Effects of Insulin on Perfused Liver from Streptozotocin-Diabetic and Untreated Rats: ^{13}C NMR Assay of Pyruvate Kinase Flux

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ABSTRACT: The effects of insulin in vitro on perfused liver from streptozotocin-diabetic rats and their untreated littermates during gluconeogenesis from either [3-^{13}C]alanine + ethanol or [2-^{13}C]pyruvate + NH_4Cl + ethanol were studied by ^{13}C NMR. A ^{13}C NMR determination of the rate of pyruvate kinase flux under steady-state conditions of active gluconeogenesis was developed; this assay includes a check on the reuse of recycled pyruvate. The preparations studied provided gradations of pyruvate kinase flux within the confines of the assay's requirement of active gluconeogenesis. By this determination, the rate of pyruvate kinase flux was 0.74 ± 0.04 of the gluconeogenic rate in liver from 24-h-fasted controls; in liver from 12-h-fasted controls, relative pyruvate kinase flux increased to 1.0 ± 0.2. In diabetic liver, this flux was undetectable by our NMR method. Insulin's hepatic influence in vitro was greatest in the streptozotocin model of type 1 diabetes: upon treatment of diabetic liver with 7 nM insulin in vitro, a partial reversal of many of the differences noted between diabetic and control liver was demonstrated by ^{13}C NMR. A major effect of insulin in vitro upon diabetic liver was the induction of a large increase in the rate of pyruvate kinase flux, bringing relative and absolute fluxes up to the levels measured in 24-h-fasted controls. By way of comparison, the effects of ischemia on diabetic liver were studied by ^{13}C NMR to test whether changes in allosteric effectors under these conditions could also increase pyruvate kinase flux. A large increase in this activity was demonstrated in ischemic diabetic liver.

It is generally accepted that three futile cycles operate in hepatic glucose metabolism (Katz & Rognstad, 1976). Cycling that causes ATP hydrolysis without a corresponding change in reactants occurs at irreversible stages where there are two opposing pathways, in which the forward and reverse directions are catalyzed by separate enzymes. In the phosphoenolpyruvate (PEP) cycle (pyruvate → PEP → pyruvate) in rat liver, the path in the gluconeogenic direction from pyruvate to PEP is catalyzed by a complex sequence of enzymes, whereas in the glycolytic direction a single enzyme, pyruvate kinase, catalyzes the reaction PEP → pyruvate (Figure 1). Pyruvate kinase has been shown to be a target site for hormonal control of gluconeogenesis (Kraus-Friedmann, 1984). Evidence is now abundant indicating that although pyruvate kinase activity is greater in the fed state, there is considerable flux through the enzyme under conditions of active gluconeogenesis in the fasted state (Freidmann et al., 1971; Katz & Rognstad, 1976; Rognstad & Katz, 1977). In this study we develop a ^{13}C NMR assay of the activity of the PEP cycle in perfused liver that is carrying out gluconeogenesis. This assay is used to test the effect of administration of a physiological level of insulin in vitro upon the activity of pyruvate kinase in liver from fasted control rats and from the streptozotocin-treated rat model of insulin-dependent diabetes.

In this ^{13}C NMR determination, livers are perfused under steady-state conditions with a ^{13}C-labeled gluconeogenic substrate ([3-^{13}C]alanine is shown in Figure 1) that enters the pathway as specifically labeled pyruvate. As Figure 1 shows schematically, label that was randomized in the tricarboxylic acid (TCA) cycle becomes incorporated into PEP prior to its appearance in either glucose via the usual route or pyruvate via the action of pyruvate kinase. As shown, pyruvate bearing the randomized label of its PEP precursor is interconverted to alanine under our conditions. This randomized label will be effectively trapped as alanine if a sufficiently large pool of alanine with the original label ([3-^{13}C]alanine in this example) is present. The flux of PEP through pyruvate kinase as a fraction of the flux of PEP → glucose is estimated from an expression relating these quantities to the relative ^{13}C enrichments at the randomized carbons in alanine as compared with the relative enrichments at the corresponding carbons in glucose. Because reuse of pyruvate with randomized label in the liver cell would cause an underestimation of pyruvate kinase flux, a monitor of the extent of this recycling in perfused liver was developed. This monitor uses the ^{13}C enrichment at glutamate C-4 and C-5 to estimate the flux from recycled pyruvate into the mitochondrial acetyl-CoA pool.

Materials and Methods

All experimental conditions were as given previously (Cohen, 1987a), including the preparation of the animals, NMR conditions, and method of liver perfusion. As before, for each perfused liver the data collected included ^{31}P NMR spectra of the perfused liver before and after the measurement of ^{13}C NMR data, ^{13}C NMR spectra of the perfused liver before and after the addition of labeled substrate, the ^{13}C NMR spectrum of the perfusion fluid after the termination of the perfusion, and ^{13}C NMR spectra of the perchloric acid extract prepared from the freeze-clamped liver. Because in all cases the pulse repetition rate was less than $5T_1$, intensity corrections for T_1 effects were required for all ^{13}C NMR spectra. It is emphasized that all spectral intensities used in calculations here were corrected for nuclear Overhauser effect (NOE) and T_1, or saturation, effects, exactly as described previously for our perfused liver, perfusate, and extract NMR conditions. Thus, three sets of intensity correction factors were determined for the various carbons in various metabolites for the corresponding three sets of NMR conditions. For each perfused liver, the rate of glucose synthesis was measured as described in detail before. As given previously, estimates of absolute

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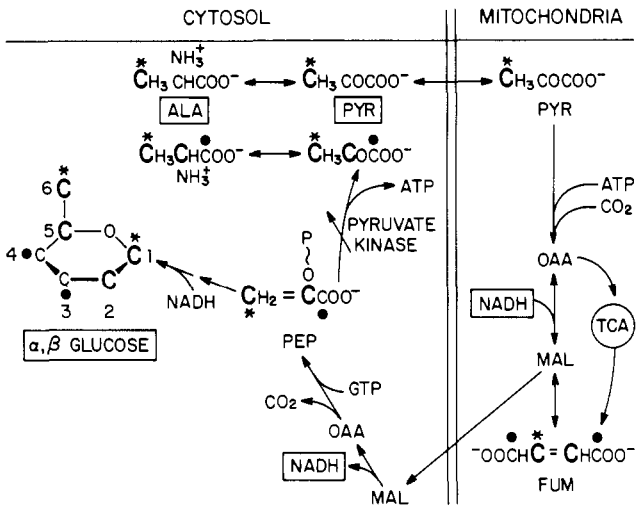

FIGURE 1: Simplified model gluconeogenic pathway. The original label at C-3 of alanine (boldface C with asterisk) is followed into the TCA cycle where randomization by malate dehydrogenase and fumarase exchange occurs; thus, in fumarate (FUM) the label is found with equal probability at *either* of the two middle carbons (boldface C's). Further randomization in TCA cycle introduces a small amount of label into the terminal carbons of FUM (● over C). MAL is malate and OAA is oxalacetate.

concentrations of metabolites present in perfused liver were based on the concentration of glucose measured in perfusate aliquots taken every 40 min, the percentage ^{13}C enrichment of the given reference carbon in glucose, and the relative NOE values (Cohen, 1987a).

