Ascorbic acid, a vitamin, is observed by in vivo $^{13}$C nuclear magnetic resonance spectroscopy of rat liver

EKKEHARD KÜSTERMANN, JOACHIM SEELIG, AND BASIL KÜNNECKE
Biocenter of the University, CH-4056 Basel, Switzerland

Ascorbic acid, a vitamin, is observed by in vivo $^{13}$C nuclear magnetic resonance spectroscopy of rat liver. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E65–E71, 1998.—The first in vivo detection of a vitamin with nuclear magnetic resonance (NMR) is reported for mammalian liver. Vitamin C, also known as ascorbic acid, was monitored noninvasively in rat liver by “whole body” $^{13}$C NMR spectroscopy at high field after infusion of [1,2-$^{13}$C$_2$]glucose into anesthetized rats. Generally, the carbon resonances of ascorbic acid overlap with those of other highly abundant cellular metabolites, thus precluding their observation in situ. This problem was resolved by taking advantage of the $^{13}$C-$^{13}$C spin couplings introduced by the two covalently bound $^{13}$C nuclei in [1,2-$^{13}$C$_2$]glucose. During glucose metabolism, [5,6-$^{13}$C$_2$]ascorbic acid was synthesized, which also exhibited characteristic $^{13}$C homonuclear spin couplings. This feature enabled the spectral discrimination of ascorbic acid from overlapping singlet resonances of other metabolites. Quantitative analysis of the spin-coupling patterns provided an estimate of the turnover rate of hepatic ascorbic acid in vivo ($1.9 \pm 0.4$ nmol·min$^{-1}$·g$^{-1}$) and a novel approach toward a better understanding of optimal ascorbic acid requirements in humans. The results obtained in vivo were confirmed with high-resolution proton and $^{13}$C NMR spectroscopy of liver extracts.

Ascorbic acid turnover; glucose metabolism; isotopomer analysis; spin coupling

VITAMIN C (ASCORBIC ACID) is an indispensable metabolite in the mammalian metabolism (for reviews, see Refs. 8, 10, 28). Its deficiency leads to a severe disease of the connective tissue known as scurvy (11, 27). In recent years, the concept of ascorbic acid as a mere antiscorbutic agent has been thoroughly revised (12). Ascorbic acid is a water-soluble reductant that is reversibly oxidized to dehydroascorbate. Apart from its participation in collagen formation, ascorbic acid is a cosubstrate for a variety of mono- and dioxygenases for redox reactions in biochemical processes such as the conversion of dopamine to norepinephrine and for the metabolism of cholesterol and carnitine (10, 12, 28). Furthermore, it serves as a radical scavenger and general antioxidant for cellular metabolites including unsaturated fatty acids, vitamins A and E, and carotenoids (1, 12, 14, 27). Effects of ascorbic acid on cancer, tissue development and differentiation, the common cold, asthma, and a variety of other diseases have been reported, but the respective biochemical mechanisms have mostly remained elusive (1, 4, 6, 9, 13, 23, 28). Nevertheless, the protective potentiality for a variety of common diseases has renewed the interest in ascorbic acid. Ascorbic acid, in combination with other vitamins, is extensively used for preventive medication. This recent development has stimulated a vast number of studies for assessing the interrelations of ascorbic acid availability with metabolism. However, one of the key questions that has remained unanswered is the requirement of ascorbic acid beyond the prevention of scurvy (12, 21, 24, 31).

All mammals, with the exception of primates, guinea pigs, and a few other species, are able to synthesize ascorbic acid endogenously (27). Ascorbic acid is a product of glucose metabolism in the glucuronate pathway (30). Even though the biochemical pathway for its formation has been known in detail for almost half a century, ascorbic acid metabolism in situ has been difficult to follow in the intact organism (24). $^{13}$C nuclear magnetic resonance (NMR) spectroscopy offers the unique possibility to noninvasively assess the carbon metabolism (for a review, see Ref. 16). The low natural abundance of $^{13}$C nuclei allows a carbon position to be labeled selectively. In analogy to $^{13}$C tracer techniques, the label can be followed throughout the metabolism, however, without the need for metabolite extraction, purification, and carbon-by-carbon degradation. The extension of this labeling concept to the concurrent $^{13}$C tagging of two adjacent carbon positions allows two carbon species to be observed simultaneously (7, 17). Moreover, the detection of $^{13}$C homonuclear spin couplings, a property that is induced by the interaction of two covalently bound $^{13}$C nuclei, provides novel information on the fate of chemical bonds during metabolism (7, 17, 19, 25). This latter strategy was employed in the present study with [1,2-$^{13}$C$_2$]glucose as a cellular fuel. $^{13}$C doubly labeled molecular species can be easily distinguished from singly labeled molecules on the basis of the typical homonuclear spin couplings (17, 19). Increased spectral resolution and sensitivity achievable at high magnetic field strengths thus allow small amounts of de novo synthesized cellular products, originating from glucose metabolism, to be detected unambiguously even in the presence of metabolites that would otherwise give rise to overlapping resonances.

