Estimates of Krebs cycle activity and contributions of gluconeogenesis to hepatic glucose production in fasting healthy subjects and IDDM patients

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Summary Normal subjects, fasted 60 h, and patients with insulin-dependent diabetes mellitus (IDDM), withdrawn from insulin and fasted overnight, were given phenylacetate orally and intravenously infused with [3-^{14}C]lactate and ^{13}C-bicarbonate. Rates of hepatic gluconeogenesis relative to Krebs cycle rates were estimated from the ^{14}C distribution in glutamate from urinary phenylacetylglutamine. Assuming the ^{13}C enrichment of breath CO_2 was that of the CO_2 fixed by pyruvate, the enrichment to be expected in blood glucose, if all hepatic glucose production had been by gluconeogenesis, was then estimated. That estimate was compared with the actual enrichment in blood glucose, yielding the fraction of glucose production due to gluconeogenesis. Relative rates were similar in the 60-h fasted healthy subjects and the diabetic patients. Conversion of oxaloacetate to phosphoenolpyruvate was two to eight times

Krebs cycle flux and decarboxylation of pyruvate to acetyl-CoA, oxidized in the cycle, was less than one-30th the fixation by pyruvate of CO_2. Thus, in estimating the contribution of a gluconeogenic substrate to glucose production by measuring the incorporation of label from the labelled substrate into glucose, dilution of label at the level of oxaloacetate is relatively small. Pyruvate cycling was as much as one-half the rate of conversion of pyruvate to oxaloacetate. Glucose and glutamate carbons were derived from oxaloacetate formed by similar pathways if not from a common pool. In the 60-h fasted subjects, over 80 % of glucose production was via gluconeogenesis. In the diabetic subjects the percentages averaged about 45 %. [Diabetologia (1995) 38: 831–838]

Key words Gluconeogenesis, Krebs cycle, fasting, insulin-dependent diabetes mellitus, liver.

It is a continuing challenge to develop a method for reliably estimating the contribution of gluconeogenesis to glucose production in humans, so that it can be applied under both physiological and pathological conditions [1, 2]. Measurements of incorporation of label into glucose on administration of a labelled gluconeogenic substrate are limited by the need to

know the amount of label in the substrate entering the gluconeogenic process and the extent of dilution of label during that process, notably at the level of oxaloacetate, an intermediate common to the Krebs cycle and gluconeogenesis.

Phosphoenolpyruvate (PEP) is the necessary intermediate formed from oxaloacetate via pyruvate in the production of glucose (Fig. 1). We therefore gave ^{14}C-labelled bicarbonate to fasted normal subjects, assuming the specific activity of expired $^{14}CO_2$ to be the specific activity of ^{14}C fixed in the conversion of oxaloacetate to pyruvate [3]. We did that because of evidence that expired $^{14}CO_2$ is a good measure of mitochondrial $^{14}CO_2$. 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
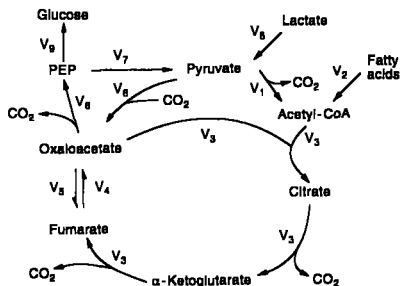
Fig. 1 Scheme of pathways of gluconeogenesis and Krebs cycle metabolism in the liver. Rates are designated V_1, V_2, etc. in keeping with previous usage [4]

in glutamine conjugated to phenylacetate. Our method, while time consuming, provides details on the metabolic rates occurring in liver while offering a direct measure of the contribution of gluconeogenesis. We have now used that approach to examine the rate of gluconeogenesis relative to Krebs cycle flux and the contribution of gluconeogenesis to glucose production in insulin-dependent diabetic (IDDM) patients, withdrawn from insulin so as to develop mild ketosis. These findings have been compared with those observed in fasted normal subjects also in mild ketosis.

Subjects, materials and methods

Subjects. A healthy man and woman, ages 35 and 28 years, with body mass indices 26.0 and 22.0 kg/m^2, respectively, and fasted for 60 h, were studied. Five males with IDDM, ages 19 to 23 years, with a mean body mass index of 22.9 kg/m^2 (range 18.0 to 26.8) were also studied. The haemoglobin A_{1c} concentration in four of the diabetic subjects ranged from 6.9 to 8.0 % and in one subject, MP, it was 13.9 %. Insulin was withdrawn 24 h before the study and food was withheld for 12 h. The diabetic subjects were encouraged to drink water throughout the period of insulin withdrawal. Approval for the study was obtained from the Human Investigation Committees at the Karolinska Hospital, Stockholm and University Hospitals of Cleveland. Informed consent was obtained from each subject.

Materials. [3-^{14}C]Lactate was prepared as previously described [4] from D-[6-^{14}C]glucose (purchased from Dupont-New England Nuclear, Boston, Mass., USA). ^{13}C-labelled sodium bicarbonate, 99 % enriched, was purchased from Merck Isotopes Inc., St. Louis, Mo., USA. The [3-^{14}C]lactate infused was dissolved in 50 or 100 ml of a sterile 0.154 mol/l sodium chloride solution. The 5 g of bicarbonate infused was dissolved in 500 ml of a sterile 0.077 mol/l sodium chloride solution. The lactate and bicarbonate solutions were shown to be pyrogen-free.

