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NMR in Physiology and Biomedicine

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I. INTRODUCTION

The structural organization of the liver is adapted to its special function as an intermediary control element between the dietary load from the gastrointestinal tract and the extrahepatic tissues. In that capacity, the liver handles large amounts of amino acids, carbohydrates, lipids, and other molecules (such as xenobiotics) that enter the body in nutrients and water. Major functions of the liver involve the biosynthesis of urea, glutamine, ketone bodies, triacylglycerols, and plasmaproteins as well as the important processes of the glucose homeostasis, gluconeogenesis, glycogenesis, and glycogenolysis. In addition it plays an important role in transformation of xenobiotic pollutants and drugs.

The hepatocyte is the most abundant cell in the liver and carries out the major functions of the organ. There are around 250 billion hepatocytes in the adult human liver. The average hepatocyte diameter is 20–22 μm and these parenchymal cells represent 60% of the cells in the liver. Since they are larger than other cells, they occupy about 80% of the parenchymal volume. The liver has a unique microcirculation, apparently defining parenchymal zones of a different isometabolic potential. This metabolic organization is termed metabolic zonation.

Thus, despite the fact that each hepatocyte has the same genome, the expression of many key enzymes depends on whether the cell is located in the upstream (periportal) or downstream (pericentral) zones. This heterogeneity of gene expression is likely to be regulated by factors which display a gradient along the sinusoids, notably oxygen tension and hormones, but at present there is no clear understanding either of the regulation of zonation nor of the role of zonation as a regulatory factor of hepatic metabolism.

Nature has produced a very complicated organ with a large number of integrated metabolic functions, yet we have only a partial knowledge of the mechanisms applied in the control and regulation of these pathways and an even lesser understanding of the regulation of the metabolic role played by the liver in its interaction with other organs of the body. The advantages of NMR methods to further investigate liver metabolism are numerous, since NMR provides a unique noninvasive window permitting an "on-line" monitoring of a wide variety of metabolic processes including cellular energetics; amino acid, carbohydrate, and lipid metabolism; pH regulation; and compartmentation. In addition, the method is applicable on isolated subcellular organelles (mitochondria), isolated cells (hepatocytes), perfused

liver, and liver *in vivo* in animals and human beings. Consequently, the applications of NMR are continually growing as different aspects of this rich methodology become adapted to use under a variety of experimental conditions.

The majority of hepatic NMR metabolic studies has focused on the phosphorus-31 and carbon-13 nuclei. They included investigations of isolated rat liver mitochondria and hepatocytes (Ogawa *et al.*, 1978; Cohen *et al.*, 1978) and perfused liver (McLaughlin *et al.*, 1979; Salhany *et al.*, 1979; Iles *et al.*, 1980; Iles and Griffiths, 1982; Cohen, 1983a; Desmoulin *et al.*, 1987a) and *in vivo* studies of animal and human livers (Quistorff *et al.*, 1983; Malloy *et al.*, 1986; Schmidt *et al.*, 1986; Dufour *et al.*, 1991; Cox *et al.*, 1992). ^{31}P NMR spectra can be obtained within few minutes and interpreted very readily since only seven to nine signals are observed. However, these signals give an excellent reflection of organ metabolism, e.g., energy content (ATP) and intracellular pH. Carbon-13 is only 1.1% abundant in nature and, consequently, only certain metabolites occurring at high concentrations (e.g., glycogen, lipids) can be detected at natural abundance (Canioni *et al.*, 1983; Jue *et al.*, 1989; Quistorff *et al.*, 1993). In order to record other ^{13}C NMR signals, it is necessary to use ^{13}C -enriched substrates, and, in this way, it is possible to carry out experiments which are analogous in many aspects to radioactive tracer experiments (Cohen *et al.*, 1979, 1981; Cohen, 1983b; Pahl-Wostl and Seelig, 1986). Other nuclei have been observed from the liver including ^1H , ^{15}N (which can be applied experimentally in a manner similar to ^{13}C), ^{23}Na , and ^{19}F (Cabanac *et al.*, 1988; Farrow *et al.*, 1990; Rafter *et al.*, 1991; Orii *et al.*, 1992). Some examples are given in this chapter. Finally, an important feature of the NMR method is the possibility of obtaining spatially resolved liver spectra leading to localized metabolic information, thus opening a new window to study pathology and the effects of therapy.

II. PHOSPHORUS-31 NMR OF LIVER

In this section, we discuss the different types of information that are available from ^{31}P NMR spectra of the liver which include the direct measurement of intracellular free concentrations of mobile metabolites and their NMR visibility (since phosphorylated metabolites with restricted molecular motion can lead to very broad or nondetectable NMR signals). Intracellular metabolic compartmentation such as cytosolic and intramitochondrial pH are also accessible under particular experimental conditions. Finally, other information on the cellular environment, such as the concentration of free and

ATP-bound Mg^{2+} , may be estimated by the ^{31}P NMR method.

A. Phosphorus-31 NMR Spectrum of the Liver

1. Resonance Assignments

A typical ^{31}P NMR spectrum of an excised perfused rat liver is shown in Fig. 1A. The apparent resolution in the 0–10 ppm region is increased by switching the temperature of the perfusate from 37 to 4°C (Fig. 1B). The three major resonances of the 0 to –20 ppm region are

FIGURE 1 Phosphorus-31 NMR spectra of an isolated rat liver perfused at 37°C (A) or 4°C (B). Spectra were recorded at 162 MHz without proton decoupling (perfusion flow rate: 3 ml/min g liver wet weight, Wistar rats of 80–100g body weight, perfusion with Krebs-Henseleit buffer, pH 7.4, 25 mM bicarbonate). 5000 Hz spectral width, 10 Hz line broadening, 240 scans, 25 μs (47.5°) pulse width, 0.5 s interpulse delay. Abbreviations: MDPA, methylene diphosphonic acid taken as reference at 18.4 ppm; PME, phosphomonoesters identified as mainly phosphorylcholine and sn-glycerol-3-phosphate; Pi, inorganic phosphate; GPC, glycerol-3-phosphorylcholine and GPE, glycerol-3-phosphorylethanolamine; NTP, NDP, nucleoside tri- and di-phosphates; DPDE, diphosphodiester, mainly NAD $^{+}$, NADH and uridine 5'-diphosphoglucose and derivatives (Reprinted from Delmas-Beauvieux *et al.*, 1992a, with permission).

