation of blood glucose.

Analyses. Glucose concentration was determined using a Glucose Analyzer (YSI 2300 Analyzer, Yellow Springs Instrument, Yellow Springs, OH). To isolate the glucose, protein was precipitated from the 60 ml of blood by addition of twice the volume of water and equal volumes of 0.3 N Ba(OH)\(_2\) and 0.3 N ZnSO\(_4\). The supernatant was deionized by passage through a column of 25 g each of ion exchange resins, AG 1-X8 in the formate form and AG 50W-X8 in the H\(^{+}\) form. The effluent was evaporated, and glucose in the residue was purified by HPLC by use of the HPX-87P column with water at 80°C as solvent.

Enrichments of the hydrogens bound to carbons 2, 5, and 6 of the glucose were determined as previously detailed (2, 8, 9). One milligram of the glucose was oxidized with periodate to yield formaldehyde containing the two hydrogens bound to carbon 6. Two milligrams were converted to pentitol phosphates, which were oxidized to yield formaldehyde containing the hydrogen bound to carbon 2. Three milligrams were converted to xylulose, which was oxidized to yield formaldehyde containing the hydrogen bound to carbon 5. The formaldehydes were condensed with ammonia to form hexamethylene tetramines (HMTs), which were assayed for \(^{3}H\) excess. Assay was performed on a gas chromatograph-mass spectrometer (HP-5985, Hewlett-Packard, Palo Alto, CA). The HMT was injected directly. Electron impact ionization was used, with the ions selectively monitored (2).

The hydrogen bound to carbon 1 was also collected and assayed in HMT via formaldehyde formation. Three milligrams of the glucose were converted to glucose 6-P by incubation with ATP and hexokinase. The glucose 6-P was reduced to sorbitol 6-P with NaBH\(_4\). The sorbitol 6-P was also oxidized with periodate to yield the formaldehyde containing carbon 1 of the glucose with its hydrogen. Both the glucose 6-P and sorbitol 6-P in these procedures were purified by application in a 6.5 x 1-cm column to 2 ml of Ag 1-X8 resin in the formate form. After being washed with water, the sugar 6-P was eluted in 1 N formic acid and the eluate evaporated.

HMTs of known enrichments provided standards for the assays. They were prepared by reducing the \(1-{\(^{3}H\})\)glucose, diluted to known enrichments, to sorbitol using NaBH\(_4\). The sorbitols were oxidized with periodate to yield the formaldehydes for conversion to HMTs.

After the above determinations, blood glucose remaining from each collection, usually ~20 mg, was diluted with unlabeled glucose to 36 mg. Twelve milligrams were oxidized, and the formaldehyde formed, again containing carbon 6 of glucose with its hydrogens, was condensed with dimethylhydrosorcinol to form formaldimedone (16). Eighteen milligrams were converted to sorbitol 6-P via glucose 6-P, as described above, and oxidized to yield carbon 1 with its hydrogen. That formaldehyde was also converted into a formaldimedone. The remaining 6 mg were reduced to sorbitol, and the sorbitol was oxidized to form formaldehyde, as just described, and from the formaldehyde again a formaldimedone was made. That formaldimedone then contained the hydrogens bound to both carbon 1 and carbon 6 of glucose. Known weights of the formaldimedones, with a range of 5.3–23.8 mg, were assayed for \(^{3}H\) activity in a liquid scintillation counter (Tri-Carb, model 1600 TR, Packard Instrument, Meriden, CT) with Ecolume as the scintillant cocktail (ICN, Costa Mesa, CA). Counting was for 50 min, resulting in probable error in the assay of \(^{3}H\) radioactivity at carbon 6 of the glucoses of <3%.

Calculations. The fraction gluconeogenesis contributed to glucose production was taken as the ratio of \(^{3}H\) enrichment at carbon 5 to that at carbon 2 of the blood glucose. The ratio of the enrichment at carbon 1 of the glucose was compared with that in the enrichment of the two hydrogens bound to carbon 6 and to the enrichment of the hydrogen bound to carbon 5. From the dpm in the weight of dimeredone assayed and the extent the blood glucose was diluted with unlabeled glucose, the specific radioactivities of the hydrogens bound to carbon 1 and to carbon 6 of the glucose were calculated. The sum of those specific radioactivities was compared with the specific radioactivity of the formaldimedone prepared from the sorbitol and hence from formaldehyde from both carbons 1 and 6. This was done to support the adequacy of the procedures for the isolation of the individual carbons with their hydrogens.

