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^{13}C NMR study of gluconeogenesis from labeled alanine in hepatocytes from euthyroid and hyperthyroid rats

(pyruvate kinase flux/triiodothyronine/microcompartmentation/ketone bodies)

S. M. COHEN*†, P. GLYNN†, AND R. G. SHULMAN†‡

*Merck Institute for Therapeutic Research, Rahway, New Jersey 07065; and †Bell Laboratories, Murray Hill, New Jersey 07974

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ABSTRACT **Metabolism of [3-^{13}C]alanine in the presence and absence of β-hydroxybutyrate or ethanol has been followed at 25°C by ^{13}C NMR at 90.5 MHz in primary hepatocytes from untreated rats and rats treated with triiodothyronine and not allowed to eat for 24 hr. The phospho*enol*pyruvate/pyruvate futile cycle was followed *in situ* by comparing the concentration of ^{13}C at the scrambled alanine C2 position with that at glucose C5. In the absence of ethanol, the flux through pyruvate kinase was 60% of the gluconeogenic flux in hepatocytes from hyperthyroid rats, compared with 25% in the controls. Incubation with ethanol reduced the pyruvate kinase flux in the hyperthyroid state to that measured in the controls. Under all conditions, the relative concentration of label at the aspartate C2 and C3 sites was 1:2, whereas at the corresponding carbons in glutamate, randomization was almost complete. These observations, which require flux of unscrambled label into aspartate, are consistent with intramitochondrial synthesis of aspartate only if there is incomplete mixing of the intramitochondrial oxaloacetate pool. The ^{13}C enrichment measured in the ketone bodies is increased by the presence of exogenous β-hydroxybutyrate. The greater labeling that we observe at C2 of β-hydroxybutyrate compared with C4 under this condition is explained by the flow through 3-hydroxy-3-methylglutaryl-coenzyme A synthase.**

In a recent ^{13}C NMR study of suspensions of isolated hepatocytes from euthyroid and hyperthyroid rats, we measured the flux through the gluconeogenic pathway from labeled glycerol into glucose, determined the pentose cycle activity, and examined the pathway through the nonoxidative pentose branch (1). In another study, in which we followed the metabolism of ^{13}C-labeled alanine in perfused mouse liver, the competition between ethanol and alanine into the tricarboxylic acid cycle was investigated, and it was also suggested that the ^{13}C NMR spectra provided a unique probe of the *in situ* flux through pyruvate kinase (2).

The present ^{13}C study shows the effect of thyroid hormone treatment upon the flux through pyruvate kinase during gluconeogenesis from labeled alanine in primary rat hepatocytes. The results confirm our previous studies in perfused mouse liver of the competition between ethanol and alanine into the tricarboxylic acid cycle.

METHODS AND MATERIALS

Liver parenchymal cells were isolated from male Sprague–Dawley rats (190–240 g) that had not eaten for 24 hr. The animals received intraperitoneal injections of either L-3,3',5-triiodothyronine (T3; Calbiochem, B grade) dissolved in isotonic saline at pH 9.8, 8 μg of T3 per 100 g of body weight per day (T3-treated, hyperthyroid), or isotonic saline alone (or no injections) (euthyroid control) for 4 days prior to cell preparation.

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The liver cell preparation, the resuspension medium, and the oxygenation of the cells in the NMR tube have been described (1). [3-^{13}C]Alanine and [2-^{13}C]ethanol (90% isotopic purity) were from Merck. 2,4-Dihydroxybutyrate was a kind gift from R. Rognstad.

The NMR spectra were measured on a Bruker HX-360 spectrometer at 90.5 MHz for ^{13}C nuclei. All spectra were recorded at 25 ± 1°C in 10-mm-diameter NMR tubes. Unless other conditions are specified, each ^{13}C spectrum is the Fourier transform of the accumulation of 900 radiofrequency pulses of 60° free induction decays of 16,000 data points each, with 2-sec recovery between pulses. ^{13}C chemical shifts are given relative to tetramethylsilane at 0 ppm.

