ELSEVIER

Biochimica et Biophysica Acta 1272 (1995) 113–118

BBA Biochimica et Biophysica Acta

Cirrhosis of the human liver: an in vitro ^{31}P nuclear magnetic resonance study

Simon D. Taylor-Robinson [a,b,*], E. Louise Thomas [a], Janet Sargentoni [a], Claude D. Marcus [a], Brian R. Davidson [c], Jimmy D. Bell [a]

[a] *NMR Unit Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK*
[b] *Department of Gastroenterology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK*
[c] *University Department of Surgery, The Royal Free Hospital and School of Medicine, Hampstead, London NW3 2QG, UK*

Received 7 November 1994; revised 28 March 1995; accepted 16 May 1995

Abstract

Human livers with histologically proven cirrhosis were assessed using in vitro ^{31}P NMR spectroscopy. Spectra were compared with those from histologically normal livers and showed significant elevations in phosphoethanolamine (PE) and phosphocholine (PC) and significant reductions in glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC). There were no significant differences in spectra from livers with compensated and decompensated cirrhosis. These results help to characterise the alterations in membrane metabolism in cirrhosis of the liver.

Keywords: NMR, ^{31}P; Cirrhosis; Phospholipid; Human; Liver

1. Introduction

The human liver responds to injury in broadly the same way, irrespective of the original causal agent [1]. Persistent alcohol abuse, viruses such as hepatitis B and hepatitis C, genetic disorders including haemochromatosis, Wilson's disease and α_1-antitrypsin deficiency, cholestatic conditions such as primary biliary cirrhosis and primary sclerosing cholangitis, certain drugs and autoimmune diseases all may provoke a series of events that ultimately lead to cirrhosis or irreversible liver damage [2].

Cirrhosis of the liver is a diffuse process, characterised by the formation of fibrous tissue and regrowth of hepatocytes in an abnormal nodular pattern [3]. Current assessment methods of the functional state of liver injury in cirrhosis are not entirely satisfactory, usually depending on a severity index obtained from a collection of laboratory parameters and clinical findings [4–7].

Nuclear magnetic resonance (NMR) spectroscopy is a non-invasive technique, which can be used to provide localised biochemical information on hepatic metabolic processes in vivo. A typical ^{31}P NMR spectrum of the human liver in vivo contains resonances which may be assigned to phosphomonoesters (PME), phosphodiesters (PDE), inorganic phosphate (Pi) and nucleotide triphosphates (NTP) [8–12].

The PME and PDE resonances in hepatic spectra are multicomponent and the constituents cannot as yet be completely resolved at the magnetic field strengths employed in human in vivo NMR studies, despite the use of proton-decoupling techniques [13]. The PME resonance includes contributions from cell membrane precursors [14] and glycolytic intermediates [15]. The PDE resonance is also composite, containing information from cell membrane breakdown products [14] and from endoplasmic reticulum [16].

Previous human in vivo NMR studies have reported on

Abbreviations: FID, free induction decay; GPC, glycerophosphorylcholine; GPE, glycerophosphosphorylethanolamine; MDP, methylene diphosphonate; NMR, nuclear magnetic resonance; NTP, nucleotide triphosphates; PC, phosphocholine; PCA, perchloric acid; PCr, phosphocreatine; PDE, phosphodiesters; PE, phosphoethanolamine; Pi, inorganic phosphate; PME, phosphomonoesters.

* Corresponding author. Fax: +44 181 7403038.

SSDI 0925-4439(95)00074-7

the elevation in PME/ATP and the reduction in PDE/ATP with increasing functional severity of cirrhosis [17,18]. However, the underlying metabolic abnormalities responsible for these observations have not been fully investigated.

In vitro NMR techniques on human tissue extracts have been successfully used to study the metabolite changes responsible for the in vivo PME and PDE signals in hepatic tumours and normal liver [15,19]. Although Menon and colleagues [18] reported on in vitro NMR findings from a small number of livers from patients with chronic liver disease, no systematic approach has been applied to the characterisation of the cirrhotic liver.

