special communication

Quantifying gluconeogenesis during fasting

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Chandramouli, Visvanathan, Karin Ekberg, William C. Schumann, Satish C. Kalhan, John Wahren, and Bernard R. Landau. Quantifying gluconeogenesis during fasting. *Am. J. Physiol.* 273 (*Endocrinol. Metab.* 36): E1209–E1215, 1997.—The use of ^2H_2O in estimating gluconeogenesis' contribution to glucose production (%GNG) was examined during progressive fasting in three groups of healthy subjects. One group ($n = 3$) ingested ^2H_2O to a body water enrichment of ≈0.35% 5 h into the fast. %GNG was determined at 2-h intervals from the ratio of the enrichments of the hydrogens at C-5 and C-2 of blood glucose, assayed in hexamethylenetetramine. %GNG increased from 40 ± 8% at 10 h to 93 ± 6% at 42 h. Another group ingested ^2H_2O over 2.25 h, beginning at 11 h ($n = 7$) and 19 h ($n = 7$) to achieve ≈0.5% water enrichment. Enrichment in plasma water and at C-2 reached steady state ≈1 h after completion of ^2H_2O ingestion. The C-5-to-C-2 ratio reached steady state by the completion of ^2H_2O ingestion. %GNG was 54 ± 2% at 14 h and 64 ± 2% at 22 h. A 3-h [6,6-$^2H_2$]glucose infusion was also begun to estimate glucose production from enrichments at C-6, again in hexamethylenetetramine. Glucose produced by gluconeogenesis was 0.99 ± 0.06 mg·kg^{-1}·min^{-1} at both 14 and 22 h. In a third group ($n = 3$) %GNG reached steady state ≈2 h after ^2H_2O ingestion to only ≈0.25% enrichment. In conclusion, %GNG by 2 h after ^2H_2O ingestion and glucose production using [6,6-$^2H_2$]glucose infusion, begun together, can be determined from hydrogen enrichments at blood glucose C-2, C-5, and C-6. %GNG increases gradually from the postabsorptive state to 42 h of fasting, without apparent change in the quantity of glucose produced by gluconeogenesis at 14 and 22 h.

deuterium oxide; glucose; glycogen; hexamethylenetetramine

AFTER ^2H_2O IS GIVEN to fasted humans and when steady state is attained, the ratio of enrichment of deuterium bound to C-5 of blood glucose to enrichment in body water equals the fraction gluconeogenesis contributes to glucose production (9). That is because a hydrogen atom from body water is bound to C-5 of every molecule of glucose formed via gluconeogenesis and none via glycogenolysis. Because a hydrogen atom from body water is added at C-2 of glucose formed via both gluconeogenesis and glycogenolysis, the ratio of enrichment at C-5 to that at C-2 also provides a measure of that fraction. To allow the giving of a well-tolerated, safe dose of ^2H_2O to humans, enrichments in the hydrogens bound to C-2 and C-5 were determined by isolating them in formaldehyde and assaying the formaldehyde by mass spectrometry using hexamethylenetetramine (HMT) (7, 9, 10). HMT is an adduct of six molecules of the formaldehyde with four molecules of ammonia, and hence the enrichments in the hydrogens of the glucose are in essence magnified sixfold for assay.

The present study had three purposes. The first was to determine with short time intervals ratios of enrichments at C-5 and C-2 during fasting to define more closely how the fractional contribution of gluconeogenesis changes with time. The second was to determine how soon after giving ^2H_2O the ratio of enrichment at C-5 to that at C-2 and in body water reaches constancy, in particular, to determine whether the contribution of gluconeogenesis can be measured within a few hours after ^2H_2O ingestion. That seemed possible because the enrichments at C-2 and C-5 should give the measure of the fractional contribution by gluconeogenesis before the attainment of steady state in glucose turnover and before the mixing of ^2H_2O with body water. The third was, by measuring glucose production, to determine during that same period of time the quantity of glucose formed via gluconeogenesis. For that purpose [6,6-$^2H_2$]glucose was given together with ^2H_2O. Enrichment of the hydrogens bound to C-6 of blood glucose was determined, again through their isolation in formaldehyde and the formation of HMT from the formaldehyde. With the accomplishment of these purposes, a further definition of the role of gluconeogenesis in glucose production during fasting was obtained.

METHODS

Twenty healthy subjects, 15 women and 5 men, ages 29.9 ± 1.9 yr, body mass index 23.3 ± 0.4 kg/m², were studied (Table 1). As recorded in dietary diaries, each subject ingested at least 200 g of carbohydrate for 4 days before the study. They reported no change in diet or weight during the prior 3 mo. The study was approved by the Human Investigation Committees at the Karolinska Hospital and the University Hospital of Cleveland. All subjects gave informed, written consent.