due to the three phosphate groups of nucleoside triphosphates (NTP), mainly ATP, with 0 ppm defined by the resonance of a 80% phosphoric acid. Additional peaks arise from diphosphodiester (NAD⁺/NADH and uridinediphosphoglucose (UDPG) and derivatives). Inorganic pyrophosphate (PP_i) is located about 1 ppm upfield of γ -NTP, but appear to be NMR invisible *in vivo* even when present at high concentrations, although it is observable in perchloric acid (PCA) extracts of the liver (Vecch *et al.*, 1986). In the 0 to 10 ppm region, the resonances are from phosphodiester (mainly glycerophosphocholine, GPC, and glycerophosphoethanolamine, GPE) inorganic phosphate (P_i), and the large group of phosphomonoesters (PME) which include compounds like phosphorylcholine (PC), AMP, and *sn*-glycerol-3-phosphate, Gro-3-P. In certain circumstances like fructose metabolism the PME peak may increase very significantly due to the accumulation of fructose-1-phosphate (Van den Berghe, 1978; Iles *et al.*, 1980). Note that the resonances of glucose-1-phosphate and ribose-1-phosphate do not belong to the PME peak, but at normal pH will be hidden under the P_i peak (Quistorff *et al.*, 1988). The assignments of most metabolite resonances detected in the liver spectrum have already been well-documented and are summarized in Table I (see also Appendix). Chemical-shift values are given relative to the resonance of methylene diphos-

phonic acid in a sealed capillary, used as a standard at 18.4 ppm. In spectra of perfused liver (or spectra recorded *in vivo*), the α - and β -phosphate resonances of NDP overlap with the α - and γ -phosphate resonances of NTP; these signals are sufficiently well-resolved in spectra recorded from perchloric acid extracts (Desmoulin *et al.*, 1987a; Iles *et al.*, 1985); likewise, the PME peak may be clearly resolved into its components in PCA extracts measured in a high-resolution NMR instrument. Knowledge of pH in the extract is very important for the interpretation, since the chemical shift of especially the components of the PME region are sensitive to changes in pH (see Appendix). At increased resolution induced by lowering the perfusate temperature (Fig. 1B), some resonances become well-resolved, in particular the PME group centered at 4–5 ppm. In contrast, hypothermia induces a broadening of the β -NTP resonance, probably due to a decreased rate of ATP-Mg²⁺ exchange (Gallis *et al.*, 1991).

2. Relaxation Parameters of Phosphorylated Metabolites

The longitudinal relaxation time (T_1) has been measured in liver by the standard inversion-recovery method and/or progressive saturation sequence, at various static magnetic fields (Table II). First, T_1 values determined under the same conditions of external magnetic field for NTP and intracellular P_i are much shorter in liver than in other tissues or organs, probably due to the higher concentration of paramagnetic ions in the liver than in other tissues. Second, T_1 values are dependent on external field strength and they increase as the field strength becomes higher, compatible with a dominant dipolar relaxation mechanism for these compounds. It is also noteworthy that temperature changes have small effects on T_1 relaxation times, in the 0–40°C range (Gallis *et al.*, 1991).

TABLE I Chemical-Shift Values and Intracellular Metabolite Contents as Determined by NMR from Perfused Liver Spectra and Biochemical Analysis of Liver Perchloric Acid Extracts

Compound (resonance)	Chemical shift (ppm)	Liver content	
		(μ mole/g dry wt)	
		NMR	Extract
NTP			
β	–18.6	6.5–8.0	6.9–8.9
α	–10.0		
γ	–4.9		
NDP			
α	–10.0	0.8–1.2	1.8–3.0
β	–4.9		
P _i	2.7	5.1–7.0	9.8–19.2
PME			
PC	3.19		
AMP	4.5		0.6–1.1
Gro-3P	4.7		0.5–2.3
DPDE			
NAD(H)	–10.7		
UDPG	–12.2		
PDE			
GPC	0.5		
GPE	1.0		
MDPA	18.4		

TABLE II Longitudinal Relaxation Time Values (s) for Intracellular NTP and P_i under Various External Static Magnetic Fields

Frequency (MHz)	β -NTP	P _i
25 ^a	0.35	0.97
72 ^b	0.10	0.30
81 ^c	0.15	0.60
162 ^d	0.35	0.75

Note. Data from ^a Meyerhoff *et al.* (1990); ^b Iles *et al.* (1980); ^c Desmoulin *et al.* (1987a); ^d Delmas-Beauvieux *et al.* (1992a).

3. Liver Metabolite Content, NMR Visibility, and Metabolite Compartmentation

Absolute content of phosphorylated metabolites of the liver can be determined with accuracy under conditions of high homogeneity of the radiofrequency field in the NMR cell. The metabolite peak areas are then proportional to the amount present within the detection volume of the rf coil (Tofts and Wray, 1988). Quantitative *in vivo* measurements using surface coils are more difficult to attain (Tofts, 1988). The general method consists in comparing the area of the resonance of interest with the area of an endogenous or an exogenous phosphorus-containing compound (i.e., methylene diphosphonic acid at 18.4 ppm or hexachlorocyclo-triphosphazene at 20.6 ppm (Gard and Ackerman, 1983)). Alternatively tissue water may be used as an internal concentration reference, applying a $^1\text{H}/^{31}\text{P}$ double-tuned probe and assuming a constant amount of water in the tissue (Wray and Tofts, 1986; Masson and Quistorff, 1992).

In order to optimize the signal-to-noise ratio in an experiment with a large number of acquisitions, pulse length and interpulse delay are deliberately selected so that some signal saturation occurs. Consequently appropriate saturation factors must be applied in the postprocessing of the data in order to evaluate the absolute content of each metabolite from the area of its NMR resonances (Tofts and Wray, 1988; Tofts, 1988). The accuracy of the determination is primarily dependent on the spectral resolution. As a matter of fact, NTP content can be easily estimated from the β -NTP peak which is well-resolved at -18.6 ppm. Quantification is more difficult for other metabolites which resonate in more crowded parts of the spectrum such as P_i , PME, and DPDE. An estimation of NDP content can be performed by measuring the difference between the area of the combined (γ -NTP + β -NDP) and the β -NTP peak, but this method is rather inaccurate, since NDP is calculated as a small difference between two relatively large numbers. In perfused rat liver, the NDP value obtained corresponds to 0.1 – 0.3 $\mu\text{mol/g}$ liver (Desmoulin *et al.*, 1987a; Tanaka *et al.*, 1989). Some chemical-shift data are summarized in Table I, including metabolite levels (given in $\mu\text{mol/g}$ dry wt assuming 1 g of liver dry weight = 3.75 g liver wet wt).