Results

Effects of Insulin in Vitro upon Perfused Liver from Normal Control Rats and Streptozotocin-Diabetic Rats As Studied by ^{13}C NMR Spectroscopy. This study emphasizes the acute effects of insulin and the use of ^{13}C NMR to estimate pyruvate kinase flux. The four ^{13}C NMR spectra shown in Figures 2 and 3 compare metabolism in liver from 24-h-fasted normal control rats perfused in the absence (Figure 2) and presence (Figure 3) of 7 nM insulin, at the same time intervals postsubstrate. Figures 2a and 3a contrast spectra recorded 22–33 min after the administration of 9.1 mM [2-^{13}C]pyruvate, 5.5 mM NH_4Cl, and 7.3 mM unlabeled ethanol. Both Figures 2a and 3a show labeled alanine, lactate, glutamate, glutamine, and aspartate. Under our conditions, two reproducible differences were observed in the first 0.5 h postsubstrate in liver incubated with insulin in vitro: significantly higher levels of PEP and 3-phosphoglycerate, both labeled at C-2 and C-3, were seen in the presence of insulin (Figure 3a). Regardless of the level of precursor aspartate, intense resonances arising from C-2 and C-3 of *N*-carbamoylaspartate are clearly resolved in both Figures 2b and 3b. Insulin did not have a significant effect on the rate of gluconeogenesis (Cohen, 1987a).

To approximate steady-state conditions (Cohen, 1983, 1987a), substrate was maintained close to the initial level throughout the perfusion period by addition of small increments of labeled pyruvate + NH_4Cl + ethanol to the reservoir at frequent intervals, typically every 15 min (Figures 2 and 3). ^{13}C enrichment at alanine C-2, which can be traced back directly to the [2-^{13}C]pyruvate substrate, was generally maintained at approximately 5.5 mM under these conditions. The ratio of ^{13}C intensity at alanine C-3, a randomized carbon, to that at C-2 reached a NOE-corrected, steady-state value of approximately 0.16 about 40 min after the initial administration of substrate. As Figures 2b and 3b indicate, ^{13}C enrichment at alanine C-3 relative to that at C-2 was not significantly affected by the addition of insulin with the substrate. A control liver that was preincubated with insulin for 35 min before the administration of substrate did, however, show a 38% increase in the relative ^{13}C enrichment at the randomized alanine carbon.

The spectra in Figure 4 compare metabolism in livers from diabetic rats; these livers were perfused with 10 mM [3-

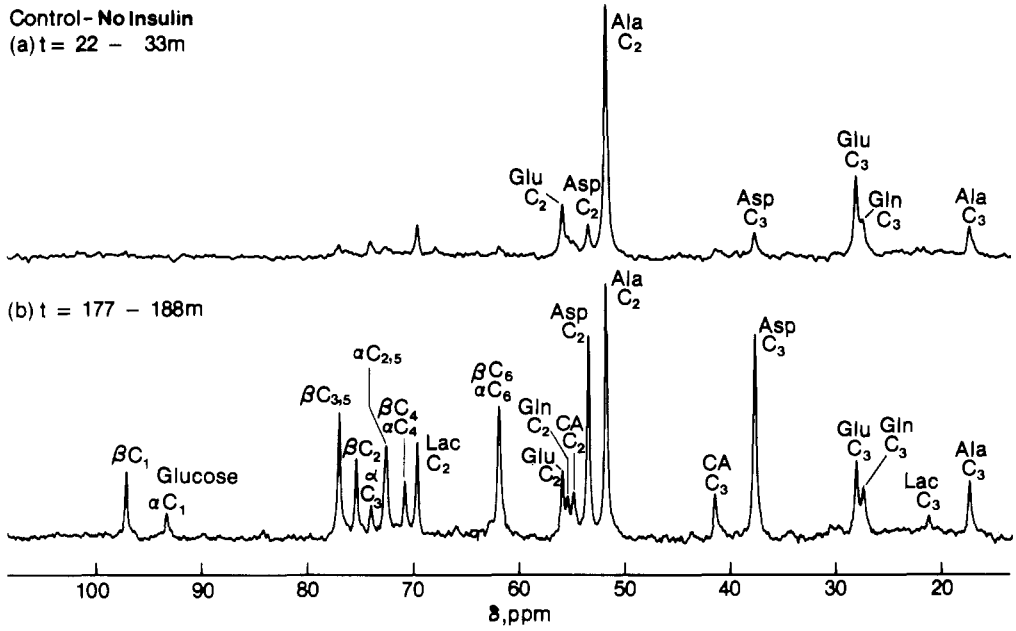

FIGURE 2: ^{13}C NMR spectra of isolated perfused liver from a 24-h-fasted normal control rat at 35 ± 1 °C. (a) Spectrum measured during the period 22–33 min after the addition of 9.1 mM [2-^{13}C]pyruvate, 5.5 mM NH_4Cl, and 7.3 mM unlabeled ethanol. Substrate was maintained at or near these levels throughout the perfusion. This spectrum represents 800 scans acquired as described in the text. A spectrum of the ^{13}C natural abundance background of this liver was accumulated under identical conditions before substrate was added. For clarity of presentation, this background spectrum was subtracted from the spectrum shown. Spectrum b was measured 177–188-min postsubstrate. The labeled ^{13}C NMR peaks include those due to the α and β anomers of D-glucose: βC-1, αC-1 to βC-6, αC-6. Other abbreviations include the following: Lac C_2, lactate C-2; Glu C_2, glutamate C-2; Gln C_2, glutamine C-2; Asp C_2, aspartate C-2; Ala C_2, alanine C-2; CA C_2, C-2 of *N*-carbamoylaspartate.