The purpose of the present study was to provide quantitative insight into ascorbic acid metabolism in situ. Here we report on the first noninvasive detection of ascorbic acid by high-field in vivo $^{13}$C NMR spectroscopy. The analysis of $^{13}$C homonuclear spin-coupling patterns allowed the turnover rate of hepatic ascorbic acid to be determined in intact rats. Extrapolations to the human metabolism provided an estimate of the optimal daily ascorbic acid uptake.
EXPERIMENTAL PROCEDURES

Animals, infusion techniques, and extraction procedures. All experiments were carried out with well-fed male Sprague-Dawley rats (200 ± 10 g) that had access to a standard laboratory rat diet and drinking water ad libitum. For the experiments, the individual rats were anesthetized with 1.8% isoflurane in N₂O-O₂ (3:1) and cannulated in the tail vein, and a small sample (200 µl) of venous blood was collected. The animal was placed in prone position on a specially designed Plexiglas holder, so that the liver was in the sensitive volume of the surface coil used for signal acquisition. A water-operated warming pad was placed on the back of the rat to maintain normal body temperature (measured rectally) throughout the experiment. Subsequently, [1,2,3-13C]glucose or [1-13C]glucose in isotonic NaCl solution was infused over 2 h at a rate of 8 µmol·min⁻¹·100 g body wt⁻¹, and the liver was assessed in the intact animal by in vivo 13C NMR spectroscopy. At the end of the infusion period, the animal was killed, blood samples were taken with a heparinized syringe, and the liver was removed and freeze-damped between aluminum tongs precooled in liquid nitrogen. Individual frozen liver specimens were powdered and subsequently extracted with 6% perchloric acid (7, 19). The acid extracts were neutralized, lyophilized, and redissolved in 99.9% deuterated water (H₂O-D₂O) for further analysis by high-resolution 13C and 1H NMR spectroscopy. The pH of the solution was adjusted to 7.0 (uncorrected pH reading) using deuterated perchloric acid and potassium deuteroxide (KOD); [2,2,3,3-D₄]-3-(trimethyl)-propionic acid sodium salt (TSP) was added to provide an internal concentration standard and chemical shift reference.

The infusion experiments with labeled glucose were repeated seven times. Furthermore, control experiments were carried out under identical experimental conditions using pure saline solution and unlabeled glucose in saline.

In vivo 13C NMR spectroscopy. In vivo 13C NMR spectroscopy (at 75.5 MHz) was performed on a Bruker BioSpec 70/20 equipped with a 7-T horizontal bore magnet suitable for “whole body” examinations of laboratory animals. A home-built concentric surface coil setup with an inner 13C coil (diam 2.3 cm, single turn) and an outer 1H coil (diam 3.5 cm, single turn) was used for signal excitation and acquisition. Blocks of 600 scans (9 min) were acquired in the pulse-acquire mode (0.9-s interpulse delay) with 1H decoupling during the acquisition only. Liver spectra were collected with 4 kwords and 250-ppm spectral width. The excitation pulse (40 µs) was adjusted to 180° at the coil center to minimize signals from superficial tissues (7, 19). Decoupling was performed using a WALTZ16 sequence with a power of 5 W, thus limiting the average power deposition in the animal to well below 8 W/kg. Typically, 4 spectra were acquired during the preinfusional period followed by 13 spectra collected during the infusion. If required, two to three subsequent acquisition blocks were summed to obtain better signal-to-noise ratios. All spectra were processed with a 10-Hz exponential apodization before Fourier transformation. Chemical shifts were referenced relative to glucose C18 at 96.7 ppm. Peak assignments were based on literature values (7, 22), reference spectra of pure compounds, and spiking experiments, in which the appropriate authentic compounds were added to the extracts.

