Use of ^2H_2O for Estimating Rates of Gluconeogenesis

Application to the Fasted State

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Abstract

A method is introduced for estimating the contribution of gluconeogenesis to glucose production. ^2H_2O is administered orally to achieve 0.5% deuterium enrichment in body water. Enrichments are determined in the hydrogens bound to carbons 2 and 6 of blood glucose and in urinary water. Enrichment at carbon 6 of glucose is assayed in hexamethylenetetramine, formed from formaldehyde produced by periodate oxidation of the glucose. Enrichment at carbon 2 is assayed in lactate formed by enzymatic transfer of the hydrogen from glucose via sorbitol to pyruvate. The fraction gluconeogenesis contributes to glucose production equals the ratio of the enrichment at carbon 6 to that at carbon 2 or in urinary water. Applying the method, the contribution of gluconeogenesis in healthy subjects was 23–42% after fasting 14 h, increasing to 59–84% after fasting 42 h. Enrichment at carbon 2 to that in urinary water was 1.12±0.13. Therefore, the assumption that hydrogen equilibrated during hexose-6-P isomerization was fulfilled. The $^3H/^{14}C$ ratio in glucose formed from [3-^{3}H,3-^{14}C]lactate given to healthy subjects was 0.1 to 0.2 of that in the lactate. Therefore equilibration during gluconeogenesis of the hydrogen bound to carbon 6 with that in body water was 80–90% complete, so that gluconeogenesis is underestimated by 10–20%. Glycerol's contribution to gluconeogenesis is not included in these estimates. The method is applicable to studies in humans of gluconeogenesis at safe doses of ^2H_2O. (*J. Clin. Invest.* 1995. 95:172–178.) Key words: glucose • mass spectrometry • deuterium • deuterated water • hexamethylenetetramine

Introduction

A challenge has been to develop a method for estimating the contribution of gluconeogenesis to glucose production in humans, so that method can be applied under physiological conditions (notably fasting) and pathological conditions (notably diabetes mellitus). Net splanchnic uptake of gluconeogenic substrates can be measured using catheter techniques and has been used to provide a measure of hepatic gluconeogenesis (1, 2). Estimates from the incorporation of labeled carbon into glucose on administering a labeled gluconeogenic substrate have been made, but are not quantitative because the specific activity or enrichment of hepatic pyruvate is unknown and the label is diluted at the level of oxaloacetate, an intermediate common to the Krebs cycle and gluconeogenesis (3). Estimates of gluconeogenesis made from the incorporation of ^{14}C from [2-^{14}C]-acetate into glucose and into hydroxybutyrate, presumed to overcome those limitations, are unacceptable (4, 5). That is because of extensive metabolism of acetate in tissues other than liver and dilution of the specific activity of hydroxybutyrate formed in liver by unlabeled hydroxybutyrate formed in muscle. Contributions of gluconeogenesis during fasting and in type II diabetes have been quantified using [6-^{3}H]glucose to measure total glucose production and ^{13}C-NMR and magnetic resonance imaging to measure changes in hepatic glycogen content (6, 7). Those estimates then depend upon differences between measurements of glucose production and changes in hepatic glycogen content over a period of time.

Phosphoenolpyruvate is the necessary intermediate, formed from pyruvate via oxaloacetate, in the synthesis of glucose. Therefore, we gave ^{14}C-labeled bicarbonate to fasted subjects, assuming the specific activity of expired CO_2 to be that of CO_2 fixed in the conversion of pyruvate to oxaloacetate (8). We estimated the extent of dilution at the level of oxaloacetate from the distribution of ^{14}C from [3-^{14}C]lactate in hepatic α-ketoglutarate, estimated from the distribution of ^{14}C in glutamine conjugated to phenylacetate. That approach is time consuming and also suffers from several uncertainties (9).

In animals a simple method for quantifying gluconeogenesis depends upon the binding of ^3H from ^3H_2O to carbon 6 of glucose during its synthesis from pyruvate (10). Thus, when ^3H_2O is administered, ^3H exchanges with the protons bound to carbon 3 of the pyruvate that become those bound to carbon 6 of glucose in the gluconeogenic process. The fraction of glucose produced via gluconeogenesis can then be quantified from the ^3H activity at carbon 6 of glucose, knowing the specific activity of ^3H_2O in the circulation. Since ^3H from ^3H_2O is bound to carbon 2 of glucose formed via both gluconeogenesis and glycogenolysis, the amount of ^3H bound to carbon 6 relative to that bound to carbon 2 can also provide the measure of the fraction of glucose formed via gluconeogenesis (11).