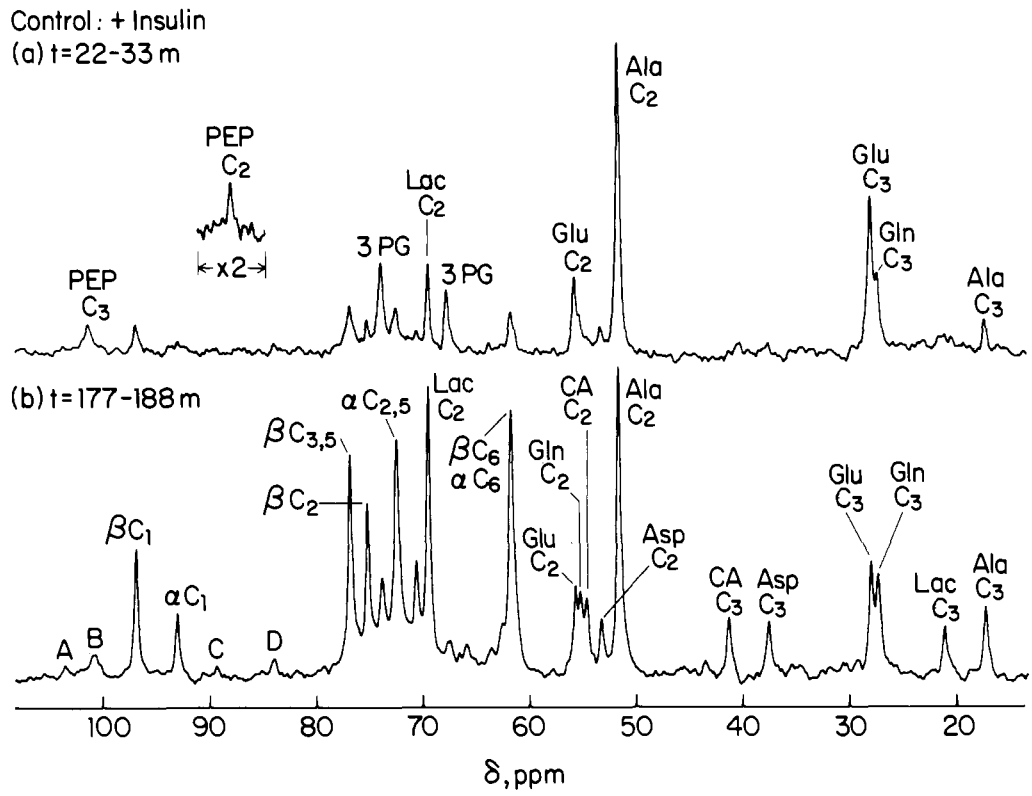

FIGURE 3: ^{13}C NMR spectra of perfused liver from a 24-h-fasted normal rat. Insulin was maintained in the perfusate at the 7 nM level. Spectrum a was measured 22–33 min after the addition of 9.1 mM [2-^{13}C]pyruvate, 5.5 mM NH_4Cl, and 7.3 mM unlabeled ethanol. Spectrum b was measured 177–188-min postsubstrate. Other conditions are as given in Figure 2. Abbreviations are as given in Figure 2, with the addition of PEP C_3 = phoshoenolpyruvate C-3. The insert shows the signal of PEP C-2 at 150.70 ppm; the notation ×2 indicates that the gain on the vertical axis was increased 2-fold when this region of the spectrum was read out. 3PG is 3-phospho-D-glycerate. The peaks labeled A, C, and D are assigned to base C-5 and ribose moiety C-1 and C-4, respectively, of UTP, UMP, and uridine. Peak B is in the region of glycogen C-1 and CH_2 of PEP.

^{13}C]alanine and 7.3 mM ^{13}C-labeled ethanol in the absence (Figure 4a) or presence (Figure 4b) of 7 nM insulin. As before, steady-state conditions were approximated by maintaining substrate close to the initial level by the frequent addition of small increments of both labeled compounds. Spectra a and b of Figure 4 contrast spectra measured at the same time interval (170–180 min) after the initial administration of substrates and after addition of essentially the same total quantity of gluconeogenic substrate, 2.48 and 2.43 mmol of [3-^{13}C]alanine, respectively. As shown in Figure 4a, in the absence of insulin *no* ^{13}C enrichment was detectable at the randomized carbon of alanine (C-2 for [3-^{13}C]alanine as substrate) in diabetic liver. When diabetic liver was perfused in the presence of insulin, ^{13}C enrichment was measured at the randomized alanine C-2 position. Preincubation of diabetic liver with insulin in vitro for 35 min before the addition of substrate (Figure 4b) brought the relative ^{13}C enrichment at the randomized alanine carbon into the range observed in liver from 24-h-fasted control rats (Figures 2 and 3; Cohen, 1987a). As observed previously, rapid consumption of substrate by diabetic liver made it difficult to keep substrate levels constant throughout the measurement of all spectra. Although the alanine C-3 level decreased during the accumulation of the spectrum shown in Figure 4a, which was selected to cover the same time interval as Figure 4b, labeled substrate levels were monitored continually, and such excursions were brief. We emphasize that in such cases relative ^{13}C enrichment at the randomized alanine carbon was measured in other spectra, from the same time sequential series, in which the level of the original alanine label was at its high, steady-state value.

While the most important in vitro effect of insulin on diabetic liver observed here was the promotion of ^{13}C label at the randomized alanine carbon, other effects are in evidence in Figure 4. In particular, aspartate and *N*-carbamoylaspartate rose to detectable levels in the presence of insulin. Surprisingly, β-hydroxybutyrate is observed in the presence (Figure 4b) but not the absence (Figure 4a) of insulin here. Variations noted previously (Cohen, 1987a) for β-hydroxybutyrate production in this model of experimental diabetes in the absence of insulin suggest that this may not be a significant difference.

Ischemia is known to enhance the rate of glycolysis in many preparations. Therefore, the effects of a 35-min period of low-flow ischemia (details given in Figure 5) were studied in perfused liver from diabetic rats (n = 4) to test whether under these conditions the rate of flux through pyruvate kinase would be increased to the NMR observable range. This protocol for inducing partial ischemia caused a decrease of 57% in the rate of gluconeogenesis typically measured in diabetic liver under our conditions, that is, from 51.7 ± 7.7 μmol (g of liver wet weight)$^{-1}$ h^{-1} (normoxic diabetic liver, n = 5) to 22.1 ± 3.3 μmol (g of liver wet weight)$^{-1}$ h^{-1} (ischemic diabetic liver ± insulin, n = 4). Figure 5 shows the ^{13}C NMR spectrum of the perchloric acid extract of a diabetic liver that was freeze-clamped after perfusion under our conditions of partial ischemia; substrate was [3-^{13}C]alanine and [2-^{13}C]ethanol. Figure 5 faithfully echoes the last ^{13}C NMR spectrum of the liver before freeze-clamping and, in addition, reveals useful information that was obscured by the broader peaks measured in spectra of liver. Figure 5 shows several pronounced changes in metabolism in diabetic liver subjected to our ischemic conditions as compared with normoxic diabetic liver perfused in the absence of insulin. First, there is appreciable labeling of the randomized alanine C-2; this labeling was not detectable in extracts of normoxic diabetic liver perfused in the absence