Experimental protocol. The protocol was similar to that previously used [3, 4] except that the subjects were given ^{13}C-labelled bicarbonate together with [3-^{14}C]lactate. The two normal subjects and three of the diabetic subjects ingested 4.8 g of phenylacetate, divided into three 1.6-g portions, at 30-min intervals. At the time the second portion was ingested (designated zero time) an infusion was begun of the labelled lactate and bicarbonate. The [3-^{14}C]lactate (40 μCi) was infused in trace quantity, 8 μCi as a bolus and then the rest infused at a con-

stant rate for the next 6 h. Labelled bicarbonate (5 g) was infused at a constant rate over the 6-h period. In the other two diabetic subjects infusion was for 8 h. Eighty μCi of the [3-^{14}C]lactate was given, 14 μCi as a bolus and the rest at a constant rate over the 8 h. Phenylacetate (4.8 g), was ingested in divided portions between the second and third hour. Five grams of the labelled bicarbonate was infused, but the rate of infusion for the first hour was 55 % and the second hour 90 % of the rate over the next 6 h.

Blood (5 to 10 ml) was drawn at hourly intervals for determinations of plasma glucose and D-β-hydroxybutyrate concentrations. Blood for the isolation of glucose (70 to 110 ml), was drawn 90 min before and at the end of the infusion. Urine, for the isolation of urea and glutamate from excreted phenylacetyl-glutamine, was collected at 90-min intervals over the last 3 h of infusion. Respiratory CO_2 was collected at hourly intervals by having the subjects breathe into balloons. Subjects were encouraged to drink 180–240 ml of water hourly during the infusion.

Measurements. Blood glucose, urinary urea, and glutamate from the excreted phenylacetylglutamine were isolated and degraded as previously described [4]. The urea carbon was collected as $BaCO_3$ by using urease. Portions of the glucose and glutamate were diluted with known quantities of unlabelled glucose and glutamate. Portions of these were then combusted to CO_2 and the remainder degraded to yield each of the carbons of the glucose and glutamate as CO_2 that was also collected as $BaCO_3$. Carbon 1 of the undiluted glutamate was isolated as $BaCO_3$ by treatment of a portion of the glutamate with chloramine T. Carbons 3 and 4 of the undiluted glucose were isolated as $BaCO_3$ by incubating the glucose with *Lactobacillus delbrueckii* to form lactate and decarboxylating the lactate. Respiratory CO_2 was collected as barium carbonate by bubbling the expired breath through carbonate-free sodium hydroxide and then adding barium chloride.

^{14}C-specific activities of the barium carbonates from urea, from the combustion of the diluted glucose and the diluted and undiluted glutamate, from the individual carbons of the diluted glucose and glutamate, and from breath CO_2 were determined by liquid scintillation spectrometry. The excess enrichment of ^{13}C in the $BaCO_3$ from urea, carbon 1 of the undiluted glutamate, the carboxyl group of lactate formed from the undiluted glucose and breath CO_2 was determined by mass spectrometry [5]. Barium carbonates from glucose, urea glutamate and respiratory CO_2 collected at zero time were assayed for the measure of ^{13}C natural abundance.

Calculations. Correction was made for the portion of the ^{14}C incorporated into glutamate that was due to the fixation of $^{14}CO_2$ formed from the [3-^{14}C]lactate. The method for estimating the portion is based upon evidence that the bicarbonate utilized in urea formation and formation of oxaloacetate from pyruvate derive from the same pool of CO_2 in hepatic mitochondria [3]. Carbon in CO_2 fixed by pyruvate becomes carbon 1 of α-ketoglutarate and hence carbon 1 of glutamate. $^{14}CO_2$ from [3-^{14}C]lactate should then be incorporated into carbon 1 of glutamate relative to its incorporation into urea in the same ratio as for ^{13}C-bicarbonate. Therefore, for a given collection period: Specific Activity in Carbon 1 of Glutamate due to $^{14}CO_2$ Fixation/Specific Activity in Urea = ^{13}C Excess in Glutamate/^{13}C Excess in Urea. The enrichment in carbon 1 of glutamate and urea having been measured as well as the ^{14}C specific activity of urea, the specific activity in carbon 1 of glutamate due to $^{14}CO_2$ fixation was calculated. Because breath CO_2 would be expected to reflect hepatic mitochondrial CO_2, its specific activity and enrichment were also used