Therefore, the aim of this study was to characterise the metabolic changes observed by in vitro ^{31}P NMR in cirrhosis of the liver. The results are discussed in the context of previous in vivo hepatic ^{31}P NMR findings.

2. Materials and methods

Standard percutaneous liver biopsies do not yield enough tissue for in vitro NMR studies, and therefore samples of cirrhotic liver were taken during surgery for orthotopic hepatic transplantation. Liver tissue was obtained from 25 patients with histologically proven cirrhosis. Ten patients (40%) had primary biliary cirrhosis, seven (28%) post-viral cirrhosis, six (24%) primary sclerosing cholangitis, one (4%) Wilson's disease and one (4%) alcoholic cirrhosis.

The severity of liver dysfunction was assessed using the Pugh's score [4], obtained from clinical and biochemical data, acquired on the day of liver transplantation. This is the standard scoring system, which is used clinically, grading liver injury from 5 (best function) to 15 (worst function), taken from information comprising serum bilirubin, plasma albumin levels, prothrombin time and the presence/severity of ascites and hepatic encephalopathy.

The 25 liver samples were categorised into two groups: functionally compensated cirrhosis with a Pugh's score ≤ 7 ($n = 10$) and functionally decompensated cirrhosis with a Pugh's score ≥ 8 ($n = 15$)(Table 1).

Permission for this study was obtained from the Ethics Committees of the Royal Postgraduate Medical School, London, and the Royal Free Hospital and School of Medicine, London. All patients provided written, informed consent.

2.1. Sample collection

Two investigators were present in the operating theatre to obtain tissue samples from each recipient liver. In every case, 6–8 representative sugar lump sized pieces of liver were freeze-clamped in liquid nitrogen with minimum possible ischaemic time (2–7 min). This was performed ex vivo within 3 min of hepatectomy in 22 cases. All samples were stored separately in a liquid nitrogen dewar until further processed.

Table 1
Laboratory data on the patients from whom the liver samples were collected

Tissue type	Serum bilirubin (μmol/l) (5–17) [+]	Plasma albumin (g/l) (35–50) [+]	Prothrombin time (s) (12–14) [+]	Pugh's score [a] (5–15)
Compensated ($n = 10$)	177 (35–460)	40 (31–48)	14 (13–16)	7 (6–7)
Decompensated ($n = 15$)	143 (34–388)	31 (22–40)	17 (13–25)	10 (8–12)

Data are means (range values).
[a] Pugh's score [4] = functional severity of cirrhosis. Score ≤ 7, compensated cirrhosis. Score ≥ 8, decompensated cirrhosis.
[+] limits of reference range.
All information was obtained preoperatively, on the day of liver transplantation.

2.2. Reference data

Reference data were obtained from wedge biopsy samples of liver, taken from 6 patients undergoing laparotomy for surgical treatment of pancreatitis. In each case, contiguous samples of liver tissue were found to be histologically normal on examination [15].

2.3. Tissue extract preparation

The wet weight of each sample was between 560 mg and 2310 mg. Twelve per cent perchloric acid (PCA) was added to the still-frozen samples, in a ratio of 5 ml/g of liver tissue. Each sample was ground down under liquid nitrogen with a mortar and pestle and then allowed to thaw, before centrifugation at 3000 rpm for 10 min. The supernatant was separated, neutralized with 3 M KOH, freeze-dried and reconstituted in D_2O. The pH was readjusted to 7.5, after the addition of 100 mmol/l of EDTA to chelate any paramagnetic metal ions present. Absolute quantification of metabolites was achieved by adding known amounts of methylene diphosphonate (MDP) and/or phosphocreatine (PCr) to the perchloric acid extracts. These acted as internal reference standards for chemical shift assignments of the resonances observed.

2.4. NMR methods

All NMR spectroscopy measurements were performed at room temperature. Proton-decoupled ^{31}P NMR spectra were obtained using a high resolution NMR spectroscopy system (operating at 11.7T), from the perchloric acid extracts of liver tissue, with 16 K data points and a 45° pulse angle applied at intervals of 1 s. Corrections for T_1 relaxation were made using samples run with a repetition time of 20 s. Metabolites were assigned using the methods

we have previously described [15]. The chemical shift of each metabolite was found and subsequently confirmed by the use of 'spiking' with known compounds [15].

