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Ketogenesis in the Living Rat Followed by ^{13}C-NMR Spectroscopy. Infusion of [1,3-^{13}C]Octanoate

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Summary: ^{13}C-NMR spectroscopy was used as a noninvasive approach to study the metabolism of [1,3-^{13}C]octanoate in rat liver. Using a properly adjusted surface coil a liver selection of better than 90% was achieved in the intact animal without abdominal surgery. After infusion of [1,3-^{13}C]octanoate via the jugular vein different patterns of metabolites were observed depending on the physiological state of the rat. In the fasted animal, the major metabolites were those of the Krebs cycle while in the diabetic animal ketogenic end products were predominant. As a fatty acid of medium chain length octanoate is imported into the inner mitochondrial space without control by the carnitine acyl transferase system. Hence, the metabolic differences observed between diabetic and fasted rats result from an intramitochondrial control mechanism. The in vivo ^{13}C-NMR results therefore support previous biochemical in vitro studies which concluded that a major control of ketone body production occurs in the inner mitochondrial space, presumably via the redox potential of the liver. As an unexpected result, ^{13}C-NMR provides evidence for the transitory esterification of the infused ^{13}C-labeled octanoic acid. The corresponding ^{13}C-NMR chemical shifts are typical for glycerides.

^{13}C-NMR-Spektroskopische Untersuchungen der Ketogenese bei der Ratte in vivo. Infusion von [1,3-^{13}C]Octanoat

Zusammenfassung: Mittels der ^{13}C-NMR Spektroskopie wurde der Metabolismus von [1,3-^{13}C]-Octanoat in der Leber von fastenden und diabetischen Ratten auf nicht-invasive Weise untersucht. Durch geeignete Wahl des Spulendurchmessers und der Meßbedingungen und durch sorgfältige Positionierung der Oberflächenspule konnte ohne chirurgischen Eingriff eine Leberselektion von besser als 90% erreicht werden. Nach Infusion von [1,3-^{13}C]Octanoat durch die Halsvene der Ratte wurden abhängig vom Zustand des Tieres unterschiedliche Metabolite beobachtet. Im fastenden Tier dominierten Metabolite des Krebszyklus, während im diabetischen Tier die Ketonkörper die wichtigste Rolle spielten. Octansäure wird unter Umgehung des Carnitin-Acyl-Transferase-Systems in die Lebermitochondrien transportiert. Die Produktion unterschiedlicher Metabolite im fastenden und diabetischen Tier kann daher nur durch einen intramitochondrialen Kontrollmechanismus erklärt werden. Die In-vivo-^{13}C-NMR-Messungen sind daher im Einklang mit biochemischen in-vitro-Unterschungen, aus denen eine Kontrolle der Ketonkörperproduktion durch das Redoxpotential der Leber abgeleitet wurde. Die ^{13}C-NMR-Experimente zeigen zusätzlich eine transitorische In-vivo-Veresterung der markierten Octansäure der Leber. In der biochemischen Literatur wurde eine direkte Veresterung von Octansäure bisher ausgeschlossen. Die beobachteten chemischen Verschiebungen stimmen mit Literaturdaten für Glycerol-ester überein.

Key words: Ketogenesis in vivo, ^{13}C-NMR spectroscopy, octanoate metabolism, triacylglycerol esters, diabetic rat liver.

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The production of ketone bodies in the mammalian liver is closely related to the availability of fatty acids as substrates for β-oxidation. Free fatty acids are released from the adipose tissue and transported to the liver. Two major regulatory steps can be recognized in the liver for the control of ketogenesis:

1) the regulation of the entry of free fatty acids into the mitochondria via the acyl carnitine transferase system,
2) the regulation of the relative fluxes of acetyl-CoA through the pathways of oxidation to CO_2 (via the citric acid cycle) and of ketogenesis.

In order to investigate the intrahepatic regulatory mechanisms for ketogenesis we have studied the metabolism of ^{13}C-labeled fatty acids in the liver of diabetic and fasted rats in vivo. As a non-invasive approach we have used the recently developed technique of in vivo ^{13}C-NMR spectroscopy which allows the study of ^{13}C-labeled metabolites in rat liver without the need for abdominal surgery. A variety of metabolites can be identified and quantitated with ^{13}C-NMR and the time-course of their synthesis and degradation can be followed in a single experiment.

We have reported earlier on experiments with fasted and diabetic rats using ^{13}C-labeled butyrate as a substrate and have also described in detail the ^{13}C-NMR technique[1,2]. Here we present experiments obtained with a fatty acid of medium chain length, i.e. 1,3-^{13}C-labeled octanoate. Common to medium and short chain fatty acids is their property to bypass the acyl carnitine transferase system. Both enter freely into the mitochondrial inner space and the regulatory mechanism governing their metabolism is determined exclusively by the pathways of β-oxidation.

The present octanoate studies revealed clear differences in the fate of the substrate depending on the physiological state of the animal. Whereas in the diabetic rat liver the major part of the ^{13}C-labeled acetyl-CoA was converted into ketone-bodies, [1-^{13}C]acetyl-CoA produced in the fasted rat liver was directed preferentially towards oxidation to CO_2 via the citric acid cycle.

^{13}C-labeled glucose was observed under in vivo conditions only in the diabetic rat but not in fasted animals indicating a high activity of the malate shuttle in the former. NADH which is produced in excess by the oxidation of fatty acids is exported via the malate shuttle from the mitochondria into the cytosol where it is consumed in gluconeogenesis. The enhanced gluconeogenesis encountered in the diabetic state allows an increased β-oxidation of fatty acids which may be otherwise soon limited by respiratory control.