Table 1. Specific activity and distribution of ^{14}C in glutamate corrected for ^{14}C incorporation from $^{14}CO_2$ into carbon 1

| Subject | Time (h) | Specific activity (dpm/μmol) | Recovery (%) | Correction to carbon 1 (%) | % ^{14}C in carbon | | | | | Carbon 2 |
					1	2	3	4	5	Carbon 1
Normal subjects										
HJ	3–4½	17.3	103.1	30.5 (24.3)	5.5	45.2	41.9	5.9	1.5	8.2
	4½–6	12.9	108.1	32.6 (32.1)	6.3	43.7	40.9	7.8	1.5	6.9
JE	3–4½	21.7	92.7	26.8 (23.1)	8.8	44.2	42.1	3.9	1.1	5.0
	4½–6	24.1	97.1	28.6 (30.5)	7.0	44.8	41.7	5.2	1.3	6.4
IDDM subjects										
KR	4½–6	15.1	91.6	33.7 (26.8)	6.6	49.7	36.1	6.4	1.2	7.5
MP	3–4½	8.6	80.4	40.0 (27.2)	6.2	49.7	37.8	4.9	1.4	8.0
	4½–6	9.0	90.1	37.5 (30.1)	5.8	53.8	34.0	4.9	1.7	9.3
RL	3–4½	16.3	95.0	15.7 (9.1)	7.3	50.5	34.9	7.0	0.4	8.2
	4½–6	22.5	96.5	20.3 (16.7)	7.2	49.7	35.6	6.8	0.6	6.9
JG[a]	6½–8	22.6	96.4	57.5 (53.2)	3.7	48.0	41.6	4.9	1.8	13.0
TS	5–6½	21.8	101.4	33.8 (29.3)	9.5	51.2	32.5	5.9	0.9	5.4
	6½–8	11.4	90.0	31.8 (27.9)	8.6	52.6	32.8	4.9	1.1	6.1

[a] JG was unable to provide a urine for collection from 5–6½ h. The specific activity of glutamate from phenylacetylglutamine excreted from 6–6½ h was 25.1 dpm/μmol

to calculate the correction due to $^{14}CO_2$ fixation. Thus, Specific Activity in Carbon 1 of Glutamate due to $^{14}CO_2$ Fixation/Specific Activity in Breath $CO_2 = ^{13}C$ Excess in Glutamate/^{13}C Excess in Breath CO_2.

For the estimation of the incorporation of ^{14}C from $^{14}CO_2$ into carbon 3 and 4 of glucose, the measurements of the specific activity and excess enrichment of urea were again employed along with the measurement of the excess enrichment of ^{13}C in glucose. Since carbon from CO_2 that is fixed is only incorporated into carbons 3 and 4 of glucose [4]: Specific Activity of Carbons 3 and 4 of Glucose due to $^{14}CO_2$ fixation/Specific Activity of Urea = ^{13}C Excess in Glucose/^{13}C Excess in Urea. Corrections were similarly made using the specific activities and enrichments in breath CO_2 rather than those for urea.

From the distribution of ^{14}C from [3-^{14}C]lactate in the carbons of glutamate from urinary phenylacetylglutamine, assumed to be an adequate reflection of the distribution in hepatic α-ketoglutarate, relative rates were estimated for the reactions in gluconeogenesis and the Krebs cycle (Fig. 1), i.e. pyruvate to acetyl-CoA, fatty acids to acetyl-CoA, fumarate to oxaloacetate, oxaloacetate to fumarate, oxaloacetate to PEP, PEP to pyruvate, lactate to pyruvate and PEP to glucose. The equations expressing those relationships, as well as an examination of the assumptions made in their derivation, have been presented [4].

The ^{13}C enrichment of carbon 1 of PEP, designated E_1, relative to that of the CO_2 fixed by pyruvate, depends upon the equilibration of oxaloacetate with fumarate, the rate of gluconeogenesis relative to Krebs cycle flux and the extent of pyruvate cycling, i.e. pyruvate → oxaloacetate → PEP → pyruvate, and/or Cori cycling, i.e. pyruvate → glucose → pyruvate [3]. The equation, previously derived [3], used to estimate E_1, assuming no pyruvate or Cori cycling, is

$$E_1 = \frac{0.5\,V_5 V_6 D}{(V_4 + V_6)^2 - (V_4 V_5 + V_5 V_6)}$$

and in the presence of significant pyruvate and/or Cori cycling

$$E_1 = \frac{V_6[0.5\,DV_5 + P_1(V_4 + V_6 - 0.5\,V_5)]}{(V_4 + V_6)^2 - (V_4 V_5 + V_5 V_6)}$$

where V_4 is the rate of conversion of fumarate to oxaloacetate, V_5 that of the conversion of oxaloacetate to fumarate and V_6 that of the conversion of oxaloacetate to PEP (Fig. 1), all relative to the rate of Krebs cycle flux. P_1 is the enrichment of carbon 1 of pyruvate, equal to $V_7E_1/(V_7 + V_8)$ where V_7 is the relative rate of the conversion of PEP to pyruvate and V_8 that of the conversion of lactate to pyruvate. D is the enrichment of the CO_2 fixed by the pyruvate, assumed to be that in breath CO_2. The enrichment in carbon 1 of PEP is then half the enrichment to be expected in glucose, i.e. in its carbons 3 and 4, if all glucose production were from gluconeogenesis via PEP. Then $100(^{13}C$ Excess Enrichment in Glucose)/$2E_1$ equals the % of glucose production by gluconeogenesis [3]. That equals 100(Enrichment in Carbon 1 of Lactate obtained by incubating the glucose with *Lactobacillus delbrueckii*)/E_1.