Subjects 1-3 at 8 PM ate during 1 h a meal of 12–14 kcal/kg body weight, composed of 48% carbohydrate, 19% protein, and 33% fat. They then fasted for 42 h except for water ingestion. Five hours after beginning the fast, they drank 3.5 ml of ^2H_2O (99.9% ^2H, Isotec, Miamisburg, OH) per kilogram body water. Body water was calculated to be 50% of body weight in women and 60% in men. Percentages used would have been less if the subjects had been obese. Water ingested

Table 1. *Age, sex, weight, height, BMI, and plasma glucose concentration*

Subject No.	Age, yr	Sex	Weight, kg	Height, m	BMI, kg/m²	Glucose, mM*
1	26	M	79	1.81	24.1	5.4, 4.1
2	26	F	54	1.60	21.1	4.3, 3.2
3	42	F	60	1.63	22.6	4.8, 3.9
4	38	F	64	1.67	22.9	5.2, 5.4
5	46	M	84	1.92	22.8	5.5, 5.6
6	23	M	72	1.85	21.0	5.1, 5.1
7	23	M	74	1.69	25.9	5.4, 5.4
8	24	M	85	1.93	22.8	5.3, 5.3
9	29	M	92	1.96	23.9	5.2, 5.3
10	43	M	85	1.84	25.1	4.8, 5.3
11	39	F	76	1.71	26.0	5.1, 4.8
12	30	F	80	1.76	25.8	4.8, 4.1
13	23	F	66	1.67	23.7	4.2, 4.3
14	18	F	54	1.68	19.1	4.4, 4.3
15	31	M	69	1.73	23.1	4.6, 4.6
16	26	M	85	1.84	25.1	4.4, 4.3
17	33	M	90	1.93	24.2	5.7, 5.1
18	22	M	91	1.95	23.9	5.8, 5.4
19	36	F	52	1.58	20.8	5.2, 5.2
20	19	F	66	1.74	21.8	4.7, 5.0

BMI, body mass index; M, male; F, female. * First concentration is that in plasma from blood drawn at the time ^2H_2O was first given except it was 12 h into the fast for *subjects 1–3*. Second concentration is that in plasma from blood drawn at end of fast.

ad libitum during the fast was enriched to 0.35% with ^2H_2O to maintain isotopic steady state. Peripheral vein blood was drawn every 2 h from the 10th to 22nd h and then at the 25th, 33rd, 36th, and 42nd h of the fast. The concentration of glucose in plasma from the blood samples and moles percent excess enrichments of the hydrogens at C-2 and C-5 of blood glucose were determined.

Subjects 4-10 at 7 PM in 1 h ingested meals of the same content as the first three subjects and then began to fast (Fig. 1). They ingested 1.25 ml of ^2H_2O/kg body water every 45th min for a total dose of 5 ml ^2H_2O/kg body water, starting at 11 h into the fast. *Subject 7* complained of dizziness after the 3rd dose and therefore was not given a 4th dose. Water ingested ad libitum was enriched to 0.5%. [6,6-$^2H_2$]glucose (98%, Cambridge Isotope Laboratories, Andover, MA) was infused through a peripheral vein, 83 mg as a priming dose at 11 h and then continuously at 50 mg/h for 3 h (6). Blood was drawn hourly from a peripheral vein from the 14th to the 16th h in two of the subjects and from the 12th h in the others, and glucose concentration in the plasma and enrichments at C-2 and C-5 of glucose from the blood was determined. Blood was collected at 10-min intervals from 13.5 to 14 h, and enrichment at C-6 of the blood glucose was determined. Enrichments in plasma water and in urinary water from urine collected at 12.5–14 h and 14–16 h were also determined in five of the subjects.

Subjects 11-17 were treated identically to *subjects 4-10*, except they ate dinner 3 h earlier; i.e., they began fasting at 5 PM (Fig. 1). [6,6-^2H]glucose infusion, the ingestion of ^2H_2O, and sampling were begun at 19 h rather than at 11 h into the fast. Urine was then collected at 20.5–22 h and 22–24 h. With two subjects, enrichment values at C-2 and C-5 at 23 and 24 h were not determined.

To determine if it is possible to obtain accurate measurements of the fractional contribution of gluconeogenesis with only one-half the usual dose of ^2H_2O, *subjects 18-20* ingested 1.25 ml of ^2H_2O/kg body water at 12 and 12.75 h into the fast. Water ingested ad libitum was enriched to 0.25%. Blood was

drawn hourly between 12 and 16 h of the fast for determination of plasma glucose concentration and enrichment in the hydrogens at C-2 and C-5 of blood glucose.

Analyses. Plasma glucose concentration was determined using glucose oxidase (Beckman Instrument, Fullerton, CA). Enrichments of the hydrogens at C-2 and C-5 of blood glucose were determined as previously described (10). Protein was precipitated from the blood by the addition of 5% $ZnSO_4$ and 0.3 N $Ba(OH)_2$, and the mixture was centrifuged. The supernatant was deionized by passage through a column of anion and cation exchange resins and glucose in the effluent isolated using high-performance liquid chromatography. An aliquot of the glucose was converted to xylose, and the xylose oxidized with periodate to yield C-5 of glucose with its hydrogen in formaldehyde.[1] HMT was prepared from the formaldehyde and assayed for mass M_1. Another aliquot of the glucose was converted to ribulose-5-phosphate, which was reduced to a mixture of ribitol-5-phosphate and arabitol-5-phosphate. They were also oxidized with periodate, yielding formaldehyde containing C-2 with its hydrogen. Again the formaldehyde was condensed to form an HMT, which was assayed for mass M_1. The assay of HMT was done on a gas chromatograph-mass spectrometer (HP-5985, Hewlett Packard, Palo Alto, CA) (7, 9, 10). The HMT was injected directly. Electron impact ionization was used, and ions were selectively monitored.