In earlier studies performed with ^{14}C-labeled octanoate on the perfused livers of diabetic and fasted rats, essentially the same amount of labeled ketone-bodies was observed for both conditions. The authors therefore concluded that the major control of ketogenesis was exerted by the regulation of the entry of free fatty acid into the mitochondria[3,4]. In contrast, the present results support the concept of Lehninger[5] and Wieland[6,7] that the availability of free oxaloacetate and hence the redox-state of the liver represents the major regulatory factor in ketogenesis.

Materials and Methods

NMR

Fourier transform NMR spectra were obtained in vivo with a Bruker BNT-80 spectrometer with a 1.9 T horizontal magnet having a clear bore of 24 cm. A Waltz-8 pulse sequence was used to obtain complete ^1H-decoupling with minimal radio frequency power. The liver selection was achieved with a surface coil of 1.8 cm diameter and a special rat support. The experimental conditions where identical to those detailed previously[1,2]. The methyl groups of phosphatidylcholine were used as an internal standard for the quantitative comparison of the ^{13}C-NMR spectra. Partial saturation of the non-protonated carbonyl resonances compared to the faster relaxing protonated carbon atoms was taken into account by an experimentally determined correction factor of 2[2].

Chemicals

[1,3-^{13}C]octanoate (90% enrichment) was prepared from pentyliodide by three chain elongations with CO_2[8]. In the first and third elongation step $^{13}CO_2$ (90%) was employed. The overall yield in the 8-step synthesis was 5–10% based on the amount of $Ba^{13}CO_3$ employed in the first Grignard reaction.

Animals

Male rats (Sprague-Dawley-strain, 250–300 g) were used for all experiments. They were fed ad libitum with standard rat chow. Fasted rats were deprived of food for 24 h prior to use. Experimental diabetes was induced by the intraveneous injection of streptozotocin (90 mg/kg body weight). Animals were used 48 h later if they proved to be acutely ketotic as determined by an immediate strongly positive urine test for ketones (ketomerckognost, Merck, Darmstadt). They were fed ad libitum until they were used. No octanoate infusion experiments were performed with well-nourished rats. In previous experiments with labeled butyric acid we noted only weak ^{13}C metabolite signals with well-

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nourished rats presumably because of the overriding abundance of non-labeled endogenous fatty acids[2].

Infusion experiments

Rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally). A catheter was placed in the right jugular vein and maintained patent with isotonic saline solution infused at 0.5 m*l*/h. The labeled octanoate was infused as a 0.2M solution (pH 7.3) with 3% albumin. The infusion rate was adjusted to 6 m*l*/h, the total volume infused per experiment was 10 m*l* corresponding to 2 mmol of labeled octanoate. The in vivo liver spectra were recorded with a surface coil. For the extracts and for the in vitro spectra the liver was excised after termination of the infusion and immediately frozen in liquid nitrogen. The lipids were extracted according to the procedure of Folch[9]. For high resolution ^{13}C-NMR spectra at 75 MHz the liver was sliced and transferred into a 10 mm sample tube. Saline in $^{2}H_2O$ was added as lock signal. The temperature was kept at 37 °C. All experiments were performed at least in duplicate and the scatter between experiments of the same series was surprisingly small as judged from the scatter in the intensities of corresponding resonances.

Lipase treatment

Slices of excised livers from diabetic rats which had been infused with [1,3-^{13}C]octanoate were incubated in an NMR tube with lipase (from *Rhizopus delemar*, 600 U/mg, Fluka AG). This enzyme cleaves selectively the carbonyl ester bond of triacyl glycerols. The temperature was maintained at 37 °C and the enzymatic degradation of the glycerol esters was followed by NMR.

Results

Fig. 1 displays in vivo ^{13}C-NMR spectra of the liver of a fasted rat. The resonance of the phosphatidylcholine methyl groups at 55 ppm was used as an internal standard to scale the different spectra[2]. Fig. 1A represents the natural abundance ^{13}C signals at the beginning of the infusion period, while Fig. 1B shows the same liver after infusion of [1,3-^{13}C]octanoate. Each spectrum was acquired during a period of 40 min. A 180° flip angle in the center of the coil was applied to suppress signals from superficial tissue. Fig. 1C shows the difference spectrum B-A