2.5. Data processing

The free induction decay (FID) was zero filled to 32 K and Fourier transformed after line-broadening of 5 Hz. Peak areas for PE, PC, GPE, GPC, MDP and/or PCr were obtained, using the NMR1® spectral processing program (New Methods Research, E. Syracuse, USA) on a SUN SPARCstation 10 (Sun Microsystems, Mountain View, CA, USA). The data were fitted to Lorentzian functions.

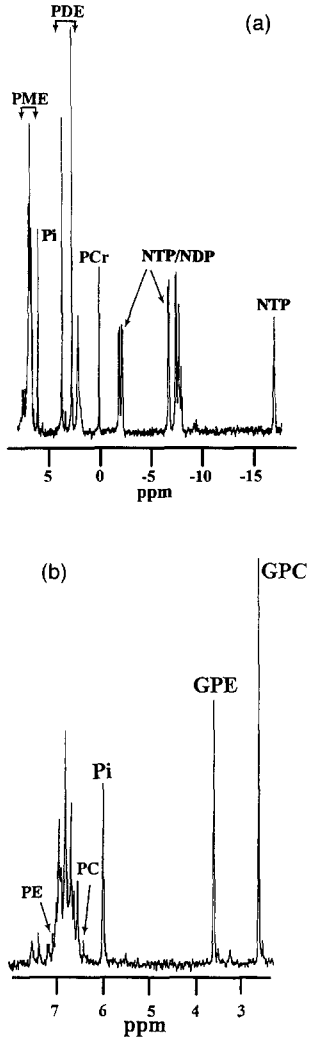

Fig. 1. Typical proton-decoupled ^{31}P NMR spectrum of perchloric acid extract prepared from histologically normal liver tissue. (a) Full spectrum; (b) PME and PDE regions. Abbreviations: PME, phosphomonoesters; PDE, phosphodiesters; NAD, NADH + NAD; NTP, nucleotide triphosphates; NDP, nucleotide diphosphate; PE, phosphoethanolamine; PC, phosphocholine; GPE, glycerophosphorylethanolamine; GPC, glycerophosphorylcholine; PCr (phosphocreatine) was added as an internal reference standard. This figure is modified from Bell et al. [15].