Results

Distribution of ^{14}C in glutamate. Carbon 1 of the glutamate from the 6½–8 h collection of subject had a ^{13}C excess enrichment about one-half that from the 5–6½ h collection (Table 1). However, the specific activities and enrichments of his breath CO_2, reflecting the enrichments in the CO_2 fixed in the incorporation of ^{13}C into carbon 1 of the glutamate were almost the same in the two periods. Therefore, there was dilution of phenylacetylglutamine or glutamate between the time of the 6½–8 h urine was collected and the total combustion, but we have been unable to determine how that occurred.

The distributions of ^{14}C in the glutamate carbons have been corrected for the incorporation of ^{14}C into carbon 1 of glutamate due to fixation of ^{14}C from $^{14}CO_2$ formed from the [3-^{14}C]lactate. The percentage corrections not in parentheses are those estimated using the specific activity of urea. It was those percentages that were used in calculating the distribu-

Table 2. Glucose and hydroxybutyrate concentrations, glucose specific activity, and distribution of ^{14}C in glucose corrected for ^{14}C incorporation from $^{14}CO_2$ into carbons 3 and 4

Subject	Time (h)	Glucose concentration (mmol/l)	Hydroxybutyrate concentration (mmol/l)	Specific activity (dpm/μmol)	Recovery (%)	Correction to carbons 3 and 4 (%)	%^{14}C in carbons						Carbons 1 + 6 / Carbons 3 + 4
							1	2	3	4	5	6	
Normal subjects													
HJ	6	4.0 (3.3)	2.8	152.3	96.0	29.0 (27.6)	25.6	21.3	2.6	2.6	21.7	26.1	9.9
JE	6	3.8 (3.3)	3.1	155.9[a]	93.8	33.3 (34.8)	24.8	22.7	2.5	2.4	21.0	26.4	10.4
IDDM subjects													
KR	4½	13.1 (13.9)		45.5	100.3	12.7 (19.1)	22.2	18.3	2.8	3.1	23.1	30.6	8.9
	6			63.6	99.2	12.1 (19.1)	22.8	18.9	2.8	2.1	23.9	28.5	8.7
MP	4½	18.4 (20.1)	1.1	27.3	100.2	11.9 (16.6)	19.4	17.4	3.7	4.4	25.2	30.0	6.1
	6			32.4	98.3	12.4 (20.4)	20.3	16.6	3.2	4.3	25.1	30.5	6.8
RL	4½	14.8 (20.4)	3.1	69.5	98.6	31.4 (18.2)	22.7	18.6	2.1	2.5	23.6	30.6	11.6
	6			93.4	99.4	21.2 (17.5)	23.6	18.4	2.8	3.0	23.3	29.0	9.1
JG	6½	19.6 (15.7)	2.3	107.9	94.6	31.4 (26.6)	24.0	20.5	2.8	3.1	23.4	26.0	8.5
	8			123.5	98.0	33.2 (29.6)	24.0	20.4	2.7	3.0	22.1	27.8	9.1
TS	6½	17.7 (20.2)	1.5	64.8	97.2	35.3 (30.6)	22.2	17.9	2.1	2.7	24.7	30.3	10.9
	8			85.5	97.7	34.0 (29.8)	23.3	18.8	2.4	2.7	23.5	29.1	9.9

[a] 144.3 dpm/μmol at 4½ h

tions. Thus, for the glutamate from subject HJ collected at 3–4½ h, 30.5 % of the ^{14}C formed in its carbon 1 was due to $^{14}CO_2$ fixation. Therefore, the % of ^{14}C in carbon 1 before correction was 7.9 % and after correction the 5.5 % recorded. The % corrections in parentheses are those calculated using the specific activities and enrichments in breath CO_2 rather than urea. The corrections calculated using the average specific activities and enrichments in breath CO_2 during the period of the collection are about 15 % lower on average than those using urea.

The ^{14}C in each of the five carbons of glutamate are recorded as a % of the sum of the ^{14}C in the five carbons. The distributions in different collection periods for a given subject are similar. Over 80 % of the ^{14}C in glutamate was in carbons 2 and 3. The ratios of ^{14}C in carbon 3 to carbon 2 of glutamate from the diabetic patients, except JG, range from 0.63 to 0.76. The ratios for JG and the subjects fasted for 60 h range from 0.86 to 0.95.

Distribution of ^{14}C in glucose. After 60 h of fasting, normal subjects, as in our previous experience [3, 4], had plasma glucose concentrations of about 4.0 mmol/l and 6 h later about 3.3 mmol/l (Table 2). The plasma concentration of β-hydroxybutyrate at the completion of infusion provides evidence for mild ketosis in all the subjects, except in subject KR for whom the concentration was not determined.

Specific activities of glucose were highest in the normal subjects fasted for 60 h and next in subject JG. The specific activity of glucose 1½ h before completing the infusion was 72 to 84 % of that at its completion. For the correction for the incorporation of ^{14}C into carbon 3 and 4 of glucose, the measurements of the specific activity and excess enrichment of urea were again employed along with the measurement of the excess enrichment of ^{13}C in glucose. The values in parentheses are the corresponding % corrections using the specific activities and enrichments in breath CO_2. Percent corrections estimated using breath CO_2 are generally somewhat lower than those using urea except for subjects KR and MP where the corrections using urea are about one-third less than those using breath CO_2.