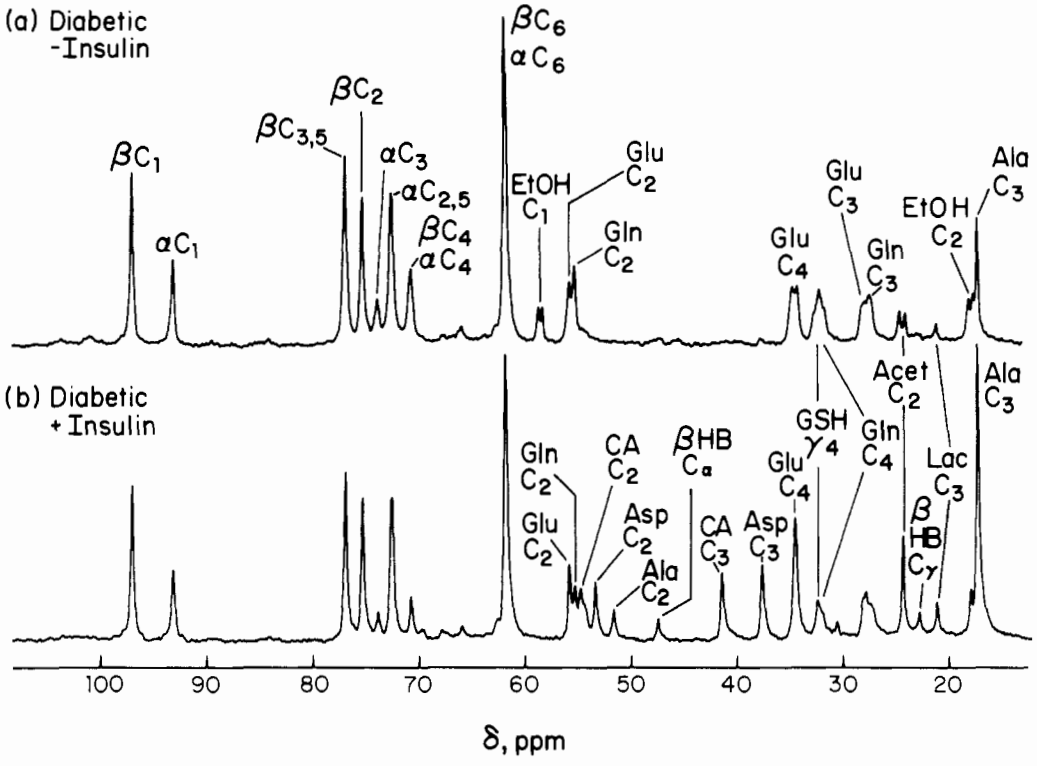

FIGURE 4: ^{13}C NMR spectra of livers from diabetic rats; livers were perfused in the absence (a) or presence (b) of insulin. Spectrum b is part of a time sequence of spectra and was accumulated during the period 170–180 min after the initial addition of 10 mM [3-^{13}C]alanine and 7.3 mM [2-^{13}C]ethanol. Insulin had been present at 7 nM during a 35-min period before substrate was added; insulin was maintained at 7 nM throughout the perfusion. Spectrum a is taken from a similar series of spectra taken of another liver from a diabetic rat and was measured 170–180 min after the initial addition of 10 mM [3-^{13}C]alanine and 7.3 mM [1,2-^{13}C]ethanol. This liver was treated exactly the same as the liver of spectrum b, except that insulin was absent from the perfusion fluid. Other conditions are as given for Figure 2. Abbreviations are given in Figure 2, with the following additions: EtOH C_2, ethanol C-2; βHB β-hydroxybutyrate C-2; GSH γ4, glutathione γ-glutamyl C-4; Acet C_2, acetate C-2.