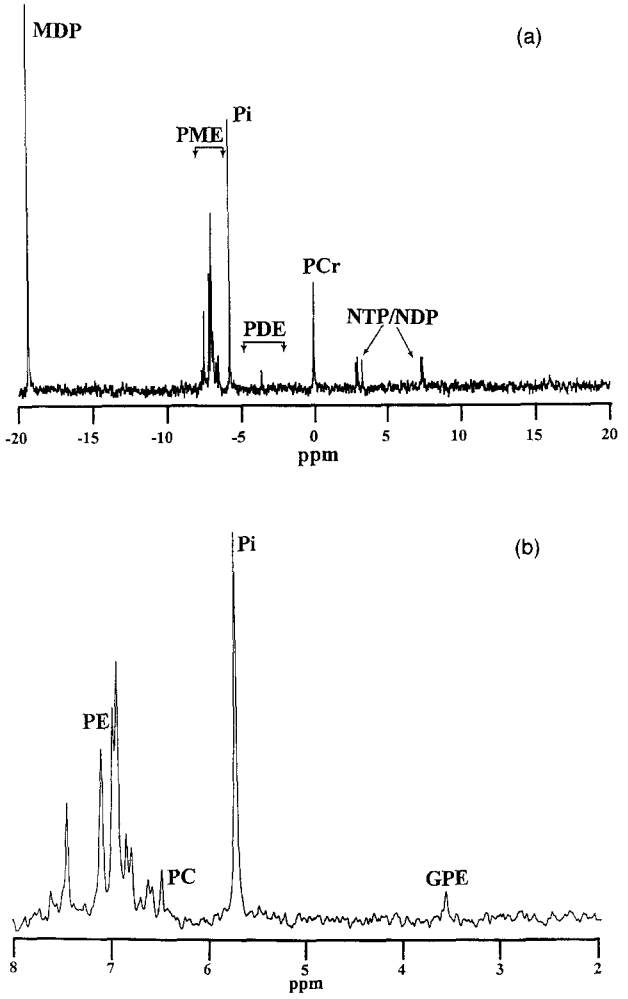

Fig. 2. Typical proton decoupled ^{31}P NMR spectrum of perchloric acid extract from liver tissue with histologically proven cirrhosis. (a) Full spectrum, (b) PME and PDE regions. Abbreviations: PME, phosphomonoesters; PDE, phosphodiesters; NAD, NADH + NAD; NTP, nucleotide triphosphates; NDP, nucleotide diphosphate; PE, phosphoethanolamine; PC, phosphocholine; GPE, glycerophosphorylethanolamine; GPC, glycerophosphorylcholine; PCr (phosphocreatine) and MDP (methylene diphosphonate) were added as internal reference standards.

2.6. Statistical analysis

Since the data were not normally distributed, non-parametric statistical analysis was applied. Values for metabolite concentrations in the patient and reference populations were compared using the Mann-Whitney U-test. A P-value of < 0.05 was considered significant. All metabolite concentrations are quoted as mean values ± 1 standard deviation.

3. Results

A typical ^{31}P NMR spectrum from a PCA extract of normal liver contains resonances arising from PME, PDE,

Table 2
Concentrations of metabolites obtained from in vitro ^{31}P NMR spectra from histologically normal and cirrhotic liver tissue

Tissue type	Metabolite concentrations (μmol/g wet weight)			
	PE	PC	GPE	GPC
Normal liver ($n = 6$)	0.16 ± 0.03	0.16 ± 0.04	2.35 ± 0.46	2.46 ± 0.37
All cirrhosis ($n = 25$)	1.04 ± 0.75 [a]	0.41 ± 0.37 [b]	0.29 ± 0.37 [a]	0.14 ± 0.26 [c]
Compensated cirrhosis ($n = 10$)	1.28 ± 0.70 [a]	0.38 ± 0.22 [b]	0.27 ± 0.31 [a]	0.13 ± 0.12 [c]
Decompensated cirrhosis ($n = 15$)	0.88 ± 0.76 [d]	0.44 ± 0.45 [e]	0.30 ± 0.42 [d]	0.14 ± 0.29 [d]

Data are mean values $\pm$ 1 S.D.
Significant difference from the reference population: [a] $P < 0.0005$, [b] $P < 0.05$, [c] $P < 0.0001$, [d] $P < 0.001$, [e] $P < 0.01$.

NTP, NDP and Pi (Fig. 1). The PME region of the spectrum consists of over 10 resonances, including signal from PE, PC, AMP,2,3-DPG, coenzyme A, glucose 6-phosphate, glycerol 1-phosphate, 3-phosphoglycerate and ribose 5-phosphate [15–19]. The PDE region contains two major resonances, GPE and GPC [15,19–21].

Most of these resonances vary markedly with ischaemia and it was therefore only sensible to quantify the more stable compounds, namely PE and PC from the PME region and GPE and GPC from the PDE region of the spectrum [22–24].

The signal intensity of the PE and PC resonances was increased and the GPE and GPC resonances reduced in spectra from liver with histologically proven cirrhosis (Fig. 2) when compared to spectra from histologically normal liver. The metabolite concentrations (μmol/g wet weight of liver tissue) are summarised in Table 2.

All cirrhotic livers showed significantly higher PE (1.04 ± 0.75 vs 0.16 ± 0.03; $P < 0.0005$) and PC concentrations (0.41 ± 0.37 vs 0.16 ± 0.04; $P < 0.05$) and significantly lower GPE (0.29 ± 0.37 vs 2.35 ± 0.46; $P < 0.005$) and GPC concentrations (0.14 ± 0.26 vs 2.46 ± 0.37; $P < 0.0001$) than normal tissue (Table 2).