Over 90 % of the ^{14}C in glucose was in its carbons 1,2,5 and 6, with about 80 % as much ^{14}C in carbon 2 as 1 and in carbon 5 as 6. The difference in the % of ^{14}C in carbon 6 to carbon 1 among the subjects is lowest for the two normal fasted subjects and next lowest for subject JG. Similarly, the differences are smallest between carbons 5 and 2 for the normal subjects and then subject JG. While the percentages of ^{14}C in carbons 3 and 4 are small, only in the normal subjects is the activity in carbon 3 equal to that in carbon 4. Thus, in the diabetic subjects, ^{14}C in carbon 4 exceeds that in carbon 3, except for subject KR at 6 h and that is undoubtedly in error, since ^{14}C in carbon 6 exceeds that in carbon 1 and in carbon 5 that in carbon 2.

Relative rates and gluconeogenesis. Rates estimated from the distributions in Table 1 are presented in Table 3. These rates are relative to the rate of Krebs cycle flux set to 1.0, i.e. the rate acetyl-CoA condensed with oxaloacetate to form citrate. More than nine times as much of the acetyl-CoA condensing with oxaloacetate was from fatty acids as from pyruvate in both the fasted normal and the diabetic subjects. The rate of the conversion of oxaloacetate to fumarate in the 60-h fasted subjects was more than 50 times the

Table 3. Rates of reactions in gluconeogenesis and the Krebs cycle relative to Krebs cycle flux

Subject	Time (h)	Pyruvate→ acetyl-CoA	Fatty acids → acetyl-CoA	Fumarate → oxaloacetate	Oxaloacetate → fumarate	Oxaloacetate → PEP	PEP → pyruvate	Lactate → pyruvate	PEP → glucose
Normal subjects									
HJ	3–4$^1/_2$	0.08	0.92	68.4	67.4	4.18	1.99	2.37	2.19
	4$^1/_2$–6	0.10	0.90	70.2	69.2	3.16	1.23	2.20	1.93
JE	3–4$^1/_2$	0.05	0.95	57.9	56.9	2.14	1.20	0.99	0.94
	4$^1/_2$–6	0.07	0.93	54.4	53.4	2.97	1.45	1.58	1.52
IDDM subjects									
KR	4$^1/_2$–6	0.08	0.92	13.6	12.6	3.46	1.52	2.02	1.94
MP	3–4$^1/_2$	0.06	0.94	14.8	13.8	4.11	2.42	1.76	1.70
	4$^1/_2$–6	0.07	0.93	7.1	6.1	5.16	3.85	1.37	1.31
RL	3–4$^1/_2$	0.07	0.93	12.8	11.8	2.65	0.43	2.29	2.22
	4$^1/_2$–6	0.07	0.93	13.7	12.7	2.75	0.65	2.17	2.10
JG	6$^1/_2$–8	0.07	0.93	46.8	45.8	7.98	4.94	3.11	3.04
TS	5–6$^1/_2$	0.06	0.94	6.5	5.5	2.06	0.91	1.21	1.15
	6$^1/_2$–8	0.06	0.94	6.1	5.1	2.67	1.57	1.15	1.09

Table 4. Comparison of breath CO_2 ^{14}C specific activities and ^{13}C enrichments at 3 h relative to 6 h into the infusions of [3-^{14}C] lactate and ^{13}C-labelled bicarbonate

Subject	Ratio at 3 h to 6 h	
	^{14}C Specific activity	^{13}C Excess enrichment
HJ	0.81	0.88
JE	0.71	0.91
KR	0.75	0.80
MP	0.78	0.83
RL	0.79	0.85
JG	0.84	0.92
TS	0.81	0.85

Table 5. Ratio of the incorporation of ^{14}C into carbons 2 and 5 to carbons 1 and 6 of glucose divided by the ratio of the incorporation into carbons 3 to 2 of glutamate

Subject	Time (h) Glucose/Glutamate	Glucose ratio / Glutamate ratio
Normal subjects		
HG	6/4$^1/_2$–6	0.83/0.94 = 0.88
JE	6/4$^1/_2$–6	0.85/0.93 = 0.91
MH[a]	4$^1/_2$/3–4$^1/_2$	0.82/0.71 = 1.15
	6/4$^1/_2$–6	0.83/0.81 = 1.02
AK[a]	4$^1/_2$/3–4$^1/_2$	0.83/0.81 = 1.02
	6/4$^1/_2$–6	0.84/0.70 = 1.20
IDDM subjects		
KR	6/4$^1/_2$–6	0.83/0.73 = 1.14
MP	4$^1/_2$/3–4$^1/_2$	0.86/0.76 = 1.13
	6/4$^1/_2$–6	0.82/0.63 = 1.30
RL	4$^1/_2$/3–4$^1/_2$	0.79/0.69 = 1.14
	6/4$^1/_2$–6	0.79/0.72 = 1.10
JG	8/6$^1/_2$–8	0.81/0.90 = 0.90
TS	6$^1/_2$/5–6$^1/_2$	0.81/0.63 = 1.29
	8/6$^1/_2$–8	0.81/0.63 = 1.31