In vitro two-dimensional 13C NMR spectroscopy. In vitro two-dimensional homonuclear 13C-1H chemical shift correlated spectroscopy (COSY) of tissue extracts was performed using a [90°-t₁-180°-acquire(t₂)] sequence with broad-band proton decoupling. Spectra were collected into a 128 × 2,048 data-point time-domain matrix with spectral widths of 20 kHz (200 ppm) along the t₁ and t₂ directions. A total recycle delay of 3 s was used, and the signal was averaged over a 16-step phase cycle for every t₁ value. Sine-Bell window functions were used along t₁ and t₂ directions before two-dimensional Fourier transformation.

In vivo high-resolution 1H NMR spectroscopy. High-resolution proton spectroscopy (at 400 MHz) of extracts was carried out on the same Bruker MSL 400 NMR spectrometer. The acquisition conditions were as follows: 27°C, H₂O lock, 90° pulses, 5,000-Hz spectral width, and 32 kwords. The residual deuterium signal of water was eliminated by a DANTE presaturating pulse train consisting of 10,000 9° pulses with 200-µs interpulse delay. A total recycle delay of 7 s was used to minimize the effects of different relaxation times of protons bound to 13C and 1H. Chemical shifts were referred to internal TSP at 0.0 ppm.

In vivo two-dimensional proton-detected 13C-correlated NMR spectroscopy. A pulse sequence for heteronuclear multiple-bond correlation (HMBC) spectroscopy as originally proposed by Bax and Summers (3) was implemented on the same Bruker MSL 400 spectrometer. Direct 13C-1H correlations were suppressed in the leading J filter, which was set to 135 Hz. Multiple-bond correlations were emphasized by setting the mixing time to 72 ms, corresponding to 2JCH = 7 Hz. The selection of the desired coherence pathway was achieved with a 16-step phase cycle. The residual signal was further suppressed by using a DANTE presaturating pulse train consisting of 5,000 9° pulses with 200-µs interpulse delay. Spectra were collected using a 256 × 4k data points time-domain matrix. For the t₁ dimension (13C) a spectral width of 20 kHz (200 ppm) was used, whereas the data in the t₂ direction (1H) was acquired with a spectral width of 4 kHz (10 ppm). A total recycle delay of 4 s was used. Sine-Bell window functions were applied along the t₁ and t₂ directions before two-dimensional Fourier transformation.

Quantifications. Blood glucose concentrations and hepatic glucose concentrations were quantified immediately after sample collection using the Trinder glucose assay kit (Sigma Chemical, Switzerland), which is based on a colorimetric reaction coupled to the oxidation of glucose by glucose oxidase. 13C satellites detected in 1H NMR spectra and resonance integrals determined in 13C NMR spectra were used to ascertain the position-specific fractional 13C enrichments of hepatic glucose as described previously (7, 19).

Hepatic ascorbic acid concentrations were determined colorimetrically in liver extracts according to Beutler (5). Briefly, ascorbic acid and other reducing agents present in liver tissue were oxidized rather unspecifically by the reaction mixture allowing the formation of a dye. In a parallel assay, the tissue extract was treated with l-ascorbate oxidase to selectively oxidized ascorbic acid and only then subjected to the colorimetric reaction. This second assay determined the blank value due to the unspecific oxidation of reducing agents other than ascorbic acid.

Hepatic glucose 6-phosphate concentrations were determined enzymatically by exploiting the selective and quantitative conversion of glucose 6-phosphate to gluconate 6-
phosphate with concomitant reduction of NADP⁺ to NADPH (26). The change in the NADPH concentration associated with the oxidation of glucose 6-phosphate was determined spectrophotometrically at 339 nm.

All values are expressed as means ± SE calculated from seven experiments.

Materials. [1,2-13C]glucose (99% 13C enriched) and 2H₂O (99.9% deuterium enriched) were obtained from Isotec (Miamisburg, OH), and [1,2-13C]glucose (99% 13C enriched) was purchased from Omicron Biochemicals (South Bend, IN).