At the time of each assay ($n = 8$), HMTs of 0.0625, 0.125, 0.25, 0.50, and 1.00% enrichments were made from [1-^2H]sorbitol (9, 10). Coefficients of variation in their measured enrichments were, respectively, 4.8, 3.1, 2.4, 1.3, and 0.4%. Enrichments at C-2 and C-5 were then determined using those standards. Enrichments at C-2 and C-5 of glucose from blood of a subject fasted overnight were determined five times. The mean enrichment at C-2 was 0.46%, with a coefficient of variation of 1.1%. The mean enrichment at C-5 was 0.19%, with a coefficient of variation of 2.6%. Assays of HMTs prepared from glucose collected before subjects were given ^2H_2O were used to correct for natural abundance.

Glucose isolated from the blood samples collected at 10-min intervals, beginning 2.5 h after the start of the [6,6-$^2H_2$]glucose infusion, was also oxidized with periodate. The formaldehyde formed, which contained C-6 of the glucose with its hydrogens, was converted into HMT, which was then assayed for mass M_2. Masses were 9–14 times natural abundance. HMTs from [6,6-$^2H_2$]glucose of 0.125, 0.25, 0.50, 0.75, and 1.0% enrichments provided a standard curve. When subjects were given [6,6-$^2H_2$]glucose without ^2H_2O, no detectable increase above natural abundance was found in M_1 of HMTs from C-2 and C-5 of blood glucose. HMTs were prepared from [1-^2H]sorbitol and [6,6-$^2H_2$]glucose separately and mixed. Whether or not formaldehyde from the sorbitol enriched in M_1 to 0.85% was mixed with formaldehyde from the glucose enriched in M_2 to 0.65%, the assay of M_2 in the HMT remained 0.65%. Thus the formation of M_1 at C-6 of glucose on the ^2H_2O administration to a body water enrichment of 0.5% did not affect the measurement of M_2 enrichment from the [6,6-$^2H_2$]glucose infused. A negligible amount of M_2 at C-6 of glucose could be formed on ^2H_2O administration, theoretically at 0.5% only 1:40,000 molecules.

Enrichment of plasma and urinary water were determined by Dr. David Wagner, Metabolic Solutions, Merrimack, NH, using an isotope ratio mass spectrometer. Plasma water weight was assumed to be 94% of plasma volume (2).

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[1] In Fig. 2 of Ref. 9, C-1 of xylose should have been shown to be oxidized by periodate to form formic acid and not CO_2.

Hours of fasting, subject 4-10	11	12	13	14	15	16
Hours of fasting, subject 11-17	19	20	21	22	23	24
^2H_2O, 1.25 ml/kg body water	x	x	x	x		
$(6,6-^2H_2)$glucose, 83 mg prime, 50 mg/h	◄———————————————►					
Blood for ^2H at C5/C2*		x	x	x	x	x
Blood for ^2H at C6				xxxx		
Urine for water ^2H			◄————►◄————►			

Fig. 1. Protocol for administration of ^2H_2O and $[6,6-^2H_2]$glucose and blood and urine sampling during fasting of *subjects 4-10* and *11-17*.

*Blood was also drawn for determination of glucose concentration and enrichment in plasma water

Calculations. Glucose production, i.e., the rate of appearance (R_a) of glucose (in mg·kg body wt^{-1}·min^{-1}) was set equal to the enrichment of the $[6,6-^2H_2]$glucose infused, i.e., 98%, divided by the moles percent excess enrichment at C-6 of blood glucose and the rate of infusion of the $[6,6-^2H_2]$glucose (in mg·kg body wt^{-1}·min^{-1}) and then minus that rate. The fraction of glucose production from gluconeogenesis was set equal to the ratio of the enrichment of the hydrogen bound to C-5 to that of C-2. The quantity of glucose produced that was contributed by gluconeogenesis was calculated by multiplying that fraction by the R_a of glucose.

Statistics. Data are presented as means ± SE. Student's *t*-test was used and $P < 0.05$ was considered significant.

RESULTS

The enrichment values at C-5 to C-2 of blood glucose from the three subjects fasted for 42 h are presented in Fig. 2. As expected, the enrichment at C-5 increased with time of fasting, whereas the enrichment at C-2 remained constant (Fig. 2*A*). Thus the fractional contribution of gluconeogenesis estimated as the ratio of the

Fig. 2. Data for 3 subjects given ^2H_2O the 5th h of fasting, from 10th to 42nd h of fasting. *A*: enrichments at C-2 (○) and C-5 (●) of blood glucose; *B*: ratios of C-5 to C-2 (C-5/C-2).

enrichment at C-5 and C-2 (C-5/C-2) increased gradually (Fig. 2*B*) from 40.4 ± 8.0% at 10 h to 92.9 ± 5.7% at 42 h. At 14 h it was 47.9 ± 11.9% and at 22 h 62.6 ± 9.5%.