Concerning the β -NTP resonance, nucleoside triphosphates other than ATP contribute to this signal, including GTP, UTP, ITP, and CTP. In isolated rat liver, under well-oxygenated perfusion and at 37°C , intracellular ATP determined by HPLC analysis of perchloric acid extracts is about 80% of the total NTP content (Gallis *et al.*, 1991). The (GTP + UTP)/ATP ratio is 0.18 – 0.20 , in agreement with other biochemical studies

(Gruber *et al.*, 1974; Lawson *et al.*, 1976). This ratio can be much higher, especially under ischemic or anoxic conditions (Gallis *et al.*, 1991). While it is generally agreed that metabolites like P_i and ADP are not fully NMR visible in the liver, there are conflicting reports on the visibility of ATP, especially during metabolic transitions. This is of significant interest since the NTP peak is often used as an internal concentration reference for relative quantification of *in vivo* data. ATP was reported to be fully visible in normoxic perfused rat liver (Masson & Quistorff, 1992), during a 4-h interval of cold ischemia (Gallis *et al.*, 1991) or by *in situ* spectroscopy of the rat liver (Iles *et al.*, 1985). Also, in isolated rat liver mitochondria, ATP was fully visible over a wide range of intra- and extramitochondrial nucleotide concentrations (Hutson *et al.*, 1989). However, a number of studies involving metabolic transitions, in particular ischemia, have reported decreased visibility of ATP (Murphy *et al.*, 1988; Humphrey and Garlick 1991). One study (Murphy *et al.*, 1988) reported that only ca. 45% of the ATP was NMR visible in perfused rat liver subjected to brief intervals of ischemia. These experiments have been repeated, however, including a range of different metabolic states, and it was shown that liver ATP remains about 90% visible and is unaffected by the metabolic perturbations (Masson and Quistorff, 1992) in agreement with studies applying cryo-NMR (Ikai *et al.*, 1990). It was furthermore concluded, that the results of Murphy *et al.* (1988) might have a technical explanation, due to insufficient temperature control of the perfused organ. That NTPs are not redistributed to the mitochondrial compartment with a subsequent decrease in NMR visibility was evident from cryo-NMR measurements (Ikai *et al.*, 1990), where disruption of the mitochondrial compartment did not change the NTP signal.

Possible factors which may cause NMR invisibility in tissue include microviscosity, chemical exchange, binding to macromolecules or membranes, and association to paramagnetic ions. NDP has been shown to be essentially undetectable in heart, muscle, brain, and kidneys and only barely detectable in liver (Cunningham *et al.*, 1986; Desmoulin *et al.*, 1987a; Tanaka *et al.*, 1989). Assuming values of 1.2 and 0.6 $\mu\text{mol/g}$ liver dry wt for mitochondrial and cytosolic ADP, respectively (Akerboom *et al.*, 1978), it can be suggested that NMR visibility of NDP reflects restricted motion of this metabolite, particularly in the intramitochondrial compartment.

Likewise, intracellular P_i is also not fully detected in the spectrum of the perfused liver (Desmoulin *et al.*, 1987a; Tanaka *et al.*, 1989) and is known to be strongly compartmented with 3.5 $\mu\text{mol/g}$ dry wt in the mitochondrial fraction and 6.7 $\mu\text{mol/g}$ dry wt in the cytosol.

Is the restricted visibility of around 70% for the NDP and 50% for the P_i related to the intracellular compartmentation of these compounds? In other words, is the mitochondrial fraction of phosphorylated metabolites only partially detectable whereas all cytosolic compounds are totally visible? This has not yet been demonstrated either *in vivo* or under experimental conditions. It should be noted, however, that in isolated rat liver mitochondria, matrix ATP appear to be fully visible over a wide concentration range (Hutson *et al.*, 1989). In the particular case of inorganic phosphate, during low-temperature perfusion (from 4 to 12°C) and in the presence of low extracellular P_i (100 μ M), it has been demonstrated in perfused mouse liver (Cohen, 1992) or rat liver (Thiaudiere *et al.*, unpublished data) that two poorly resolved resonances are present in the spectrum, in the P_i spectral region, which may originate from cyto-

solic and mitochondrial P_i , as suggested by the valinomycin (K^+ membrane ionophore) effect on their chemical shifts (Cohen, 1992) or the effect of an alkaline load (Fig. 2).

Divalent cations of Mg^{2+} (and also Ca^{2+} and Zn^{2+}) are able to produce large shifts in the signals of the β -resonance of the NTP molecules. The γ -resonance is less affected whereas the α -resonance is practically not shifted (Cohn and Hughes, 1962). The extent of ATP-Mg complexation and the concentration of intracellular free Mg^{2+} according to the fast exchange equilibrium,

can be determined by analyzing the NMR spectrum. It is possible to calculate the free Mg^{2+} content *in vivo* from the chemical shift of the ATP signals, provided that the dissociation constant K_D is known under intracellular conditions and under the assumption that no factors other than pH or Mg^{2+} binding affect the ATP resonance frequencies (Gupta, 1986). Using K_D values of about 30–50 μ M, intracellular free Mg^{2+} concentrations in the range of 400–800 μ M were calculated (Desmoulin *et al.*, 1987a). Values of 0.46 and 0.7 mM have been reported for perfused liver and liver *in vivo*, respectively (Malloy *et al.*, 1986; Cohen, 1983b).

FIGURE 2 Intracellular P_i compartmentation in the perfused rat liver. The liver was perfused as in Fig. 1, except the medium which was a bicarbonate-free Krebs–Henseleit buffer, containing 0.1 mM inorganic phosphate (4°C and pH 6.5); under these conditions, cytosolic pH is 6.6 and mitochondrial pH is 7.6 (A). An alkaline load (20 mM NH_4Cl) was then performed for 10 min, inducing an increase of cytosolic pH value to 6.8, without affecting mitochondrial pH (B) (from Thiaudiere *et al.*, unpublished results).

B. Liver pH Homeostasis

All mammalian cells are able to maintain a higher intracellular pH (pH_{in}) than predicted from the Nernst equilibrium (Calvey, 1971). The pH gradient can be maintained both by a high buffering capacity of the cytosol and by ion-transport mechanisms (Madhus. 1988; Gores *et al.*, 1989). In hepatocytes, the pH_{in} -regulatory systems have been only partly characterized: (i) the amiloride-sensitive Na^+/H^+ -exchange system located on the basolateral membrane (Henderson *et al.*, 1987; Grinstein *et al.*, 1989), (ii) the Cl^-/HCO_3^- -exchange inhibited by stilbene derivatives present on the canalicular membrane (Meier *et al.*, 1985), and (iii) the Cl^- -independent Na^+ -dependent HCO_3^- cotransport, as reported more recently and located on the basolateral membrane (Renner *et al.*, 1989). Intracellular pH of the intact liver can be determined directly, while preserving tissue integrity, by NMR spectroscopy. The chemical shift of nuclei proximal to rapidly exchanging chemical groups can be used to measure the pH of the system when it is near the pK_a of the exchanging group.