RESULTS

Figure 1 displays a typical in vivo 13C NMR spectrum of the liver of an intact rat after 2 h of [1,2-13C₂]glucose infusion. For clarity, only the aliphatic region is presented. The in vivo spectrum is dominated by the natural abundance (na) resonances of lipids. Smaller signals were obtained from the infused [1,2-13C₂]glucose (resonances 2–5). Products of the hepatic glucose metabolism were also clearly visible and assigned according to previous work (7, 19, 20, 22). The aliphatic region contains resonances of de novo synthesized glycogen C₁ (resonance 1), lactate C₂ (resonance 8), glycerol C₁ plus C₃ (resonance 9), glycerol C₆ (resonance 10), lactate C₃ (resonance 11), and alanine C₃ (resonance 12), which all show characteristic multiplet structures due to 13C homonuclear spin couplings of covalently bound 13C nuclei initially introduced by [1,2-13C₂]glucose. In addition to these well-known hepatic metabolite resonances, a novel signal was observed at 70.4 ppm. Figure 1 (inset) depicts an expansion of the spectral region at 70.4 ppm. The novel resonance reveals a pseudotriplet structure consisting of two outer lines separated by 42 Hz (resonance 6) and an inner line (resonance 7) shifted off-center to higher frequencies by mere 0.027 ppm.

To identify the molecular origin of the resonance pattern at 70.4 ppm, liver extracts were prepared and analyzed with high-resolution one- and two-dimensional 13C and 1H NMR spectroscopy. Figure 2A displays relevant portions of a 13C NMR spectrum acquired from a perchloric acid extract of the liver obtained from a rat infused with [1,2-13C₂]glucose. Figure 2B shows the same extract spectrum, processed with a 15-Hz exponential line broadening to approximate line widths typically obtained in vivo spectra. For comparative purposes, Fig. 2C presents corresponding parts of an in vivo 13C NMR spectrum of the rat liver used for preparing the extract. A remarkable similarity between the spectral features obtained in vivo and from the extract can be ascertained. The high spectral resolution obtained in the extract spectrum (Fig. 2A) allowed the main components of the region at 70.4 ppm to be identified as a doublet and an inner singlet...
NMR spectroscopy (13C-COSY) was carried out. [1,2-13C2]glucose. The off-diagonal correlations are of acquired from the extract of a liver of a rat infused with 13C nuclei in a highly enriched molecular species. 13C homonuclear spin couplings, a feature uniquely available to NMR techniques, provide novel information on the covalently linked coupling partners. In the present study, spin couplings were exploited to further analyze the molecular origin of the doublet resonance detected at 70.4 ppm. Careful spectral analysis furnished a potential coupling partner that gave rise to a doublet at 63.5 ppm (see Fig. 2A, right). Unfortunately, this novel resonance partially overlapped with a more intense doublet signal previously identified as arising from carbons C1 and C3 of [2,3-13C2]glycerol (20). Spiking experiments suggested that the resonances at 70.4 and 63.5 ppm belonged to carbons C5 and C6 of ascorbic acid, respectively. To substantiate these latter assignments two-dimensional 13C-13C correlated 13C NMR spectroscopy (13C-COSY) was carried out.

Figure 3 displays the relevant portion of a 13C-COSY acquired from the extract of a liver of a rat infused with [1,2-13C2]glucose. The off-diagonal correlations are of primary interest, since they reflect 13C-13C connectivities in molecular species. For simplicity, we shall focus on the upper off-diagonal correlations only (upper and lower off-diagonal correlations essentially contain the same information). Major correlations attributable to glucose C5a-C6a (resonance D), glucose C5β-C6β (resonance F), glycerol C2-C3 (resonance E), and glycerol 3-phosphate C2-C3 (resonance C) are clearly discernible. A strong cross peak at 70.4/63.5 ppm (resonance A) indicates that the doublet resonances detected in 13C NMR spectra at 70.4 and 63.5 ppm indeed arise from the same molecular species, as expected from the structure of the C5-C6 moiety of ascorbic acid.