The results from the seven subjects given ^2H_2O beginning at 11 h and ending at 13.25 h and the seven subjects beginning at 19 h and ending at 21.25 h are presented in Fig. 3. The enrichment at C-5 of blood glucose increased initially, reaching near constancy by the 14th and 22nd h, respectively (Fig. 3*A*). Figure 3*B* presents the corresponding enrichments at C-2. The pattern of increase was similar to that at C-5. In Fig. 3*C* the C-5/C-2 values are plotted. At 13 and 21 h the ratios were 100.8 ± 2.9 ($n = 6$) and 97.5 ± 2.3% ($n = 6$), respectively, of those at 14 and 22 h. Ratios at 14 h were 97.0 ± 3.6% ($n = 7$) of those at 16 h. Ratios at 22 h were 97.1 ± 4.1% ($n = 5$) of those at 24 h. In Fig. 3*D* the enrichment of plasma water in the ten subjects in whom it was measured are recorded. By 14 and 22 h, 45 min after the last dose of ^2H_2O, enrichment was constant.

Enrichments in urinary water collected from 12.5–14 h and 14–16 h and from 20.5–22 h and 22–24 h from the ten subjects are recorded in Table 2. The means of the enrichments in plasma water (plotted in Fig. 3*D*) ± SE, along with the enrichment at 16 h at C-2 (plotted in Fig. 3*B*), are recorded for comparisons. The remarkable similarity in the enrichment in urinary water in the second collection, in plasma water and at C-2 is evident. Enrichment in urinary water in the first collection periods was 93.0 ± 1.8% of those in the second collection periods.

The enrichment of mass M_2 at C-6 of glucose was stable during the sampling period, with an average coefficient of variation of 3%, indicating that steady-state conditions were achieved (Fig. 4). The R_a of glucose, calculated from each of the four 10-min measurements for each of the subjects, in Table 3, were higher in the subjects fasted 16 h compared with those fasted 22 h [1.84 ± 0.05 and 1.54 ± 0.09 mg·kg^{-1}·min^{-1} ($P < 0.02$), respectively]. Also recorded in Table 3 are the C-5/C-2 values (plotted in Fig. 3*C*) for the 14th to 16th h and 22nd to 24th h. The small standard errors emphasize the relative constancy of those ratios. The ratio of 0.539 ± 0.023 at 14–16 h is significantly different from the ratio of 0.640 ± 0.022 at 22–24 h ($P <$
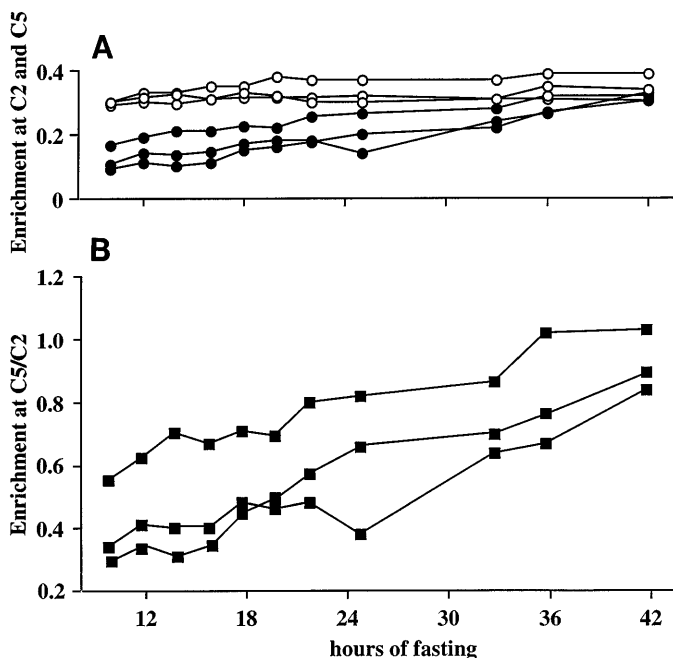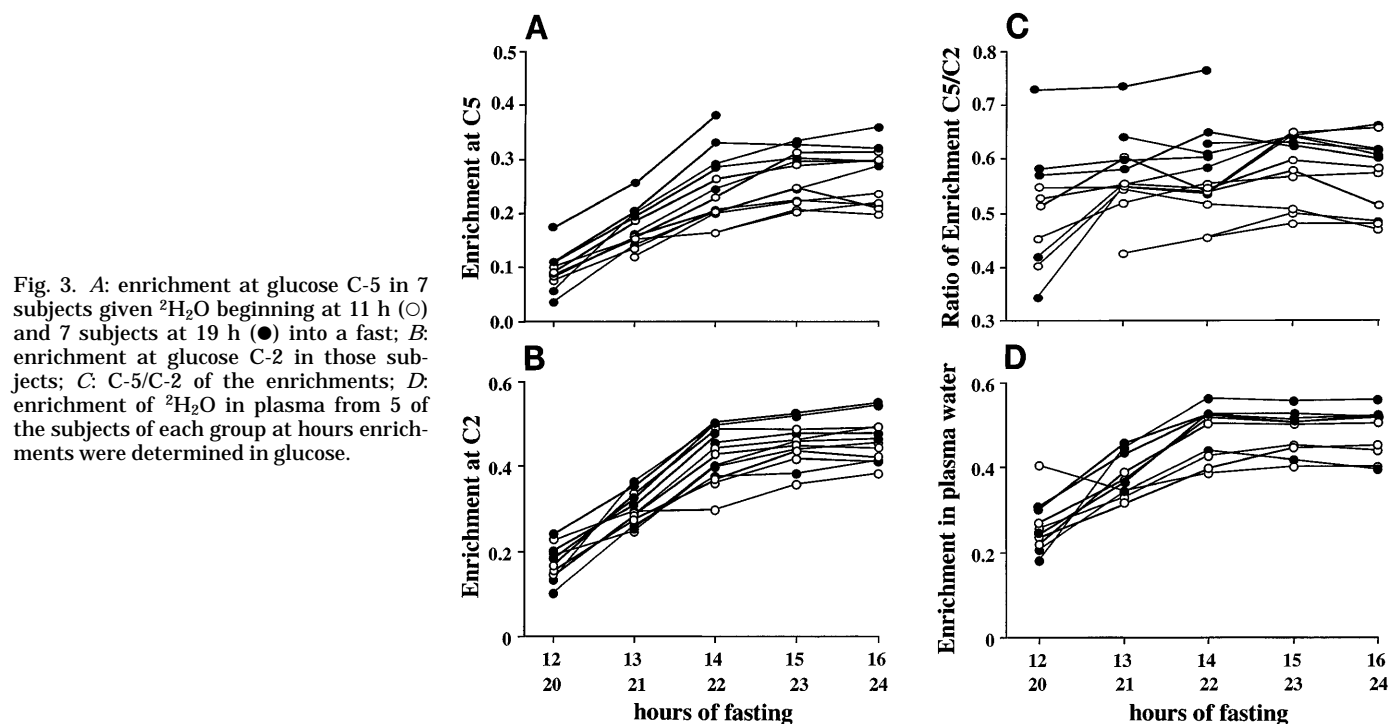
Fig. 3. *A*: enrichment at glucose C-5 in 7 subjects given ^2H_2O beginning at 11 h (○) and 7 subjects at 19 h (●) into a fast; *B*: enrichment at glucose C-2 in those subjects; *C*: C-5/C-2 of the enrichments; *D*: enrichment of ^2H_2O in plasma from 5 of the subjects of each group at hours enrichments were determined in glucose.