EXPERIMENTAL RESULTS

Fig. 1 compares the ^{13}C spectra of hepatocytes from normal and T3-treated rats; the cells were incubated with [3-^{13}C]alanine and unlabeled β-hydroxybutyrate. There were two major differences between these spectra. (*i*) In the cells from the hyperthyroid rat there was more ^{13}C enrichment at the scrambled alanine C2 position compared with that at the corresponding glucose C5; this ratio is a direct measure of the bifurcation of the flux from phospho*enol*pyruvate (*P-e*Prv). (*ii*) The *in situ* synthesis of ketone bodies was depressed in the cells from the T3-treated rat; however, in both spectra there was more enrichment at C2 of β-hydroxybutyrate than at C4.

Fig. 2 shows the competition of ethanol with alanine into the tricarboxylic acid cycle in hepatocytes from control rats for labeled and unlabeled substrates in all combinations of interest. Fig. 2*a* shows that when [3-^{13}C]alanine was the only carbon source, C2, C3, and C4 of glutamate were all appreciably labeled. In accordance with previous results on perfused mouse liver (2), when unlabeled ethanol and [3-^{13}C]alanine were introduced (Fig. 2*b*), C4 of glutamate and C2 and C4 in the ketone bodies were not labeled, whereas the ^{13}C enrichments at C2 and C3 of glutamate were not reduced. However, when [2-^{13}C]ethanol and [3-^{13}C]alanine were added (Fig. 2*c*), C4 of glutamate and C2 and C4 in the ketone bodies were intensely labeled. Finally, when [2-^{13}C]ethanol and unlabeled alanine were added (Fig. 2*d*), those carbons directly derived from [2-^{13}C]acetyl-CoA (namely, C4 of glutamate and glutamine and C2 and C4 in β-hydroxybutyrate and acetoacetate) were strongly labeled. The ^{13}C enrichment measured in the ketone bodies and at C4 and C2 of glutamate is given in Table 1.

The ^{13}C spectra of hepatocytes from T3-treated rats incubated with either [3-^{13}C]alanine and β-hydroxybutyrate or [3-^{13}C]alanine and [2-^{13}C]ethanol are shown in Fig. 3. Fig. 3*b*

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; T3, L-3,3',5-triiodothyronine; *P-e*Prv, phospho*enol*pyruvate.

‡ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511.

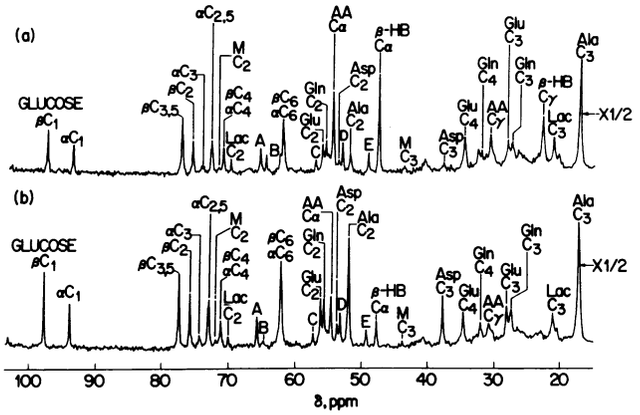

FIG. 1. ^{13}C NMR spectra at 25°C. (*a*) Hepatocytes from a euthyroid rat. This spectrum is the sum of 3000 scans accumulated over the interval 140–230 min after addition of 28 mM [3-^{13}C]alanine and 8 mM D,L-β-hydroxybutyrate to the suspension of cells. (*b*) Hepatocytes from a T3-treated rat. The spectrum was taken under the same conditions as in *a*. βC_1, αC_1–βC_6, and αC_6, carbons of the glucose anomers; MC_2, malate C2; $LacC_2$, lactate C2; AACα, acetoacetate C2; β-HB Cα, D-β-hydroxybutyrate C2; AACγ, acetoacetate C4; β-HB Cγ, D-β-hydroxybutyrate C4. Peaks A–E are due to the buffer.

shows that the oxidation of ethanol abolished the enhanced enrichments of label, seen in Figs. 1*b* and 3*a*, at the scrambled alanine C2 position in the hyperthyroid state. These spectra show considerably more labeling at C1, C2, C5, and C6 than at C3 and C4 of glucose (see Table 2), in accordance with our previous measurements on perfused mouse liver (2).