Further evidence for the assignment of the resonances at 70.4 and 63.5 ppm to ascorbic acid C5 and C6, respectively, was obtained by proton NMR spectroscopy. Two-dimensional 1H-detected 13C-COSY, optimized for detecting long-range heteronuclear couplings (HMBC), was carried out on liver extracts prepared from rats infused with [1-13C]glucose. Figure 4 shows a contour plot of the corresponding region of interest where a large number of correlations can be detected. In particular, those of glycerol dominate the spectral region at 63.5 ppm. The cross peaks at 4.05/63.5 and 4.53/63.5 ppm were ascribed to the correlations of ascorbic acid H5-C6 and H4-C6, respectively. These correlations corresponded well with the chemical shifts of pure ascorbic acid as determined by one-dimensional 13C and 1H NMR spectroscopy (Table 1). It should be noted for the interpretation of these latter experiments with [1-13C]glucose that ascorbic acid is exclusively labeled in position C6 (refer to DISCUSSION and Ref. 30). Due to this, no further strong H-C correlations can be anticipated for ascorbic acid under the prevailing conditions, where direct 1JHC couplings were attenuated and the 12C concentration in positions other than C6 was too low to be detected.

The quantitative analysis of the labeling patterns observed with 13C NMR spectroscopy furnishes an estimate of the turnover of ascorbic acid in situ, provided that hepatic glucose accurately reflects the labeling of the glucose 6-phosphate pool as was shown previously (18). In a first step, the fraction of de novo...
could readily be evaluated in situ as is exemplified in enrichment of \([5,6-^{13}C_2]\) ascorbic acid compared with the relative concentrations of \(^{13}C\)-containing metabolites \((7, 19, 17, 25)\). The integrated intensities of \(^{13}C\) has been replenished with freshly formed molecular not the entire but only a part of the ascorbic acid pool for glucose C1 and C2 reflect the hepatic pool of molecules originating from the precursor \([1,2-^{13}C_2]\)glucose and \([5,6-^{13}C_2]\)ascorbic acid isotopomers among the overlap of its NMR resonances with those of cellular metabolites.\(^1\) The doublet resonances detected for ascorbic acid including \([1,2-^{13}C_2]\) glucose 6-phosphate, indicates that not the entire but only a part of the ascorbic acid pool has been replenished with freshly formed molecular species \((7, 19, 17, 25)\). The integrated intensities of \(^{13}C\) NMR resonances are a direct measure of the abundance of \(^{13}C\) nuclei and can thus be used to determine the relative concentrations of \(^{13}C\)-containing metabolites.\(^1\) The doublet resonances detected for ascorbic acid C5 and C6 are due exclusively to de novo synthesized molecules originating from the precursor \([1,2-^{13}C_2]\)glucose. Similarly, the doublet resonances detected for glucose C1 and C2 reflect the hepatic pool of \([1,2-^{13}C_2]\)glucose administered to the animal.\(^2\) Hence, the ratio of \([5,6-^{13}C_2]\) ascorbic acid to \([1,2-^{13}C_2]\)glucose could readily be evaluated in situ as is exemplified in synthesized ascorbic acid (relative turnover\(_{ASCA}\)) was calculated according to Eq. 1

\[
\text{Relative turnover}_{ASCA} = \frac{p_{ASCA}}{p_{Glc12}} = \frac{[5,6-^{13}C_2] \text{ ascorbic acid} \cdot [\text{total glucose}]}{[1,2-^{13}C_2] \text{ glucose} (1)
\]

where \(p_{ASCA}\) and \(p_{Glc12}\) are the respective fractional enrichments of \([5,6-^{13}C_2]\) ascorbic acid, i.e., the relative occurrence of \([5,6-^{13}C_2]\) ascorbic acid isotopomers among the total pool of ascorbic acid and \([1,2-^{13}C_2]\) glucose (including \([1,2^{13}C_2]\) glucose 6-phosphate). The rationale of this approach is the fact that a reduced fractional enrichment of \([5,6-^{13}C_2]\) ascorbic acid compared with that of its direct precursor, hepatic \([1,2-^{13}C_2]\) glucose (including \([1,2^{13}C_2]\) glucose 6-phosphate), indicates that not the entire but only a part of the ascorbic acid pool has been replenished with freshly formed molecular species \((7, 19, 17, 25)\). The integrated intensities of \(^{13}C\) NMR resonances are a direct measure of the abundance of \(^{13}C\) nuclei and can thus be used to determine the relative concentrations of \(^{13}C\)-containing metabolites.\(^1\) The doublet resonances detected for ascorbic acid C5 and C6 are due exclusively to de novo synthesized molecules originating from the precursor \([1,2-^{13}C_2]\)glucose. Similarly, the doublet resonances detected for glucose C1 and C2 reflect the hepatic pool of \([1,2-^{13}C_2]\)glucose administered to the animal.\(^2\) Hence, the ratio of \([5,6-^{13}C_2]\) ascorbic acid to \([1,2-^{13}C_2]\)glucose could readily be evaluated in situ as is exemplified in