0.01). In the last column of Table 3 is the product of the fractional contribution of gluconeogenesis and the R_a of glucose, i.e., the quantities of glucose produced by gluconeogenesis. The quantities are not significantly different between the two time periods, averaging ~1.0 mg·kg^{-1}·min^{-1}.

The enrichments at C-2 and C-5 of blood glucose from the three subjects given only two doses of ^2H_2O, at 12 and 12.75 h of fasting, showed the same pattern as the enrichments from the subjects given twice the amount of ^2H_2O (Fig. 5). Thus there was a gradual increase in the calculated contribution of gluconeogenesis between the 13th and 14th h (means 40.1 and 48.0%), and then it was constant for the next 2 h (mean at 15 h 48.7% and 49.4% at 16 h). Plasma water enrichments were similar to those at C-2 by the 15th h. In one subject, plasma water enrichment declined from 0.34% in the 13th h to 0.25% in the 14th h, presumably due to incomplete

mixing, plasma being collected at the 13th h, only 15 min after the second dose of ^2H_2O.

DISCUSSION

Steady state in plasma water enrichment was attained within 3 h after beginning the ingestion of ^2H_2O, emphasizing the relatively rapid absorption of ^2H_2O and its mixing with body water. An enrichment in body water of 0.25% still allowed reliable measurements of C-5/C-2. Thus two doses and the resulting achievement of steady state in body water enrichment by 2 h can be accomplished without compromising estimates of gluco-

Table 2. *Percent enrichments in urinary water, plasma, and hydrogen at C-2 of blood glucose*

Subject No.	Urine		Plasma	C-2
	12.5–14 h	*14–16 h*	*14–16 h*	*16 h*
6	0.440	0.453	0.438 ± 0.007	0.455
7	0.385	0.383	0.397 ± 0.005	0.381
8	0.490	0.496	0.508 ± 0.000	0.505
9	0.389	0.411	0.420 ± 0.011	0.402
10	0.480	0.523	0.526 ± 0.004	0.455
	20.5–22 h	*22–24 h*	*22–24 h*	*24 h*
13	0.497	0.535	0.560 ± 0.002	0.542
14	0.358	0.418	0.418 ± 0.012	0.415
15	0.485	0.524	0.534 ± 0.001	0.472*
16	0.488	0.513	0.543 ± 0.004	0.505*
17	0.422	0.517	0.525 ± 0.004	0.522

Values are means ± SE for Plasma. *At 22 h.

