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A High Resolution Proton Nuclear Magnetic Resonance Approach to the Study of Hepatocyte and Drug Metabolism

Application to Acetaminophen

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SUMMARY

^1H spin echo NMR spectra of intact hepatocytes, isolated from rat liver, showed resonances for glucose, mobile fatty acids, and $^+N(CH_3)_3$ groups including choline headgroups of phosphoglycerides. Spectra from extracts of the same cells contained many more well resolved resonances due to low M_r metabolites. These included signals for free amino acids, ketone bodies, glucose, lactate, and acetate. ^1H NMR spectra from suspensions of intact hepatocytes incubated with acetaminophen showed no resonances for drug metabolites, although changes in sugar resonances were observed. However, spectra of extracts from acetaminophen-treated hepatocytes contained resonances for both acetaminophen itself and its major metabolites, the glucuronide and sulfate conjugates. Results on the extent of acetaminophen metabolism as measured by ^1H NMR compared well with previously reported chromatographic studies. The rate of metabolism of acetaminophen by hepatocytes was much slower in ^2H_2O buffer compared to H_2O buffer and selective deuteration of several metabolites including the ketone bodies, glucose, and acetaminophen glucuronide was observed. The deuteration of glucose C_2H appeared to be due to futile cycling of the glycolytic pathway to at least fructose 6-phosphate, and incorporation of deuterium by the enzyme phosphoglucoisomerase. This work demonstrates that ^1H NMR studies of intact hepatocytes and cell extracts together can provide considerable insight into the metabolism of acetaminophen *in vitro*. Little pretreatment of samples is required, results can be obtained rapidly, and both normal and drug metabolites can be observed simultaneously. Similar studies should be applicable to a wide range of other drugs.

INTRODUCTION

We have recently described the use of ^1H NMR spectroscopy for the rapid monitoring of the excretion of the analgesic drug acetaminophen [*N*-acetyl-4-aminophenol, *I* (structures shown on next page)] and its metabolites by human subjects (1). The major metabolites are the sulfate and glucuronide conjugates, *II* and *III*, together with smaller amounts of thiol conjugates such as the cysteinyl and *N*-acetylcysteinyl derivatives (2). The latter are of particular interest as possible indicators of the occurrence of toxic side reactions during acetaminophen metabolism (3). The biotransformation of acetaminophen occurs mainly in the liver (4). Studies of acetaminophen metabolism by isolated rat and mouse hepatocytes have been reported previously. High drug concentrations were used (10 mM) and metabolites were detected by liquid chromatography (5). In this paper, we explore the use of ^1H NMR spectroscopy for the investigation of the metabolism of acetaminophen by hepatocytes *in vitro*, with the aim of observing not only the drug metabolites but also the affect of the drug on normal metabolites. This is possible because NMR experiments can be carried out with the minimum of sample pretreatment and there is no need to preselect for detection of particular molecules, provided the metabolites possess protons suitable for observation by NMR spectroscopy.

There appear to be only two previous reports of *in vitro* NMR studies on hepatocytes, in which pH differences between mitochondrial and cytosolic compartments were determined by ^{31}P NMR (6), and the metabolism of ^{13}C-labeled glycerol was studied by ^{13}C NMR (7). Metabolic pathways in intact mammalian liver have been investigated recently by ^{13}C NMR also using isotopically enriched compounds administered to perfused livers in a conventional spectrometer (8) or to live rats in a topical magnetic resonance instrument (9).

Some workers have argued that severe background interferences from broad resonances of macromolecules

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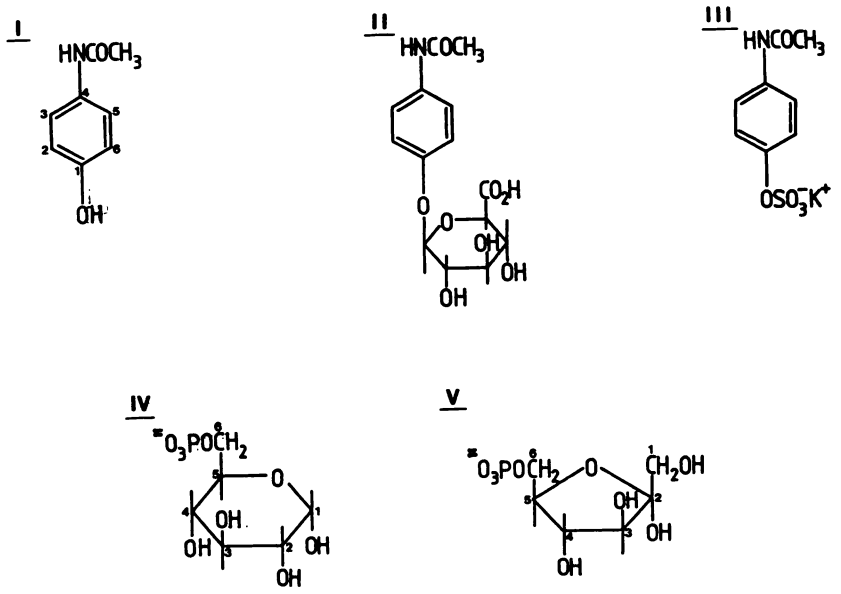