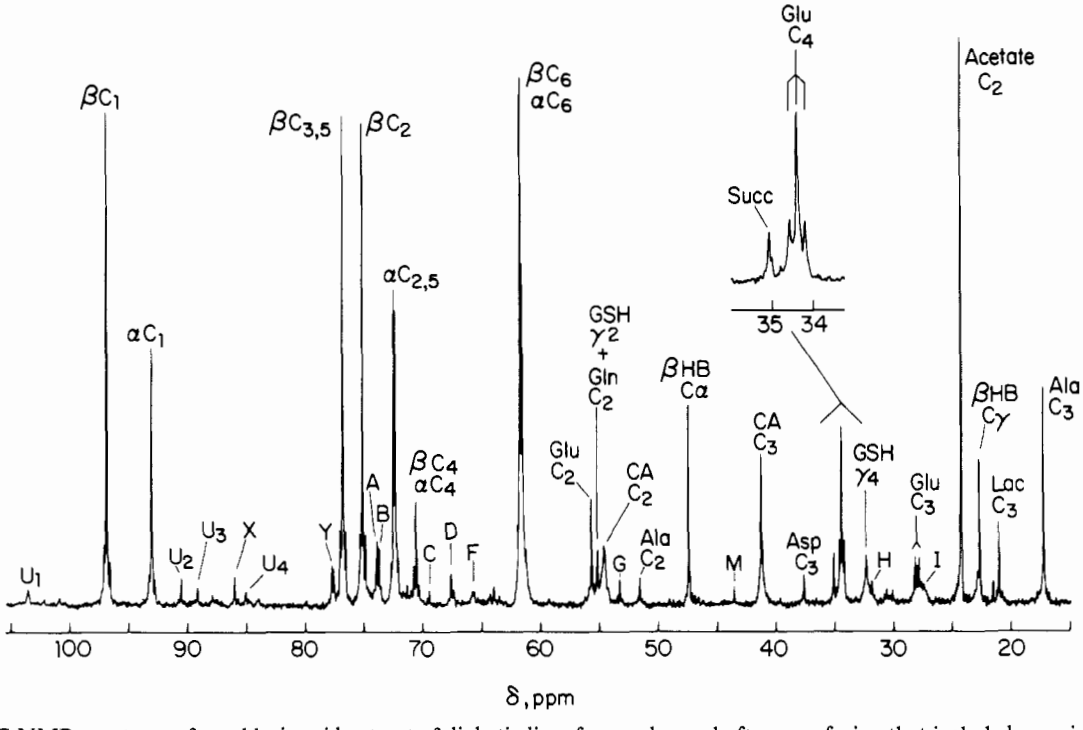

FIGURE 5: ^{13}C NMR spectrum of perchloric acid extract of diabetic liver freeze-clamped after a perfusion that included an episode of partial ischemia. The diabetic liver was perfused in the presence of 10 mM [3-^{13}C]alanine and 7.3 mM [2-^{13}C]ethanol under our standard conditions, with the exception that, beginning 40-min postsubstrate, there was a 35-min period of partial ischemia, during which flow was reduced to $^1/_{10}$ the normal rate, after which normal flow was restored. At the end of the perfusion period, which in this case was the standard period plus 35 min, the liver was freeze-clamped at liquid nitrogen temperature. The spectrum is the Fourier transform of the accumulation of 14 400 pulses of 30° free-induction decays, with 2.3-s recovery time between pulses. The pH of the extract was 7.94, and the NMR sample temperature was 12 °C. The abbreviations are as given in Figures 2–4, with the following additions: Succ, succinate C-2 and C-3; U_1, U_2, U_3, and U_4, due to UTP, UMP, and uridine; X and Y, unknown; A and D, 3-phospho-D-glycerate C-2 and C-3, respectively; B, glucose α-C-3; C, lactate C-2; F, L-glycerol 3-phosphate C-3; G, aspartate C-2; H, glutamine C-4; I, glutamine C-3 + glutathione γ-glutamyl C-3.

Table I: ^{13}C NMR Measurement of Isotopic Distribution in Carbons of Glucose Formed from ^{13}C-Labeled Substrates by Isolated Perfused Liver from Streptozotocin-Diabetic Rats and from Fasted, Untreated Control Rats[a]

donor rat	substrate[b]	relative ^{13}C enrichment of glucose						(C-1 + C-2 + C-3)/ (C-4 + C-5 + C-6)
		C-1	C-2	C-3	C-4	C-5	C-6	
diabetic								
A	Ala	95	84	21	20	85	100	0.98
B	Pyr	93	98	18	20	100	96	0.97
C	Pyr	92	100	19	17	100	95	1.00
control								
A	Ala	93	78	19	20	78	100	0.96
B	Pyr	85	91	19	19	100	92	0.92
C	Pyr	87	90	17	14	100	90	0.95

[a] ^{13}C distributions are from the integrated intensities of the glucose lines in the NMR spectra of the perfusates recorded under nonsaturating conditions. The estimated error is ±2 units. Control rats were fasted 24 h. [b] Ala denotes 10 mM [3-^{13}C]alanine + 7.3 mM unlabeled ethanol. Pyr denotes 9.1 mM [2-^{13}C]pyruvate + 5.5 mM NH_4Cl + 7.3 mM unlabeled ethanol.

of insulin. Second, a pronounced increase in succinate is seen. In normoxic liver from either diabetic or control rats and in the corresponding extracts, either no ^{13}C-labeled succinate is detected, or a succinate signal that rises only slightly above the noise level is seen. [A longer period of ischemia caused an even greater increase in the level of succinate (not shown).] Increase in succinate is consistent with inhibition at level of succinate dehydrogenase. Third, aspartate and *N*-carbamoylaspartate rose to observable levels. Note the characteristic broad line width of the *N*-carbamoylaspartate C-2 resonance (Cohen, 1987a). With the exception of elevated succinate levels, these changes parallel the effects of insulin in vitro upon diabetic liver (Figure 4).

Other notable changes observable in NMR spectra of postischemic perfused liver (not shown) under these conditions include a brief, transient increase of about 30% in ^{13}C label at lactate C-3 and, during the period 30–90-min postischemia, a transient 2-fold increase in the ^{13}C enrichment at glutamate C-4, whereas glutamate C-2 and C-3 showed little change. Increase in ^{13}C enrichment at C-4 of the γ-glutamyl moiety of glutathione, which typically occurs 80–90-min postsubstrate, was delayed until 120–160-min postischemia. ^{31}P NMR spectra measured postischemia, after the final ^{13}C spectrum was recorded, showed a large (ca. 55%) decrease in the liver ATP as compared with the preischemia level.

^{13}C NMR Determination of Pyruvate Kinase Flux during Gluconeogenesis in Perfused Liver. After steady-state conditions are established in perfused rat liver carrying out active gluconeogenesis from [3-^{13}C]alanine, the input of substrate units to the liver is approximately equal to twice the output of glucose units from the liver. These steady-state conditions were maintained by keeping substrate concentration in the large volume (65 mL) of perfusion fluid approximately constant at 10 mM so as to support a large intracellular trapping pool of alanine with the original unscrambled label at C-3. Measurement of the relative ^{13}C enrichments at the randomized alanine carbons under these conditions provides the basis of a ^{13}C NMR assay of the flux from PEP through pyruvate kinase compared to the flux from PEP to glucose.

The pathway from [3-^{13}C]alanine into the TCA cycle is followed in Figure 1. In the TCA cycle the original label is randomized with the result that the two middle carbons of fumarate and, hence, malate and oxalacetate are strongly labeled (Cohen, 1983). Consequently, the two olefinic carbons of PEP are both strongly labeled. As shown schematically in Figure 1, the middle carbon of PEP is labeled *before* any label appears at the corresponding carbon C-2 of pyruvate. Consequently, pyruvate kinase activity can introduce a label into C-2 of pyruvate. [A similar circumstance obtains for the lesser amount of label found at the carboxylic carbons (C with ● in Figure 1) of PEP and pyruvate]. This pyruvate is converted to alanine by the active liver alanine aminotransferase. Thus, with the maintenance of a large trapping pool of unscrambled [3-^{13}C]alanine, the ^{13}C enrichments at alanine C-2 and C-1 are directly related to the pyruvate kinase flux in the whole perfused organ. The flux from PEP through pyruvate kinase as compared with the input of substrate or, equivalently, as compared with twice the flux from PEP to glucose can be estimated from the expression

$$\frac{\text{pyruvate kinase flux}}{2(\text{gluconeogenic flux})} = \text{Ala(C-2/C-3)}[1 + (1-\phi)\text{Glc(C-6/C-5)} + (1-\phi')\text{Glc(C-4/C-5)}]/\{1 - [\text{Glc(C-6/C-5)}][\text{Ala(C-2/C-3)}]\} \quad (1)$$

As is customary, we assume that the ^{13}C enrichment in C-4, C-5, and C-6 of glucose reflects the ^{13}C enrichment in PEP. Operation of the pentose cycle in liver can modify the label distribution in glucose as compared with that which obtained for precursor PEP. However, the data in Table I of this paper and Table II of the previous one (Cohen, 1987a) indicate that pentose cycle activity was low under our conditions. In addition, the ^{13}C enrichments in C-4, C-5, and C-6 of glucose are used in our model because operation of the pentose cycle affects the ^{13}C distribution in C-1, C-2, and C-3 of glucose to a much greater extent (Katz & Rognstad, 1967; Cohen et al., 1981b).