As shown in Fig. 3*a* and generally observed in these spectra, C2 and C3 of glutamate were labeled with almost equal probability. The broadening of glutamate and glutamine C3 peaks in the presence of [2-^{13}C]ethanol (Fig. 3*b*) is probably due to a multiplet structure arising from spin–spin interaction between ^{13}C at C3 and C4 in the same molecule. When aspartate is seen in these spectra (e.g., Figs. 1*b*, 2 *b* and *c*, and 3), the labeling at C2 is consistently below that at C3. The observed ratios of the ^{13}C enrichment at C2 to that at C3 of aspartate and the enrichment at C3 to that at C2 of glutamate are summarized in Table 3. These ratios are presented together because, in the absence of scrambling, the flow of the original alanine C3 label would be

Table 1. ^{13}C enrichment in ketone bodies and ratio of enrichment at C4 to that at C2 of glutamate during metabolism of [3-^{13}C]alanine in hepatocytes from euthyroid fasted rats

Substrate	β-HB/AA*	β-HB(C2:C4)†	Glu(C4:C2)
[3-^{13}C]Ala + [2-^{13}C]EtOH	1.4 ± 0.3	1.02 ± 0.03	1.20 ± 0.06
[3-^{13}C]Ala + β-HB	‡	2.2 ± 0.09	0.65 ± 0.20
[3-^{13}C]Ala	0.33 ± 0.07	§	0.88 ± 0.12

Results are given as the means ± SD of the integrated intensities of the corresponding ^{13}C NMR peaks. Each result is the average of three to five experiments. All data are from spectra recorded under nonsaturating NMR conditions. EtOH, ethanol; β-HB, β-hydroxybutyrate. Concentrations used: alanine, 28 mM; D,L-β-hydroxybutyrate, 4–20 mM; ethanol, 20 mM.

* Ratio of ^{13}C enrichment at C2 of D-β-hydroxybutyrate to that at C2 of acetoacetate for ketone bodies synthesized *in situ*.

† Ratio of ^{13}C enrichment at C2 to that at C4 of D-β-hydroxybutyrate synthesized *in situ*. No NMR signal was visible from the unlabeled D,L-β-hydroxybutyrate administered under these NMR conditions.

‡ This ratio for ketone bodies synthesized *in situ* ranged from 1 to 2.5, increasing with the amount of exogenous β-hydroxybutyrate added.

§ Ratio was ≈1, but was unreliable due to low signal-to-noise ratio in these spectra.