STRUCTURES *I–V*. *I, acetaminophen (N-acetyl-4-aminophenol); II, acetaminophen glucuronide (4-glucuronosidoacetanilide); III, acetaminophen sulfate (N-acetyl-4-aminophenol sulfate); IV, glucose 6-phosphate; V, fructose 6-phosphate*

and H_2O present insuperable problems during ^1H NMR studies of cell metabolism, and have resorted to ^2H- or ^{13}C-labeling experiments (10). However, our own work on blood, plasma, and urine (11–13), and the work of others on red cells (14, 15), Friend leukemia cells (16), and hybrid neuroblastoma × glioma cells (17), has shown that these difficulties can be largely overcome by the use of high frequency spectrometers (360–500 MHz) combined with either ^2H_2O-H_2O exchange, secondary irradiation of H_2O, saturation transfer, spin echo, or selective excitation pulse sequences.

A further aim of the present work was to examine possible differences in the NMR picture of cell metabolism provided by studies of intact hepatocytes in comparison with cell extracts. Macromolecules tumble slowly in cells and give rise to broad resonances. However, there are several mechanisms which can lead to severe broadening of the resonances of low molecular weight metabolites including local field inhomogeneities, viscosity effects, interaction with paramagnetic centers, and immobilization through binding to lipids or proteins. Clearly, the biochemical picture of metabolism obtained from NMR studies on whole cells could be severely misleading if "NMR-invisible" molecules are present. The work presented here is therefore of interest in the wider context of NMR applications to intact biological systems.

We show here that, with careful design of the NMR experiments, considerable insight into the metabolism of acetaminophen by hepatocytes and the influence of acetaminophen on cell metabolism can be gained. Studies of this type may provide a convenient test system for a range of other widely used drugs and for experimental chemotherapeutic agents.

EXPERIMENTAL PROCEDURES

Hepatocyte suspensions. Hepatocytes were isolated from Sprague-Dawley rats (150–200 g, Olac, Bicester, Oxon, U.K.) using essentially the procedure of Moldeus *et al.* (18). After pentobarbital anesthesia and cannulation of the hepatic portal vein, the liver was perfused with Hanks' buffer containing EGTA[1] (0.6 mM) and albumin (6.67 mg/ml), followed by Hanks' buffer containing collagenase (0.5 mg/ml, Boehringer) and $CaCl_2$ (4 mM). The liver was then dispersed into Krebs-Henseleit buffer containing HEPES (12.6 mM) and albumin (10 mg/ml). The suspension was filtered through gauze and the hepatocytes were allowed to settle. To remove all traces of albumin and collagenase, the cells were washed twice with Krebs-Henseleit buffer made up in ^2H_2O at pH* 7 [where pD = 0.4 + pH*, the meter reading (19)] or in H_2O (pH 7.4) and then resuspended in the same buffer. Cell numbers were determined using a hemocytometer slide and cell viability was measured using the trypan blue exclusion assay. An initial cell viability of 86–90% was obtained and suspensions containing between 6×10^6 and 1×10^7 cells/ml were used. NMR measurements were then carried out as follows.

Control cells. Two 1-ml aliquots of the cell suspension in deuterated buffer were placed in 5-mm NMR tubes, and spectra were obtained immediately. One tube was then maintained at 37 ± 1° for 240 min and gassed with 95% O_2/5% CO_2; the other was kept at 25° with no gassing. The NMR spectra of both samples were recorded at frequent time intervals.

The cells in a further 1-ml aliquot of the cell suspension were lysed by addition of an equal volume of ^2H_2O followed by a rapid freezing and thawing cycle. After centrifugation to remove cell debris, the supernatant was lyophilized and redissolved in 1 ml of deuterated 0.1 M phosphate buffer (pH* 7) for NMR measurement.