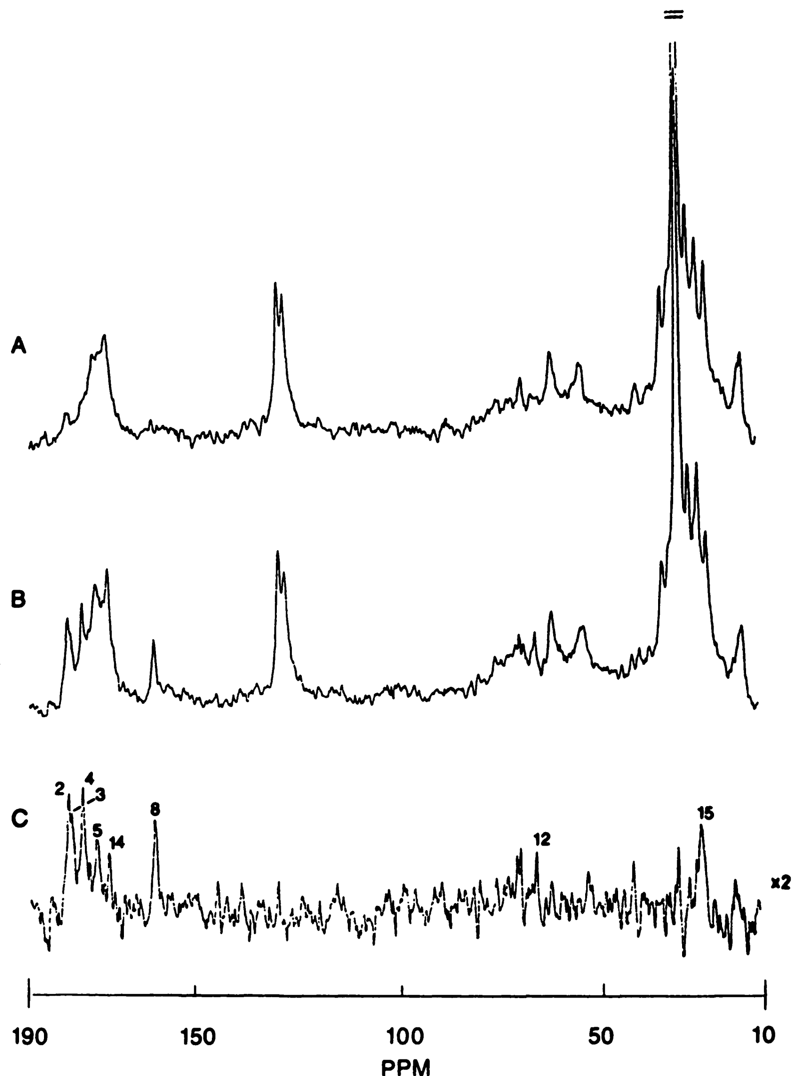

Fig. 1. ^{13}C-NMR spectra of in vivo rat liver observed in a fasted animal.

Spectra were obtained at 20.1 MHz with a surface coil and a ^{13}C 180° surface nulling pulse. Proton decoupling was employed only during the 100 ms aquisition time. [1,3-^{13}C]-octanoate was infused via the jugular vein over a period of 110 min. (A) Spectral acquisition from 20 min before to 20 min after start of infusion; (B) spectral aquisition for the period 80–120 min after start of infusion; (C) difference spectrum (B) – (A) showing only resonances of metabolized [1,3-^{13}C]octanoate. Assignment of resonances according to Table 1. Each spectrum represents 2000 aquisitions. An artificial line broadening of 4 Hz was used.

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in which the natural abundance ^{13}C background is eliminated and where only those resonances are retained which result from the metabolism of [1,3-^{13}C]octanoate. Most signals are clustered in the region of the carbonyl groups around 170 ppm and the assignment of the resonances is summarized in Table 1. The most intense resonances in Fig. 1C arise from metabolites of the citrate cycle, in particular glutamate C(5) (resonance 2 at 181.4 ppm), glutamine C(5) (resonance 4 at 177.8 ppm), and hydrogen carbonate (resonance 8 at 160 ppm). Resonances of the free substrate were not observed; however, resonances 14 at 172 ppm and 15 at 24.9 ppm must be assigned to C(1) and C(3) of octanoate bound as glycerolester (cf. below).

Corresponding spectra were obtained for diabetic animals and are shown in Fig. 2. Fig. 2A

Table 1. Assignment of ^{13}C resonances.

Resonance	Chemical shift [ppm]	Assignment
1	184.0	C-1 butyrate
2	181.4	C-5 glutamate
3	180.5	C-1 β-hydroxybutyrate
4	177.8	C-5 glutamine
5	175.0	C-1 acetoacetate
6	174.7	C-1 glutamate
7	174.2	C-1 glutamine
8	160	carbonate
9	76.1	C-3 β-glucose
10	73.2	C-3 α-glucose
11	70.1	C-4 α,β-glucose
12	65.7	C-3 β-hydroxybutyrate
13	19.4	C-3 butyrate
14	172.0	C-1 octanoylglyceride
15	24.9	C-3 octanoylglyceride
16	210.0	C-3 acetoacetate