The above approach cannot be applied to humans because of the large amount of ^3H_2O that would have to be administered to achieve adequate incorporation into glucose. The use of ^2H_2O offers an alternative. However, there are also limits to the quantity of ^2H_2O that can be administered and a method adequate to quantify enrichment at carbon 6, as well as at carbon 2, at an acceptable dose of ^2H_2O, is yet to be reported (12–14). The

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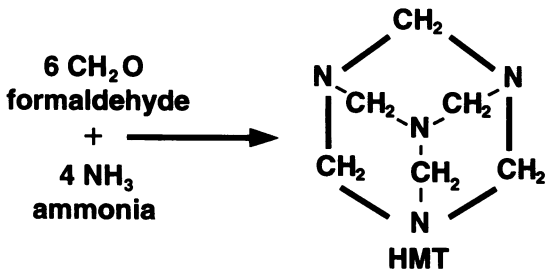

Figure 1. Formation of HMT from formaldehyde and ammonia.

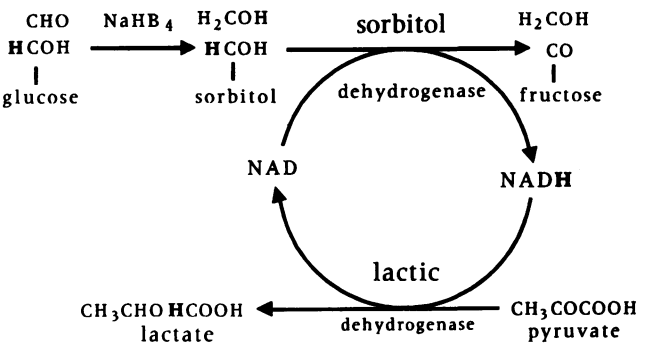

Figure 2. Reactions in the transfer to pyruvate of the hydrogen (***bold***) bound to carbon 2 of glucose.

present study reports such a method along with a presentation of its application to the fasted state.

When glucose is oxidized by periodic acid, formaldehyde is formed bearing the hydrogens bound to carbon 6 of glucose. Six molecules of formaldehyde in the presence of ammonia, through a series of additions of ammonia and ammonia-addition products to the carbonyl of successive formaldehyde molecules, form a molecule of hexamethylenetetramine (HMT)[1] (Fig. 1). All 12 hydrogens of the HMT are those from the six molecules of formaldehyde (15). Therefore, we have used HMT to measure the enrichment of deuterium bound to carbon 6 of glucose. The enrichment in the hydrogen bound to carbon 2 of glucose is obtained by reducing glucose to sorbitol and coupling its oxidation to fructose to the reduction of pyruvate to lactate, and then measuring enrichment in the lactate (Fig. 2).

Methods

Subjects. 16 healthy subjects, 12 women and 4 men, ages 24 to 46 years (31.1±6.6), were studied. Their weights ranged from 49 to 79 kg (Body Mass Index 22.0±1.7). They had ingested, by dietary history, weight maintaining diets containing at least 200 g of carbohydrate daily.

Experimental protocol. At 5 PM on the day of the study, 12 of the subjects ingested dinner over a period of 1 h. They then fasted, except for water ingestion, until completion of the study. 5 h after beginning the fast, they ingested 2.5 g of ^2H_2O (99.9% ^2H; Isotec Inc., Miamisburg, OH) per kg body water and four hours later ingested a second dose of 2.5 g/kg body water of the ^2H_2O. Body water was calculated at 50% of body weight in women, and at 60% in men (16). The only other fluid ingested throughout the fast was water that was enriched 0.5% with ^2H_2O, in order to maintain isotopic steady state. Four subjects fasted for 70 h and blood was drawn at 68, 69, and 70 h into the fast, and urine collected between 64–67 h and 67–70 h. These individuals were studied first, since if the assumption held of complete exchange of ^2H with the protons that become those bound to carbon 6 of glucose, after prolonged fasting almost all glucose produced should have been found to be by gluconeogenesis. In three subjects fasting was for 64 h, with blood drawn at 16, 40, and 64 h, and urine collected at 13–16 h, 37–40 h and 61–64 h. The remaining five subjects fasted for 42 h, blood was drawn at 14, 18, 22, 26, 38, and 42 h, and urine collected at 14–18, 22–26, 36–38, and 38–42 h. Their dinner was of 12–14 kcal/kg.

Two other subjects had dinner of 12–14 kcal/kg, but between 8 and 9 PM, and then began a fast of 42 h. Beginning at 7 AM each ingested 1.0 g of the ^2H_2O per kg body water at 45-min intervals five times, for a total dose of 5.0 g/kg body water. Again, the only other fluid ingested during the fast was 0.5% ^2H enriched water. Blood was drawn at 14, 18, 22, 26, 38, and 42 h into the fast. Urine was collected between 14–18, 18–22, 36–38, and 38–42 h.

To obtain a measure of the extent of equilibration of the hydrogens bound to carbon 3 of pyruvate with the hydrogens of body water, two subjects were fasted for 60 h and then 200 μc of [3-^3H]lactate and 40 μc of [3-^{14}C]lactate were infused intravenously, one third as a bolus and the rest over a 3 h period (17). Whole body radiation exposure from the ^{14}C and ^3H was estimated to be ~ 50 mrem (18). Blood was drawn at hourly intervals.