There was no significant difference between PE, PC, GPE and GPC concentrations from livers with functionally compensated cirrhosis and those from livers from functionally decompensated cirrhosis (Table 2).

There were regional variations in metabolite concentrations when liver samples from different areas of the same liver were analysed. Table 3 illustrates these variations in metabolite levels in a patient with compensated cirrhosis.

There was no correlation between individual biochemical indices (serum bilirubin, plasma albumin and prothrombin time) or clinical parameters of liver dysfunction (presence of ascites and hepatic encephalopathy), measured on the day of the transplant operation, and PE, PC, GPE and GPC concentrations from the liver extracts.

Table 3
In vitro ^{31}P NMR: Variations in metabolite concentrations obtained from different regions of the same liver

Metabolite concentration	Region 1	Region 2	Region 3	Region 4
PE (0.09–0.24) *	1.34	1.76	3.72	1.85
PC (0.11–0.23) *	0.43	0.77	0.91	1.13
GPE (1.79–2.71) *	0	0	1.00	0.15
GPC (2.09–2.83) *	0	0	0	0

All values expressed as μmol/g wet weight of liver tissue.
* Reference range.

4. Discussion

This study used in vitro ^{31}P NMR to describe the changes in aqueous soluble membrane components in livers with histologically proven cirrhosis, compared to normal human liver tissue.

Several human in vivo ^{31}P NMR studies of the liver have shown abnormalities in PME, PME/ATP, PME/PDE and PDE/ATP in patients with cirrhosis [17,18,25–27]. Two of these studies have correlated the functional severity of liver injury in cirrhosis with an elevation in PME/ATP and a reduction in PDE/ATP [17,18].

Our study attempted to investigate the underlying metabolic changes responsible for these in vivo spectral appearances in man. Unfortunately, a limitation of human tissue characterisation by in vitro methods is the unavoidable period of ischaemia during biopsy collection. Only quantification of PE, PC, GPE and GPC was attempted, as the other metabolites that comprise the PME and PDE peaks are known to alter radically from the in vivo situation during periods of ischaemia [15,19]. Hachisuka and colleagues [28] noted that in rat liver subjected to prolonged periods of ischaemia beyond 30 min, PC and PE were relatively stable, while GPE and GPC decreased. However, post-mortem studies of human brain and animal liver have indicated that the levels of PE, PC, GPE and GPC are not significantly affected by periods of ischaemia of up to one hour [22–24]. In our study much shorter periods of ischaemia were encountered. Twenty-two of the 25 tissue samples from cirrhotic liver were collected within 3 min of hepatectomy, while in the three tissue samples the ischaemic period was up to 7 min.

Comparison of the ^{31}P NMR spectra of PCA extracts from cirrhotic liver and histologically normal tissue showed increased concentrations of PE and PC and decreased concentrations of GPE and GPC from the diseased tissue.

Regional variations in metabolite concentrations were observed from samples obtained from different areas of each individual liver.

Our results suggest that increased concentrations of PE and PC may be responsible for elevation in PME/ATP observed in vivo [17,18,27]. Similarly, the reduction of PDE/ATP seen in vivo [17,18] may be explained, at least in part by the reduction in GPE and GPC which we have noted. Endoplasmic reticulum is also an important component of the PDE resonance in vivo [16,29], but its relative contribution in the human cirrhotic liver is unclear and requires further study.

The predominant contribution of PC and PE are as intermediates on the pathway of phospholipid biosynthesis [14]. GPE and GPC are phospholipid breakdown products [14]. Increased PE [30–33] and PC [34] have been observed in the regenerating rat liver and in other conditions of rapid cellular proliferation, such as in hepatic tumours [15,19,35]. Lymphomatous infiltration of the liver is also associated with elevated PE levels [35].

The hallmark of cirrhosis is abnormal regrowth of liver tissue in a nodular pattern. This occurs in the presence of increased fibroblastic activity [3]. The increase in PE and PC in our study may therefore be due to increased cell turnover as the cirrhotic liver attempts to regenerate. Either hepatocyte regeneration or the laying down of fibrous tissue, during the cirrhotic process, may be responsible for this phenomenon.