Acetaminophen metabolism. The ^1H NMR spectrum of 0.9 ml of an oxygenated suspension of hepatocytes (obtained from a second preparation) in deuterated Krebs-Henseleit buffer (pH* 7) was first recorded. Acetaminophen (*I*) was then added (final concentration, 3.3 mM; added as 0.1 ml of a 33 mM solution in the same buffer) and rapidly mixed, and the NMR spectrum was recorded immediately. The suspension was then maintained at 37° and gassed with 95% O_2/5% CO_2, and the spectrum was recorded periodically over 230 min. A second aliquot of cells without added acetaminophen was treated similarly as a control. Hepatocytes in a further 5 ml of the same suspension were allowed to sediment (about 10 min under gravity) and the NMR spectrum of 0.5 ml of packed cells was recorded.

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate.

Cell extracts. These were prepared from both control and acetaminophen-treated cells which had been incubated in H_2O or ^2H_2O buffers. All cell suspensions were adjusted so as to contain 10^7 cells/ml with a cell viability of 80%. Suspensions of hepatocytes (2 ml) were incubated in four rotating round-bottomed flasks maintained at 37° in a water bath and constantly gassed with 95% O_2/5% CO_2. Two samples contained H_2O and two contained ^2H_2O Krebs-Henseleit buffer. Acetaminophen was added to one of each to give a final concentration of 3.3 mM. The other two flasks of cells acted as controls. After an incubation time of 105 min, 0.75 ml of suspension was removed from each flask and the cells were lysed and centrifuged; the supernatant was lyophilized, as described above, and redissolved in ^2H_2O for NMR measurement.

This process was repeated on the remaining 1.25 ml after an incubation time of 180 min. Cell viabilities at this time were: control cells, 69% (H_2O) and 70% (^2H_2O); and acetaminophen-treated cells, 69% (H_2O) and 78% (^2H_2O).

For comparison, two other methods of extracting molecules from control cells were used. For these, 5-ml aliquots of cell suspensions in H_2O or ^2H_2O buffers were treated with either an equal volume of 30% trichloroethanoic acid, to disrupt cells and precipitate proteins, or were subjected to mechanical trauma with a high speed homogenizer (Polytron) to lyse the cells. Cell debris was removed by centrifugation and the extracted substances were lyophilized and redissolved as before.

NMR measurements. Spectra were recorded at 27° on Bruker WH400 and AM500 spectrometers, at 400 and 500 MHz, respectively, using quadrature detection. The H_2O signal was suppressed with continuous secondary irradiation when necessary. No special arrangements were made to oxygenate the cell suspensions in the NMR probe since they were well oxygenated prior to NMR measurement. Accumulations were begun less than 2 min after introduction of the sample into the probe. The tube was spun relatively rapidly (approximately 30 Hz), and the total spectral accumulation times were very short (<5 min). In metabolism experiments involving acetaminophen, results were discounted if sedimentation did occur because of possible hypoxia. However, control cells that had sedimented, and were therefore probably hypoxic, gave similar ^1H NMR spectra to freshly oxygenated suspensions (e.g., see Fig. 1: spectra of a dense suspension of hepatocytes, about 10^8 cells/ml, that had been gassed with 95% O_2/5% CO_2 for 45 min and then allowed to sediment for 5 min, and 0.5 ml of the slurry was transferred to the NMR tube). Normal (single-pulse) spectra were acquired using 60–70° pulses repeated every 4 sec, to allow proton magnetization to recover by T_1 relaxation. Spin echo spectra were recorded using the Hahn sequence (90°-τ-180°-τ-collect) (20, 21) with a τ value of 60 msec and a delay between cycles of 3 sec. This pulse sequence causes resonances to become phase-modulated according to their multiplicity and coupling constants (J); e.g., with τ = 60 msec, singlets and triplets are upright, whereas doublets appear inverted if J = 8 Hz ($\tau = 1/2J$). Spectra of cell suspensions were collected into 8,192 computer points and were zero-filled to 16,384 points. Spectra of cell extracts were collected into 16,384 points.

^1H NMR spectra of acetaminophen (*I*) and its major metabolites (glucuronide, *II*; sulfate, *III*; cysteinyl; *N*-acetylcysteinyl; and glutathionyl conjugates) were recorded in deuterated phosphate buffer (0.1 M, pH* 5.8) with sodium trimethylsilyl[$^2H_4$]propionate as an internal chemical shift reference. For other assignments, standards of various candidate compounds in ^2H_2O solution were added to cells or lysates to give a final concentration of about 1 mM.