Analytical procedures. Enrichment of deuterium in urinary water was determined by Dr. David Wagner (Metabolic Solutions, Inc., Merrimack, NH). The urines were diluted 1:20 with tap water. The ^2H enrichment of the diluted urine sample was measured, following zinc reduction at 490°C to produce deuterium gas, as deviations of $^2H/H$ ratio in per mil (δ^2H ‰ parts per thousand) from that of the reference water ($^2H/H = 0.0001558$) on an isotope ratio mass spectrometer (19).

To prepare the HMT containing the hydrogens bound to carbon 6 of blood glucose, blood was diluted with an equal volume of water and deproteinized using the same volume of 5% $ZnSO_4$ and of 0.3 N $Ba(OH)_2$. The supernatant was passed through a mixed column of AG 1-X8 in the formate form and AG 50W-X8 in the H^+ form. The column was washed thoroughly with water and the effluent was evaporated to dryness. The quantity of glucose in the effluent was determined using glucose oxidase in an automated analyzer (20).

The procedure for oxidizing the glucose to formaldehyde has been previously detailed (21). In initial runs 4.3 ml of 0.37 M sodium meta periodate was added to 50 mg of glucose in 5 ml of water. The solution was capped and allowed to stand overnight. Then 1 ml of 10 N HCl was added and enough 1.2 M sodium arsenite to discharge all the iodine color, ~ 11 ml. The solution was made basic to phenol red with NaOH and distilled almost to dryness. The distillate was collected on ice and concentrated NH_4OH was added in large excess. The solution was capped and allowed to come to room temperature and stand overnight. Then it was evaporated to dryness at 50°C in an air stream. The white residue of HMT could be purified by sublimation. In these preliminary trials, when [6-^{14}C]glucose was used, 50–70% of the radioactivity added was recovered in the distillate and ~ 25% in the HMT.

In other initial tests, [6-^3H,6-^{14}C]glucose was oxidized to form formaldehyde and the formaldehyde converted to the HMT derivative. The $^3H/^{14}C$ ratio in the HMT on the three occasions determined was 91.9, 81.0, and 95.3% of that in the glucose. The [6-^3H]glucose used had been purified by HPLC and the ^3H and ^{14}C activities were determined after oxidation of the HMT derivative to $^{14}CO_2$ and ^3H_2O (Tricarb Model 306 Sample Oxidizer; Packard Instrument Co., Meriden, CT). Thus, perhaps through a secondary isotope effect, the enrichment of deuterium in the HMT derivative may be somewhat less than that in the glucose. However, since standards used in assaying the deuterium content in the HMT are made from [1-^2H]glucose, as will be described, that is without consequence.

After the preliminary runs, the procedure for preparing the HMT

1. *Abbreviations used in this paper:* HMT, hexamethylenetetramine; MPE, moles percent excess.

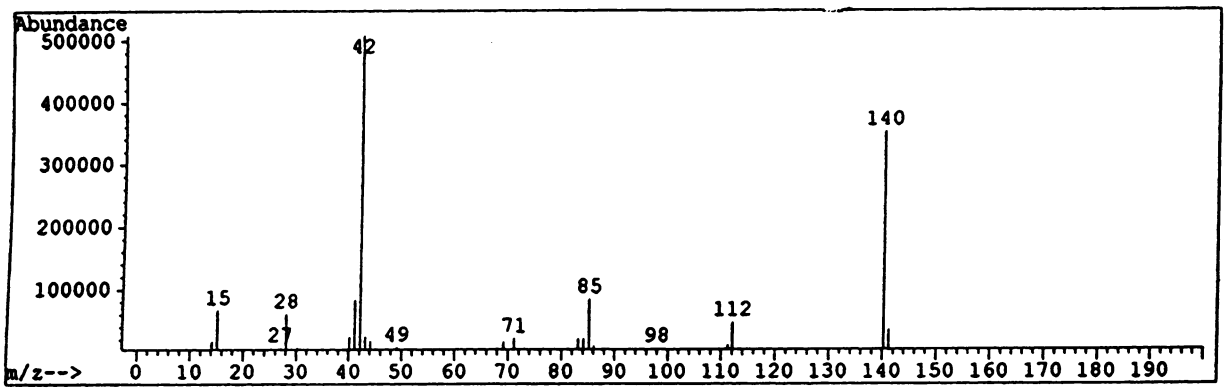

Figure 3. Electron Impact Mass Spectrum of Hexamethylenetetramine. The base-peak m/z 140 represents the molecular ion.

was taken to small scale. 2 mg of glucose was dissolved in 0.2 ml of water and 0.6 ml of the 0.37 M sodium meta periodate, and then 1 ml of 1 N HCl and ~ 2 ml of 1.2 M sodium arsenite were added. The basic solution was distilled and 0.5 ml of concentrated NH_4OH was added to the distillate. The residue after evaporation was then taken up in methylene chloride. The deuterium enrichment of the HMT was measured on a gas chromatograph–mass spectrometer (HP5985; Hewlett Packard, Palo Alto, CA) system. HMT (140 kD) is sufficiently volatile so that it can be injected directly into the gas chromatograph without derivatization. A glass column (3.2 mm × 1.8 m) packed with 3% OV-17 on the gas chrom Q, 100–120 mesh (Applied Science, College Station, PA) was used. The injection port was kept at 170°C with the gas chromatograph/mass spectrometer interphase at 250°C. Helium was used as the carrier gas at a flow rate of 21 ml/min. Oven temperature was 120°C. Electron impact ionization (60 meV) was used and ions m/z 140 and 141, corresponding to unenriched and enriched (m + 1) molecular ions, were monitored using selected ion monitoring. The retention time for HMT was 5 min. The electron mass spectrum of HMT is shown in Fig. 3.