Fig. 4. Enrichment of the hydrogens at C-6 (M_2) at 10-min intervals beginning 2.5 h after initiating [6,6-$^2H_2$]glucose infusion, at 14 (○) and 22 h of fast (●).
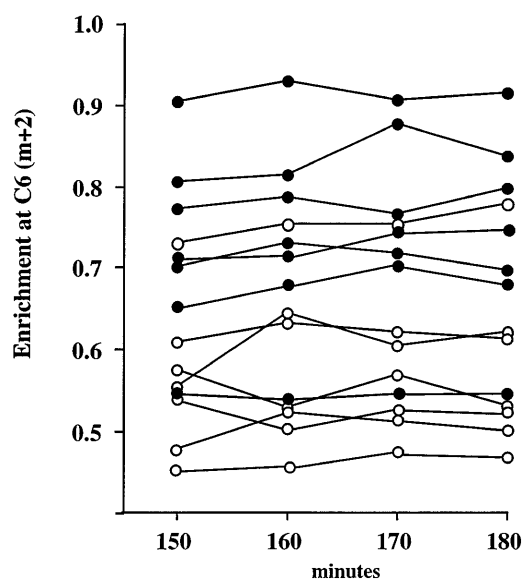
Table 3. *Rate of appearance of glucose, fractional contribution of gluconeogenesis, and calculated rate of gluconeogenesis*

Subject No.	R_a of Glucose, $mg \cdot kg^{-1} \cdot min^{-1}$	C-5/C-2	Gluconeogenesis, $mg \cdot kg^{-1} \cdot min^{-1}$
	13.5–14 h	*14–16 h*	
4	1.73 ± 0.02	0.479 ± 0.013	0.83
5	1.81 ± 0.04	0.471 ± 0.008	0.85
6	1.88 ± 0.01	0.498 ± 0.014	0.94
7	1.78 ± 0.01	0.565 ± 0.005	1.00
8	2.05 ± 0.02	0.584 ± 0.012	1.20
9	1.70 ± 0.02	0.544 ± 0.023	0.92
10	1.90 ± 0.03	0.634 ± 0.040	1.20
Means ± SE	1.84 ± 0.04	0.539 ± 0.023	0.99 ± 0.06
	21.5–22 h	*22–24 h*	
11	1.40 ± 0.01	0.619 ± 0.011	0.87
12	1.14 ± 0.01	0.624 ± 0.005	0.71
13	1.74 ± 0.02	0.629 ± 0.023	1.09
14	1.82 ± 0.04	0.599 ± 0.033	1.09
15	1.61 ± 0.02	0.608*	0.98
16	1.43 ± 0.02	0.770*	1.10
17	1.68 ± 0.01	0.631 ± 0.014	1.06
Means ± SE	1.54 ± 0.09	0.640 ± 0.022	0.99 ± 0.06

Values are means ± SE. R_a, rate of appearance; C–5/C–2, ratio of C–5 to C–2. *At 22 h.

neogenesis. Possibly, an even shorter period to steady state could be achieved by giving the ^2H_2O intravenously, but oral absorption seems so rapid that other routes seem unlikely to prove advantageous. Oral administration should result in hepatic water enrichment exceeding that in the systemic circulation for a period before complete equilibration, but that should not alter the C-5/C-2 generated.

The ratio of the deuterium enrichment at C-5 to that of body water, once steady state is attained, is a direct measure of the fraction gluconeogenesis contributes to glucose production (9). The C-5/C-2 does not require steady state, and water enrichments need only be sufficient to allow reliable determinations of enrich-

Fig. 5. Enrichments in plasma water (▲), and enrichments at C-5 (●) and C-2 (○) of blood glucose (*left* scale) and ratios of those enrichments (■, *right* scale) for subjects (n = 3) given ^2H_2O beginning 12 h into a fast to attain an enrichment of ≈0.25%.

ments at C-2 and C-5. The C-5/C-2 was essentially constant from the 2nd to the 5th h after beginning ^2H_2O ingestion. The rapid attainment of constancy in the ratio is presumably because the enrichments at C-2 and C-5 proceed through small, rapidly turning over intermediates in the pathways of gluconeogenesis and glycogenolysis, e.g., phospho*enol*pyruvate, the triose phosphates and hexose phosphates. An increase in the contribution of gluconeogenesis to glucose production during the 3 h was too small to be detected.

^2H_2O ingested in a single dose to give an enrichment in body water of 0.5% may produce severe vertigo (20). By dividing that dose into several equal portions ingested over 2–2.25 h or by giving ^2H_2O to attain an enrichment of only 0.35% at bedtime in a single dose, that side effect is prevented, as evidenced by our prior and present experience (1, 9, 10). One subject complained of mild dizziness, which we attribute to more rapid absorption relative to mixing with body water in him than the other subjects. Thus, in this subject, enrichment in plasma water after a second dose was already as high as enrichments reached at later times in the other subjects (Fig. 3D). The times selected for ^2H_2O administration depend on the times rates of gluconeogenesis are to be measured, the possible need to interrupt sleep pattern, and the time of the last meal to avoid deposition of labeled glycogen. The latter could be a particular concern when the rate of food absorption is slow.