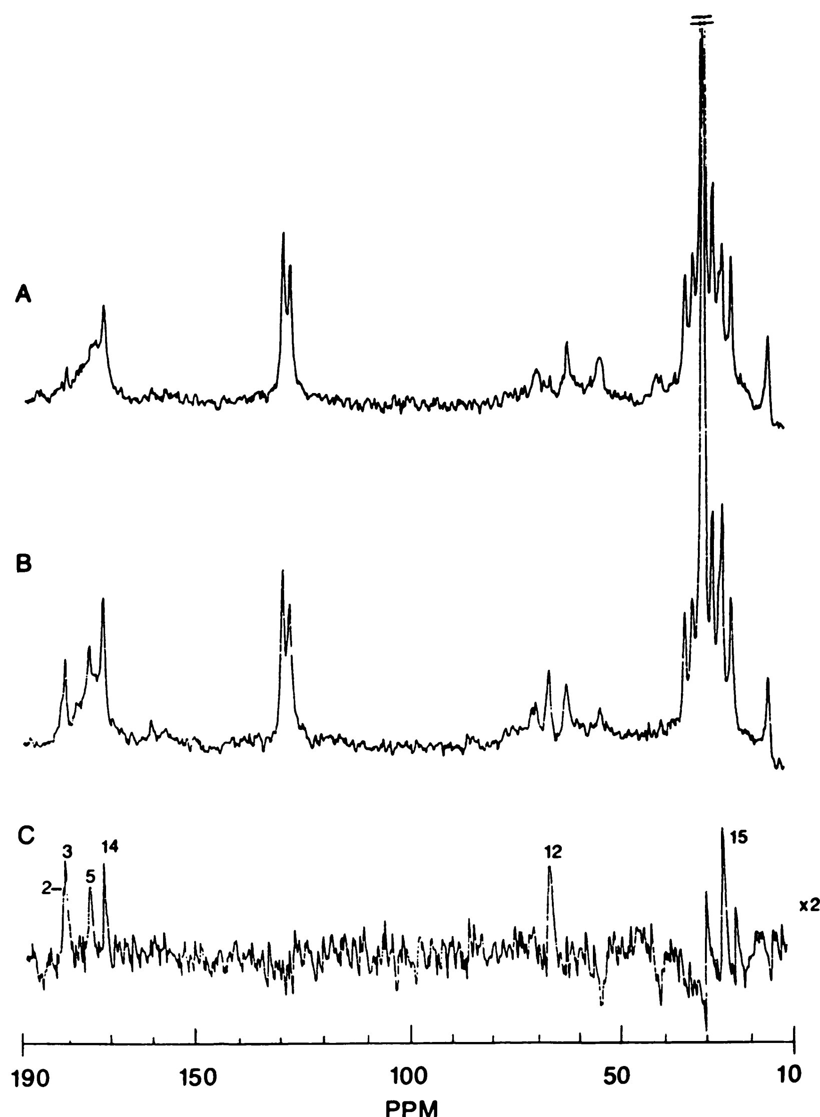

Fig. 2. ^{13}C-NMR spectra of in vivo rat liver observed in a diabetic animal.
Infusion of [1,3-^{13}C]octanoate. Other experimental conditions as in Fig. 1. (A) Spectral aquisition from 20 min before to 20 min after start of infusion; (B) 80–120 min after start of infusion; (C) difference spectrum (B) – (A) showing only intrahepatic metabolites derived from [1,3-^{13}C]octanoate (cf. Table 1 for assignment).

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shows the natural abundance ^{13}C liver spectrum of the diabetic rat, Fig. 2B represents the spectrum after infusion of [1,3-^{13}C]octanoate, and Fig. 2C gives the difference spectrum B-A. Compared to the fasted animal, resonances from glutamine and carbonate are missing; instead new resonances appear which can be assigned to the ketone bodies β-hydroxybutyrate C(1): resonance 3 at 180.5 ppm; C(3): resonance 12 at 65.7 ppm) and acetoacetate (C(1): resonance 5 at 175 ppm). Again a considerable fraction of [1,3-^{13}C]octanoate occurs in the form of its glycerol ester (resonance 14 at 172 ppm and resonance 15 at 24.5 ppm).

The ^{13}C labeling pattern observed after infusion of [1,3-^{13}C]octanoate is in broad agreement with previous results obtained with [1,3-^{13}C]-butyrate as a substrate[2]. In fasted rats, both studies demonstrate that the ^{13}C-labeled fatty acids are converted essentially to products of the citrate cycle, i.e. glutamate, glutamine, and hydrogen carbonate. Likewise, the major metabolite in the liver of the diabetic animal was found to be 3-hydroxybutyrate in both cases. However, an interesting difference should also be noted. The formation of acetoacetate under in vivo conditions could only be detected with [1,3-^{13}C]octanoate but not with [1,3-^{13}C]butyrate. Even though acetoacetate was also produced from butyrate, its concentration in the liver was too low to be seen in the in vivo experiment. Only when perchloric acid extracts of the liver were prepared was it possible to demonstrate the formation of this metabolite. In contrast, the C(1) resonance of acetoacetate was readily observable both in diabetic and fasted rat liver in vivo with [1,3-^{13}C]octanoate as substrate. In fact, with a long enough measuring time it was also possible to detect the C(3) resonance of acetoacetate at 210 ppm (cf. insert in Fig. 3) which is characterized by an inherently low sensitivity due to a long T_1 relaxation time.

A further similarity in the metabolism of infused butyrate and octanoate was the formation of ^{13}C labeled glucose in the diabetic rat. Fig. 3 shows the in vivo liver spectrum of a diabetic rat acquired over a measuring period of 1 h with simultaneous infusion of [1,3-^{13}C]octanoate. In addition to the resonances already discussed, a new resonance appeared at 70.1 ppm (resonance 11) and was assigned to C(4) of α- and β-glucose. At a later stage of the same experiment a further resonance appeared at 76.1 ppm and was assigned to the C(3) resonance of β-glucose. Since the glucose signals in the in vivo spectra are rather weak further assignments were made with perchloric acid extracts of liver slices. The spectra of the extracts are represented in Fig. 4.