The results to be reported are for assays using glucose from < 3 ml of blood. With each run of unknowns, standards of HMT of known deuterium enrichments (0.125, 0.25, 0.50, 1.00, and 2.00%) were also run. The standards were prepared by diluting [1-^{2}H]glucose (98% ^{2}H; Cambridge Isotope Labs, Woburn, MA), with unlabeled glucose to a deuterium enrichment twice that desired for the standards. The deuterium enrichment of the commercially obtained [1-^{2}H]glucose was confirmed by mass spectrometric analysis. The glucose was reduced to sorbitol by sodium borohydride (22). The sorbitol was oxidized with periodate and the HMT derivative then prepared from the formaldehyde formed. Each molecule of sorbitol yields two molecules of formaldehyde, one containing carbon 1 and the other carbon 6 of the sorbitol, and hence of glucose. Thus, the enrichment of deuterium in the formaldehyde and hence the HMT will be half that bound to carbon 1 of the glucose. As evidence for this, [1-^{3}H,6-^{14}C]glucose was reduced to sorbitol and an HMT derivative was made from that sorbitol. The ^{3}H/^{14}C ratios in the glucose and sorbitol were the same, determined by oxidizing aliquots to ^3H_2O and $^{14}CO_2$ and analyzing them in a liquid scintillation counter (Tricarb Model 1600 TR; Packard Instrument Co.), while the ^{3}H/^{14}C ratio in the HMT was 105% of that ratio.

The deuterium enrichment of the samples, in moles percent excess (MPE), was calculated from the linear regression equation of the standard curve. Since only one H on glucose would be labeled, based upon probability, moles percent excess would be the same as atoms percent excess. The coefficient of variation for the measurement of moles percent excess for the enriched standards ranged from 9% at < 0.25% enrichments and 5.5–8% at enrichments greater than 0.5% (n = 9).

To measure deuterium enrichment at carbon 2 of blood glucose, modification of the method of Muntz and Carroll (22) for converting glucose to fructose was used. 3 mg of glucose from the blood was reduced with sodium borohydride to sorbitol and the sorbitol was incubated at 37°C for 3 h in 0.7 ml of 0.02 M sodium phosphate buffer, pH 8.0, containing 0.3 mg of NAD, 2.1 mg of sodium pyruvate, 10 U of sorbitol dehydrogenase and 220 U of lactic dehydrogenase (Sigma Chemical Co., St. Louis, MO). The incubate was then acidified and continuously extracted with ether for 24 h. After neutralizing the lactic acid in the extract with sodium hydroxide, the ether was evaporated and the residue of sodium lactate was subjected to HPLC using an Aminex HPX-87H column (Bio Rad Laboratories, Richmond, CA) with 0.01 N H_2SO_4 as solvent. About 1.2 mg of lactate was recovered in the fraction containing lactate as determined spectrometrically. The unknown lactate samples were analyzed along with known standards of lactate of 0.25, 0.50, 0.75, and 1.00% MPE prepared from [2-^{2}H]glucose (Cambridge Isotope Labs). The deuterium enrichment of the lactate was determined by gas chromatograph–mass spectrometric analysis (23). The reproducibility of the mass spectrometric analysis has been reported previously (23). Preparation of lactate from glucose by the coupled enzymatic reaction gave a coefficient of variation of 13–15% at the low, < 0.5%, enrichments.

The procedure for determining the ^{3}H/^{14}C ratio in glucose and lactate in the blood of the two subjects infused with [3-^{3}H,3-^{14}C]lactate was the same as previously described (17). $ZnSO_4$ and $Ba(OH)_2$ were added to the blood and the precipitated protein was removed by centrifugation. Lactate was isolated from the acidified supernatant by ether extraction and purified by HPLC using the HPX-87H system. The acidified supernatant after ether extraction was deionized by passage through the ion exchange resins. Glucose in the effluent was purified by HPLC using an Aminex HPX-87P column with water at 80°C as solvent. The DPM of ^{3}H and ^{14}C in the glucose and lactate was determined by liquid scintillation spectrometry.