**Galactose Administration**

Two men and two women, 19–28 yr old, with body mass indexes between 19.1 and 23.8, also continued to fast for 15 h. Each subject was infused continuously via an antecubital vein beginning at 11 h into the fast with 13.3–15.7 \(\mu\)Ci/h of \(1-{\(^{3}H\})\)galactose, 6.0–7.7 \(\mu\)Ci/h of \(1-{\(^{14}C\})\)galactose, and 99–108 mg/h of the \(1-{\(^{3}H\})\)galactose in saline at a rate of 12 ml/h. At 14 h and 15 h, blood was collected (60 ml) from an antecubital vein of the other arm for isolation of blood glucose.

Analyses. Glucose concentration and the isolation of blood glucose were done as described for the labeled succinate administration. An aliquot of the labeled galactose infusate was diluted 100-fold with unlabeled galactose. Three milli-
grams were reduced with NaBH$_4$ to galactitol, which was isolated using HPLC and oxidized to yield formaldehyde and, hence, an HMT containing carbons 1 and 6 of the galactose with their hydrogens. The procedure was then identical to that in forming the HMT from glucose via reduction to sorbitol, as just described. The enrichment of the hydrogen bound to carbon 1 of the galactose was calculated by multiplying the HMT's enrichment by 2, because formaldehyde containing carbon 6 with its unlabeled hydrogens diluted the formaldehyde containing carbon 1 with its labeled hydrogen.

Another 10 mg of the diluted galactose infusion was also reduced to galactitol that was oxidized to formaldehyde, but that formaldehyde was reacted to form a formaldehyde. An HMT was prepared from 3 mg of blood glucose, after its conversion to sorbitol 6-P, to yield the enrichment at its carbon 1.

The remaining blood glucose, ~25 mg, was diluted to 40 mg by addition of unlabeled glucose. Aliquots were used to determine the $^3$H and $^{14}$C specific activities at carbon 1, carbon 6, and carbons 2 and 5 together. Formaldehyde from 1) 20 mg of the glucose via sorbitol 6-P, 2) 10 mg of the glucose, and 3) 5 mg of the glucose reduced to sorbitol. In assaying the HMTs, we prepared HMTs of known enrichment from formaldehyde made by oxidation of galactitol made by diluting the purchased [1-$^2$H]glucose with unlabeled glucose. Again, known weights of the formaldehyde, ranging from 5.4 to 8.0 mg, were assayed for $^3$H and $^{14}$C activities in the liquid scintillation counter. Counting was 25-50 min, resulting in a probable error of <1% in the assays of $^{14}$C radioactivity at carbon 1 of galactose and glucose. From those radioactivities, the weights of the formaldehyde, and the dilution of the labeled galactose with unlabeled galactose and the blood glucose with unlabeled glucose, specific radioactivities were calculated.

Calculations. The ratio of the $^3$H-to-$^{14}$C specific radioactivities at carbon 1 of the blood glucose to that in the galactose infused × 100 was taken as the percentage of the $^3$H retained relative to $^{14}$C in the conversion of the [1-$^3$H,1-$^{14}$C]galactose to [1-$^2$H,1-$^{14}$C]glucose. The ratio of $^3$H enrichment to $^{14}$C specific activity at carbon 1 of blood glucose to that in the galactose infused × 100 was taken as the percentage of the $^3$H retained in the conversion of the [1-$^3$H,1-$^{14}$C]galactose to [1-$^2$H,1-$^{14}$C]glucose. The sum of the $^3$H enrichments and $^3$H and $^{14}$C specific activities at carbons 1 and 6 of glucose was compared with the sum of the enrichments and specific radioactivities at those carbons obtained by oxidation of the sorbitols. Enrichment of the hydrogen at carbon 6 was below the sensitivity of the assay and therefore was taken as 0.