[a] From reference 4

rate of Krebs cycle flux. In the diabetic subjects, again with the exception of JG, it was 5 to 14 times more. The rate of conversion of oxaloacetate to PEP was two to five times more, except for JG where it was eight times more, and with no discernible difference between the groups. About half of the PEP was converted to glucose and the remainder to pyruvate and again with no discernible difference between the groups. Thus, the rate of carboxylation of pyruvate to oxaloacetate, equal to the rate of conversion of oxaloacetate to PEP, was 30 times or more than the rate of its decarboxylation to acetyl-CoA. About 50 % of the PEP formed was cycled.

The specific activity and enrichment in breath CO_2 approached a plateau by the third hour (Table 4). At 5 h, the specific activity of the breath CO_2 was 88 % or more and of ^{13}C 93 % or more of that at 6 h. Thus, while it was only a small difference, the specific activity of breath CO_2 approached a plateau more slowly than its enrichment.

The ratio of incorporation of ^{14}C into carbons 2 and 5 to carbons 1 and 6 of glucose (Table 2) divided by the ratios of the incorporations of ^{14}C into carbons 3 to 2 of glutamate (Table 1) are about 1.0 (Table 5). The ratios for the diabetic subjects average 1.16.

Estimates of E_1 during the last 1$^1/_2$ h of infusion, using for D the average ^{13}C enrichment in breath CO_2 for that period, are recorded in columns 3 and 4 of Table 6. The mean of the enrichments in carbon 3 and 4 of circulating glucose, determined by conversion of the glucose to lactate and decarboxylation of the lactate, is recorded in the fifth column. The estimated percentage of glucose production due to gluconeogenesis is then 100 times E_1 divided by the mean of the enrichments in carbons 3 and 4 of the glucose. Those estimates are recorded in the last two columns of Table 6.

Table 6. [13]C Excess estimated in carbon 1 of PEP (E_1) and half that observed in carbons 3 and 4 of glucose; estimated % glucose formation via gluconeogenesis

Subject	Time (h)	Estimated excess ^{13}C in E_1 Pyruvate cycling		Observed Excess ^{13}C in carbons 3 and 4 of glucose/2	% Gluconeogenesis Pyruvate cycling	
		Absent	Present		Absent	Present
Normal subjects						
HJ	$4^1/_2$–6	0.316	0.370	0.268	84.8	72.4
JE	$4^1/_2$–6	0.396	0.489	0.346	87.4	70.8
IDDM subjects						
KR	$4^1/_2$–6	0.326	0.413	0.105	32.2	25.4
MP	$4^1/_2$–6	0.190	0.354	0.075	39.5	21.9
RL	$4^1/_2$–6	0.301	0.336	0.096	31.0	28.6
JG	$6^1/_2$–8	0.294	0.433	0.191	65.0	44.1
TS	$6^1/_2$–8	0.139	0.198	0.121	87.1	61.1

Table 7. Excess ^{13}C enrichments in carbon 1 of glutamate and carbons 3 and 4 of glucose at times of collection

Subject	Carbon 1 of glutamate						Carbons 3 and 4 of glucose			
Normal subjects										
HJ	3–$4^1/_2$ h	0.062	$4^1/_2$–6 h	0.065			$4^1/_2$ h	0.496	6 h	0.536
JE	3–$4^1/_2$ h	0.123	$4^1/_2$–6 h	0.152			$4^1/_2$ h	0.676	6 h	0.692
IDDM subjects										
MP	3–$4^1/_2$ h	0.063	$4^1/_2$–6 h	0.066						
RL	3–$4^1/_2$ h	0.022	$4^1/_2$–6 h	0.054						
JG	3–6 h	0.099	6–$6^1/_2$ h	0.107	$6^1/_2$–8 h	0.121	$6^1/_2$ h	0.316	8 h	0.382
TS	3–5 h	0.091	5–$6^1/_2$ h	0.107	$6^1/_2$–8 h	0.047	$6^1/_2$ h	0.176	8 h	0.242

In the 60-h fasted normal subjects blood glucose was similarly enriched at $4^1/_2$ and 6 h (Table 7). Enrichments in blood glucose in the diabetic subjects $1^1/_2$ h before the end of infusion were still 20 to 30 % less than at the end of the infusion.