Calculations. The fraction of blood glucose formed by gluconeogenesis was calculated in two ways: (1) by multiplying by one-half the MPE deuterium in the HMT (^{2}H on carbon 6 of glucose) synthesized from glucose in blood collected at a given time, divided by the MPE of deuterium in urinary water collected during that time, and (2) by multiplying by one-half the MPE deuterium in the HMT synthesized from glucose in blood collected at a given time divided by the MPE deuterium in lactate (^{2}H on carbon 2 of glucose) synthesized in the oxidation of sorbitol formed from glucose in the blood collected at that time. The rationale for using half the MPE in the HMT in the calculations is as follows. Since two hydrogens are bound to carbon 6, theoretically, if all the glucose was formed by gluconeogenesis, the enrichment in each of those hydrogens should equal the enrichment of a hydrogen in water or the hydrogen bound to carbon 2 of the glucose, and enrichment of HMT represents enrichment of both hydrogens bound to carbon 6 of glucose.

All data are reported as mean±SD. Coefficients of variation, were determined using SPSS package (Statistical Package for Social Sciences, Chicago, IL) on a personal computer.

Table I. MPE Deuterium in HMT from Blood Glucose, at Carbon 2 of Blood Glucose and in Urinary Water and Estimates of Percent Gluconeogenesis in Subjects Fasted for 64 to 70 h

	Blood glucose			Urinary water			Percent gluconeogenesis		
Subject	68 h	69 h	70 h	64–67 h	67–70 h		68 h	69 h	70 h
ML	0.36	0.37		0.26	0.27		65	69	
YH	0.68	0.73	0.60		0.48		71	76	63
AJ		0.45	0.50	0.38	0.39			58	64
AH	0.67	0.63	0.66	0.50	0.52		64	61	63
	(0.55)*	(0.48)	(0.57)				(61)‡	(66)	(58)
Subject	16 h	40 h	64 h	13–16 h	37–40 h	61–64 h	16 h	40 h	64 h
BR	0.40	0.54	0.63	0.40	0.43	0.44	50	63	72
	(0.56)*	(0.59)	(0.45)				(36)‡	(46)	(70)
LD	0.25	0.58	0.58	0.41	0.40	0.42	30	73	69
SJ	0.33	0.58		0.40	0.41	0.41	41	71	

* Values in parenthesis are MPE deuterium at carbon 2 of glucose when that was determined. ‡ Values in parenthesis under percent gluconeogenesis are the estimates calculated from the enrichment at carbon 2.

Results

Table I presents in its top half the results for the four subjects in whom blood samples were drawn at 68, 69, and 70 h, and urine collected between 64 and 70 h. Deuterium enrichment in urinary water would be expected to be the same in the two urine samples collected. They were, with the enrichment of about 0.5% as expected from the dose of (99.9%) ^2H_2O ingested and the 0.5% ^2H_2O ingested as the sole fluid source during the fast. That is so except for ML who in error was given only one-half the dose of ^2H_2O. The enrichment in the two hydrogens bound to carbon 6 of blood glucose, measured by using the HMT derivative, would also be expected to be the same at 68, 69, and 70 h. Percent gluconeogenesis, estimated from the enrichments in HMT and urinary water, ranged from 58 to 76%, with an average of 60%.

The results for the three subjects fasted for 64 h is presented in the lower half of Table I. By 16 h after the beginning of the fast, 7 h after the ingestion of the second dose of (99.9%) ^2H_2O, equilibration with body water would be expected (24), so that the enrichment of deuterium in body water as reflected in urinary water would be constant and it was. The fraction of glucose production via gluconeogenesis has been estimated, again from the enrichment in HMT and urinary water. The percentages at 16 h fasting averaged 40%, and increased to an average of 70% at 64 h.

Table II shows the results for the timed studies in the 7 subjects fasted for 42 h, 5 of whom were dosed with ^2H_2O at the 5th and 9th h of fasting and 2 (subjects LL and AD) at the 10th to 13th h. There was an increase with time in the percent contribution by gluconeogenesis estimated from the enrichments in HMT and urinary water. During the 14–22-h period, the percentages range from 24 to 45% (Fig. 4). By 42 h, similar to the subjects fasted at 64 to 70 h, the values ranged from 59 to 84%. Again body water deuterium enrichment was constant for each subject, ranging from 0.40 to 0.51%, as measured by the enrichment in urinary water.

The enrichments in the hydrogen bound to carbon 2 of glucose, when it was determined, are recorded in parentheses in Tables I and II along with the percent gluconeogenesis calculated from the enrichments at carbons 6 and 2. The enrichments at carbon 2, as expected, are similar to those in urinary water. The ratio of ^2H enrichment at carbon 2 of blood glucose and in urinary water in simultaneously measured samples (n = 32) was 1.12±0.13. The fraction (5) of glucose produced via gluconeogenesis calculated using ^2H enrichment on carbon 2 of glucose was somewhat lower than that calculated using ^2H enrichment in urinary water. The reason for this is not known. It may be related to the technical limitation in the preparation and analysis of lactate. The contribution of gluconeogenesis to glucose production during fasting is displayed in Fig. 4. There was a progressive increase in the contribution, approaching a plateau by 38 h of fasting.