Table 1. $^3$H enrichment (atom percent excess) of hydrogen bound to carbons 1, 2, 5, and 6 of glucose normalized to 0.5% body water enrichment

<table>
<thead>
<tr>
<th>Hours of Fasting</th>
<th>$^3$H Enrichment</th>
<th>Carbon 1</th>
<th>Carbon 2</th>
<th>Carbon 5</th>
<th>Carbon 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0.33 ± 0.01</td>
<td>0.44 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.18 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>0.40 ± 0.02</td>
<td>0.47 ± 0.02</td>
<td>0.35 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>0.45 ± 0.01</td>
<td>0.50 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.34 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 5). Enrichments in urinary water, the measure of enrichments of body water, for each of 5 subjects have been reported, 0.451 ± 0.014% (8). Normalization for each subject was made by multiplying the $^3$H enrichment measured at the carbons in that subject's glucose by 0.5% divided by the enrichment in that subject's urinary water.

Table 2. Enrichments and specific radioactivities of hydrogen bound to carbons of blood glucose from fasted subjects ingesting $^3$H$_2$O and infused with [2,3-$^3$H]glucose

<table>
<thead>
<tr>
<th>Subject's Initials</th>
<th>Hours of Fasting</th>
<th>$^3$H Enrichment</th>
<th>Carbon 1</th>
<th>Carbon 2</th>
<th>Carbon 5</th>
<th>Carbon 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM</td>
<td>14</td>
<td>0.30</td>
<td>0.46</td>
<td>0.26</td>
<td>0.17</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.35</td>
<td>0.56</td>
<td>0.27</td>
<td>0.16</td>
<td>1.3</td>
</tr>
<tr>
<td>EA</td>
<td>14</td>
<td>0.33</td>
<td>0.43</td>
<td>0.24</td>
<td>0.19</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.31</td>
<td>0.44</td>
<td>0.21</td>
<td>0.17</td>
<td>1.4</td>
</tr>
<tr>
<td>SV</td>
<td>14</td>
<td>0.21</td>
<td>0.31</td>
<td>0.13</td>
<td>0.11</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.24</td>
<td>0.34</td>
<td>0.17</td>
<td>0.13</td>
<td>1.1</td>
</tr>
<tr>
<td>KL</td>
<td>14</td>
<td>0.29</td>
<td>0.42</td>
<td>0.28</td>
<td>0.19</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.30</td>
<td>0.46</td>
<td>0.31</td>
<td>0.20</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Enrichments are expressed in %excess $^3$H, and radioactivities are expressed in dpm/µmol.

Statistics

Means ± SE are recorded. Significance of differences has been determined using Student's t-test.

RESULTS

Recorded in Table 1 are enrichments of the hydrogens bound to carbon 1 and to carbons 2, 5, and 6 (previously reported in Ref. 9) in blood glucose from the subjects fasted 42 h. Enrichments at carbon 1 were, respectively, 1.8, 1.7, and 1.3 times the average of enrichments of the two hydrogens at carbon 6 at 14 h ($P < 0.01$), 22 h ($P < 0.01$), and 42 h ($P < 0.01$) of fasting. Enrichment at carbon 1 was 1.3 times that at carbon 5 at 14 h ($P < 0.05$), and the enrichments were the same at 42 h. The ratio of the enrichment at carbon 5 to that at carbon 2 increased from 0.59 to 0.74 and to 0.92 from 14 h to 22 h and to 42 h, in keeping with increasing contributions of gluconeogenesis to glucose production as fasting progresses.

Table 2 records for the four subjects given $^3$H$_2$O and [2,3-$^3$H]glucose the enrichments of the hydrogens bound to carbons 1, 2, 5, and 6; and 2) the $^3$H specific activities of the hydrogens bound to carbons 1 and 6 of the blood glucose collected at 14 and 15 h of fasting. Enrichments and specific radioactivities at 14 and 15 h for each subject are similar. Therefore, the average for the 2 h has been used in calculations. The sum of the specific radioactivities of the hydrogens at carbons 1 and 6 was 100 ± 2% of the specific radioactivity of those
hydrogens collected together via sorbitol formation (see METHODS), in support of the analytical methods used (data not shown). The ratios of the enrichments and specific radioactivities in Table 2 are recorded in Table 3 with their means ± SE. Gluconeogenesis, determined by the enrichments at carbon 5/carbon 2, contributed 55 ± 6% to glucose production. That is in accord with the results in Table 1 after 14 h of fasting and with our previous measurements (2, 9). Also in accord with the results in Table 1, at 14 h the enrichment of the hydrogen bound to carbon 1 was 1.8 times the average of the enrichment of hydrogens bound to carbon 6, and the hydrogen at carbon 1 had 1.3 times the enrichment at carbon 5. The specific radioactivity of the hydrogens bound to carbon 6 was 34 times that of the hydrogen bound to carbon 1.