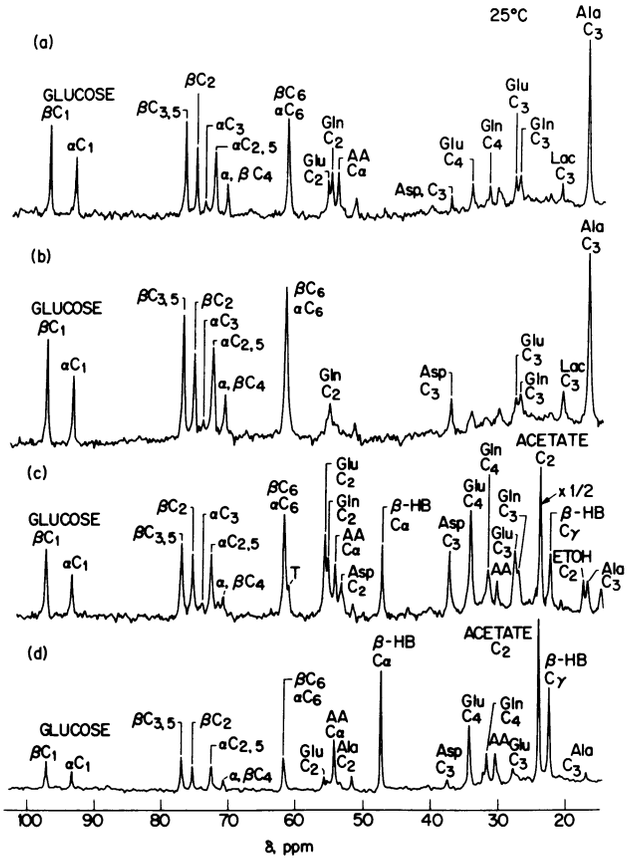

FIG. 2. ^{13}C NMR spectra at 25°C of hepatocytes from euthyroid rats. One spectrum from a sequence of spectra is shown for each of four different cell samples. Substrates are: (*a*) [3-^{13}C]alanine; (*b*) [3-^{13}C]alanine and unlabeled ethanol; (*c*) [3-^{13}C]alanine and [2-^{13}C]ethanol; and (*d*) unlabeled alanine and [2-^{13}C]ethanol. Each spectrum was taken 145–175 min after addition of substrate. Alanine was administered at 28 mM and ethanol at 20 mM. Abbreviations are given in the legend of Fig. 1 except that ETOH C_2 is ethanol CH_3, T is due to the buffer, and AA is acetoacetate C4.

into the methylene carbon of oxaloacetate and, thence, into C3 of aspartate via aspartate aminotransferase and into C2 of glutamate via the tricarboxylic acid cycle and action of glutamate dehydrogenase.

DISCUSSION

Pyruvate Kinase and Gluconeogenesis. As shown in Figs. 1 and 3 and Table 4, the ^{13}C NMR spectra provide simultaneous observations of the flux from *P-e*Prv through pyruvate kinase and the flux from *P-e*Prv to glucose in hepatocytes during the metabolism of alanine. The glucose data in Table 2 indicate that C2 and C3 of cytosolic oxaloacetate are both strongly labeled (2). Presumably this is because of fumarase exchange, which has almost completely scrambled the original alanine C3 label in cytosolic oxaloacetate. Consequently, labeled C2 of *P-e*Prv introduces a label into C2 of pyruvate through pyruvate kinase activity. Subsequently, through the activity of alanine aminotransferase, this pyruvate is converted to alanine. In the presence of a large trapping pool of unscrambled [3-^{13}C]alanine, the ^{13}C enrichment at alanine C2 is a direct measure of the rate of pyruvate kinase flux. Measurement of the corresponding glucose C5 intensity gives the flux from *P-e*Prv to glucose in the same units. In the absence of ethanol, the relative flux through pyruvate kinase is 2.6–4.5 times greater in the hyperthyroid state (see Table 4).