RESULTS AND DISCUSSION

Intact hepatocytes. A "normal" single-pulse ^1H NMR spectrum of a dense suspension of hepatocytes is shown in Fig. 1a. The spectrum contains broad overlapping resonances with few sharp features. The most prominent of the broad resonances can be assigned to protons of unsaturated fatty acids (11, 12, 22, 23) (F_1–F_7). Fig. 1b shows the spin echo ^1H NMR spectrum of the same cell suspension. The spin echo pulse sequence results in the elimination of many of the broad resonances. These are mainly due to protons of macromolecules and have short spin-spin relaxation times (T_2 values). The sharper resonances due to protons with longer T_2 relaxation times are revealed. However some broad signals, at 0.9, 1.3, and 2.2 ppm (F_1, F_2, and F_5), still remain and can be assigned (22, 23) to the terminal —CH_3, —$(CH_2)n$-, and —(CH_2)-CO— protons, respectively, of mobile fatty acids, probably part of triglycerides and phospholipids. Their broad appearance in spin echo spectra may be due to chemical shift heterogeneity rather than relaxation effects. We have previously observed such signals in ^1H NMR spectra of mammalian plasma and they are especially intense in cases of hyperlipidemia (12). Very low density lipoproteins that are rich in triacylglycerides are synthesized and secreted by the liver (24). Canioni *et al.* (9) attributed some sharp ^{13}C NMR signals from liver observed by topical magnetic resonance to fatty acids in phospholipids. Triglycerides account for about 9–30% of the total lipids in liver, and phospholipids are about 65% (9).

The remainder of the NMR spectrum is comprised of lower intensity resonances from glucose that are phase modulated in the spin echo experiment and two sharp singlet resonances in the region 3.1–3.3 ppm. The ^1H NMR spectrum of a [$^2H_4$]methanol extract of lyophilized liver cells contained intense characteristic resonances for the phosphatidylcholine headgroup and fatty acyl chains of phospholipids (22, 23). This suggests that the singlet observed at 3.2 ppm in intact cells is also from a choline headgroup. Other possible ^+N-$(CH_3)_3$ groups with resonances in this region include betaine and carnitine.

Over a period of 240 min, these control cells showed a reduction in the intensities of the glucose resonances, presumably due to glycolysis. However, no resonances for other metabolites appeared. Glycolysis in erythrocytes, on the other hand, leads to an increase in the intensities of the ^1H NMR signals for lactate (25), but for hepatocytes, lactate is further metabolized in the mitochondria to acetyl-CoA and CO_2 (24).

A similar suspension of hepatocytes was incubated in the presence of 3.3 mM acetaminophen (*I*) and monitored by ^1H NMR at intervals over a period of 220 min. Fig. 2, a and b, shows the normal and spin echo NMR spectra recorded after 210 min. By this time, the intensities of the doublet resonances at 7.25 and 6.90 ppm due to the C_3H, C_5H and C_2H, C_6H aromatic protons of acetaminophen, and its *N*-acetyl resonance at 2.15 ppm had decreased. Surprisingly, no new peaks appeared in the spectrum corresponding to drug metabolites. A standard addition of acetaminophen glucuronide (*II*) was made to this cell suspension at 225 min. Characteristic, well resolved resonances for *II* were observed in the NMR spectrum, suggesting that their nonappearance during the experiment was not due to adverse line broadening resulting from (extracellular) diffusion in inhomogeneous fields created by the cells. Resonances for extracellular acetaminophen metabolites, glucuronide and sulfate (*II* and *III*), were seen in spectra of supernatants removed from cells before they were lysed. However,