GPE and GPC levels are reduced in rapidly proliferating cells [15,19,32,33] and, in conditions of increased cell turnover such as the failing cirrhotic liver, it may be reasonable to expect reduced levels of these cell membrane degradation products.

Unlike the in vivo studies where there was an elevation in PME/ATP and PDE/ATP, correlated with the functional severity of liver injury [17,18], there was no statistical difference between metabolite levels from functionally compensated and functionally decompensated cirrhotic liver in our study. This may partially reflect the arbitrary nature of the clinical grading system [4], which is subject to a number of extrahepatic influences. Furthermore, the regional variation in metabolites concentrations that we observed within each individual liver highlights the fact that cirrhosis is not a uniform process. Therefore, the lack of distinction between liver samples from patients with compensated and decompensated cirrhosis may also be a reflection of the varying composition of these tissue samples.

Further studies correlating in vivo ^{31}P NMR spectral abnormalities with in vitro ^{31}P NMR appearances and electron microscopy of liver tissue to assess the NMR contribution of endoplasmic reticulum are required. However, the results of this study suggest that the changes in PE, PC, GPE and GPC are responsible, to a large extent, for the PME/ATP and PDE/ATP abnormalities seen in patients with cirrhosis of the liver.

Acknowledgements

This study was supported by the U.K. Department of Health and the Medical Research Council.

We would like to thank the MRC Biomedical NMR Centre, Mill Hill and Birkbeck College, ULIRS for their NMR and technical support; Brenda Hayter, Linda Selves and Debbie Marshall, the liver transplant coordinators at the Royal Free Hospital, London, for their assistance in sample collection and Dr David Menon for helpful advice.

References

[1] Sherlock, S. and Dooley, J. (1993) Diseases of the Liver and Biliary System, pp. 357–369, Blackwell Scientific Publications, Oxford.
[2] Erlinger, S. and Benhamou, J.-P. (1991) in: Oxford Textbook of Clinical Hepatology (McIntyre, N., Benhamou, J.-P., Bircher, J., Rizzetto, M. and Rodes, J., eds), pp. 380–390, Oxford University Press, Oxford.
[3] Rojkind, M. and Greenwel, P. (1991) in: Oxford Textbook of Clinical Hepatology (McIntyre, N., Benhamou, J.-P., Bircher, J., Rizzetto, M. and Rodes, J., eds), pp. 357–369, Oxford University Press, Oxford.
[4] Pugh, R.N.H., Murray-Lyon, I.M., Dawson, J.L., Pietroni, M.C. and Williams, R. (1972) Br. J. Surg. 60, 646–649.
[5] Shapiro, J.M., Smith, H. and Schaffner, F. (1979) Gut 20, 137–140.
[6] Albers, I., Hartmann, H., Bircher, J. and Creutzfeldt, W. (1989) Scand. J. Gastroenterol. 24, 269–276.
[7] Dickson, E.R., Grambsh, P.M., Fleming, T.R., Fischer, L.D. and Langworthy, A. (1989) Hepatology 10, 1–7.
[8] Oberhaensli, R.D., Hilton Jones, D., Bore, P.J., Hands, L.J., Rampling, R.P. and Radda, G.K. (1986) Lancet ii, 8–11.
[9] Cox, I.J., Bryant, D.J., Collins, A.G., George, P., Harman, R.R., Hall, A.S., Hodgson, H.J.F., Khenia, S., McArthur, P., Spencer, D.H. and Young, I.R. (1988) J. Comput. Assist. Tomogr. 12, 369–376.
[10] Meyerhoff, D.J., Boska, M.D., Thomas, A.M. and Weiner, M.W. (1989) Radiology 173, 393–400; erratum (1990) Radiology 176, 584.
[11] Angus, P.W., Dixon, R.M., Rajagopalan B, Ryley N.G., Simpson, K.J., Peters, T.J., Jewell, D.P. and Radda, G.K. (1990) Clin. Sci. 78, 33–38.
[12] Oberhaensli, R., Rajagopalan B., Galloway, G.J., Taylor, D.J. and Radda, G.K. (1990) Gut 31, 463–467.
[13] Lenkinski, R.E. (1989) Invest. Radiol. 24, 1034–1038.
[14] Ruiz-Cabello, J. and Cohen, J.S. (1992) NMR Biomed. 5, 226–233.
[15] Bell, J.D., Cox, I.J., Sargentoni, J., Peden, C.J., Menon, D.K., Foster, C.S., Watanapa, P., Iles, R.A. and Urenjak, J. (1993) Biochem. Biophys. Acta 1225, 71–77.
[16] Murphy, E.J., Rajagopalan, B., Brindle, K.M. and Radda, G.K. (1989) Magn. Reson. Med. 12, 282–289.
[17] Munakata, T., Griffiths, R.D., Martin, P.A., Jenkins, S.A., Shields, R. and Edwards, R.H.T. (1993) NMR Biomed. 6, 168–172.
[18] Menon, D.K., Sargentoni, J., Taylor-Robinson, S.D., Bell, J.D., Cox, I.J., Bryant, D.J., Coutts, G.A., Rolles, K., Burroughs, A.K. and Morgan, M.Y. (1995) Hepatology 21, 417–427.
[19] Cox, I.J., Bell, J.D., Peden, C.J., Iles, R.A., Foster, C.S., Watanapa, P. and Williamson, R.C.N. (1992) NMR Biomed. 5, 114–120.
[20] Iles, R.A., Stevens, A.N. and Griffiths, J.R. (1982) Prog. Nucl. Magn. Spectrosc. 15, 49–200.
[21] Cohen, S.M. (1983) J. Biol. Chem. 258, 14294–14308.
[22] Dawson, R.M.C. (1955) Biochem. J. 60, 325–328.
[23] Perry, T.L., Hansen, S., Berry, K., Mok, C. and Lesk, D. (1971) J. Neurochem. 18, 521–528.