The 3H-to-14C and 2H-to-14C ratios at carbon 1 of the blood glucose collected at 14 and 15 h of fasting from the four subjects infused with the labeled galactose are recorded in Table 4. They are recorded as a percentage of the ratios in the [1-2H,1-3H,1-14C]galactose. The 14C specific activity of the galactose infused was 25,030 ± 520 dpm/µmol, and the 14C specific activity of the blood glucose at 15 h was 202 ± 23 dpm/µmol. The 3H specific activity of the hydrogen bound to carbon 6 is recorded

<table>
<thead>
<tr>
<th>Subject’s</th>
<th>Hours of Fasting</th>
<th>Glucose* Galactose</th>
<th>Carbon 6† Carbon 1 + Carbon 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL</td>
<td>14</td>
<td>80.1 78.1</td>
<td>0.8 2.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>78.1 70.6</td>
<td>1.0 2.3</td>
</tr>
<tr>
<td>HU</td>
<td>14</td>
<td>77.0 71.6</td>
<td>1.3 2.7</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>76.5 66.9</td>
<td>1.5 3.2</td>
</tr>
<tr>
<td>HN</td>
<td>14</td>
<td>71.2 73.5</td>
<td>1.1 3.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>79.5 64.8</td>
<td>1.2 4.0</td>
</tr>
<tr>
<td>AB</td>
<td>14</td>
<td>76.5 51.2</td>
<td>0.5 2.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>73.8 44.2</td>
<td>0.7 2.7</td>
</tr>
<tr>
<td>Means ± SE</td>
<td></td>
<td>76.9 ± 0.9</td>
<td>65.1 ± 5.9</td>
</tr>
</tbody>
</table>

*3H/14C and 2H/14C, ratios of 3H to 14C and 2H to 14C, respectively.
†3H/14C or 2H/14C at carbon 1 of blood glucose.

3H-to-14C and 2H-to-14C ratios at carbon 1 of the blood glucose collected at 14 and 15 h of fasting from the four subjects infused with the labeled galactose are recorded in Table 4. They are recorded as a percentage of the ratios in the [1-2H,1-3H,1-14C]galactose. The 14C specific activity of the galactose infused was 25,030 ± 520 dpm/µmol, and the 14C specific activity of the blood glucose at 15 h was 202 ± 23 dpm/µmol. The 3H specific activity of the hydrogen bound to carbon 6 is recorded.

Fig. 1. Deuterium (D) labeling from 2H2O at carbon 3 of oxaloacetate (OAA). A: pyruvate’s equilibration with alanine results in equal labeling of its three hydrogens, but because equilibration is not complete, enrichment in each hydrogen is less than that in the 2H2O. B: pyruvate yields OAA, with the two hydrogens at carbon 3 having the enrichment of the hydrogens in the pyruvate. C: OAA also equilibrates with fumarate via malate, and to that extent one of the two hydrogens at carbon 3 of OAA takes on the enrichment of the 2H2O, that hydrogen then becoming more enriched than the other hydrogen.
as a percentage of the sum of the 3H specific activities of the hydrogens bound to carbons 1 and 6. Percentages at 14 and 15 h for each subject are similar, and therefore for statistical analysis again the average of the 14-h and 15-h values for each subject has been calculated, and the mean ± SE for the four averages is presented.

Relative to 14C, 76.6% of the 3H and 65.5% of the 2H bound to carbon 1 of the galactose were retained in glucose. Only 1.0% as much 3H and 2.9% as much 14C were at carbon 6 as the sum at carbons 1 and 6 of blood glucose. The sums of the 3H specific activities of the hydrogens at carbons 1 and 6 and of their 14C specific activities, individually determined, were 99 ± 2 and 93 ± 3%, respectively, of the specific radioactivities of those hydrogens and carbons isolated together as formaldehyde by the oxidation of sorbitol formed from the glucose. Enrichment of the hydrogen bound to carbon 1 of the glucose was 107 ± 3% of the enrichments of the hydrogens bound to carbons 1 and 6 of the glucose, again determined via sorbitol formation (data not shown).