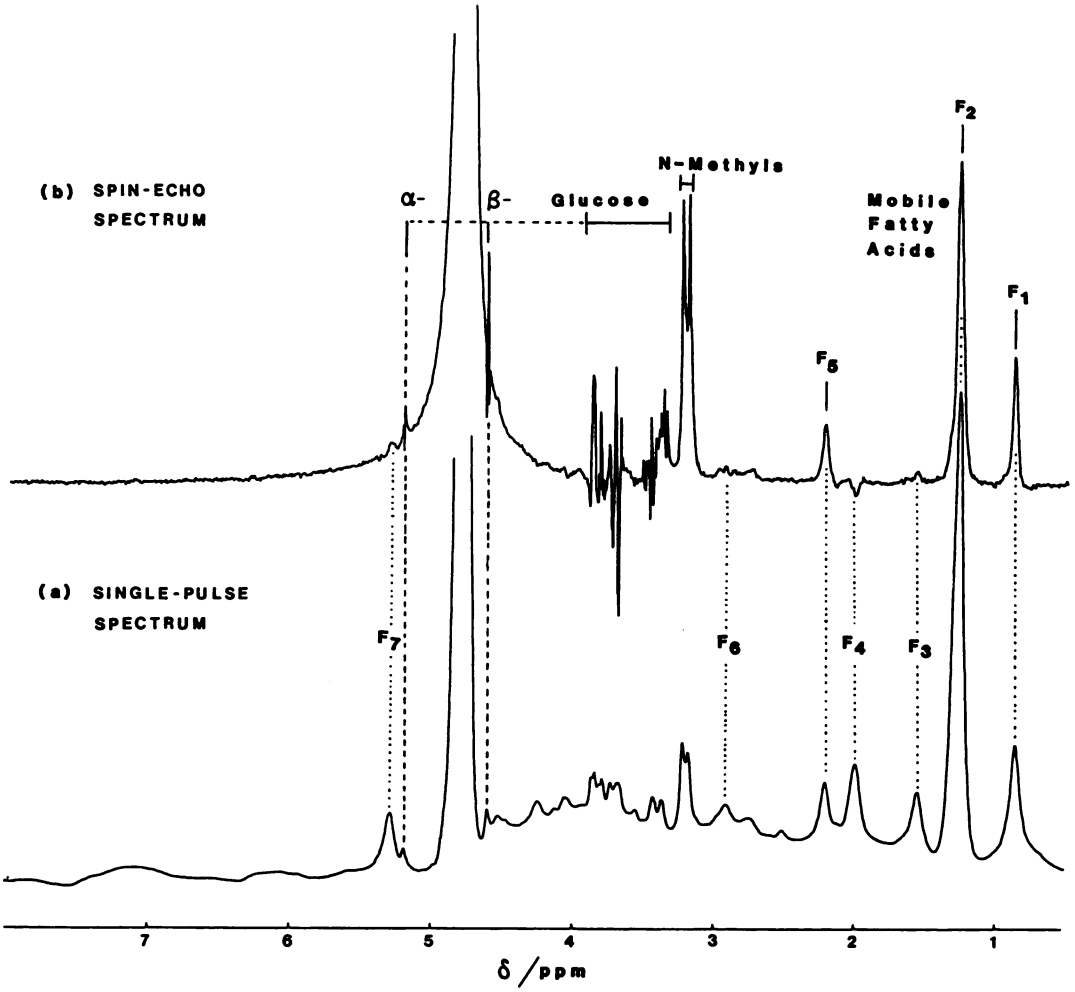

FIG. 1. *500-MHz ^{1}H NMR spectra of a packed suspension of hepatocytes in ^2H_2O, obtained using (a) single pulses (normal acquisition) and (b) a spin echo pulse sequence (τ = 60 msec)*

Peaks have been assigned for glucose, *N*-methyl groups (including choline headgroup of phospholipid at 3.2 ppm), mobile fatty acids (F_1, —CH_3; F_2, —$(CH_2)_n$—; F_3, -CH_2CH_2CO-; F_4, -CH═CHCH_2-; F_5, -CH_2CO-; F_6, -HC═CH-CH_2-CH═CH-; F_7, -CH═CH-); and, in *a*, a broad envelope of protein resonances. Note that F_3, F_4, F_6, and F_7 resonances are suppressed in the spin echo spectrum due to phase modulation and relaxation effects.

these were very weak and would have been difficult to observe in the presence of cells when the signal-to-noise ratios were lower, partly due to a loss of signal intensity due to T_2 relaxation during the evolution period of the spin echo experiment.

A major difference between the ^{1}H NMR spectra of acetaminophen-treated and control cells was that resonances for glucose increased, and that new resonances appeared between 3.5 and 4.0 ppm with a doublet at 5.41 ppm (Fig. 2, *S*), whereas for control cells, resonances in this region decreased in intensity. These are within the usual chemical shift range of sugars. The doublet splitting of about 4 Hz is similar to that expected for an α-anomeric proton of a sugar. There is no evidence of a corresponding β-anomer, i.e., no new resonance at about 4.7–4.8 ppm, although this could be obscured by the ^{2}HOH resonance. Various standard additions, including glucose 1-phosphate, glucuronic acid, and galactose, failed to characterize this new "sugar" molecule. $\alpha(1\rightarrow 4)$-linked glucose disaccharides, e.g., maltose and sucrose, give a similar doublet resonance at 5.4 ppm, but these two particular sugars had differing resonances in the region 3.5–4.0 ppm from those observed. The production of the new sugar by hepatocytes appeared to be induced by the presence of acetaminophen. However, we have also detected it in cell lysates and some other preparations of liver cells which had not been treated with acetaminophen. The exact circumstances of its production are therefore currently unclear.