The intracellular liver P_i signal is readily observable and its resonance is particularly sensitive to pH changes on the 6.7 to 7.5 pH unit range. In liver perfused with a Krebs–Henseleit medium (25 mM bicarbonate, pH 7.4),

at 37°C and under normoxic conditions, the P_i resonance reflects essentially the cytosolic pH_{in} of hepatocytes; the measured pH_{in} value is 7.18 ± 0.03 ($n = 32$) (Durand *et al.*, 1993). The effect of an isobutyric acid load in the pH_{in} under various experimental conditions (bicarbonate-free medium, low sodium content, presence of inhibitors as amiloride and the stilbene derivative SITS) has been reported (Durand *et al.*, 1993). An illustration of the effect of amiloride and bicarbonate in the liver response to an acid load is given in Fig. 3: pH_{in} remained constant, even in the presence of amiloride (Fig. 3A) indicating that the amiloride-sensitive Na^+/H^+ exchanger is inactive at external physiological pH. In the absence of bicarbonate (or with bicarbonate, but in presence of stilbene derivatives), the acid load induced a dramatic fall of pH_{in} (Fig. 3B), indicating that the $Na^+:HCO_3^-$ symport is involved in pH homeostasis. NMR studies have also demonstrated that the Na^+/H^+ exchanger is activated under systemic acidosis as soon as pH_{in} reached a pH_{in} set point value of around 7.0 (Durand *et al.*, 1993).

FIGURE 3 Effect of amiloride on liver response to an acid load. Liver was perfused with a Krebs-Henseleit buffer at pH 7.4 in the presence or absence of bicarbonate. Livers were perfused for 15 min with 1 mM amiloride before an acid load consisting of 12 min perfusion with 35 mM isobutyric acid. Ionic composition of the perfusate was constant during the entire experiment: 147.4 mM Na^+ ; 25 mM (A) or 0 mM (B) HCO_3^- ; 127.3 mM Cl^- ; 1.2 mM SO_4^{2-} ; 1.2 mM $H_2PO_4^-/HPO_4^{2-}$; 1.3 mM Ca^{2+} ; 5.9 mM K^+ ; 1.2 mM Mg^{2+} ; 0.3 mM pyruvate; 2.1 mM lactate (reprinted from Durand *et al.*, 1993, with permission).

C. Ethanol Metabolism

Hepatic ethanol metabolism has been extensively documented and it is now well-established that ethanol administration causes a two- to threefold increase in the cytosolic and mitochondrial redox state and fall of ATP both *in vitro* and *in vivo* (Williamson *et al.*, 1969; Cunningham 1986; Masson *et al.*, 1993). The decreased redox state is probably the reason for the observed accumulation of *sn*-glycerol-3-phosphate (Desmoulin *et al.*, 1987a; Masson *et al.*, 1993), which in turn may explain the concomitant decrease of P_i which occur within minutes after exposure to an ethanol load (Williamson *et al.*, 1969). In perfused rat liver, changes in the metabolic state produced by the presence of 70 mM (0.4%) ethanol, i.e., at a concentration on the order of blood levels found in heavy alcohol drinkers, have been followed by NMR (Desmoulin *et al.*, 1987b). The main result is a quick initial decrease in cellular P_i , the magnitude of which depends on the alcohol concentration, followed by a slow recovery phase which, however, is complete only at relatively low ethanol concentrations (<2 mM). The ATP depletion has a different time course with a much slower decrease and little recovery (Masson *et al.*, 1992, 1993). These changes are qualitatively similar to what may be observed after fructose (Van den Berghe, 1978; Iles *et al.*, 1980) or xylitol load (Vincent *et al.*, 1989) and a combination of fructose and alcohol clearly aggravates the metabolic stress of the liver with almost complete exhaustion of the β -NTP signal (Quistorff *et al.*, 1988).

The mechanism of ethanol- or fructose-induced nucleotide and P_i loss appears to be explainable by the initial loss of P_i to the PME pool (*sn*-glycerol-3-P or Fru-1-P) and the subsequent deinhibition of the AMP deaminase causing the adenine-nucleotide pool loss primarily as uric acid (Van den Berghe, 1978; Masson *et al.*, 1993). Liver oxygen consumption is affected only slightly by alcohol (Masson *et al.*, 1992), although there are disagreements about this in the literature (Thurman and Sholz, 1977).

As judged by the chemical shift of P_i , intracellular pH of the liver is decreased by about 0.1 pH unit in response to ethanol infusion (Desmoulin *et al.*, 1987a) and by about 0.2 units by a fructose load of 10 mM (Iles *et al.*, 1980). Also, an upfield shift of the β -NTP has been reported in an *in vivo* experiment on rat liver, suggesting that the alcohol load caused a decreased free magnesium (Quistorff *et al.*, 1983).

In the chronically alcohol-fed rat, the P_i /ATP ratio and, to a lesser extent, the PME/ATP ratio, are higher than those in controls. Concomitant morphological studies suggest that the decrease in energy state is the primary effect of ethanol intake (Cunningham, 1986; Takahashi *et al.*, 1990). The intracellular pH of the liver

in vivo in chronically alcohol-fed rats is stable (Takahashi *et al.*, 1990; Desmoulin *et al.*, 1990), while there is a slight but continual deterioration of the ATP/ P_i ratio over a 55-day interval of ethanol feeding (Ling and Brauer, 1990).

D. Liver Preservation

Studies have demonstrated that livers can be safely preserved for more than 24 h. The current method of preservation includes flushing the donor liver with a cold preservation solution followed by hypothermic storage (Belzer and Southard, 1988). The nondestructive nature of NMR spectroscopy should be of great interest for studying the organ's ability to restore metabolic function upon flow after a period of long cold storage. Combined with near infrared (NIR) spectroscopy which monitors organ hemoglobin (and myoglobin) saturation (Chance *et al.*, 1990) and surface fluorescence measurements (Chance *et al.*, 1962) providing information on the redox state, very detailed information could be made available on the metabolic state of the liver. However, no studies combining all of these methods are available yet, and only a few have been performed using ^{31}P NMR to investigate the effect of cold ischemia and subsequent normothermic reperfusion on the energetic parameters of the liver (Lanir *et al.*, 1987; Busza *et al.*, 1988). In particular, the method is very well suited to test the performance of various preservation media (Delmas-Beauvieux *et al.*, 1992a). It has been demonstrated that the rate of decrease of NTP and pH_m is strongly dependent on the nature of the preservation medium. In contrast NTP recovery was not significantly affected after 18-h postischemic reperfusion in various preservation media. Accordingly, intracellular NTP values after 8 h ischemia were chosen to estimate the performance of various preservation media (Delmas-Beauvieux *et al.*, 1992a,b). In a study on human liver transplants (Tanaka *et al.*, 1993) it was shown that the liver oxygenation as measured with preoperative NIR spectroscopy (Chance *et al.*, 1990) recovered quickly following reperfusion when connected to recipient circulation. However, the redox state of the liver tissue as measured by redox ratio scanning (Quistorff *et al.*, 1986) on freeze-clamped biopsies and arterial β -hydroxybutyrate/acetoacetate ratios recovered only slowly over the course of 8–16 h. So far there appear to be no studies correlating the NMR-determined P_i /ATP with mitochondrial redox state.