**DISCUSSION**

Enrichments from 2H2O of the hydrogens bound to carbon 1 and carbon 6 of fructose 6-P in the process of gluconeogenesis originate in the reactions forming oxaloacetate. Because the hydrogens at carbons 1 and 6 then have the same precursor, i.e., the hydrogens bound to the methylene carbon of the oxaloacetate, they should have the same enrichments. The fact that enrichments of the hydrogen at carbon 1 of glucose have 1.3–1.8 times the average of the enrichment of the two hydrogens at carbon 6 then indicates that 1) one of the two hydrogens bound to carbon 1 and to carbon 6 of fructose 6-P had a higher enrichment than the other hydrogen and 2) it was the hydrogen with the higher enrichment at carbon 1 that became the hydrogen bound to carbon 1 of blood glucose. The pathways responsible for that higher enrichment and the consequences for estimations of contributions of gluconeogenesis will now be examined.

When pyruvate equilibrates with alanine, catalyzed by transaminase in a medium containing 2H2O, the three hydrogens bound to carbon 3 of pyruvate are enriched to the same extent (14) (Fig. 1A). Hence, the methylene hydrogens of oxaloacetate formed from that pyruvate are equally enriched (Fig. 1B). Therefore, that equilibration cannot be the explanation for the greater enrichments at carbon 1 of the blood glucose. The rate of equilibration of fumarate with oxaloacetate is rapid relative to the rate of gluconeogenesis (11), and the hydration of fumarate to form malate is stereospecific (1, 4). Thus, in the presence of 2H2O, one of the two hydrogens of the methylene group of the fructose is removed in the isomerization to glucose 6-P and hence in glucose. The hydrogen bound to carbon 2 of GAP is labeled by the 2H2O and becomes that bound to carbon 5 of glucose formed via the condensing of the GAP with DHAP. In the isomerization of F-6-P to G-6-P, the hydrogen bound to carbon 2 of the glucose is also labeled.

![Fig. 2. Conversion of [2,3-3H]succinate to OAA in the presence of 2H2O. Tritium (T) labels both hydrogens of fumarate formed from the succinate. Addition of 2H2O to malate formed from the fumarate results in carbon 3 of OAA having one hydrogen labeled with D and the other with T.](image)

![Fig. 3. Conversion to glucose of OAA formed from [2,3-3H]succinate and unlabeled pyruvate in the presence of 2H2O. OAA formed by the reactions depicted in Figs. 1 and 2 yields phosphoenolpyruvate (PEP) and hence the triose phosphates (P), glyceraldehyde 3-P (GAP) and dihydroxyacetone 3-P (DHAP), with D at both hydrogens and T at one hydrogen of their carbon 3. The triose phosphates then condense to yield fructose 6-P (F-6-P). The hydrogen bearing T at carbon 1 of the F-6-P is removed in the isomerization to glucose 6-P (G-6-P) and hence in glucose. The hydrogen bound to carbon 2 of GAP is labeled by the 2H2O and becomes that bound to carbon 5 of glucose formed via the condensing of the GAP with DHAP. In the isomerization of F-6-P to G-6-P, the hydrogen bound to carbon 2 of the glucose is also labeled.](image)

![Fig. 4. Labeling from 2H2O at carbon 1 of G-6-P through phosphoglucoisomerase and phosphomannoisomerase activities. M-6-P, mannose 6-phosphate. Deuterium will also be bound to carbon 2 in the process.](image)
The evidence that is the case is found in the results of the [2,3-3H]succinate with 2H2O administration. Thus hydration of [2,3-3H]fumarate from [2,3-3H]succinate forms [3-3H,2,3-3H]malate.2 One of the two methylene hydrogens of the oxaloacetate from that malate is then labeled with 3H and the other with 2H (Fig. 2).3