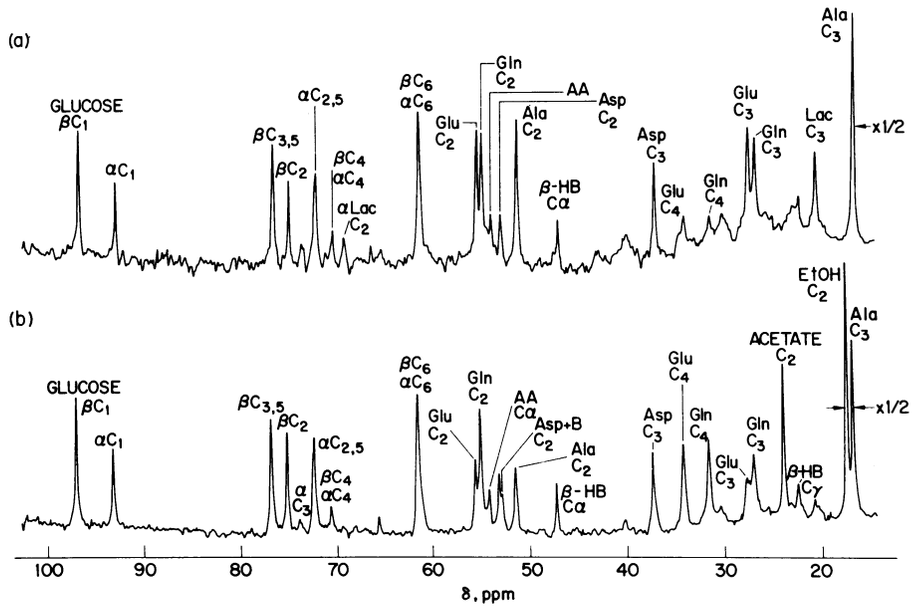

FIG. 3. ^{13}C NMR spectra at 25°C of hepatocytes from hyperthyroid rats. (*a*) Spectrum accumulated 85–115 min after addition of 28 mM [3-^{13}C]alanine and 10 mM D,L-β-hydroxybutyrate. (*b*) Spectrum of another suspension of hepatocytes from a hyperthyroid rat, treated exactly as the sample in *a* except that in *b*, the substrate was 28 mM [3-^{13}C]alanine and 20 mM [2-^{13}C]ethanol. Abbreviations are given in the legends of Figs. 1 and 2; B is due to the buffer. Note that the $AspC_2$+B arrow points to the buffer line; $AspC_2$ is the larger overlapping peak.

An enhanced rate of glucose production from alanine was observed qualitatively in these studies in hepatocytes from T3-treated rats, in accord with the quantitative results of Singh and Snyder (3), who reported an increased glucose production of 160% in perfused liver from fasted hyperthyroid rats. This increase in the rate of gluconeogenesis agrees with measurements of increased activities of hepatic pyruvate carboxylase, *P-e*Prv carboxykinase, and glucose-6-phosphatase (4, 5) in the hyperthyroid rat and means that the increased flux ratios given in Table 4 indicate *a fortiori* absolute increases in flux through pyruvate kinase. Because the malic enzyme interconverts malate and pyruvate, the possibility that enhancement of the activity of this enzyme in the hyperthyroid state (6) contributed to the flux of label into C2 of pyruvate was examined by use of the malic enzyme inhibitor, 2,4-dihydroxybutyrate (7). The data in Table 4 show that the presence of this inhibitor did not alter the pyruvate kinase flux ratio, indicating negligible contributions of the malic enzyme.

Table 2. ^{13}C distribution in carbons of glucose formed from labeled alanine in hepatocytes from control or T3-treated fasted rats compared with ^{13}C distribution in glutamate in the same sample

T3-treated	Substrate	Glucose C1	C2	C3	C4	C5	C6	Glutamate C3:C2
−	[3-^{13}C]Ala	89	64	23	22	83	100	1.02
+	[3-^{13}C]Ala + [2-^{13}C]EtOH	95	78	18	18	83	100	0.82
+	[3-^{13}C]Ala + [2-^{13}C]EtOH	95	77	19	17	80	100	0.81
−	[3-^{13}C]Ala + [2-^{13}C]EtOH	86	72	16	17	77	100	0.99
−	[3-^{13}C]Ala + [2-^{13}C]EtOH	81	85	27	27	86	100	0.98
−	[3-^{13}C]Ala + unlabeled EtOH	90	72	22	18	75	100	0.93
−	[3-^{13}C]Ala + unlabeled EtOH	76	63	22	22	77	100	0.82
+	[3-^{13}C]Ala + β-HB	93	73	24	25	82	100	0.74
−	[3-^{13}C]Ala + β-HB	78	78	18	18	80	100	1.05
−	[3-^{13}C]Ala + β-HB	79	69	20	18	75	100	0.89

The ^{13}C distributions are from the integrated intensities of the NMR peaks in spectra recorded under nonsaturating conditions. The estimated error for the relative specific activities in glucose is ±2 units. The estimated error in the glutamate C3:C2 ratio is 7%. Substrate concentrations and abbreviations are given in Table 1.

In general, the flux through pyruvate kinase is greatly decreased in hepatocytes from fasted, as compared with fed, rats (8), presumably because of elevated glucagon levels (9) and depressed insulin levels (10, 11) in the fasted state. By using ^{14}C tracer techniques, Rognstad (12) determined that the rate of pyruvate kinase flux was nearly 50% of the rate of gluconeogenesis from lactate in hepatocytes from T3-treated, fasted rats, which is similar to the ratio measured under the same conditions in hepatocytes from fed euthyroid rats (8). Alanine, the substrate in the present studies, has been reported to be a strong allosteric inhibitor of pyruvate kinase (13). Nevertheless, as shown in Table 4, the rate of pyruvate kinase flux is 60% of the rate of gluconeogenesis in hepatocytes from T3-treated, fasted rats in the presence of high concentrations of alanine, providing ethanol is absent. This indicates that alanine has a negligible effect upon pyruvate kinase under our conditions.