E. Clinical Studies

^{31}P NMR of the human liver has been used in a number of centers to obtain spectra from patients with

FIGURE 4 Phosphorus-31 chemical-shift imaging of the human liver. Spectra were obtained using a 2D-CSI sequence at 1.5 T and with a surface coil of 16 cm diameter (8×8 matrix, 240 mm field of view, slice thickness 20 mm, voxel volume 19.5 ml). (A) Schematic representation; (B) muscle spectrum, voxel b; (C) muscle plus liver spectrum, voxel c; (D) liver spectrum, voxel d (180° pulse angle at the center of the coil, 1 s interpulse delay, 10 mm spectrum) (from Biran *et al.*, unpublished data).

various liver diseases (Oberhansli *et al.*, 1986, 1990; Meyerhoff *et al.*, 1989; Segebarth *et al.*, 1991; Cox *et al.*, 1991, 1992). To obtain metabolic information from the liver, extrahepatic signal from muscles overlying the liver must be avoided. Spectra specifically localized to the liver can be obtained by using chemical-shift imaging methods as illustrated in Fig. 4. The human liver spectrum shows the resonance of NTP, inorganic phosphate, PME, and phosphodiester (PDE). The determination of absolute metabolite concentrations *in vivo* is however difficult and most of the investigative centers report their finding as ratios of peak areas.

The study of Cox *et al.* (1992) reports that the reproducibility of the results in healthy adult subject spectra is within a coefficient of variation between 35% (intersubject variability) and 15% (intraexamination variability) for various ratios including: PME/ATP, PDE/ATP, and P_i /ATP. With the precision of the technique, only pathological changes differing from normal

in their metabolite ratios by at least 30% are likely to be interpretable. In a study of 17 patients, an increase in the PME/PDE ratio was found in spectra from primary or secondary tumors. These results invariably presented an increase in PME/ATP ratio, and in some cases, a reduction of PDE/ATP ratio. In general the spectral pattern did not distinguish between diseases of various etiologies (Cox *et al.*, 1992). In another investigation on a patient with a history of alcohol abuse, the PME/ATP and PME/P_i ratio was raised and showed a significant positive correlation with the severity of alcoholic hepatitis (Angus *et al.*, 1990). Alcoholic liver diseases were also studied by Meyerhoff *et al.* (1989), who did not observe the changes in metabolite ratio. They found an absolute decrease of metabolite concentration in both alcoholic hepatitis and alcoholic cirrhosis. Such disparate observations between investigative centers highlight the difficulties arising with NMR technology. The present lack of standardized methods obstructs comparison of the data from different centers. Masson *et al.* (1994) suggested the use of fructose infusion for the evaluation of the metabolic function of the human liver.

III. CARBON-13 NMR OF LIVER

A. ¹³C Natural Abundance NMR Spectrum

1. Resonance Assignments in Excised Liver Spectra

The occurrence of carbon-13 isotope is very low in nature (1.1%) and ¹³C NMR studies are restricted to investigations of highly concentrated metabolites. In addition, the heteronuclear coupling with the proton—which must be suppressed by proton irradiation—and the low intrinsic sensitivity of the ¹³C nucleus (6% of the proton sensitivity) make it difficult to obtain good quality spectra, especially *in vivo*. In liver, some metabolites have very high intracellular levels. They include carbohydrates such as glycogen, triglycerides, and circulating phospholipids and they can be observed in natural abundance by ¹³C NMR. A typical excised rat liver spectrum is shown in Fig. 5 and 6: the resonances have been assigned on the basis of chemical shifts previously reported from various carbohydrates and lipid compounds (Canioni *et al.*, 1983) and the assignments to specific lipid or carbohydrate carbons are given in Table III (see also Appendix).

Despite its large molecular weight, stored glycogen gives rise to relatively narrow resonances as seen in the 60–100 ppm region of the excised liver spectrum. The resonance from the C1 of glycogen is shifted 7.8 ppm downfield from its position in the α -glucose ring (92.7 ppm); this shift is due to the $\alpha(1 \rightarrow 4)$ linkage of glyco-

FIGURE 5 High-resolution carbon-13 NMR spectra of rat liver (A) and rat adipose tissue (B). The spectra recorded at 90.5 MHz represent 1024 free-induction decays accumulated at 24°C in a 20-mm (liver) and 10-mm (adipose tissue) sample cell with a 45° radiofrequency pulse and an interpulse delay of 0.5 s. Resonances have been numbered from 1 to 11 for carbons of fatty acyl chains and from 12 to 15 for phospholipids and free carboxylate groups. The region between 55 and 105 ppm shows the signals from carbohydrates (16 and 19–28) and the glycerol backbone in triglycerides and phospholipids (17 and 18). The assignments of resonances are reported in Table III. Chemical shifts are in ppm from tetramethylsilane (reprinted from Canioni *et al.*, 1983).

syl units to form glycogen. For the same reason, glycogen C4 resonates 7.5 ppm downfield from its position in α -glucose. Spectra recorded from purified glycogen, extracted from rat liver, display a shoulder at 99.2 ppm on the glycogen C1 resonance that comes from the C1 of glucose residues linked $\alpha(1 \rightarrow 6)$ in glycogen. From a comparison of the integral of this signal with that of C1 in the $\alpha(1 \rightarrow 4)$ links, it is possible to estimate the proportion of $\alpha(1 \rightarrow 6)$ links in the structure (Sillerud and Shulman, 1983).

Storage lipids such as triacylglycerols are present in the liver as neutral fat droplets and therefore give rise to sharp resonance lines assigned to specific carbon atoms in triacylglycerols. As illustrated in Fig. 5, resonances 1 to 8 arise from saturated carbons of the fatty acyl chains, resonances 9 and 10 come from the unsaturated

FIGURE 6 Carbon-13 natural abundance CP-MAS solid-state NMR spectroscopy of frozen excised rat liver. The liver was perfused with Krebs–Henseleit buffer at 4°C, pH 7.4, for 10 min. While superfusing with ice-cold perfusate, a roughly cylindrical tissue sample was cut and frozen in a cylindrical form (5.8 mm inner diameter). The frozen pellet (5.8 mm diameter, 13 mm length) was placed in the MAS sample holder at -100 to -150°C . The $^1\text{H}/^{13}\text{C}$ CP-MAS probe was tuned to 299.1 and 75.4 MHz, respectively. The spectrum represents an average of 2500 scans obtained at -150°C with cross polarization via spin lock and appropriate proton decoupling, displayed with 35-Hz line broadening. Spinning frequency was 4.55 kHz, corresponding to 60.4 ppm (reprinted from Quistorff *et al.*, 1993).

carbons, and resonance 11 comes from the carboxyl group at the ester linkages. The signals arising from the glycerol backbone of triacylglycerols (and some phospholipids) are also observed (17, C1 and C3, and 18, C2 of glycerol esters). The resonances 14 and 15 appear at positions characteristic of the methyl carbons of the phosphatidylcholine head groups and the methylene units from the head groups of both phosphatidylcholine and phosphatidylethanolamine.