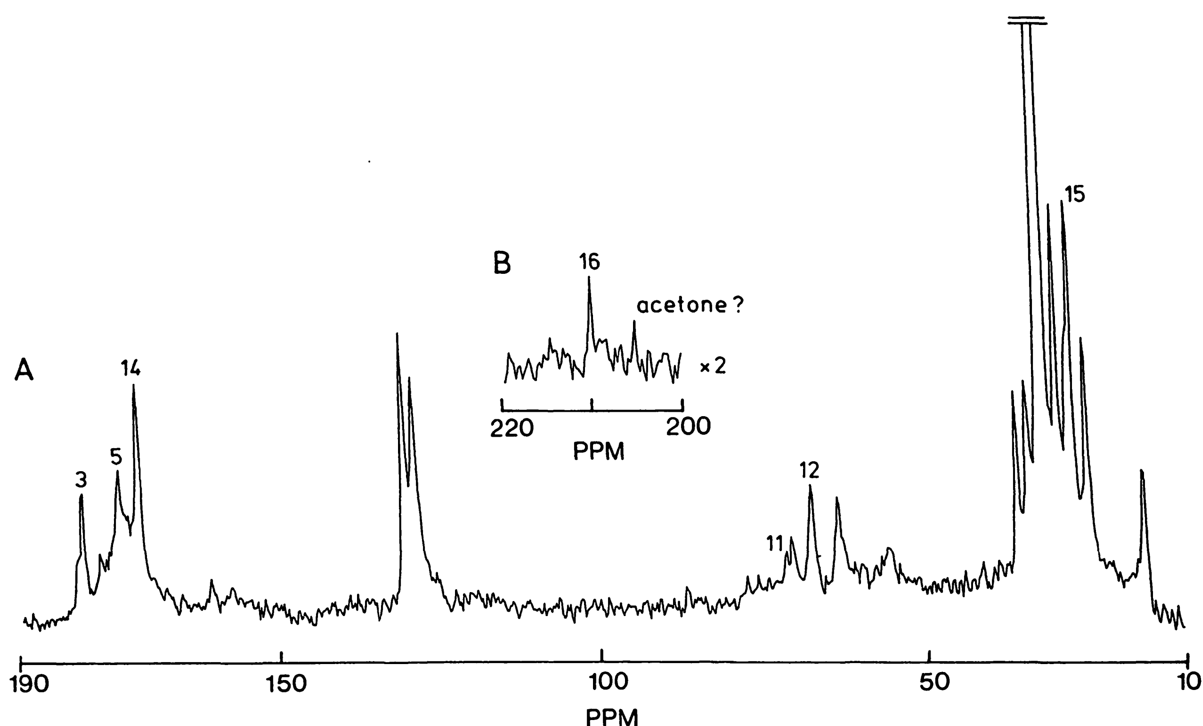

Fig. 3. ^{13}C-NMR spectrum of in vivo rat liver of a diabetic animal.

The spectrum was aquired over a period of 1 h during the infusion of [1,3-^{13}C]octanoate. (B) Shows the expanded region between 200 and 220 ppm. Assignment of resonances according to Table 1. 3000 aquisitions. An artificial line broadening of 2 Hz was used.

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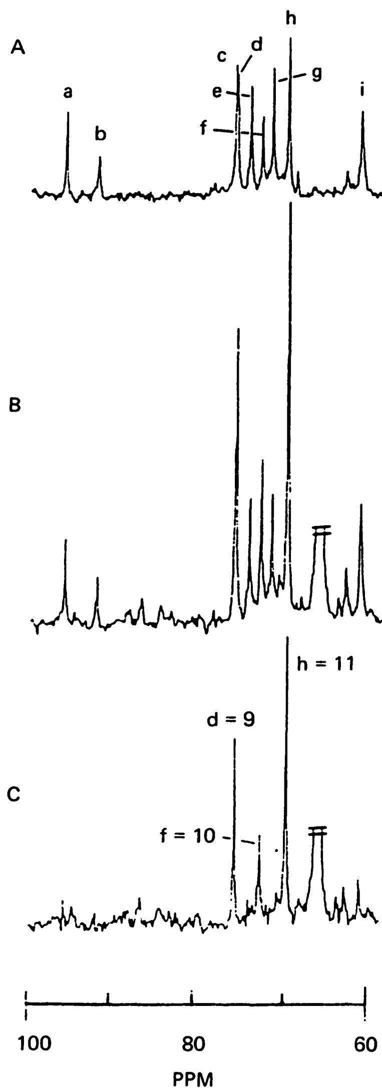

Fig. 4. ^{13}C-NMR spectra (at 75.5 MHz) of the perchloric acid extracts of rat liver.

(A) Obtained from a control animal, (B) from a diabetic animal after infusion of [1,3-^{13}C]octanoate; (C) difference spectrum (B) – (A) showing only those glucose resonances which have been ^{13}C-labeled during the infusion of [1,3-^{13}C]octanoate. In (B) and (C) the intense signal of 3-hydroxybutyrate is truncated. Resonance assignment of the glucose signals in (A):

a at 96.1 ppm	C(1)	β-glucose
b at 92.0 ppm	C(1)	α-glucose
c at 76.2 ppm	C(5)	β-glucose
d at 76.0 ppm	C(3)	β-glucose
e at 74.3 ppm	C(2)	β-glucose
f at 73.0 ppm	C(3)	α-glucose
g at 71.7 ppm	C(2,5)	α-glucose
h at 70.0 ppm	C(4)	α, β-glucose
i at 60.9 ppm	C(6)	α, β-glucose

Resonance assignment in (C) according to the signals observed in vivo (cf. Table 1). Each spectrum represents 5000 aquisitions with a recycle delay of 6 s and a flip angle of 40°. An artificial line broadening of 2 Hz was used.

Only the region between 60 and 100 ppm is shown which is characteristic of the glucose resonances. Fig. 4A is the spectrum of a diabetic control rat without infusion, Fig. 4B that of a diabetic rat after infusion of [1,3-^{13}C]octanoate while Fig. 4C is the difference spectrum B-A. The most intense signal is that of the C(3) resonance of 3-hydroxybutyrate at 65.4 ppm which has been truncated in Fig. 4. The assignment of the other resonances to the various glucose carbon atoms is summarized in Table 1 and is based on a comparison with standards. Fig. 4C then provides evidence for a distinct flow of ^{13}C label into carbon atoms C(3) and C(4) of α- and β-glucose and for a relatively high rate of gluconeogenesis in the diabetic state.