The new sugar may be an intermediate in glycogenolysis which is needed for glucuronide formation. Sillerud and Shulman (8) have assigned some sharp ^{13}C NMR resonances in spectra of mammalian liver to glycogen. However, the ^{1}H NMR spectrum of a solution of glycogen in ^2H_2O was found to be broad and featureless.

Cell extracts. Fig. 3, a and b, shows the normal and spin echo 500-MHz ^{1}H NMR spectra, respectively, of a cell extract obtained (by the freeze-thaw method) from control cells that had been incubated in H_2O buffer for 3 hr. The normal spectrum contains a broad envelope of overlapping resonances, due to protons within proteins, and superimposed on this are sharper resonances for

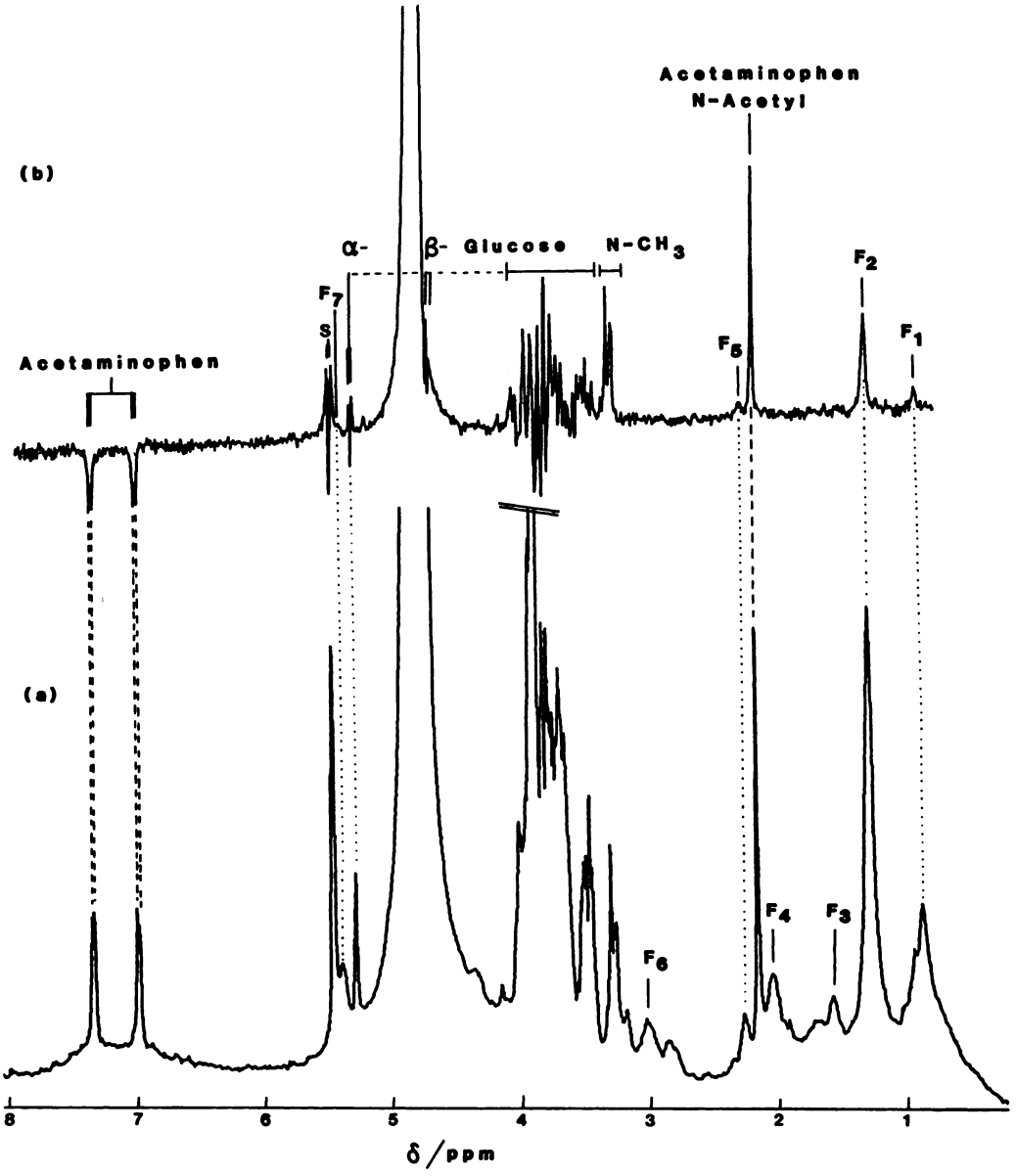

FIG. 2. *400-MHz ^1H NMR spectra of a packed suspension of hepatocytes after 3.5 hr in ^2H_2O buffer containing 3.3 mM acetaminophen*
a, single-pulse NMR spectrum; b, a spin echo spectrum (τ = 60 msec). Resonances are labeled for acetaminophen and a new sugar (*S*) in addition to those seen in Fig. 1.

several low M_r metabolites. In the spin echo spectrum, the protein envelope is eliminated and the resonances for the low M_r metabolites are phase modulated. It is clear that the spectra of extracts of lysed cells contain many more well resolved resonances for cellular metabolites than spectra of intact cells (cf. Figs. 1b and 3b). These include resonances for amino acids (valine, leucine, isoleucine, tyrosine, and phenylalanine), ketone bodies (3-D-hydroxybutyrate and acetoacetate), acetate, lactate, and glucose.

Signals for the mobile fats observed in the spectra of intact cells (Figs. 1 and 2) were notably absent from the spectra of extracts. Such fats may precipitate with the cell membranes, and may be removed by centrifugation, or they might not redissolve in aqueous media after the lyophilization step. The *N*-methyl resonances, from 3.1–3.3 ppm, are now better resolved and are comprised of at least four individual signals. However, there is an overall loss of intensity at 3.2 ppm, compared to spectra of intact cells, further confirming that the resonance at this position was due to the choline headgroup of a phospholipid.

Resonances for GSH were not observed in ^1H NMR spectra of whole cells and gave only weak resonances in the spectra of extracts. GSH concentrations of at least 2.5 mM within isolated hepatocytes have been reported previously (26). Therefore, if similar concentrations of GSH are present in the hepatocytes studied here, then its resonances must be broadened, even in cell extracts, either due to binding to proteins, or chemical exchange reactions or perhaps interaction with paramagnetic species. Oxidation may have occurred but no significant resonances due to GSSG were seen in the spectra. A standard addition of a solution containing GSH was