The unique chemical structure of the polyunsaturated fatty acids permits them to be distinguished from the monounsaturated fatty acids in the ^{13}C NMR spectrum. The monounsaturated fatty acids, such as oleic acid, have a single double bond, and both of that acid's olefinic carbons resonate at 130.0 ppm. The polyunsaturated fatty acids have two or more double bonds, separated in pairs by a single methylene unit. In linoleic acid, which has two double bonds, the two olefinic carbons adjacent to the single methylene group resonate at 128.5 ppm, and the exterior two olefinic carbons resonate at 130 ppm. In polyunsaturated fatty acids with three or four double bonds (such as linolenic acid), only the two exterior olefinic carbons resonate at 130 ppm whereas the other double-bonded carbons appear around 128.5 ppm. A quantitative determination of the fatty acid composition of triacylglycerols and of the amount of

mono- and polyenoic fatty acids on the basis of the ^{13}C NMR spectrum is possible (Sillerud *et al.*, 1986).

Recently solid-state natural abundance ^{13}C NMR experiments on excised frozen liver was performed, demonstrating a well-resolved spectrum which allowed identification of the same resonances as described above for the liquid state (Quistorff *et al.*, 1993). Interestingly in solid state the C1 resonance of glycogen appeared as a split peak at 96.2 and 102.2 ppm in contrast to the single peak seen in liquid state (Fig. 6). This suggests that two conformational states of glycogen are populated, with rapid equilibration in the liquid state, but no apparent equilibration in the frozen state. This cross polarization-magic angle spinning (CP-MAS) solid-state method for frozen samples potentially allows a significant improvement of the signal-to-noise ratio compared with *in vivo* spectroscopy, since the time limitation imposed by the biological processes *in vivo* is eliminated in the frozen state. In some cases the method may substitute for studies of PCA extracts (Quistorff *et al.*, 1993).

2. Relaxation Parameters

Longitudinal relaxation times (T_1) and nuclear Overhauser enhancements (NOE) for ^{13}C resonances of fatty acyl chains in rat liver, at 8.4 T and 37°C have been published (Canioni *et al.*, 1983). T_1 values are in the range of 1.8 s (CH_3 head groups) to 0.2 s ($-\text{CH}_2-\text{CO}-$). A decrease in T_1 values from the ω -carbon to the carboxyl ester group is observed, suggesting a decrease in chain mobility going from the terminal methyl to the glycerol backbone. For all these carbons, the NOE ($\eta + 1$) is around 1.2–1.5. Detailed investigations on T_1 and NOE of glycogen carbons can be found in the literature and are of critical importance in the process of quantitation on the basis of signal areas (Sillerud and Shulman, 1983; Zang *et al.*, 1990). At around 37°C , T_1 values are field-dependent; for glycogen C1, they evolve from 0.3 s at 8.4 T to 0.15 s at 4.7 T and 0.07 s at 2.1 T. The NOE is nearly constant and has a ($\eta + 1$) value of 1.3 for glycogen C1, at magnetic fields varying from 2.1 to 8.4 T. The field dependence of the relaxation parameters (T_1 and, to a lesser extent, T_2), has led Zang *et al.* (1990) to propose a model of glycogen dynamics, where the fast internal motions (3.9×10^{-9} s) dominate the longitudinal relaxation, leading to the observation of narrow resonances in the NMR spectrum and consequently to the near 100% ^{13}C NMR visibility of glycogen.

3. The Natural Abundance of the *in Vivo* Liver Spectrum

Measurement of the NMR spectrum of liver *in vivo* requires the availability of specific techniques and methodologies for acquiring the NMR signal from a

TABLE III Assignments and Chemical-Shift Values of Carbon-13 Resonances of Rat Liver and Rat Adipose Tissue Spectra^a

Resonance	Assignment	Chemical shift (ppm)
1	*CH ₃ —CH ₂ —CH ₂ —, fatty acyl chain	14.20
2	CH ₃ —*CH ₂ —CH ₂ —, fatty acyl chain	23.05
3	—*CH ₂ —CH ₂ —CO—, fatty acyl chain	25.20
4	—CH=CH—*CH ₂ —CH=CH—, fatty acyl chain	25.05
5	—CH=CH—*CH ₂ —CH ₂ —, fatty acyl chain	27.60
6	—(CH ₂) _n —, fatty acyl chain	30.05
7	CH ₃ —CH ₂ —*CH ₂ —, fatty acyl chain	32.30
8	—CH ₂ —*CH ₂ —CO—, fatty acyl chain	34.15
9	—CH=*CH—CH ₂ —*CH=CH—, fatty acyl chain	128.40
10	—*CH=CH—CH ₂ —CH=*CH—, fatty acyl chain	130.00
11	—CH ₂ —CH ₂ —*CO—, fatty acyl chain	172.10
12	—CO—OR, protein, phospholipid	173.80
13	—COO—, Glu, free fatty acid	182.20
14	—CH ₂ —NH ₂ , ethanolamine (protein)	40.00
15	(CH ₃) ₃ N—, choline	54.60
16	C-6, α,β-glucose, glycogen	61.40
17	C-1, C-3, glycerol (ester)	62.20
18	C-2, glycerol (ester)	69.50
19	C-4, α,β-glucose	70.40
20	C-2, C-5, α-glucose; C-5, glycogen	72.10
21	C-3, α-glucose	73.50
22	C-3, glycogen	73.95
23	C-2, β-glucose	79.90
24	C-3, C-5, β-glucose	76.50
25	C-4, glycogen	78.05
26	C-1, α-glucose	92.70
27	C-1, β-glucose	96.60
28	C-1, glycogen	100.50

Note. Reprinted from Canioni *et al.* (1983).

^a Chemical shifts are in parts per million downfield from tetramethylsilane. The methyl group of fatty acyl chain is used as an internal reference at 14.2 ppm downfield from tetramethylsilane. The resonances are numbered as indicated in Fig. 1. Asterisk indicates the specifically assigned carbon. See also appendix.

well-defined volume within the body. A method termed "topical magnetic resonance" (TMR), which combines the use of surface coils and profiling B_0 magnetic fields with static field gradients, was developed by Gordon *et al.* (1980). ¹³C TMR spectra have been recorded with 10 min of signal averaging in living rats; the TMR spectrum (2.1 T) is not as well-resolved as the high field one (8.4 T); nevertheless, some signals characteristic of the liver are detected, for instance the resonances coming from C1 and C6 and the unresolved group of C2 and C5 of liver glycogen are present in the *in vivo* spectrum (Canioni *et al.*, 1983).