In one of the subjects given [3-^3H,3-^{14}C]lactate, the $^3H/^{14}C$ ratio in blood glucose at 1 h was 12.0%, at 2 h, 10.8%, and at 3 h, 11.7% of the ratio in the simultaneously obtained blood lactate. In the second subject, the corresponding percentages were 12.0, 12.9, and 12.6%.

Discussion

Theoretical basis for the approach. The major assumption in our approach is that there is essentially complete exchange between the deuterium in body water and the two hydrogens bound to carbon 3 of phosphoenolpyruvate (Fig. 5). Phosphoenolpyruvate is formed from pyruvate via oxaloacetate. Hence, all the phosphoenolpyruvate formed from pyruvate, alanine and lactate should then bear label from the protons of water and hence provide a measure of the rate of gluconeogenesis from those gluconeogenic substrates.

There is evidence for extensive exchange (25–27). The exchange occurs in the transamination of pyruvate with alanine (28), as well as the equilibration of oxaloacetate with fumarate before oxaloacetate's conversion to phosphoenolpyruvate (Fig. 5). However, it is not complete. Guo et al. (13) reported that on injecting rats fasted 24 h with ^2H_2O, equilibration of the ^2H_2O with total body water occurred within 1 h, but the enrichment of the hydrogen bound to carbon 6 was only 50% of that in the water. They considered the possibility that glycerol accounted for as much as 30% of glucose's production under their condi-

*Table II. MPE Deuterium in HMT from Blood Glucose, at Carbon 2 of Blood Glucose, and in Urinary Water and Estimates of Percent Gluconeogenesis from Data for Subjects Fasted for 42 h**

	Blood glucose						Urinary water				Percent gluconeogenesis					
Subject	14 h	18 h	22 h	26 h	38 h	42 h	14–18 h	22–26 h	36–38 h	38–42 h	14 h	18 h	22 h	26 h	38 h	42 h
EF	0.25	0.22	0.26	0.43	0.59	0.61	0.42	0.45	0.44	0.45	30	26	29	48	67	68
	(0.54)*	(0.51)	(0.52)	(0.52)	(0.46)	(0.52)					(23)§	(22)	(25)	(41)	(64)	(59)
AP	0.35	0.23	0.38	0.54	0.59	0.58	0.48	0.51	0.48	0.49	36	24	37	53	61	59
	(0.50)	(0.54)	(0.41)	(0.55)	(0.52)						(35)	(21)	(46)	(49)	(57)	
TA	0.35	0.37	0.41	0.53	0.53	0.60	0.46	0.47	0.47	0.47	38	40	44	56	56	64
	(0.53)	(0.47)				(0.50)					(33)	(39)				(60)
IH	0.37	0.34	0.34	0.54	0.52	0.61	0.44	0.43	0.42	0.42	42	39	40	63	62	73
	(0.44)	(0.42)				(0.50)					(42)	(41)				(61)
UM	0.24	0.39	0.35	0.43	0.54	0.57	0.43	0.44	0.44	0.45	28	45	40	49	61	63
	(0.43)	(0.54)				(0.43)					(28)	(36)				(66)
LL	0.35	0.30	0.35		0.63	0.63	0.45	0.44‡	0.45	0.44	39	33	40		70	72
	(0.58)	(0.52)				(0.51)					(30)	(29)				(62)
AD	0.26	0.34	0.28		0.51	0.69	0.40	0.40‡	0.42	0.41	33	43	35		61	84
	(0.49)	(0.50)				(0.49)					(27)	(34)				(70)

* Values in parentheses are MPE deuterium at carbon 2 of blood glucose. § Values in parenthesis under percent gluconegenesis are the estimates calculated from the enrichment at carbon 2. ‡ From urine collected between 18–22 h.

tions. Carbon 6 of glucose, formed from lactate by hepatocytes from fasted rats and hamsters in the presence of ^3H_2O, had 1.6 to 1.9 atoms of the theoretical 2.0 atoms of ^3H bound (29). A similar extent of binding of ^3H was observed when ^3H_2O was given to fasted mice (11). Except for the report of Guo et al. (13), this suggests that exchange may be incomplete by as much as 20%. The evidence in vivo in humans is that 20% is about right, since when [3-^{3}H,3-^{14}C]lactate was infused into normal subjects fasted overnight, the $^3H/^{14}C$ in glucose that formed was ~ 20% of that of blood lactate (16) and after 60 h of fasting ~ 10%. Thus, 80 to 90% of the ^3H bound to carbon 3 of the lactate had exchanged with the protons of water before conversion to glucose.

In the human fasted for as long as 60 h, there are still glycogen stores in liver, its release accounting for perhaps 5% of glucose production (6). In the 60 h fasted individual, based upon the uptake of alanine, lactate pyruvate and glycerol across the splanchnic bed, glycerol should account for ~ 10% of overall gluconeogenesis (1). In the overnight fasted individual it may account for only ~ 3% (30). Thus, with complete exchange of the hydrogens of pyruvate with those of body water, with prolonged fasting, a contribution to glucose production of ~ 85% by gluconeogenic precursors entering via phosphoenolpyruvate would have been expected. The 58–76% of contribution between 64 and 70 h of fasting in the present study is in keeping with an incomplete exchange of ~ 20%.