\(^1\) The quantitative analysis is based on the premise that the NOE/saturation is equal for the resonances of the structurally similar parts of glucose and ascorbic acid.

\(^2\) Glucose and glucose 6-phosphate usually coresonate in vivo \(^{13}C\) NMR spectra. The integrated resonance intensities of \([1,2-^{13}C_2]\)glucose were therefore corrected for the <6% contribution of glucose 6-phosphate to the sum of the pools of free and bound hepatic glucose (refer to biochemical quantifications given below).

Table 1. \(^{13}C\) and \(^1H\) NMR chemical shifts and scalar couplings of individual carbons and protons of ascorbic acid

<table>
<thead>
<tr>
<th>Position</th>
<th>Chemical Shift (^{13}C) NMR, ppm</th>
<th>Coupling (^1J_{CC}), Hz</th>
<th>Chemical Shift (^1H) NMR, ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>177.82</td>
<td>91.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>113.96</td>
<td>91.6/84.3(^*)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>175.81</td>
<td>84.3/43.1(^*)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>79.15</td>
<td>43.1/40.9(^*)</td>
<td>150.3</td>
</tr>
<tr>
<td>5</td>
<td>70.37</td>
<td>40.9/40.9(^*)</td>
<td>143.4</td>
</tr>
<tr>
<td>6</td>
<td>63.49</td>
<td>40.9</td>
<td>144.2</td>
</tr>
</tbody>
</table>

Authentic ascorbic acid, traces of \([1-^{13}C]\) glucose and \([2,2,3,3-D_4]\) 3-(trimethyl)propanoic acid sodium salt (TSP) were dissolved in 99.9% \(^2H_2O\) at pH 7.0 (uncorrected pH reading) and assessed by \(^{13}C\) and \(^1H\) nuclear magnetic resonance (NMR) spectroscopy at 100 and 400 MHz, respectively. Chemical shifts are referenced to internal glucose \((C1 \text{ at } 96.7 \text{ ppm})\) and TSP \((0.00 \text{ ppm})\) for the \(^{13}C\) and \(^1H\) spectral domain, respectively. \(^*\) Different coupling constants attributable to \(^1J_{CC}\) couplings with 2 chemically inequivalent carbon neighbors. \(^†\) \(^1H\) NMR shifts of 2 chemically inequivalent protons \(H^6\) and \(H^{6'}\).

**DISCUSSION**

Over the last few years, it has been recognized that ascorbic acid is involved in a great variety of biochemical processes beyond the prevention of scurvy \((4, 10, 12, 28)\). The prime function attributed to ascorbic acid is its antioxidant potential as a scavenger for reactive oxygen species and other oxidants \((1, 12, 27, 28)\). However, the many new beneficial roles ascribed to ascorbic acid have relaunched the debate as to what the minimal, optimal, and maximal ascorbic acid allowance should be. One of the major difficulties encountered in answering this question is the direct assessment of ascorbic acid metabolism in vivo, which has been notoriously difficult, if not impossible \((21, 24)\).

To the best of our knowledge, the present data are the first in vivo observation of a vitamin by \(^{13}C\) NMR spectroscopy in mammalian liver. Vitamins are generally thought to be required by the body in fairly small amounts and, at first sight, seem to be out of the reach of noninvasive detection by in vivo NMR spectroscopy. However, ascorbic acid is present in mammalian liver at concentrations well above 100 nmol/g, and values as high as 2.2 µmol/g have been reported for rat liver \((15, 21)\). In vivo assessment of ascorbic acid is therefore not primarily restricted by the limited availability of the vitamin but rather by the extensive overlap of its NMR resonances with those of cellular metabolites present at much higher concentrations. In the present study, this problem was resolved by taking advantage of advanced \(^{13}C\)-labeling strategies.