Finally, we need to consider the question of transitory octanoate esterification. Medium chain fatty acids such as octanoate are generally believed not to serve directly as substrates for triacylglycerol formation[3,10,11]. Hence, the correct identification of the resonances at 172 ppm and 24.8 ppm observed in in vivo liver spectra after infusion of [1,3-^{13}C]octanoate was of major importance. To this purpose, the ^{13}C-NMR spectrum of the infusion solution containing free [1,3-^{13}C]octanoate was measured first. The chemical shifts of carbon atoms C(1) and C(3) were determined as 184.3 ppm and 25.7 ppm, respectively, and were clearly different from those observed under in vivo conditions (Table 2). Thus the occurrence of free [1,3-^{13}C]octanoate in the liver could be excluded. Next, methanol-chloroform extracts of the excised livers of diabetic rats were prepared. The corresponding ^{13}C-NMR spectra of the extracted lipids (dissolved in $CDCl_3$) are shown in Fig. 5 with Fig. 5A as a control (no infusion), Fig. 5B after infusion of [1,3-^{13}C]octanoate, and 5C as the difference spectrum B-A. The difference spectrum contains strong resonances at 173.4 ppm and 24.7 which are close to those observed in vivo liver. By contrast, free [1,3-^{13}C]octanoate in $CDCl_3$ has resonances at 180.5 ppm and 24.7 ppm (cf. Table 2). The solubility of the unknown metabolite in chloroform/methanol together with the observation that the natural abundance ^{13}C-NMR spectra of triacylglycerols are characterized by resonances at 171.6 ppm (C(1)) and 24.9 ppm (C(3))[12,21] suggests an esterification of [1,3-^{13}C]octanoate in the diabetic rat liver. The formation of a thioester can be excluded since the corresponding carbonyl resonances occur at 195 ppm[22].

A final proof for the esterification could be established by incubation of rat liver slices with lipase, an enzyme which selectively cleaves the

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Table 2. Chemical shifts (in ppm) relative to TMS = 0.

Substrate	C(1)	C(3)
Infusate – 0.2M solution of [1,3-^{13}C]octanoate (pH 7.3)	184.3	25.7
Adipose tissue in vitro	172	not assigned
Triacylglycerols in sciatic nerv[12] in lipoproteins[21]	 171.6 171.5–173.9	 24.9 not determined
Observed in vivo after infusion of [1,3-^{13}C]octanoate	172	24.9
Thio-ester[22]	195	not determined
[1,3-^{13}C]octanoic acid in $CDCl_3$	180.5	24.7
Observed in lipid extracts from excised livers after the infusion of [1,3-^{13}C]octanoate	173.4	24.9

carbonyl ester linkage in triacylglycerols. The reaction could be followed in vitro with ^{13}C-NMR and Fig. 6 summarizes the spectra of the carbonyl region before (6A) and after addition of lipase (6B). The liver was excised from diabetic rat after infusion of [1,3-^{13}C]octanoate. The intense ester signal at 172 ppm in (6A) is decreased upon lipase treatment while a new resonance at 184.2 ppm is observed, which must be attributed to free octanoate. Lipase treatment also releases natural long-chain fatty acids. However, the carbonyl resonances of palmitate and oleate occur at 181–182 ppm[13] and can thus be differentiated from octanoate.

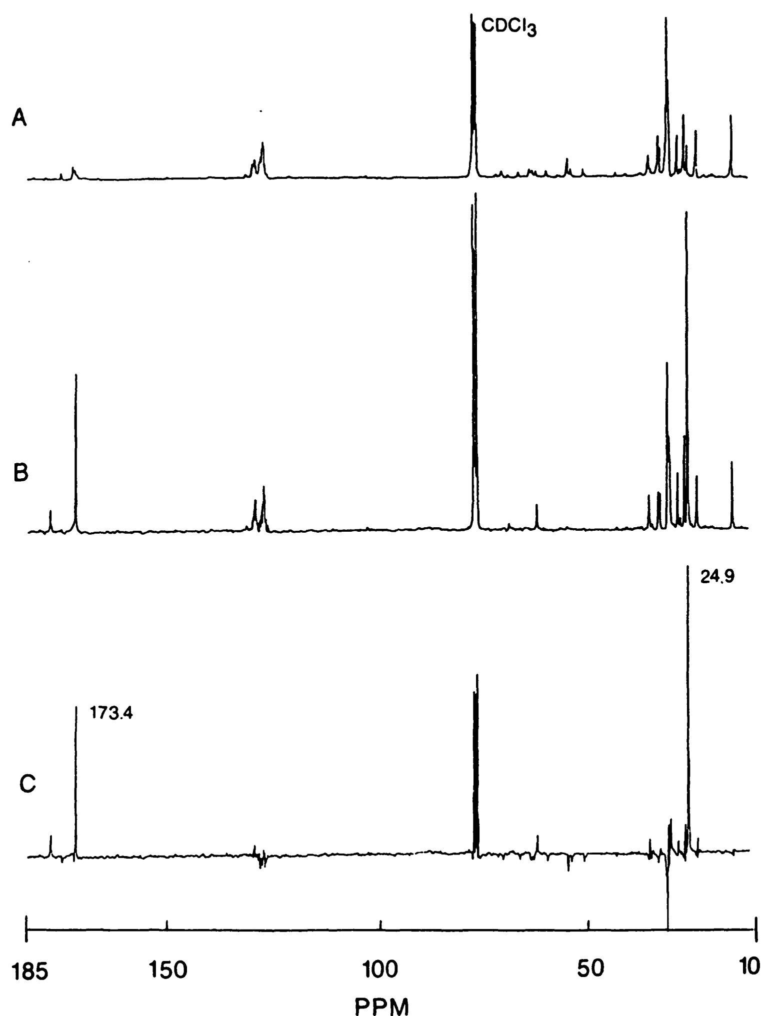

Fig. 5. ^{13}C-NMR spectra (at 75.5 MHz) of extracted lipids dissolved in $CDCl_3$.