The mechanism by which thyroid hormones stimulate pyruvate kinase is not known. By using a specific radioimmunoassay to measure intracellular concentrations of rabbit liver pyruvate kinase, Johnson and Veneziale (14) reported that, in general, hormonal control of the enzyme is through regulation of its catalytic state; however, their preliminary data for thyroxine treatment left open the possibility of induction of new, more active enzyme. Regulation by endogenous insulin activity is unlikely, even though insulin levels are elevated in mildly hyperthyroid rats (10, 11) and insulin has been shown to increase pyruvate kinase activity *in vivo* (15) and to antagonize the inhibition of pyruvate kinase by glucagon in isolated hepatocytes (16). However, studies by Okajima and Ui (11) with streptozotocin-diabetic rats and rats treated with antiserum against insulin show that the increased glucose turnover observed in hyperthyroid rats is largely independent of endogenous insulin.

The present experiments show that incubation of hepatocytes from hyperthyroid rats with ethanol reduces the pyruvate kinase flux from 63% to 25% of the rate of gluconeogenesis (see Table 4). The oxidation of ethanol is inhibitory to glycolysis in liver (17), presumably because of the effect of the greatly in-

Table 3. Ratio of ^{13}C enrichment at C2 to that at C3 of aspartate and ratio of enrichment at C3 to that at C2 of glutamate (or glutamine) during gluconeogenesis from alanine in hepatocytes from control or T3-treated fasted rats

Substrate	Euthyroid		Hyperthyroid	
	Asp C2:C3	Glu C3:C2	Asp C2:C3	Glu C3:C2
[3-^{13}C]Ala + β-HB	0.47 ± 0.14	0.98 ± 0.08	0.47 ± 0.04	0.80 ± 0.10
[3-^{13}C]Ala + [2-^{13}C]EtOH	0.50 ± 0.04	0.98 ± 0.08	0.57 ± 0.05	0.81 ± 0.05
[3-^{13}C]Ala + unlabeled EtOH	0.46 ± 0.14	0.90 ± 0.08		
[3-^{13}C]Ala	0.48 ± 0.10	0.90 ± 0.13		

Results are given as the means ± SD for the ratios of the integrated intensities of the ^{13}C NMR peaks corresponding to the indicated carbon atoms. Each result is the average of four or five experiments; usually two or three spectra from the time course of each experiment were used in the calculation. In a few spectra, the signal-to-noise ratios for glutamine C3 and C2 were more favorable than those for glutamate; in these cases Gln C3:C2 was used. All data are from spectra recorded under nonsaturating NMR conditions. Substrate concentrations and abbreviations are given in Table 1.

creased cytosolic NADH/NAD^+ ratio upon the glyceraldehyde-3-*P* dehydrogenase reaction. Earlier studies (18) of perfused rat liver showed that addition of ethanol during the metabolism of alanine increased the gluconeogenic flux with a forward crossover between 3-phosphoglycerate and glyceraldehyde-3-*P* and decreased *P-e*Prv levels by a half to a third. Because preincubation of hepatocytes with ethanol does not alter the activity of the pyruvate kinase subsequently extracted from the cells (19), it appears probable that the effect of the oxidation of ethanol upon the pyruvate kinase flux measured *in situ* is mediated by redox control of glyceraldehyde-3-*P* dehydrogenase. A possible mechanism for this control is that *P-e*Prv levels, which are reported to be 2-fold higher in liver from hyperthyroid rats during the metabolism of alanine (3), may be lowered by the increased gluconeogenic rate, resulting in the lower pyruvate kinase flux shown in Table 4. The 2-fold increase in the pyruvate kinase flux ratio noted in Table 4 for hepatocytes from control rats incubated with alanine and ethanol, as compared with alanine alone, shows ethanol operating in the opposite direction. Possibly this increase in pyruvate kinase flux can be attributed to a higher level of fructose 1,6-P_2 (18), an allosteric activator of pyruvate kinase. Both postulated changes due to the oxidation of ethanol (i.e., decreased *P-e*Prv levels in the hyperthyroid state and increased fructose 1,6-P_2 levels) could be tested experimentally.