In the context of \(^{13}C\) labeling, it is noteworthy that all mammals are able to synthesize ascorbic acid endogenously, with the exception of a few species, including primates and guinea pigs, which lack the enzyme L-gulono-\(\gamma\)-lactone oxidase, which catalyzes the uti-
mate reaction in the synthetic pathway (8, 27, 30). To better visualize the flow of \(^{13}\text{C}\) label, Fig. 5 depicts schematically the pathway of ascorbic acid synthesis in rat liver as proposed on the grounds of previous biochemical analyses (30). Briefly, glucose is converted in several steps to glucuronate and further to gulonate, gulono-\(\gamma\)-lactone, 2-oxogulono-\(\gamma\)-lactone, and finally to ascorbic acid, while retaining the original carbon skeleton of glucose. This sequence of reactions potentially allows ascorbic acid metabolism to be assessed in labeling experiments using \([^{13}\text{C}]\)glucose as substrate.

Conventional labeling strategies that make use of \([1,^{13}\text{C}]\)glucose or \([2-^{13}\text{C}]\)glucose are, however, prone to failure, since the resulting resonances of ascorbic acid (Table 1) overlap with those of metabolites that also become labeled during \([^{13}\text{C}]\)glucose metabolism (16). In contrast, \(^{13}\text{C}\) homonuclear spin couplings introduced by doubly labeled \([1,2,^{13}\text{C}_2]\)glucose are rather unusual features in \(^{13}\text{C}\) NMR spectra, which provide novel biochemical information. The occurrence of \(^{13}\text{C}\) homonuclear spin couplings at natural abundance \(^{13}\text{C}\) concentration has a negligible probability, e.g., 0.012% for a C2 segment, and thus couplings are only detected as long as two artificially introduced \(^{13}\text{C}\) nuclei are chemically bound together. Such spin coupling leads to characteristic splittings of the corresponding resonances into doublets that can be readily distinguished from the singlet resonances of monolabeled species.

According to Fig. 5, infusion of \([1,2,^{13}\text{C}_2]\)glucose should lead to the synthesis of \([5,6-^{13}\text{C}_2]\)ascorbic acid, which is characterized by doublet resonances for carbons C5 and C6. This was borne out experimentally in vivo in the livers of intact rats and in liver extracts as is exemplified in Figs. 1 and 2, respectively. In vivo \(^{13}\text{C}\) NMR spectroscopy clearly revealed a doublet resonance due to \([5,6-^{13}\text{C}_2]\)ascorbic acid C5 that framed a singlet resonance at 70.4 ppm (Fig. 1). This inner singlet resonance was found to belong to glucose C4, which remained \(^{13}\text{C}\) labeled to an extent only slightly above the natural abundance level. \(^{13}\text{C}\) NMR spectra of corresponding liver extracts, obtained at the end of the infusion, corroborated the results obtained in situ (Fig. 2). Analogous processing of the extract spectra (Fig. 2B) and in vivo spectra (Fig. 2C) with exponential windowing lead to almost identical spectral patterns. The ascorbic acid C6 signal was detectable only in extract spectra because, in vivo, it overlapped with the C3 resonance of \([2,3-^{13}\text{C}_2]\)glycerol, which was also derived from \([1,2-^{13}\text{C}_2]\)glucose (20). The doublet resonances observed for ascorbic acid, C5 and C6, the distinct pair of correlations detected at 70.4/63.5 ppm in \(^{13}\text{C}\)-COSY of tissue extracts (Fig. 3), and the absence of any singlet resonances pertaining to ascorbic acid fully support the metabolic pathway (Fig. 5) proposed previously for ascorbic acid biosynthesis from glucose (30).