In eq 1, Ala(C-2/C-3) is the ratio of ^{13}C enrichment at C-2 to that at C-3 of alanine, Glc(C-6/C-5) is the ratio of ^{13}C enrichment at C-6 to that at C-5 of glucose, and Glc(C-4/C-5) is the corresponding ratio for glucose C-4. This ^{13}C NMR assay requires the measurement of the ^{13}C enrichments at C-2 and C-3 of alanine in spectra of perfused liver acquired under steady-state conditions during active gluconeogenesis; the alanine intensities must be corrected for T_1 and NOE effects as described under Materials and Methods. The ^{13}C distributions in the glucose produced are measured with greater accuracy in spectra of the corresponding perfusates (Table I) (Cohen, 1987a). In eq 1, ϕ is the fraction of the ^{13}C-labeled alanine pool in which *both* C-2 *and* C-3 are labeled in the same molecule. ϕ can be determined conveniently in the ^{13}C NMR spectrum of the perfusate from the ratio $D/(D + S)$ for C-6 (or C-1) in the ^{13}C-labeled glucose produced by the liver, where D is the intensity of the outer C-6 (or C-1) doublet and S the intensity of the center C-6 (or C-1) singlet. (Figure 8 of the previous paper shows these doublet to singlet splittings.) ϕ' is the fraction of the ^{13}C-labeled alanine pool in which *both* C-1 *and* C-2 are labeled in the same molecule. ϕ' can be determined from the ratio $D/(D + S)$ for glucose C-4 in the perfusate spectrum. (Equation 1 also applies when the original gluconeogenic substrate is [2-^{13}C]pyruvate + NH_4Cl, in effect, [2-^{13}C]alanine, if the symbols for alanine C-2 and C-3 are exchanged, as well as those for glucose C-5 and C-6.) The

Table II: ^{13}C NMR Measurement of Effects of Insulin and Ischemia on Relative and Absolute Pyruvate Kinase Flux during Gluconeogenesis in Perfused Liver from Streptozotocin-Diabetic Rats and 12- and 24-h-Fasted Untreated Control Rats[a]

donor rat	insulin	substrate	pyruvate kinase flux/ gluconeogenic flux	pyruvate kinase flux [μmol (g of liver wet weight)$^{-1}$ h^{-1}]
control				
1	−	Ala	0.72	14
2	−	Ala	0.80	24
3	−	Pyr	0.72	
4	−	Ala	0.72	14
5	+	Pyr	0.80	16
6	+	Pyr	0.72	13
7	++	Pyr	0.96	25
8	−	Pyr	1.20	14
9	−	Ala	1.04	18
10	+	Pyr	0.82	12
diabetic				
1–6	−	Ala or Pyr	ND	ND
7	+	Ala	0.18	12
8	+	Ala	0.52	14
9	++	Ala	0.74	32
10	+	Pyr	0.14	3
11	+	Ala	0.28	7
12	−	Pyr	0.24	5
13	−	Ala	0.34	8

[a] Pyruvate kinase flux/gluconeogenic flux and pyruvate kinase flux were calculated with the appropriate form of eq 1 and the measured rates of gluconeogenesis. The relative ^{13}C distributions in the alanine signals used in eq 1 were from ^{13}C NMR spectra of perfused liver; the distributions were corrected for differences in NOE and partial saturation as described in the text. Ala denotes 10 mM [3-^{13}C]alanine plus 7.3 mM unlabeled or [2-^{13}C]ethanol. Pyr denotes 9.1 mM [2-^{13}C]pyruvate + 5.5 mM NH_4Cl + 7.3 mM unlabled ethanol. Insulin, when added, was maintained at 7 nM: (++) insulin present 35 min before substrate was added; (+) insulin addition with substrate. Control rats 1–7 were fasted 24 h; controls 8–10 were fasted 12 h. Diabetic livers 10–13 each went through a 35-min period of partial ischemia as described in Figure 5. ND means not detectable.

lower limit of detection is estimated to be a pyruvate kinase flux of about (0.05) (2) (gluconeogenic flux).

ϕ and ϕ' varied little from preparation to preparation under our conditions. For example, for donor rat diabetic 9 of Table II, $\phi = 0.15$ and $\phi' = 0.30$, whereas for control 6 $\phi = 0.16$ and $\phi' = 0.31$. Thus, using the appropriate ^{13}C enrichments in glucose, we find that neglect of the terms Ala(C-2/C-3)[(−ϕ)Glc(C-6/C-5) + (1 − ϕ')Glc(C-4/C-5)] in eq 1 typically results in an underestimation of the relative pyruvate kinase flux of only 1–2%, compared with use of the full expression. Therefore, under our conditions, an abbreviated form of eq 1, namely

$$\frac{\text{pyruvate kinase flux}}{2(\text{gluconeogenic flux})} = \frac{\text{Ala(C-2/C-3)}[1 + \text{Glc(C-6/C-5)}]}{1 - [\text{Glc(C-6/C-5)}][\text{Ala(C-2/C-3)}]} \quad (2)$$

can be used with little compromise and provides a particularly straightforward assay.

The ^{13}C NMR assay of eq 1 was used to measure the bifurcation of the flux from PEP under a number of different conditions, with the results given in Table II. In liver from 24-h-fasted rats (controls 1–4), the ratio of the pyruvate kinase flux to the gluconeogenic flux shows little variability in the absence of insulin, ranging from 0.72 to 0.80, independent of substrate. Because of variations in the rates of gluconeogenesis, the absolute rate of pyruvate kinase flux in these livers ranged from 14 to 24 μmol (g of liver wet weight)$^{-1}$ h^{-1}. This ratio tended to be higher for liver from 12-h-fasted rats (0.82 − 1.20 for controls 8–10), indicating greater relative flux through pyruvate kinase in this state. However, in absolute terms, the rate of flux was lower for liver from 12-h-fasted rats, ranging from 12 to 18 μmol (g of liver wet weight)$^{-1}$ h^{-1}, because the rate of glucose production was only half of that measured for liver from 24-h-fasted, untreated controls. Preincubation of liver from 24-h-fasted rat with insulin for 35 min (control 7) increased the relative pyruvate kinase flux to 0.96. Because the gluconeogenic rate for liver from 24-h-fasted controls was not depressed by insulin, the absolute flux through pyruvate kinase was relatively high, viz., 25 μmol (g of liver wet weight)$^{-1}$ h^{-1}, for control 7. Addition of insulin with substrate (controls 5 and 6) had little effect; however, our data on the in vitro effects of insulin on liver from fasted control rats are limited.