Lipids obtained from the liver of (A) a diabetic control rat and (B) a diabetic rat after the infusion of [1,3-^{13}C]octanoate; (C) difference spectrum (B) – (A) showing only two resonances which have been labeled during the infusion of [1,3-^{13}C]-octanoate. A chemical shift of 173.4 ppm is typical for the carbonyl group of triacylglycerol esters of fatty acids. Each spectrum represents 3000 aquisitions with a recycle delay of 3 s and a flip angle of 40°. An artificial line broadening of 0.5 Hz was used.

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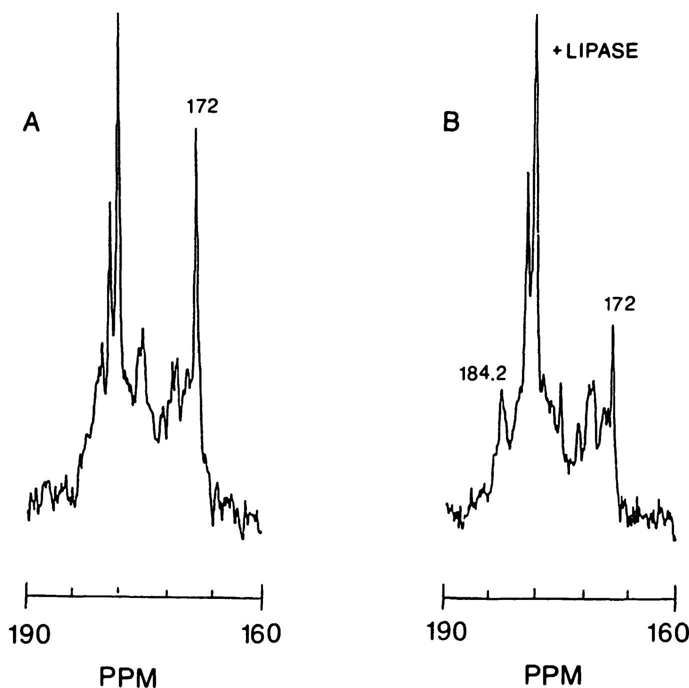

Fig. 6. In vitro spectra (at 75.5 MHz) of the liver of a diabetic rat after the infusion of [1,3-^{13}C]-octanoate before (A) and after (B) incubation with lipase.

Only the region of the carbonyl resonances is shown. The signal at 172 ppm was assigned to the triacylglycerol ester of octanoate, the signal at 184.2 ppm to free octanoate. The temperature was maintained at 37 °C throughout the measurements.

Discussion

It is a widely held view that medium chain fatty acids such as octanoic acid do not serve as substrates for triacylglycerol formation[3,10,11]. Surprisingly, the ^{13}C-NMR experiments with [1,3-^{13}C]octanoate provide unambiguous evidence for a rather rapid esterification of part of the infused fatty acid, this effect being observed in the liver of fasted as well as diabetic rats. The accumulation of the octanoate ester in the liver is only transient. The intensity of the ester resonances reaches its maximum at the end of infusion and decreases to 20% of this value within one hour. The present experiments allow no decision if the ester bond is split in the liver or if the ester is exported from the liver tissue. Treatment with triacylglycerol lipase releases free [1,3-^{13}C]octanoate indicating that the labeled octanoic acid was incorporated into a triacylglycerol. The quantitative extent of octanoate incorporation into triacylglycerol was generally in the same order as shown in Figs. 1 and 2, but was found drastically increased in one experiment. Free octanoate could not be detected in rat liver under in vivo conditions.

These results differ from those obtained with ^{13}C-labeled butyrate. Even though butyrate and octanoate were infused at the same molar concentrations, the in vivo liver spectra after butyrate infusion always showed resonances characteristic of free butyrate. On the other hand, no butyrate ester signals were observed in the previous experiments[1,2].

The in vivo ^{13}C-NMR studies with [1,3-^{13}C]-octanoate as infusate differ from in vitro studies on perfused liver using radioactively labeled [1-^{14}C]octanoate[3]. The amount of labeled ketone bodies produced from the radioactively labeled octanoate, which was applied at high loads, was approximately identical for both diabetic and fasted rats. In diabetic rats only a modest depression of the activity of the citrate cycle was observed. However, the total ketone body production (radioactively labeled plus nonlabeled ketones originating from endogenous long chain fatty acids) was twice as high in the liver of diabetic than in that of fasted rats.

In contrast, it is evident from the present in vivo study that the distribution of the ^{13}C label between the citrate cycle and the pathway of ketogenesis is strikingly different for fasted and diabetic rats. In the fasted state the major ^{13}C-labeled metabolites produced are those of the citrate cycle, in the diabetic state mainly ketogenic end products are observed. Hence, unlike the in vitro studies with perfused liver[3] in vivo ^{13}C-NMR provides evidence for a control of the ketone body production from octanoate at the level of the disposal of acetyl-CoA between the citrate cycle and the pathway of ketogenesis.

The enhanced rate of ketone body production in the diabetic state is accompanied by a concomitant increase in the rate of gluconeogenesis [14–17]. In agreement with these earlier studies we observed signals of ^{13}C-labeled glucose in vivo in the liver of diabetic animals only. In fasted rat the concentration of labeled glucose

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was too low (< 0.5mM) to be detectable at the present ^{13}C-NMR sensitivity.