The result is that on administration of 2H2O and [2,3-3H]succinate, the two hydrogens bound to carbon 1 and the two to carbon 6 of fructose 6-P, formed in the conversion of 2 oxaloacetate → fructose 6-P, will be enriched in 3H. However, one of the two hydrogens bound to each carbon, the one arising partially via the hydration of fumarate, will have the higher enrichment, while the other bears the 2H. It is the hydrogen bearing the 3H that is removed in the isomerization of fructose 6-P to glucose 6-P (Fig. 3). That is evidenced by the two hydrogens bound to carbon 6 of the blood glucose having 34 times as high a specific radioactivity as the hydrogen bound to carbon 1 of the glucose (Table 3).4

Essentially every molecule of glucose formed via gluconeogenesis bears a hydrogen from water at carbon 5 (Fig. 3). In support of that, at 42 h of fasting, enrichment at carbon 5 approaches that in body water (2, 9). Therefore, via gluconeogenesis the enrichment of the hydrogen bound to carbon 1 cannot exceed that bound to carbon 5. Accordingly, at 42 h of fasting, when glycogen stores are depleted, the enrichment at carbon 1 does not exceed that at carbon 5 (Table 1). However, it does at 14 h of fasting (Table 1), when glycolysis contributes to glucose production. Therefore, there must be a pathway, not yet considered, by which in a medium of 2H2O, the hydrogen bound to carbon 1 of blood glucose from glycogen is enriched. That means, during the conversion of glycogen to glucose, that the carbon-to-hydrogen bond at carbon 1 of the glycosyl unit of glycogen is cleaved and then reformed. The pathway is found in the equilibration of fructose 6-P with mannose 6-P. In fructose 6-P → glucose 6-P the hydrogen at carbon 1 labeled with 3H was lost. In the equilibration of fructose 6-P with mannose 6-P, the other hydrogen exchanges with the hydrogen of water (17). It is then that hydrogen that becomes the hydrogen bound to carbon 1 of glucose. Thus, when glycerol → glucose 1-P → glucose 6-P → fructose 6-P → mannose 6-P → fructose 6-P → glucose 6-P → glucose, the hydrogen bound to carbon 1 of glucose will be enriched from the 2H2O (Fig. 4). The evidence that this occurs is in the loss, relative to 14C, of 3H and 2H of the (1-2H,1-3H,1-14C)galactose in its conversion to glucose (Fig. 5). That is so because galactose will follow the same pathway as glucose 1-P formed from glycogen.5

The equilibration of mannose 6-P with fructose 6-P in addition to the hydration of fumarate then contributes to the greater enrichment of the hydrogen bound to carbon 1 of the glucose than the average of the enrichments of the hydrogens bound to carbon 6.

Enrichment of the hydrogens bound to carbon 6 of glucose continues to be used as a measure of the contribution of gluconeogenesis to glucose production (18). In the fasted state, the average enrichment of those hydrogens is only ~70% of the enrichment of the hydrogen bound to carbon 5 (Ref. 8 and Tables 1 and 2). Glycerol is released into the circulation by lipolysis of stored triglyceride and is therefore unlabeled (3). Whereas the conversion of that glycerol to glucose results in enrichment at carbon 5 and not carbon 6 (9), glycerol's contribution to gluconeogenesis can account for only a portion of the lower enrichment at carbon 6 than at carbon 5 (8, 10). The remainder is then attributable to the incomplete equilibration of pyruvate with alanine and oxaloacetate with fumarate.5

5 2H and 3H from [1-2H,1-3H]glucose 6-P are removed in the oxidative portion of the pentose pathway, but 14C from [1-14C]glucose 6-P is also lost as CO2. Hence, the 14H-to-14C and 3H-to-14C ratios in glucose would not change, except that because of an isotope effect in the removal of the 14H and 3H, higher ratios could result (7). Also, [2-2H]glucose 6-P formed on 2H2O ingestion will via the oxidative portion of the pentose pathway form [1-3H]ribulose 5-P. Hence, in the complete pathway, 3 [2-2H]glucose 6-P → 3 CO2 + 2 [1-2H]fructose 6-P + glyceraldehyde 3-P. However, one fructose 6-P will have 2H at its carbon 5, because glyceraldehyde 3-P is the precursor of its lower half. Also, after an overnight fast, only a relatively small amount of glucose 6-P is metabolized via the pathway (11).