Acetyl-CoA and Ketone Bodies. In agreement with previous studies on perfused mouse liver (2), Fig. 2 shows that during the metabolism of alanine and ethanol, acetyl-CoA is largely derived from ethanol, whereas alanine enters the tricarboxylic acid cycle almost exclusively via pyruvate carboxylation. Fig. 2 and the data in Table 1 show that, in the presence of labeled ethanol, those carbons directly traceable to C2 of acetyl-CoA (e.g., C4 of glutamate and glutamine) are labeled and that the ketone bodies are equally labeled at C2 and C4 as expected from the condensation of [2-^{13}C]acetyl-CoA via the usual route (20).

This pathway predicts a symmetrically labeled 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). However, the data in Table 1 show that the presence of unlabeled β-hydroxybutyrate during the metabolism of [3-^{13}C]alanine puts more ^{13}C at C2 than at C4 in β-hydroxybutyrate. Comparison of Fig. 1*a* with Fig. 2*a* shows greater total ^{13}C enrichment in the ketone bodies in the presence of exogenous ketone body. This observation suggests an increased turnover of ketone bodies, rather than net synthesis, in the presence of a large unlabeled pool and that, under these conditions, there is a direct pathway from acetoacetate into acetoacetyl-CoA. This route may be via succinyl-CoA:acetoacetate CoA transferase, although its activity is considered to be low in liver (20).

The nonequivalence of ^{13}C labeling at C2 and C4 of β-hydroxybutyrate produced *in situ* under these conditions is in accord with the mechanism that Miziorko and Lane (21) demonstrated for avian liver HMG-CoA synthase. In this scheme (21), unlabeled acetoacetyl-CoA, derived from added β-hydroxybutyrate, and [2-^{13}C]acetyl-CoA, derived from [3-^{13}C]alanine, go through an intermediate, enzyme-S-[^{13}C]HMG-SCoA, which is hydrolyzed to release the [^{13}C]HMG-CoA, HO_2C-$^{13}CH_2$-$C(OH)(CH_3)$-CH_2-CO-SCoA. In the HMG-CoA lyase reaction, this [^{13}C]HMG-CoA then gives [2-^{13}C]acetoacetate and unlabeled acetyl-CoA. Of course, [2,4-^{13}C]acetoacetate is also produced from the relatively abundant [2-^{13}C]acetyl-CoA. The 2:1 ratio measured for ^{13}C enrichment at C2 compared with that

Table 4. Flux through pyruvate kinase as a fraction of the flux of *P-e*Prv to glucose during gluconeogenesis from alanine in hepatocytes from control or T3-treated fasted rats

Substrate	Euthyroid		Hyperthyroid	
	No. of exps.	Pyruvate kinase flux/ gluconeogenic flux	No. of exps.	Pyruvate kinase flux/ gluconeogenic flux
[3-^{13}C]Ala + D-β-HB	5	0.24 ± 0.02	5	0.63 ± 0.09
[3-^{13}C]Ala + [2-^{13}C]EtOH	6	0.22 ± 0.04	3	0.25 ± 0.03
Ala (unlabeled) + [2-^{13}C]EtOH	3	0.24 ± 0.04	1	0.22
[3-^{13}C]Ala	3	0.11 ± 0.01	3	0.50 ± 0.11
[3-^{13}C]Ala + 2,4-dihydroxybutyrate			1	0.59

Results are expressed as the means ± SD for the ratios of the integrated intensity of the ^{13}C NMR line of the scrambled C2 position in alanine to twice the intensity of the ^{13}C label at the corresponding C5 position of glucose. In general, two or three spectra from the time course of a given experiment were used in the calculations. Number of experiments refers to the number of different hepatocyte preparations examined. All data are from spectra recorded under nonsaturating NMR conditions while a large trapping pool of unscrambled [3-^{13}C]alanine was maintained; no correction was applied for incomplete trapping. Substrate concentrations and abbreviations are given in Table 1; 2,4-dihydroxybutyrate, 12 mM.