Traditionally, ascorbic acid requirements have been evaluated using depletion and feeding experiments or epidemiological investigations (12, 31). In this study, a different approach was adopted. Rats were used on the premise that, in the unperturbed metabolism, ascorbic acid requirement and endogenous synthesis would be in balance. Rapid and extensive label incorporation into ascorbic acid, as demonstrated with in vivo \(^{13}\text{C}\) NMR spectroscopy, indicates that ascorbic acid was indeed readily synthesized in rat liver. Although \(^{13}\text{C}\) NMR spectroscopy, as such, is a noninvasive modality, increased availability of glucose during the infusion period could have driven the synthesis of ascorbic acid. Blood glucose concentrations, however, were found to be maintained at normal levels, even during prolonged glucose-infusions. Furthermore, hepatic glucose, glucose 6-phosphate, and ascorbic acid concentrations in glucose-infused animals and controls did not differ. We thus conclude that in vivo \(^{13}\text{C}\) NMR spectroscopy at high field allows ascorbic acid in the liver of intact rats to be monitored at physiological concentrations.

Apart from a mere qualitative detection of ascorbic acid metabolism, \(^{13}\text{C}\)-labeling patterns observed with \(^{13}\text{C}\) NMR spectroscopy provided a quantitative estimate of the ascorbic acid turnover in situ. At metabolic steady state, the rate of ascorbic acid synthesis (and degradation) was calculated to be 1.9 \( \pm \) 0.4 nmol \( \cdot \) min\(^{-1} \) \( \cdot \) g\(^{-1} \) liver tissue (Eq. 1). This turnover rate is 32 times faster than previously reported by Kipp and Rivers (15), but at least twofold slower than determined by Schmidt et al. (29) in experiments based on \([1-^{14}\text{C}]\)ascorbic acid administration to rats and guinea pigs. In contrast to the direct assessment of ascorbic acid metabolism, as demonstrated herein, the rate of appearance of radioactivity in excrements was used in the latter experiments as a measure of ascorbic acid turnover. This approach, however, suffers severely from the drawback that the retention of radioactivity in metabolites other than ascorbic acid remains undetec-
detected and may, in turn, significantly decrease the apparent rate of ascorbic acid metabolism.

Although overt ascorbic acid deficiency in humans is manifest by scurvy symptoms, the optimal requirement, i.e., the most suitable dosage of the vitamin that enhances body functions, has remained elusive (21). Here, a new approach is presented, which is based on the premise that ascorbic acid synthesis in rats would not be at an absolute minimum but rather at an optimum. Considering the balance between ascorbic acid requirement and synthesis in rat liver, a simple extrapolation (based on body weight) of the ascorbic acid turnover rate determined in rats (1.9 nmol·min⁻¹·g⁻¹) to the human situation suggests that a daily uptake of ~1 g ascorbic acid is optimal. Although this simple approach has obvious limitations, it is noteworthy that supplementation experiments carried out with human subjects showed significant enhancements of various body functions with ascorbic acid allowances of up to 1 g/day (for review, see Ref. 31).

In summary, the increased spectral resolution and sensitivity of in vivo ¹³C NMR spectroscopy at high magnetic field strength provided a means for observing metabolites that previously eluded detection in situ. If particular advantage is taken of ¹³C homonuclear spin couplings introduced by doubly labeled [¹,²-¹³C₂]glucose, hepatic ascorbic acid could be identified, and its de novo synthesis could be monitored noninvasively in rat liver. The quantitative analysis of ¹³C homonuclear spin-coupling patterns allowed the turnover rate of ascorbic acid to be evaluated. The potentiality of the method presented thus lends itself to a large variety of in vivo studies, where the interrelations of metabolism, disease, and ascorbic acid availability can be further assessed. Extensions to studies on human subjects should be readily feasible, since the only changes to the method needed are the replacement of [¹,²-¹³C₂]glucose for [⁵,⁶-¹³C₂]ascorbate and the detection of ascorbic acid consumption rather than synthesis.

We are indebted to Dr. Peter Scherer (Spectrospin, Switzerland) for expertise and support in the analyses of tissue extracts with the heteronuclear multiple-bond correlation spectroscopy. This work was supported by the Swiss National Science Foundation (Grant 3100.49758.96) and the Doerenkamp-Zbinden Foundation.

Present address of E. Küstermann: NMR Center, Massachusetts General Hospital, Charlestown, MA 02129.

Address for reprint requests: B. Künecke, Dept. of Biophysical Chemistry, Klingelbergstr. 70, CH-4056 Basel, Switzerland.

REFERENCES