Human liver (and muscle) glycogen is detected with 13 min resolution at 2.1 T by using a concentric proton/¹³C surface coil (11 and 8-cm coil diameters, respectively). Proton decoupling can be achieved under these condition by single-frequency irradiation of the C1-bound proton, with 10 W decoupling power, which

does not exceed the FDA guideline of 8 W/kg of tissue. Signal-to-noise ratios above 20/1 are obtained, supporting the use of ¹³C NMR as a noninvasive technique of human glycogen metabolism (Jue *et al.*, 1989).

B. Intermediary Liver Metabolism Using ¹³C-Enriched Substrates: Gluconeogenesis, Glycolysis, and the Tricarboxylic Acid Cycle

Lack of sensitivity to low concentrations of metabolites is the major drawback of the NMR technique, particularly in the case of the ¹³C nucleus, and the majority of the studies on living tissue have been carried out using ¹³C-enriched substrates. The isotopic tracer method using radioactive isotopes has provided direct evidence for the existence of complex metabolic pathways in isolated hepatocytes and perfused liver. The carbon-13 isotope approach can be considered as similar in essence to the

radioactive ^{14}C investigation; however, the method offers additional advantages including the noninvasive measurement of the flow of label through various biochemical reactions and the possible determination of the proportion of different ^{13}C isotopomers of the same metabolite by the analysis of homonuclear and heteronuclear spin coupling (see Chap. 26, Sherry and Malloy, this volume). The recording of high-resolution spectra of perchloric acid extracts from tissues or cells after incubation or perfusion with specifically labeled compounds offers an easier and rapid way to detect and quantitate a vast number of metabolites in a single spectrum (see Chap. 14, Bell *et al.*, this volume). The assignments of ^{13}C resonances for some enriched metabolites usually found in perfused livers are given in Table IV. The main limitation of the method at present is the cost of enriched precursors which must be used at millimolar concentrations to detect signals from labeled intermediates.

The interactions between gluconeogenesis and the TCA cycle have been analyzed in isolated hepatocytes (Cohen *et al.*, 1979, 1981) and in perfused rat liver (Cohen, 1983b) by using a variety of ^{13}C -enriched gluconeogenic precursors. The distribution of flux through the pyruvate dehydrogenase and the pyruvate carboxylase routes has been evaluated (Cohen *et al.*, 1979;

Cohen, 1983b) using enriched alanine and alanine plus ethanol. The main observation in the presence of ethanol was that alanine entered the TCA cycle quite exclusively through the pyruvate carboxylase reaction. The determination of the pyruvate kinase activity (phosphoenolpyruvate cycle) was also studied by Cohen using $[3-^{13}\text{C}]$ alanine as a substrate (Cohen, 1987b): the fate of the original methyl group of alanine was followed into the TCA cycle, assuming that alanine entered the cycle exclusively through pyruvate carboxylase, with a very low activity of the pyruvate dehydrogenase (DiDonato *et al.*, 1993). Owing to the symmetry of the fumarate molecule and the activity of the fumarase, the label was scrambled at either the carbon 2 or 3 of malate. These labeled carbons appeared as C1 (and C6) and C2 (and C5) in neosynthesized glucose. Any label introduced at the C2 of pyruvate is then the result of a pyruvate kinase activity. Since pyruvate is converted to alanine by the very active liver alanine aminotransferase, under the maintenance of a large trapping pool of unscrambled $[3-^{13}\text{C}]$ alanine, the enrichment at alanine C2 (and C1) can be considered as directly related to the pyruvate kinase flux (Cohen, 1987a). Pyruvate recycling was detected by studying the glutamate enrichment: the acetyl CoA labels only C4 in the α -ketoglutarate and—due to transaminase activities—C4 in glutamate. The enrichment at glutamate C5 is then an indication of the reuse of pyruvate enriched at the C2 position. The ^{13}C enrichment at glutamate C4 relative to C5 thus reflects approximation of the turnover rate of the TCA cycle.

Streptozotocin-induced diabetic rats were also used to study gluconeogenesis in the presence of $[3-^{13}\text{C}]$ alanine, $[2-^{13}\text{C}]$ ethanol, or $[2-^{13}\text{C}]$ pyruvate plus ammonia. From comparison with experiments carried out on livers from fed or fasted animals, a novel effect of insulin on the activity of pyruvate kinase flux was observed (Cohen, 1987b,c).

C. Glycogen Synthesis and Degradation

The liver plays a critical role in blood glucose homeostasis, using glycogen synthesis or degradation to modulate the blood glucose content, by reciprocal control of glycogen phosphorylase and glycogen synthase. *In vivo* studies have indicated that both activities may be simultaneously active, thus implying the probable occurrence of futile cycling between glucose-1-P and glycogen (Jungermann, 1987). Simultaneous synthesis and degradation of glycogen have been observed in the perfused rat liver, by using $[1-^{13}\text{C}]$ glucose as substrate in a pulse-chase experiment (Shulman *et al.*, 1988). Further studies carried out by the same group, using a double-label infusion protocol led the authors to the definitive

TABLE IV Chemical Shifts of Carbon-13 Resonances of Various Metabolites Detected in Liver NMR Spectra under Perfusion with Enriched Substrates (in ppm, Reference at 0 ppm: Tetramethylsilane). See also appendix.

Metabolite	Carbon	Chemical shift
Acetate	C2	24.4
Alanine	C3	17.5
	C2	51.7
Aspartate	C3	37.9
	C2	53.7
Citrate	C2, C4	46.1
Glutamate	C3	28.4
	C4	34.7
	C2	55.9
Glutamine	C3	27.7
	C2	55.4
	C4	32.7
Glutathione	C3	27.3
	C4	32.2
	C2	55.1
β -Hydroxybutyrate	C4	22.9
	C2	47.6
α -Ketoglutarate	C4	36.7
Lactate	C3	21.2
Succinate	C2, C3	35.4
Malate	C3	43.7

conclusion that in both the fed and the fasted rat, the processes of glycogen synthesis and breakdown occur simultaneously, during the period of net glycogen synthesis. This finding, at variance with the results obtained on isolated hepatocytes, suggests that the model of regulation of hepatic glycogen synthesis by simultaneous inactivation of glycogen phosphorylase and activation of glycogen synthase must be revised (William *et al.*, 1990). It should be noted, however, that interpretation of NMR data of ^{13}C labeling of glycogen depends critically on whether the last-in-first-out or random in-out principle for glycogen synthesis/degradation applies in the liver.