at C4 in β-hydroxybutyrate suggests that about one-third of the β-hydroxybutyrate synthesized *in situ* under these conditions came from reduction of [2-^{13}C]acetoacetate. Production of unlabeled acetyl-CoA in the lyase step, and possibly also by thiolase, would be expected to decrease the C4:C2 ^{13}C enrichment ratio in glutamate slightly. The C4:C2 ratio observed in glutamate, given in Table 1, is 75% of the value obtained during the metabolism of [3-^{13}C]alanine in the absence of exogenous β-hydroxybutyrate.

Aspartate and Glutamate. The data in Table 3 show that, under all conditions investigated, the ratio of the ^{13}C enrichment at C2 to that at C3 in aspartate is about 1:2, whereas the ^{13}C enrichments at the corresponding carbons in glutamate approach a ratio of 1:1, indicating almost complete randomization at C3 and C2 of glutamate. Both aspartate C3 and glutamate C2 are traceable directly to the methylene carbon of oxaloacetate, whereas aspartate C2 and glutamate C3 are derived from the carbonyl carbon of oxaloacetate. It is customary to assume that (*i*) the ^{13}C distribution in glutamate, which must be derived from labeled α-ketoglutarate formed via the tricarboxylic acid cycle, reflects that of intramitochondrial oxaloacetate and (*ii*) the ^{13}C distribution in glucose reflects that of cytosolic oxaloacetate, in which the ^{13}C label may be more randomized due to additional cytosolic fumarase exchange. The results in Table 3 show that the aspartate C2:C3 and glutamate C3:C2 ^{13}C distributions were invariant under all conditions investigated. The observation that these ratios are independent of reducing equivalents in the cytosol suggests that all aspartate was synthesized in the mitochondria. This suggestion is in agreement with the results of Meijer *et al.* (22) showing that aspartate required for ureogenesis in isolated hepatocytes is translocated from the mitochondria. The observation in Table 3 of less scrambling of label in aspartate than in glutamate implies that if, under these conditions, aspartate is synthesized only by the intramitochondrial aspartate aminotransferase, then the *in situ* fluxes of intramitochondrial oxaloacetate are such that in a significant fraction of the oxaloacetate accessible to the aminotransferase, the ^{13}C label has not been randomized. The mechanism responsible may involve a favorable intramitochondrial location of aspartate aminotransferase with respect to pyruvate carboxylase, in combination with favorable *in situ* kinetics. On the basis of extensive ^{14}C tracer studies, LaNoue and coworkers have concluded that in kidney mitochondria two functional glutamate pools exist (23) and that in liver mitochondria there is a steep gradient of aspartate within the matrix space (24). Another possible explanation of the lesser scrambling in aspartate would be some more direct cytosolic path from alanine to aspartate. However, two obvious routes seem unlikely because, first, pyruvate carboxylase is thought to be strictly an intramitochondrial enzyme in rat liver (25) and, second, the equivalence of the aspartate C2:C3 ratio in the normal and hyperthyroid states suggests that the malic enzyme contribution is negligible.

Glucose Labels. The data in Table 2 show that the randomization of label observed at C5:C6 of glucose is either about the same as (hepatocytes from T3-treated rats) or less than (controls) that measured at the corresponding carbons of glutamate. In the pathway of gluconeogenesis from alanine, the transport of malate from the mitochondria provides the carbon for glucose production from alanine (26, 27). The observation that, in hepatocytes from hyperthyroid rats, the scrambling in glucose is the same as that seen in glutamate is in accord with the usual pathway and also appears to be in accord with the rapid exchange of malate between cytosol and mitochondria measured by Rognstad in kidney cortex (28). However, as shown in Table 2, in control cells the scrambling in glucose is less than the scrambling in glutamate. A possible explanation of this is that part of the glucose flux is contributed directly by the less scrambled aspartate (see Table 3) via cytosolic aspartate aminotransferase activity, providing the flux through *P-e*Prv carboxykinase is greater than the composite fumarase exchange rate.

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