In mammals, fatty acids do not directly serve as substrates for gluconeogenesis. Therefore the pathway of ^{13}C label from the infused fatty acid to glucose needs to be considered in more detail. As precursors for glucose formation serve three-carbon substrates such as lactate, alanine, glycerol or pyruvate. In order to be converted to glucose, pyruvate must first enter into the mitochondria, where it is transformed to oxaloacetate by the action of pyruvate carboxylase. The oxaloacetate formed from pyruvate mixes with the intermediary oxaloacetate pool of the Krebs cycle. By a variety of different models the extent of mixing between the two pools has been estimated to be in the range of 5 to 30%[18–20]. By this exchange mechanism [1-^{13}C]acetyl-CoA which has been incorporated into four-carbon dicarboxylic acids of the citrate cycle can also enter the pathway of gluconeogenesis. As a second mechanism oxidation of [1,3-^{13}C]octanoate produces ^{13}C-labeled hydrogen carbonate which may, in part, be added to pyruvate. The resulting oxaloacetate is then labeled at carbon atoms C(1) as shown in Fig. 7A. This label (noted by an asterix) is scrambled via the fumarase exchange reaction and appears at carbon atoms C(1) and C(4) of malate (Fig. 7B). Malate (1) is exported into the cytosol where it is converted via oxaloacetate (2) into phospho*enol*pyruvate (3). Phospho*enol*pyruvate is further converted into glycerolaldehyd 3-phosphate (4), which is in equilibrium with dihydroxyacetone 1-phosphate (5). These substrates may then be utilized for gluconeogenesis. A ^{13}C label originally located in C(1) or C(4) of malate would thus finally end up in the C(3) and C(4) carbon atoms of glucose.

An enrichment of ^{13}C label in glucose was observed with either [1,3-^{13}C]octanoate (this work) or [1,3-^{13}C]butyrate[2] as substrates, but only in diabetic animals. At the same time only little ^{13}C-labeled hydrogen carbonate and glutamate were produced in the liver of diabetic animals and the corresponding resonances were at the limit of detectability under in vivo conditions. The increased flux of ^{13}C label into glu-

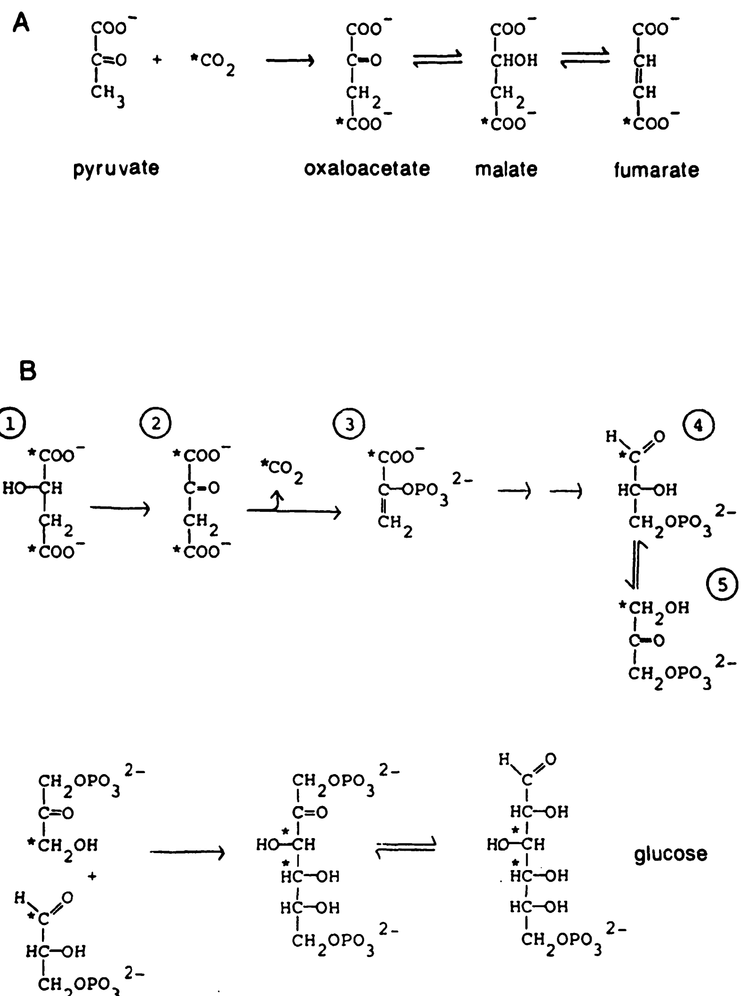

Fig. 7. Metabolic ^{13}C-labeling pattern of glucose after infusion of [1,3-^{13}C]octanoate.
The position of the ^{13}C label is indicated by an asterix. (A) Formation of ^{13}C-labeled oxaloacetate via the pyruvate carboxylase reaction and subsequent scrambling of the label in the fumarase exchange reaction; (B) formation of glucose starting from ^{13}C-labeled malate (cf. text).

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cose thus provides evidence for a concomitant increase in the activity of the malate shuttle. Via this mechanism NADH is exported from the mitochondria into the cytosol where it is consumed in gluconeogenesis. Due to the high rate of β-oxidation in the diabetic state, NADH is produced in excess in the mitochondria creating a reduced redox state. The oxidation capacity of the liver would soon be limited by respiratory control if there was not a high consumption of NADH in gluconeogenesis and if acetyl-CoA would not be directed into ketogenesis resulting in the generation of less NADH than via oxidation in the citrate cycle.

The high rate of β-oxidation in the diabetic state generates a reduced redox-state inside the mitochondria. Directing acetyl-CoA into ketogenesis and not into the citrate cycle results in the generation of less NADH and enhances the oxidation capacity of the liver which might otherwise soon be limited by respiratory control. Our studies with octanoate and butyrate[2] provide evidence that the redox-state in the mitochondria and the concentration of free oxaloacetate is of major importance for the regulation of ketogenesis as has been suggested by Wieland[6,7]. This control mechanism is probably more important than the control of the entry of fatty acids into the mitochondria via the carnitine acyl transferase system[4,20].

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