Discussion

Carbons 1, 2 and 3 of α-ketoglutarate, the precursor of glutamate, are from carbons 4, 3 and 2 of oxaloacetate [4]. [3-^{14}C]Lactate via [3-^{14}C]pyruvate forms [3-^{14}C]oxaloacetate. [14]C in carbon 2 compared to that in carbon 3 of glutamate (Table 1) is then a function of the extent of equilibration of oxaloacetate with fumarate relative to the other fates of oxaloacetate. [14]C from [3-^{14}C]lactate is incorporated into carbon 4 of oxaloacetate, and hence carbon 1 of glutamate, by the conversion of [2, 3-^{14}C]α-ketoglutarate to [1, 2, 3, 4-^{14}C]oxaloacetate via the Krebs cycle and by [14]CO$_2$ fixation by pyruvate. Since ^{14}C in carbon 1 has been corrected for incorporation due to fixation, that ^{14}C relative to the ^{14}C in carbons 2 and 3 is the measure of cycle flux relative to the conversion of oxaloacetate to PEP. [14]C in carbon 4 reflects the decarboxylation of pyruvate to acetyl-CoA relative to its other fates. [14]C in carbon 5 is a measure of pyruvate cycling, i. e. pyruvate → oxaloacetate → PEP → pyruvate.

The rates for the two 60-h fasted subjects (Table 3) agree with our previous finding on giving [3-

^{14}C]lactate in an identical manner to three other subjects, although the equilibration of oxaloacetate with fumarate was less [4]. The flux of PEP to pyruvate was about one-half the flux of pyruvate to oxaloacetate (Table 3), also in accord with previous estimates [4, 6]. The contribution of Cori cycling cannot be differentiated from that of pyruvate cycling and the estimate relies on low incorporations. [14]C in carbon 4 of glutamate from the $4^1/_2$–6 h collections of the subjects fasted 60 h, as before [4], averaged five times the ^{14}C in carbon 5. [14]C in carbon 3 of PEP was about 1.2 times that in its carbon 2 as evidenced by distributions in glucose. [14]C in carbon 3 of circulating lactate in the previous study was about nine times that in carbon 2 at 6 h. If ^{14}C in the acetyl-CoA forming carbons 4 and 5 of glutamate had come solely from PEP via pyruvate cycling a ratio of ^{14}C in carbon 4 to 5 of 1.2 would have been expected and 9 if solely from lactate. That it was 5, while assumptions are many and the percentages small, then suggests that pyruvate and Cori cycling each contributed half.

The metabolic state of the diabetic subjects was more likely to be heterogeneous than for the normal subjects. Furthermore, the rates being relative, do not provide a measure of the absolute rates of gluconeogenesis. In starvation and uncontrolled diabetes, the major hepatic energy source derives from the formation of NADH and FADH$_2$ in fatty acid oxidation to acetyl-CoA, which results in ketosis, similar in de-

gree in the two groups. Krebs cycle activity is inhibited by the reduced state of those nucleotides.

Corrections for $^{14}CO_2$ incorporation from the [3-^{14}C]lactate averaged about 30 % (Table 1). The relative specific activity and ^{13}C enrichment in urea is assumed to reflect the relative specific activity and enrichments of hepatic mitochondrial CO_2. Similar percentage corrections using urea and breath CO_2 support that assumption (Table 1). The reason the corrections estimated using breath CO_2 are about 15 % less than those using urea is unknown, but those corrections would result in only a small change in the estimated rates.

Comparisons are for breath CO_2 and urea specific activities and ^{13}C enrichments arising from ^{14}C labelled bicarbonate formed by the intracellular metabolism of [3-^{14}C]lactate and ^{13}C-labelled bicarbonate infused intravenously. For the comparisons changes in specific activities within tissues should then parallel those of enrichment. This was reasonably achieved (Table 4).

Support for use of the phenylacetylglutamine derivative is found in similar distributions of ^{14}C in the carbons of glucose and glutamate derived from oxaloacetate [4]. Since the glucose carbons arise from hepatic oxaloacetate, presumably the glutamate carbons were also derived from the same oxaloacetate source. That could only occur by the conversion of hepatic α-ketoglutarate to glutamine that conjugates with phenylacetate. Further evidence is found in a correction of about 30 % for the contribution of $^{14}CO_2$ fixation to the ^{14}C in carbon 1 of glutamate and carbons 3 and 4 of glucose. Additional support comes from similar distributions of ^{14}C from [2, 3-^{14}C]succinate, [2-^{14}C]propionate and [3-^{14}C]lactate in phenylacetylglutamine from normal subjects fasted 60 h coupled with evidence for extensive metabolism of [2, 3-^{14}C]succinate and [3-^{14}C]lactate in liver [7]. Still further support comes from estimates similar to ours from the distribution of ^{13}C in glutamate on perfusing liver from fasted rats with [3-^{13}C]lactate [8] and on isotopomer analysis of liver glutamate from rats given [U-^{13}C]lactate and [2, 3-^{13}C]lactate [9].

Correction for CO_2 fixation in the normal subjects was again generally about 30 % for both glutamate and glucose (Tables 1 and 2). For subjects KR and MP, corrections were less for glucose than glutamate. However, those were the subjects in whom corrections using breath CO_2 specific activities and enrichments were higher than those using urea. While that raised concern about the urea measurements, no error was found.