Glucose cycling, i.e., glucose → glucose 6-phosphate (G-6-P) → glucose, has been estimated to be 11–20% of glucose production in the overnight-fasted state (9). In the initial period after ^2H_2O ingestion, cycling would form [2-^2H]G-6-P from unlabeled glucose in the circulation through the isomerization of G-6-P with fructose-6-phosphate (F-6-P). Therefore, [2-^2H]glucose would be released into the circulation. The lower C-5/C-2 during the first hours may reflect that cycling. With time, as circulating glucose has more label at C-2, cycling would lose its effect, since the glucose entering the cycle would already bear a label at C-2. The conversion of glycogen to triose phosphates in the hepatocyte, and then synthesis of glucose from the triose phosphates, is included in the estimate of the contribution of gluconeogenesis rather than glycogenolysis. That is the case for any approach that depends on triose phosphate formation for the measure of gluconeogenesis (9).

Glycogen cycling could label glycogen at the same time unlabeled glucose was released from glycogen. That would result in an underestimation of gluconeogenesis' contribution to glucose production. However, we previously summarized reasons for believing glycogen cycling is not significant in the fasted state (9). Glycogen cycling was recently concluded to occur in the fasted state (3), but reference is to cycling, estimated using ^{13}C nuclear magnetic resonance (NMR), during net glycogen deposition in subjects who had fasted (11). A recent abstract (13) reports no cycling, estimated using ^{13}C NMR, after an overnight fast. Another recent abstract (5) reports cycling during fasting, but for quantitation depends on the dilution of ^2H from
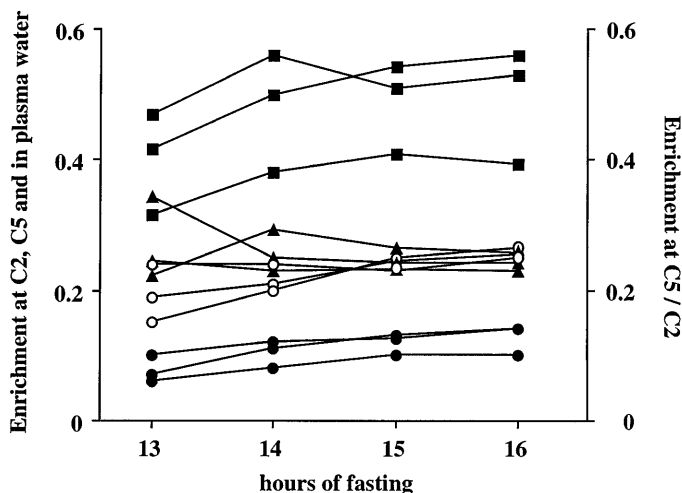
[1-^{2}H]galactose in the glucuronidation of acetaminophen. For that quantitation the reaction UTP + glucose-1-phosphate (G-1-P) → UDP-glucose + pyrophosphate is assumed to be irreversible. Data are sparse (21), but that reaction appears rather to be near equilibrium in liver (12). Schwenk and Kahl (17) similarly calculated UDP-glucose flux from enrichment of glucuronide excreted on infusion of [1-^{13}C]galactose and acetaminophen into fasted dogs, equating flux with cycling through glycogen. Their estimate of glycogen cycling's contribution is also untenable to the extent exchange occurs between G-1-P and UDP-glucose, rather than net flow. Our finding of similar percent contributions at times during fasting, whether ^{2}H$_2$O was given many or only a few hours before those times, is evidence for glycogen cycling not being of significance to the estimations.

When mixing of the ^{2}H$_2$O and glucose turnover are sufficient to give a constant enrichment at C-2, that enrichment should approximate the enrichment in body water, measured in plasma and/or urine. That was the case. The slightly lower enrichment in the urinary water collections at 12.5–14 h and 20.5–22 h than in collections over the next 2 h presumably was due to the beginning of those collections at the time the third of the four doses was ingested.

The advantage of the HMT, rather than other derivatives in measuring glucose production using [6,6-^{2}H$_2$]glucose, is that a relatively small amount of [6,6-^{2}H$_2$]glucose needs be administered. That is because of the in essence sixfold greater HMT enrichment over that of the hydrogens in the glucose and the HMT containing only C-6 and its hydrogens. The simultaneous giving of ^{2}H$_2$O to a body water enrichment of 0.5% does not interfere with the measurement of production. Also, the giving of the [6,6-^{2}H]glucose does not affect the ratio of the enrichments at C-2 and C-5. That is because the only pathway by which ^{2}H from [6,6-^{2}H$_2$]glucose can be incorporated at C-2 and C-5 is via the formation of ^{2}H$_2$O. The quantity of ^{2}H$_2$O formed from the [6,6-^{2}H$_2$]glucose is negligible compared with the amount of ^{2}H$_2$O ingested and only adds to that quantity. Thus the 83 + (50 × 3) = 233 mg of [6,6-^{2}H$_2$]glucose infused could maximally yield only 26 mg of ^{2}H$_2$O, when the smallest dose of ^{2}H$_2$O ingested by a subject was 70 g.