The contribution of the direct versus indirect pathway of liver glycogen repletion is also still under debate: experiments with radioactive tracers have shown that glycogen synthesis requires active gluconeogenesis, thus implying that the indirect pathway, e.g., trioses $\rightarrow$ glucose-1-P $\rightarrow$ glycogen, can contribute significantly to the synthesis as compared to the conventionally accepted direct route glucose $\rightarrow$ glucose-1-P $\rightarrow$ glycogen. The contributions of these two pathways were determined under conditions where $[1-^{13}\text{C}]\text{glucose}$ or $[1-^{13}\text{C}]\text{glucose}$ plus unlabeled alanine were given orally (Shulman *et al.*, 1985, 1987; Shalwitz *et al.*, 1989).

D. Urea Metabolism and Related Pathways

Perfusion of rat livers with 10 mM $[2-^{13}\text{C}]\text{acetate}$ as substrate first led to the labeling of intracellular glutamate at the C4 position. At steady state, glutamate C2 and C3 were also labeled, but to a lesser extent than C4 (Desmoulin *et al.*, 1988). This scrambling pattern is generated as flux of enriched acetyl-CoA continues through the TCA cycle. The intracellular content and ^{13}C enrichments of glutamate and glutamine in liver were dependent on the direction of perfusion. In antegrade perfusion, low levels of Gln were detected as compared to Glu, even in presence of 1 mM NH_4Cl , indicating a high glutaminase activity. In contrast, in retrograde perfusion, Glu was rapidly converted to Gln and this synthesis was stimulated by NH_4Cl . These data and the evaluation of the specific ^{13}C enrichment of glutamate and glutamine under $[2-^{13}\text{C}]\text{acetate}$ or $[3-^{13}\text{C}]\text{alanine}$ perfusion (M. Biran, unpublished data) are in good agreement with the model of metabolic zonation described by Haussinger and Sies (1979) for the glutamine and urea metabolism in the liver lobule.

E. Fatty Acid Metabolism and Ketogenesis

The metabolism of fatty acids in the liver has been studied for different nutritional or hormonal situations

(Cross *et al.*, 1984; Cerdan *et al.*, 1988). In the work of Pahl-Wostl and Seelig (1986), two pathways for hydroxybutyrate (as acetoacetyl-CoA) formation from butyrate were considered: (a) β -oxidation followed by synthesis of acetoacetyl-CoA, via the hydroxymethylglutaryl pathway, resulting in the production of β -hydroxybutyrate equally labeled at C1 and C3; and (b) direct production of acetoacetyl-CoA from butyrate, followed by the condensation of acetyl CoA from the common pool to give hydroxymethylglutaryl-CoA, which in turn would be split into acetoacetate and acetyl-CoA. In this mechanism, C1 and C2 of butyrate would be exchanged against carbons of the acetyl-CoA from the common pool whereas C3 and C4 would not be replaced. The comparison of the C1/C3 ratio should provide evidence for the contribution of the (a) and/or (b) pathways.

IV. OTHER NUCLEI

Protons are the most sensitive and the most abundant nuclei in living systems. However, because of limitations due to the presence of the water signal of tissue, and to the multiplicity of spin systems and overlapping resonances, very few studies have been devoted to liver using this nucleus. In one paper, Rafter *et al.* (1991) investigated the changes occurring in the proton NMR spectra of rat liver extracts, as a function of the age of the rat, in the first 21 days of life. Their major finding was that betaine was increasing sharply from Day 1–7 (1–2 $\mu\text{mol/g}$) to Day 19 (5–6 $\mu\text{mol/g}$), whereas minor changes occurred in taurine levels (see Chap. 14, Bell *et al.*, this volume). Other metabolites detected included lactate (at a very low level), phosphocholine, and glycerophosphocholine. From this study, it seems likely that few well-defined resonances, apart from betaine, would be detected *in vivo*, and the range of metabolite that could be detected is then very limited.

^{19}F is 100% abundant in nature and is the most sensitive nucleus after ^1H and ^3H (see Chap. 16, London *et al.*, this volume). ^{19}F NMR offers a powerful technique for monitoring the fate of fluorinated anesthetics, anticancer drugs, or labeled metabolites as fluorinated glucose derivatives. The metabolism of 5-fluorouracil (5FU) was studied in rat or mice liver (Stevens *et al.*, 1984; Cabanac *et al.*, 1988). Major metabolites detected *in vivo* on rat or in perfused mouse liver included: α -fluoro- β -alanine (FBAL) and dihydrofluoracyl (FUH2). No 5F-dUMP was detected in liver, but this metabolite—corresponding to the active antitumor compound—was present in an implanted tumor, in the

same rodent (Stevens *et al.*, 1984). Since a number of fluorinated drugs are currently in clinical use, their catabolism by the liver could be assessed by using the ^{19}F NMR method. The sensitivity of the nucleus is sufficiently high to permit ^{19}F imaging of the organ in certain circumstances of fluorinated drug treatments.

^{15}N NMR possesses an adequate sensitivity and resolution to study the incorporation of $^{15}\text{NH}_4^+$ into key metabolic pathways in a number of microorganisms, organs, and tissues (Farrow *et al.*, 1990; Nieto *et al.*, 1992). The large range of ^{15}N chemical shift (around 400 ppm) permits us to distinguish between glutamate and glutamine and then to follow the incorporation of ^{15}N into the γ - and α -nitrogen atoms of glutamine, for example. The source of ammonia used by isolated rat liver mitochondria in the production of citrulline has been investigated by Nieto *et al.* (1992) by analyzing the heteronuclear ^{15}N — ^{13}C coupling, observed by ^{13}C NMR. The approach is based on the coupling which appears in the C7 resonance of the citrulline produced after incubation of mitochondrial suspensions with $^{13}\text{CO}_3\text{H}^-$ and $^{15}\text{NH}_4^+$. The methodology can be extended to other metabolic pathways resulting in a ^{15}N — ^{13}C bond, allowing the detection of ^{15}N , with the higher metabolic versatility of ^{13}C NMR.

V. PERSPECTIVES

Despite the large number of known metabolic functions performed by the liver it has proven difficult to devise clinically useful procedures for the assessment of the metabolic "functional status" of the liver, although the clearance of galactose or of organic ions like bromosulphophthalein, fluorescein, and indocyanine may be used for the evaluation of liver elimination function (Tygstrup, 1990). One very rational approach would, of course, be to measure the gluconeogenic capacity, and while this is easily accomplished *in vitro*, it has proven much more difficult *in vivo* (Katz and Grunner, 1979; Esenmo *et al.*, 1992). It is very likely that NMR techniques in the future will be able to satisfy the needs for such procedures allowing the evaluation of the metabolic capacity of the liver, although the technique as applied clinically is still in its infancy. However, several studies on fructose metabolism seem to be pointing the way toward an assessment of metabolic function (e.g., Seegmiller *et al.* 1990; Masson *et al.* 1993b), and, in addition, studies on the mechanism of glycogen synthesis have provided new important information necessary for the interpretation of ^{13}C NMR data for the evaluation of the hepatic capacity of glycogen synthesis (Grunner *et al.*, 1993).

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