Ratios of ^{14}C in the carbons of glutamate and glucose derived from carbons 2 and 3 of oxaloacetate are similar for the normal subjects fasted 60 h (Table 5), but in the diabetic subjects, the average ratio of 1.16 suggests less isotopic equilibration of carbons 3 and 2 of the oxaloacetate used in the formation of

the glutamate than glucose. There is evidence for two pools of oxaloacetate in the liver [7–10]. One would then be mitochondrial, with relatively incomplete isotopic equilibration and the source of the glutamate carbons. The other would arise by transport of mitochondrial oxaloacetate into the cytosol and then further isotopic equilibration before conversion to glucose. Alternatively, some labelled α-ketoglutarate converted in the diabetic subjects to glutamine was not from the liver, or the difference could reflect a heterogeneity in hepatocytes [11].

The possibility of cytosolic and mitochondrial pools of oxaloacetate is tempered by the suggestion of less isotopic equilibration in the carbons from oxaloacetate in glucose than glutamate when individuals fasted overnight were infused with glucose and given [3-^{14}C]lactate and phenylacetate [4]. Lower specific activities in glutamate from glutamine (Table 1) than in glucose (Table 2) are in accord with much of the glutamine used in the conjugation of phenylacetate being unlabelled and coming from muscle [12, 13].

^{14}C in carbon 2 to 1 of glutamate overlap ^{14}C in carbons 1 and 6 to ^{14}C in carbons 3 and 4 of glucose (Tables 1 and 2), i.e. 5.0 to 9.3, excluding subject JG, compared to 5.0 to 11.6, but glucose ratios tend to be more, as was previously observed [4]. This again suggests some difference in the source of the carbons of oxaloacetate for glucose and glutamate formation. The difference in range increases, if the incorporation of ^{14}C in carbon 2 of citrate, the source of which is carbon 3 of oxaloacetate, relative to that in carbon 6 of citrate, the source of which is carbon 1 of oxaloacetate, is taken into account. The range is then 4.9 to 8.0 rather than 5.0 to 9.3.

A major reservation in estimates of rates of gluconeogenesis from the incorporation of label from a labelled substrate into glucose is uncertainty as to the extent of dilution of label at the level of oxaloacetate [1, 3, 4]. That the rate of gluconeogenesis is 2 or more times the rate of Krebs cycle flux in the uncontrolled diabetic patient means that dilution of the labelled substrate is relatively small. We previously estimated that in the 60-h fasted human, in the conversion of [3-^{14}C]pyruvate to glucose, the specific activity of PEP would be about five-sixths the specific activity of intracellular pyruvate [4]. Label is assumed for the estimates [4] only to be lost in significant quantity from the cycle in the conversion of oxaloacetate to PEP (Fig. 1). The same pattern in glutamate formed by livers of fasted rats perfused with [3-^{13}C]lactate in vitro and by livers of fasted rats infused with [3-^{13}C]lactate in vivo supports that assumption [13].

Inability to determine the specific activity or enrichment of intracellular pyruvate has in the past prevented quantitation. The use of breath CO_2 eliminates that problem, since its specific activity or enrichment appears to be a good measure of that in in-

tracellular CO_2 [3]. However, when (1-^{13}C)leucine was infused into 60-h fasted humans, enrichment of breath CO_2 was about 15 % less than CO_2 in arterial and hepatic vein blood (14).

As in our previous study [3], in the normal subjects fasted 60 h, 80 % or more of the glucose produced (assuming no cycling) was by gluconeogenesis (Table 6). This is in accord with gluconeogenesis being the only source of circulating glucose after prolonged fasting, except for small amounts from glycogen [15] and glycerol [16]. In the diabetic subjects the contribution of gluconeogenesis varied, but except for subject TS was much less, averaging about 45 %. Uptake of gluconeogenic substrates across the splanchnic bed in IDDM subjects, withdrawn from insulin, has accounted for 32–40 % of splanchnic glucose output [17, 18].

Glycogen stores remaining during the period of insulin withdrawal may explain the lower contributions of gluconeogenesis to glucose production in the first three diabetic subjects. We expected the higher contribution in subject JG because the distribution of ^{14}C and specific activity in his blood glucose were similar to that in the normal subjects fasted 60 h. The almost symmetrical labelling of glucose carbons, evidence of nearly complete isotopic equilibration between dihydroxyacetone-3-P and glyceraldehyde-3-P, has been observed when glycogen is depleted [4].

Since enrichment (Table 7) and specific activities (Table 2) in glucose 1$^{1}/_2$ h before the end of infusion was less than at the end of the infusion, steady state was not achieved. However, as the contribution of glycogen to glucose production decreases, the specific activity of glucose would be expected to increase. A limitation in achieving steady-state in circulating glucose enrichment in the diabetic subjects was probably their large glucose pool. Until steady-state is achieved, gluconeogenesis may be low because a significant portion of glucose in the blood is in the circulation prior to the administration of ^{13}C-bicarbonate. In the two 60-h fasted normal subjects, steady-state was approached as evidenced by the similar enrichments in glucose at 4$^{1}/_2$ and 6 h. In the three 60-h fasted subjects in the previous study [4], glucose and glutamate specific activities are in accord with the results for the two subjects in the present study. The achieving of steady-state for the estimate of relative rates is not a determinant as long as the distribution of ^{14}C in glutamate is not changing. The enrichment of ^{13}C in carbon 1 of glutamate would be expected to increase in time with increasing enrichment in breath CO_2.

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