[24] Perry, T.L., Hansen, S. and Gandham, S.S. (1981) J. Neurochem. 36, 406–412.
[25] Cox, I.J., Menon, D.K., Sargentoni, J., Bryant, D.J., Collins, A.G., Coutts, G.A., Iles, R.A., Bell, J.D., Benjamin, I.S., Gilbey, S., Hodgson, H.J.F. and Morgan, M.Y. (1992) J. Hepatol. 14, 265–275.
[26] Meyerhoff, D.J., Karczmar, G.S. and Weiner, M.W. (1989) Invest. Radiol. 24, 908–984.
[27] Rajanayagam, V., Lee, R.R., Ackerman, Z., Bradley, W.G. and Ross, B.D. (1992) J. Magn. Reson. Imag. 2, 183–190.
[28] Hachisuka, T., Nakayama, S., Tomita, T. and Takagi, F. (1992) J. Surg. Res. 53, 251–256.
[29] Bates, T.E., Williams, S.R. and Gadian, D.G. (1989) Magn. Reson. Med. 12, 145–150.
[30] Ferrari, V. and Harkness, R.D. (1954) J. Physiol. 124, 443–463.
[31] Murphy, E.J., Brindle, K., Rorison, C.J., Dixon, R.M., Rajagopalan, B. and Radda, G.K. (1992) Biochim. Biophys. Acta 1135, 27–34.
[32] Morikawa, S., Inubushi, T., Kitoh, K., Kidoh, C. and Nozaki, M. (1992) Biochim. Biophys. Acta 1117, 251–257.
[33] Farghali, H., Rilo, H., Zhang, W., Simplaceanu, V., Gavaler, J.S., Ho, C. and van Thiel, D.H. (1994) Lab. Invest. 70, 418–425.
[34] van Noorden, C.J., Vogels, I.M. and Houtkooper, J.M. (1988) Cell Biochem. Funct. 6, 53–60.
[35] Dixon, R.M. and Tian, M. (1993) Biochim. Biophys. Acta 1181, 111–121.