In the overnight fasted state, incomplete exchange of ^2H from water with hydrogens of pyruvate to the extent of 20%, means that an estimate of 30% should be increased to ~ 36%. Presumably the correction for incomplete exchange should be between 10 and 20% for all times between overnight and prolonged fasting. An amount of deuterium from deuterated water could be bound at the level of triose phosphate to the carbon becoming carbon 6 of glucose, because of fructose-6-P cycling (31). That amount is likely negligible in the present context.

The contribution of gluconeogenesis may also be underestimated because of glycerol's metabolism other than its conversion to glucose. The glycerol moiety of triglyceride is formed

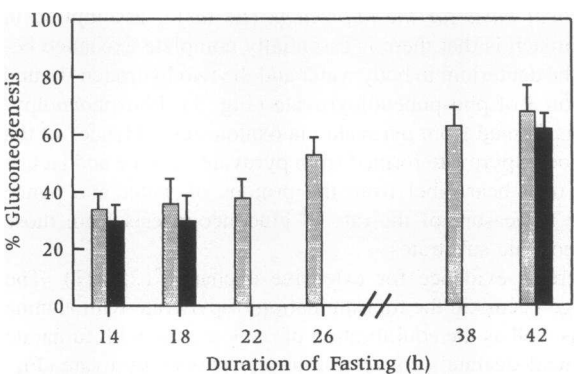

Figure 4. Estimates of gluconeogenesis during fasting over 42 h in seven normal subjects administered ^2H_2O. Percent gluconeogenesis was calculated from comparison of deuterium MPE at carbon 6 of glucose with that in urinary water (*solid bars*) or with that at carbon 2 of glucose (*shaded bars*). Data are presented as mean±SD for $n = 7$, except for 26 h where $n = 5$.

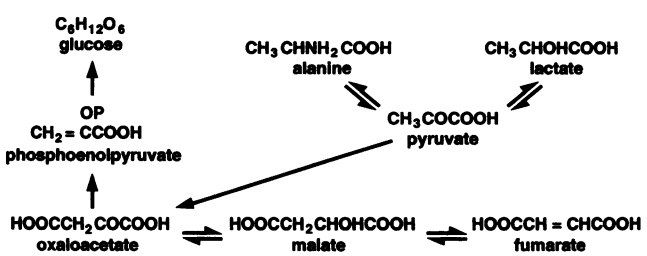

Figure 5. Pathways of formation of glucose from alanine, lactate, and pyruvate.

following glycerol's phosphorylation in liver (32, 33). Glycerol phosphate equilibrates with dihydroxyacetone phosphate. To the extent that occurs unlabeled glycerol phosphate will exchange with deuterated dihydroxyacetone phosphate, formed as intermediate in glucose's synthesis from pyruvate. The result will be less deuterium bound to carbon 6 of the glucose than if there were no exchange.

Practical limitations. A major limitation in the present approach is the amount of ^2H_2O that can safely be given. Animal studies have led to the conclusion that humans can safely ingest for prolonged periods amounts of ^2H_2O enriched to 1% (34). Deuterium in body water at an enrichment of 0.5 to 0.6% has been maintained in humans for up to 40 to 50 d without evidence of toxicity (16). However, giving deuterated water in a single dose to achieve that enrichment has been associated on occasion with marked, although transient, vertigo (16). To avoid that side effect, in the first 12 studies, we gave half the dose at bedtime and the other half 4 h later. None of the subjects experienced vertigo.

Estimating the contribution of gluconeogenesis from the ratio of enrichment of the hydrogen bound to carbon 6 of blood glucose to that in body water requires that essentially all the glucose be formed during the period after the ^2H_2O equilibrated. It may take 3 to 4 h for complete equilibration of the ^2H_2O with body water (24) and glucose present before the administration of ^2H_2O would give a falsely low estimate of the contribution of gluconeogenesis. Furthermore, because of the turnover time of the glucose, the estimates provide the measure of the contribution of gluconeogenesis not at the time blood is drawn, but over several hours preceding the time of blood drawing.

By estimating gluconeogenesis from the deuterium bound to carbon 6 to that bound to carbon 2, glucose present in blood before ^2H_2O administration and the time until ^2H_2O equilibrates with body water no longer matters. However, there is then the problem of the rate at which ^2H_2O can be ingested to give enrichments that can be reliably measured. The administration of a total of 5.0 g/kg body water in five doses over three hours did not result in vertigo or any other side effect and enrichments in glucose could be reliably measured, as evidenced by the similar estimates when ^2H_2O was given many hours before blood drawing and those drawn one hour after the ingestion of ^2H_2O. The estimate of the contribution of gluconeogenesis can then be made for the period of a few hours preceding the blood sampling.