6 During fasting, amino acids converted to glucose via α-ketoglutarate, succinate, and fumarate are released by proteolysis into the circulation and hence are also unlabeled. As fumarate → malate, 2H will be bound to the carbons of those amino acids that become carbon 6 of glucose. One of the two hydrogens at carbon 6 then will be labeled. In the hydration of phosphoenolpyruvate, 1H will also be bound to those carbons that become carbon 5 of glucose. Aspartate's conversion to glucose will result in 3H at carbon 6 to the extent oxaloacetate, formed from the aspartate, equilibrates with fumarate.

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2 In hydration of a double bond, a new carbon-to-hydrogen bond must be formed using a hydrogen from water. The exchange of a hydrogen bound to a carbon with a hydrogen from water depends on the extent of equilibration.

3 Both methylene hydrogens could be labeled with 3H via pyruvate cycling, i.e., [3-3H]phosphoenolpyruvate formed from the [3-3H]oxaloacetate would be converted to [3-3H]pyruvate, which would be recycled to oxaloacetate. However, because of equilibration of pyruvate with alanine, most of that 3H would be lost to water. Similarly, any 3H-labeled pyruvate, formed from 3H-labeled glucose synthesized from the [3-3H]oxaloacetate, would lose its 3H.

4 With only one of the two hydrogens bound to carbon 6 significantly labeled with 3H, that hydrogen will have a specific radioactivity essentially twice that of the two hydrogens.
Of the [1-3H]galactose conversion to glucose, only ~2% was via triose-P, i.e., galactose → UDP-glucose → glucose 6-P → triose-P → glucose 6-P → glucose. That is concluded, with the assumption of complete equilibration of glyceraldehyde 3-P with dihydroxyacetone 3-P (10), because only 1% of 3H in the glucose formed was bound to carbon 6 (Table 4). As much as 3% could have been converted because of the ~30% loss of 3H in the conversion of the [1-3H]galactose to [1-3H]glucose. Incorporation of 14C at carbon 6 of glucose only gives a maximum estimate of the conversion via triose-P, i.e., 2(2.9) = 5.8%, because incorporation is also via glucose 6-P → triose (lactate) → glucose 6-P → glucose. We (13) previously infused overnight-fasted women with [6-14C]galactose, and from the randomization of 14C in the carbons of blood glucose, we estimated a "triose-P cycling" of 13%. The estimate was made by approximating the portion of the incorporation at carbon 1 that went via triose. Although the women had been hyperthyroid, they had been treated, and evidence was that when studied they were in an euthyroid state.

There are three immediate consequences of these findings. First, a test of the validity of a method for quantitating the contribution of gluconeogenesis to glucose production is that it estimates nearly 100% gluconeogenesis on long-term fasting. Enrichment at carbon 5 to that at carbon 2 fulfills that requirement, but so does the enrichment at carbon 1 to carbon 2. However, in short-term fasting, because glycogenolysis contributes to the enrichment at carbon 1, substituting the enrichment at carbon 1 for that at carbon 5 will give overestimates of the contribution of gluconeogenesis. Second, the recovery in glucose of 2H from [1-2H]galactose has been used to estimate the amount of galactose converted to glycogen via UDP-glucose (6). That is an overestimate to the extent the 2H is removed in the conversion of the galactose to glucose (2), which in the present study is ~30%. Third, in the use of the enrichment of the hydrogen at carbon 5 to estimate gluconeogenesis in fasting (2, 9), glycerol is assumed not to be converted to triose-P via glucose 6-P and that triose-P is then converted to glucose. To the extent that such a conversion does occur, glucose formed from glycerol will be labeled at carbon 5 as well as carbon 2. A contribution of glycogenolysis to glucose production will then be attributed to gluconeogenesis. The extent of that triose-P cycling is very small, at least in the normal individual at 14 h of fasting. Thus triose-P cycling of 3% would decrease an estimate of a contribution of gluconeogenesis of 50 to 48.5%.

Furthermore, acetyl-CoA formed on fatty acid oxidation or from pyruvate, will have 3H bound to its methyl carbon. To the extent that acetyl-CoA condenses with oxaloacetate to form citrate, which via the Krebs cycle is oxidized to oxaloacetate, a methylene hydrogen of that oxaloacetate will contain some 4H from the acetyl-CoA.

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REFERENCES