The increase in the fractional contribution of gluconeogenesis to glucose production during the fast was to be expected. The contributions of 54% at 14 h and 64% at 22 h are in accord with our previous findings (9). The decline in hepatic glucose production was significant. With no change in the quantity of glucose produced by gluconeogenesis at 14 and 22 h, that decline is attributable to a decrease in glycogenolysis.

A difference from our previous findings (9) remains unexplained. Previously, at 14 h of fasting, the enrichment at C-2 was 11 ± 3% less than that in urinary water and 5 ± 1% less at 22 h. We attributed those percentages to incomplete equilibration of G-6-P with F-6-P during glycogenolysis and accordingly made corrections. In the present study we have not discerned

any clear difference between the enrichment at C-2 and body water (Table 2).

Other approaches were recently introduced for estimating the contribution of gluconeogenesis to glucose production during fasting. Shulman and associates' estimates rest on determining the difference between glucose production, measured using labeled glucose, and changes in liver glycogen content with fasting, measured by magnetic resonance imaging and ^{13}C NMR spectroscopy. Their estimates of contributions of ~50% in the postabsorptive state (14), ~64% during the first 22 h, and ~96% after 40 h of fasting (16) are in agreement with ours. Their approach, although elegant, is tempered by the limited number of measurements of liver glycogen content that can comfortably be made and separate measurements of glucose production. Results may be more variable than ours; e.g., although the mean contribution to gluconeogenesis in 13 subjects in the postabsorptive state was ~50%, estimates ranged from 24 to 90% (14). If over the first 4–12 h of fasting gluconeogenesis contributes 50%, over the next 10 h gluconeogenesis would have to contribute ~80% to result in a contribution during 22 h of fasting of 64% (16).

Hellerstein and associates (5) infused ^{13}C-labeled glycerol and by mass isotopomer distribution analysis estimated a contribution to gluconeogenesis of 38% at 14 h of fasting and 75–85% after 60–90 h. We found, after 60 h of fasting, a contribution of ~60% (8). The method requires the assumption of a single pool of labeled triose phosphates from which the glucose is formed. That assumption is not fulfilled using [^{13}C]glycerol (8, 15).

Tayek and Katz (18, 19) infused [U-^{13}C$_6$]glucose into normal subjects fasted overnight. From the mass distributions of isotopomers in arterial blood glucose and lactate, gluconeogenesis was estimated to contribute ~40% to glucose production, in agreement with our estimate. However, the equations they used for calculation give overestimates of gluconeogenesis, because exchange of label is not adequately differentiated from dilution of label and formation of glucose from unlabeled triose-phosphate is not equated correctly. There is underestimation to the extent gluconeogenesis occurs from substrates not converted to glucose via pyruvate in liver, and the extent of the enrichment in arterial lactate is more than in hepatic pyruvate.

Gay et al. (4) quantitated gluconeogenesis during fasting by labeling glycogen with ^{13}C-labeled carbohydrate over several days. The amount of carbohydrate being oxidized was then estimated by indirect calorimetry, ^{13}C enrichment in expired CO$_2$ was determined, and the enrichment in hepatic glycogen was calculated with the assumption that all the ^{13}CO$_2$ was from hepatic glycogen and that was the only carbohydrate oxidized to CO$_2$. From that enrichment and the enrichment in blood glucose, assuming gluconeogenesis only from unlabeled precursors, the fractional contribution of gluconeogenesis to glucose production was calculated. It was estimated again at ~50% after an overnight fast. Also assumed was that the glycogen undergo-

ing glycogenolysis was homogeneously labeled. Glucose formed with recycling of label is not included in the estimate.

In conclusion, the use of ^2H_2O eliminates many assumptions required in other methods. The method is easily applied, that is, ^2H_2O administration, blood drawing, and determination of enrichments in hydrogens from blood glucose. Those determinations are by chemical procedure now routinely performed. The contribution of gluconeogenesis to glucose production can be estimated in at least as short a time as required to measure glucose production. Both can be measured together by beginning ^2H_2O and $[6,6-^2H_2]$glucose administrations at the same time. Changes in the gluconeogenic contribution can be closely monitored. Expenses relate mainly to the use of the mass spectrometer. There are limitations to the method. The quantity of glycogen cleaved to triose phosphates before conversion to glucose is included in the estimated gluconeogenic contribution. There are possibly small corrections for glucose release by hydrolysis at the branches of glycogen (9). In addition, because of the long half-life of ^2H_2O in body water, repeat studies may not be feasible for many weeks, e.g., quantifying gluconeogenesis after treatment compared with before a treatment will not be possible if glycogen present before the ^2H_2O is to be given a second time is significantly labeled at C-5 with ^2H.

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