Total glucose production is assumed to be represented by the enrichment in the hydrogen bound to carbon 2 of glucose. In keeping with the hydrogen bound to carbon 2, and not carbon 6 of glucose released from glycogen equilibrating with the hydrogen of body water, when hepatocytes were incubated with ^3H_2O under anaerobic conditions to prevent gluconeogenesis, there was negligible incorporation of tritium at carbon 6 and extensive incorporation at carbon 2 of glucose produced by glycogenolysis (29). In the overnight fasted individual, ~ 80% of the hydrogen bound to carbon 2 of glucose formed by glycogenolysis arises by exchange with body water. That was evidenced (*a*) by the formation of glucose from [2-^{3}H,6-^{14}C]-galactose with 20% of the $^3H/^{14}C$ ratio in the galactose, and (*b*) by the formation of the glucuronide of acetaminophen with a $^3H/^{14}C$ ratio 20% of that in the blood glucose when [2-^{3}H,6-^{14}C]glucose was administered with acetaminophen (16).

There is also good evidence for a hydrogen from body water being bound to carbon 2 of every glucose molecule formed by gluconeogenesis. Thus, Rognstad et al. (29) found that hepatocytes formed glucose from pyruvate and lactate in the presence of ^3H_2O with nearly the theoretical maximum of one atom equivalent of tritium at carbon 2. Postle and Bloxham (33), on administering glucose and ^3H_2O to rats, found that glucose from liver glycogen contained 0.9 of the theoretical 1.0 atoms of tritium at carbon 2. That finding was confirmed by Golden and Katz (11). Since liver from fasted rats contain a small amount of glycogen, the amount actually found in the glycogen formed was closer to 1.0 (11). However, Guo et al. (13), on giving ^2H_2O to fasted rats, found only 0.74 atom equivalents of deuterium bound to carbon 2 of blood glucose. In accord with the hydrogen bound to carbon 2 equilibrating with hydrogen in body water in our study, after enough time for the ^2H_2O to equilibrate, the enrichment of the hydrogen bound to carbon 2 was similar to the enrichment in water.

It is not possible to give the quantities of ^3H_2O that would be required to obtain meaningful incorporations of ^{3}H into the hydrogens bound to carbon 6 of glucose. The amount of [6,6-$^2H_2$]glucose formed using ^2H_2O at an enrichment of 0.5% (10) would be too small to be measured. The dimedone derivative of formaldehyde has been used to measure the incorporation of ^{3}H from ^3H_2O (10). Since that derivative contains 22 hydrogens and 16 carbons in addition to those from the formaldehyde, low enrichments in formaldehyde hydrogens become difficult to measure. The HMT derivative has the advantage in that all its 12 hydrogens arise from those bound only to carbon 6 of glucose, which results in a six-fold increase in ^{2}H enrichment (m + 1) over that in glucose. In addition, HMT can be used directly for mass spectrometric analysis.

Determination of specific deuterium labeling in glucose, as used by Guo et al. (13), and in a similar manner by Shalwitz et al. (14) in their studies on rats, depends upon the monitoring of selected ions in the mass spectra of glucose pentacetate and then subtraction analysis. A critical assumption made by Shalwitz et al. (14) was that there was equal enrichment on carbons 3, 4, and 5 of glucose. Rognstad et al. (29) found evidence for equal labeling at carbons 3, 4, and 5 of glucose formed by hepatocytes incubated with ^3H_2O. Guo et al. (12) found much less label at carbons 3 and 4 than carbon 5 of glucose when ^2H_2O was given to fasted rats. Postle and Bloxham (35) had similar results when they examined the glycosyl units of glycogen formed by rats given glucose and ^3H_2O. Furthermore, in the studies of Guo et al. (13) and Shalwitz et al. (14) deuterium enrichments in body water were 3–4%.

Comparison of estimates. The estimated contribution of gluconeogenesis of 21–46% to total glucose production at 14–22 h of fasting and 58–76% at 64–70 h is in accord with the calculated gluconeogenic contribution by liver of ~ 30% after an overnight fast and 80% after 60 h of fasting, measured from glucose release and the uptake of glycerol, amino acid, pyruvate, and lactate across the splanchnic bed (1). Glucose production by the kidney has been estimated also by the balance technique (36) to be ~ 10% of total glucose production in the 60 h fasted individual.

Gay et al. (37) conclude that 50% of glucose production is due to gluconeogenesis after an overnight fast. Their estimate is based upon measurements of ^{13}C enrichment of plasma glucose and breath CO_2 after giving subjects a diet containing carbohydrate of low ^{13}C abundance and then of a higher ^{13}C abundance. Rothman et al. (6) have estimated that 64±5% of glucose production is by gluconeogenesis in the first 22 h of a

fast. Their estimate is based upon differences between glucose production measured using tritiated glucose and the rate of disappearance of glycogen from liver measured by NMR. Our conditions differ from those of Rothman et al. (4), in that they gave a liquid meal of 650 calories, ingested in 5 min, before beginning the fast, and we gave a solid meal of ~ 740 calories ingested over a 1-h period. Also, glucose production by liver may be overestimated using tritiated glucose to the extent glucose is produced by kidney.

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