In six normoxic livers from streptozotocin-diabetic rats (diabetics 1–6 of Table II) that were perfused in the absence of insulin, pyruvate kinase flux was undetectable by this assay. As a check on the sensitivity of our assay, perchloric acid extracts of these six livers were prepared after freeze-clamping. The ^{13}C NMR spectra of these extracts were examined for ^{13}C enrichment at the randomized carbons in alanine; no measurable enrichment was found. As an upper limit, we estimate that the relative pyruvate kinase flux in diabetic liver was at least 7-fold lower than that measured in liver from 24-h-fasted normal control rats. Addition of insulin to the perfusion medium 35 min before substrate increased both the relative flux and the absolute flux through pyruvate kinase in one diabetic liver (diabetic 9) to about the level measured in liver from 24-h-fasted control rats, viz., to 0.74 and 32 μmol (g of liver wet weight)$^{-1}$ h^{-1}, respectively. Coadministration of insulin with substrate brought the pyruvate kinase flux up to detectable levels in diabetic liver (diabetics 7 and 8), but the effect was smaller; under these conditions relative fluxes of 0.18 and 0.52 were measured, while the absolute rates of pyruvate kinase flux were 12–14 μmol (g of liver wet weight)$^{-1}$ h^{-1}. Ischemic conditions in diabetic liver also increased pyruvate kinase flux to detectable levels. Two diabetic livers (diabetics 12 and 13) were each subjected to a 35-min episode of low-flow ischemia (produced as described above) within the first hour of perfusion. The relative flux through pyruvate kinase was increased to 0.24 and 0.34, respectively, of the gluconeogenic flux in the postischemic state. The absolute fluxes were only 5 and 8 μmol (g of liver wet weight)$^{-1}$ h^{-1}, reflecting the lower rate of gluconeogenesis in postischemic liver. The effects of insulin and ischemia (diabetics 10 and 11 of Table II) do not appear to be additive in diabetic liver, but again the data are quite limited.

Reuse of pyruvate formed from ^{13}C-labeled PEP would lead to an underestimation of pyruvate kinase flux. A sensitive measure of the extent of this recycling of pyruvate is provided by the ^{13}C enrichment at C-4 and C-5 of glutamate. In the liver cell, pyruvate enters the TCA cycle by two possible paths: the pyruvate carboxylase route to oxalacetate (Figure 1) and the pyruvate dehydrogenase route to acetyl-CoA. Both routes are operative under our conditions (Cohen, 1987b). (a) The substrate [3-^{13}C]alanine is converted to [2-^{13}C]acetyl-CoA, as is [2-^{13}C]ethanol. [2-^{13}C]Acetyl-CoA labels *only* C-4 in α-ketoglutarate and, hence, glutamate. Therefore, appearance of ^{13}C label at glutamate C-5 indicates reuse of pyruvate with randomized label at C-2. The ^{13}C enrichment at glutamate C-4 relative to that at C-5 thus reflects the label distribution in the mitochondrial acetyl-CoA pool. (b) The substrate [2-^{13}C]pyruvate is converted to [1-^{13}C]acetyl-CoA, which labels only C-5 of glutamate. Consequently, for liver incubated with

[2-^{13}C]pyruvate + NH_4Cl + unlabeled ethanol, observation of ^{13}C label at glutamate C-4 is evidence of reuse of pyruvate with randomized label at C-3. For this monitor of pyruvate recycling we measured the ^{13}C distribution at C-4 and C-5 of glutamate in ^{13}C NMR spectra of perchloric acid extracts of the freeze-clamped livers; intensities were corrected for NOE and T_1 effects as described under Methods and Materials. When the gluconeogenic substrate was [3-^{13}C]alanine (alone or in the presence of unlabeled ethanol or [2-^{13}C]ethanol), no ^{13}C enrichment was measured at glutamate C-5 in any sample listed in Table II, whereas a strong label was observed at glutamate C-4 in all cases (Figures 4 and 5) (Cohen, 1987a). (In the presence of [2-^{13}C]ethanol, however, only a lower limit on pyruvate reuse is obtained because in this circumstance less than half the labeled acetyl-CoA can be traced to labeled alanine.) These data indicate that the large perfusate pool of unscrambled [3-^{13}C] alanine, maintained constant at 10 mM, provided an efficient trap for flux from PEP → pyruvate. When the substrate was [2-^{13}C]pyruvate + NH_4Cl + ethanol, appearance of label at glutamate C-4 in three samples listed in Table II indicated that the trapping of ramdomized pyruvate by the intracellular pool of [2-^{13}C]alanine was incomplete. In two normal livers (controls 7 and 8), both of which exhibited high relative pyruvate kinase fluxes, 20 ± 2% of the labeled mitochondrial acetyl-CoA pool arose from recycling of pyruvate, while [2-^{13}C]pyruvate provided the balance. Incomplete trapping was observed in only one liver from a streptozotocin-treated rat. In diabetic 10, 2% of the labeled mitochondrial acetyl-CoA pool arose from the recycling of randomized pyruvate. The pyruvate kinase fluxes given in Table II for these three livers were not corrected for incomplete trapping, but our estimates of pyruvate reuse provide a means for making this correction.

Discussion

Determination of Pyruvate Kinase Flux. Control of phosphoenolpyruvate recycling has been demonstrated to be important in the hormonal regulation of gluconeogenesis from substrates that enter the pathway prior to the triose phosphate level (Groen et al., 1983; Pilkis et al., 1978; Feliu et al., 1976). The flux through pyruvate kinase under these gluconeogenic conditions has been measured by several different approaches (Katz & Rognstad, 1976; Freidmann et al., 1971; Groen et al., 1983; Cohen et al., 1981a). A requisite feature of the ^{13}C NMR assay of the flux through pyruvate kinase is found in modified form in the earlier ^{14}C method of Rognstad (1975), viz., use of a substrate that will introduce a label into PEP prior to the labeling of pyruvate. An appealing aspect of the NMR method is its ability to measure the ^{13}C enrichment at the individual carbons of alanine in whole perfused liver nondestructively, in real time, whereas ^{14}C tracer methods require the separation of cellular extracts for the measurement of isotopic yields. While both ^{13}C NMR and ^{14}C methods can provide the percentage label at a particular carbon, only the NMR measurement also gives the distribution of labeled carbons in the *same* molecule from the ^{13}C–^{13}C splittings. Because the ^{13}C distributions in both alanine and glucose are incorporated into the present NMR assay, no modifications were required to take into account flux of additional label from [^{13}C]ethanol entering the TCA cycle as [^{13}C]acetyl-CoA (Table II). NOE and T_1 effects may influence the intensities measured for different carbon atoms to varying extents. For this reason, the validity of the correction procedures used in the NMR assay was checked by use of two different specifically labeled substrates. The data in Table II are consistent with the assay's being independent of whether the original label was on C-3 or C-2 of alanine. Randomization of the label in pyruvate is also reflected by the corresponding lactate signals. Because the lactate C-2 signal overlaps that from C-2 of the glycerol moiety of the triacylglycerols, alanine C-2 and C-3 should be used in the NMR determination unless the absence of exchange of label into the triacylglycerol pool can be demonstrated. It should be noted that this NMR assay is not applicable to livers from fed control rats; the low rate of gluconeogenesis in this preparation causes a breakdown of the steady-state approximation on which the model is based.

The absolute and relative measurements of pyruvate kinase flux given in Table II for perfused liver from fasted normal rats are in reasonable agreement with results from the ^{14}C isotopic tracer procedures of Rognstad (1982) and Grunnet and Katz (1978) for isolated rat hepatocytes and of Freidmann et al. (1971) for perfused liver, although comparisons for our exact incubation conditions are not available. ^{14}C methods have not, to our knowledge, been used to estimate pyruvate kinase flux in diabetic liver. Activity of the hepatic malic enzyme, which interconverts malate and pyruvate, is known to be low under conditions favoring gluconeogenesis (Young et al., 1964) and should therefore be a negligible source of error in our NMR assay. This view is reinforced by ^{14}C tracer (Rognstad, 1976) and ^{13}C NMR (Cohen et al., 1981a) studies, which demonstrated that L-2,4-dihydroxybutyrate, an inhibitor of the malic enzyme, had no significant effect on measured rates of pyruvate kinase flux in isolated hepatocytes .

Hepatic Effects of Insulin in Vitro. The effects of insulin in vitro on liver from 24-h-fasted control rats under our conditions were modest. The most noticeable ^{13}C NMR detectable changes were transient elevations in the levels of intracellular PEP and 3-phospoglycerate shortly after the addition of substrate and a small (30%) increase in relative pyruvate kinase flux after a 35-min preincubation with insulin. The slight effects of insulin on control liver in vitro measured here are compatible with earlier ^{31}P and ^{13}C NMR observations indicating that incubation with insulin in vitro had little effect on either glycogenesis or levels of phosphorylated intermediates in perfused liver from 16-h-fasted normal rats (Cohen, 1983).

In contrast to the minor changes observed in normal liver, preincubation of perfused liver from a streptozotocin-treated rat with insulin in vitro for 35 min before the administration of substrate induced the partial reversal of diabetes shown in Figure 4. Intracellular aspartate and *N*-carbamoylaspartate increased to observable levels, and the gluconeogenic rate decreased slightly. The most striking effect of insulin in vitro upon diabetic liver was the induction of a hugh enhancement in the flux through pyruvate kinase, sufficient to bring this flux up to the control level. This effect does not appear to have been demonstrated previously in experimental models of diabetes. Increased rate of synthesis of either pyruvate kinase or the liver malic enzyme cannot be responsible for the rapid effects we observe by ^{13}C NMR after treatment of diabetic liver with insulin in vitro. The activities of liver pyruvate kinase and the malic enzyme are known to be depressed in experimental diabetes; although substantial increases in the rates of synthesis of both enzymes have been measured in diabetic rats treated with insulin in vivo, these changes were not seen until at least 24-h posttreatment (Miyanaga et al., 1982; Parks & Drake, 1982; Thompson & Drake, 1982).

Frank expression of insulin's hepatic influence requires the presence of glucagon (Unger, 1985). Consistent with this view of insulin's major hepatic effect being the opposition of the action of glucagon, our ^{13}C NMR results show that the most pronounced effect of insulin in vitro was seen in perfused liver

from streptozotocin-diabetic rats, in which model glucagon levels are known to be about doubled (R. Saperstein, unpublished results). In particular, the large increase in the rate of pyruvate kinase flux observed by ^{13}C NMR in diabetic liver incubated with insulin in vitro (Figure 4; Table II) is consistent with the sizable body of evidence that has accumulated indicating that in isolated hepatocytes insulin antagonizes the ability of exogenous glucagon to cause the rapid inactivation of pyruvate kinase (Feliu et al., 1976; Blair et al., 1976; Claus et al., 1979).

It is well established that liver pyruvate kinase is allosterically activated by fructose 1,6-bisphosphate and H^+ ion and inhibited by ATP (Seubert & Schoner, 1971). Indeed, fructose 1,6-bisphosphate has been reported to inhibit the ability of physiological levels of glucagon to inactivate pyruvate kinase (Clause et al., 1979). Because elevation of fructose 1,6-bisphosphate and H^+ ion and decrease in ATP are conditions generally associated with ischemia (Jennings et al., 1981; Gaja et al., 1973; Wollenberger et al., 1968), the effects of ischemia on diabetic liver were followed by ^{13}C NMR to serve as a comparison with the effects of insulin in vitro upon enhancement of the rate of pyruvate kinase flux in this model. As Figure 5 and the data in Table II indicate, large increases in the rate of flux through pyruvate kinase were also demonstrated in diabetic liver under our conditions of ischemia. This effect also does not appear to have been measured previously in experimental diabetes. Endogenous insulin levels are very low in this preparation (Schein et al., 1971), and no exogenous insulin was present. Therefore, enhancement of pyruvate kinase flux under these conditions of ischemia is consistent with the anticipated changes in allosteric effectors.

A ^{13}C NMR determination of the activity of the phosphoenolpyruvate cycle under conditions of active gluconeogenesis in isolated perfused liver was developed here. Reuse of the recycled pyruvate, which can lead to an underestimation of the pyruvate kinase flux, was monitored by using the ^{13}C enrichment at glutamate C-4 and C-5. Three preparations, viz., perfused liver from streptozotocin-diabetic rats and from 24-h- and 12-h-fasted normal control rats, were examined to provide a gradation of pyruvate kinase flux while meeting the assay's requirement of active gluconeogenesis. These ^{13}C NMR studies indicate that, under our conditions, insulin exerted its major effect in vitro upon diabetic liver; in particular, insulin's direct hepatic influence in this model was evinced as a manyfold increase in the rate of pyruvate kinase flux.

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Registry No. L-Ala, 56-41-7; EtOH, 64-17-5; $MeCOCO_2H$, 127-17-3; insulin, 9004-10-8; pyruvate kinase, 9001-59-6.

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