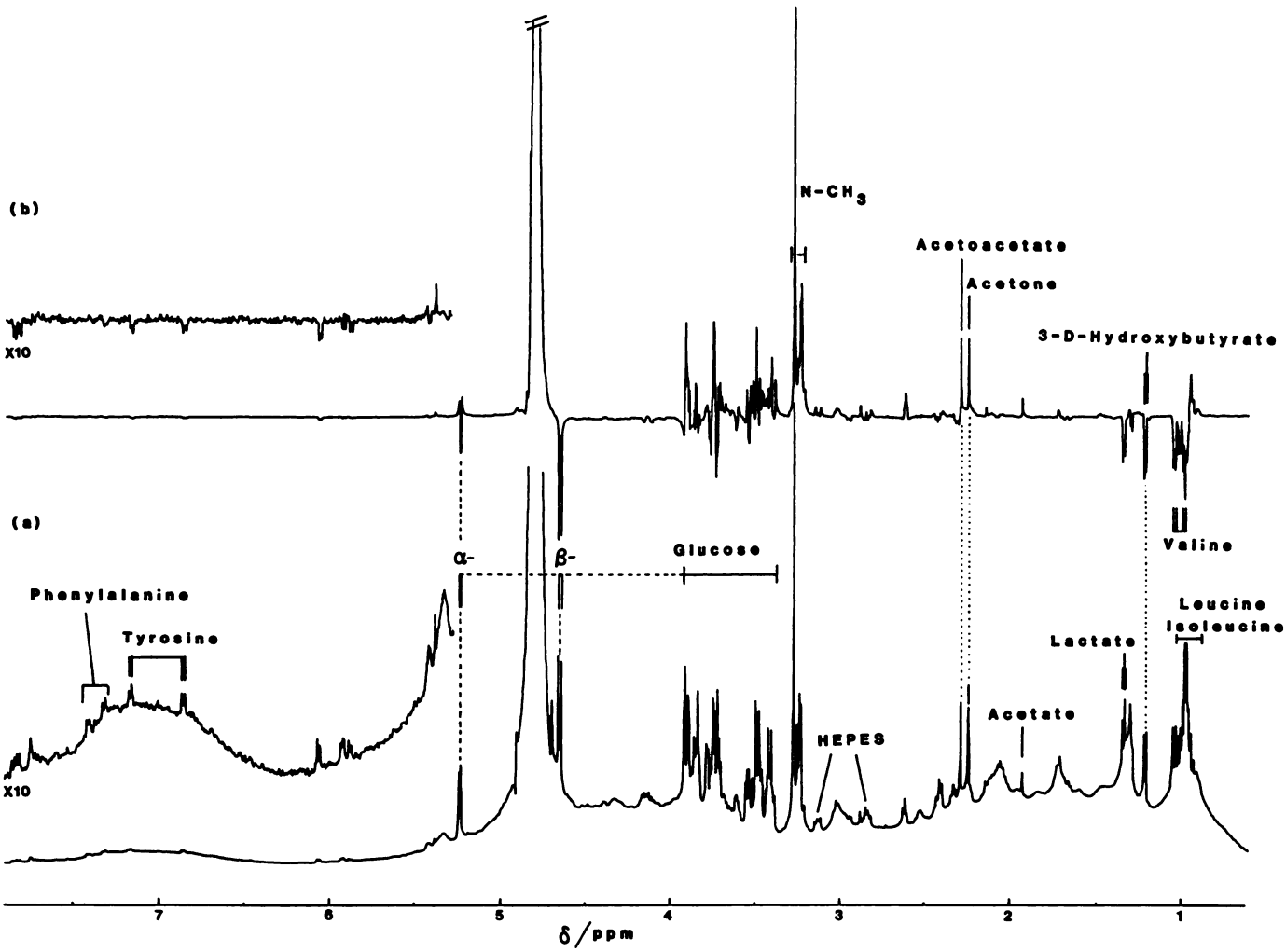

FIG. 3. *500-MHz ^{1}H NMR spectra of lyophilized extracts from hepatocytes redissolved in ^2H_2O, obtained using (a) single pulses (normal acquisition) and (b) a spin echo pulse sequence (τ = 60 msec)*

A broad envelope of protein resonances is seen in a, and resonances for many low M_r metabolites that are phase modulated are shown in b.

made to a similar cell extract and resonances due to GSH were observable in the ^{1}H NMR spectrum recorded. This suggests that broadening does not account for the non-appearance of GSH and that it was present at a low concentration in this cell preparation.

Fig. 3 also shows resonances assignable to HEPES from the buffering medium, even though the cells had been thoroughly washed (three times). This suggests that HEPES diffuses (or is transported) into the cells.

Aliquots from an incubated cell suspension were removed at two time intervals and the cells were subsequently washed, lysed, and centrifuged and the supernatant was lyophilized. Since a relatively mild technique (freeze-thaw) was used to disrupt the cells, many of the liver enzymes were still present and were presumably still functional. Samples were therefore redissolved in ^2H_2O, and NMR measurements were carried out immediately to keep the time after lysis to a minimum. For comparison, two other methods were used to lyse cells: treatment with trichloroethanoic acid, which was assumed to inhibit enzyme activities by denaturation and precipitation, and homogenization, which in a similar manner to the freeze-thaw method caused release of low M_r metabolites from compartments but did not affect the cellular proteins. The spin echo ^{1}H NMR spectra of extracts obtained after each of these different treatments were essentially the same, except for shifts due to pH differences between the samples.

The production of ketone bodies (acetoacetate, 3-D-hydroxybutyrate, and acetone) by hepatocytes as observed by ^{1}H NMR (Fig. 3) increased with time and was not affected by the presence of acetaminophen (Fig. 4a). Acetone, produced via decarboxylation of acetoacetate, is volatile; therefore, some was probably lost during the lyophilization step prior to NMR measurement. Resonances for ketone bodies were much less intense in spectra of extracts from cells which had been incubated initially in ^2H_2O buffer (see Fig. 4b). Also, the doublet methyl resonance of 3-D-hydroxybutyrate is present as a singlet, i.e., it is decoupled from its neighboring CH proton. This can be explained by incorporation of deuterium from the solvent and NAD^{2}H during the enzymatic conversion of acetoacetate to 3-D-hydroxybutyrate. Complete chemical exchange of the acidic CH_2 meth-

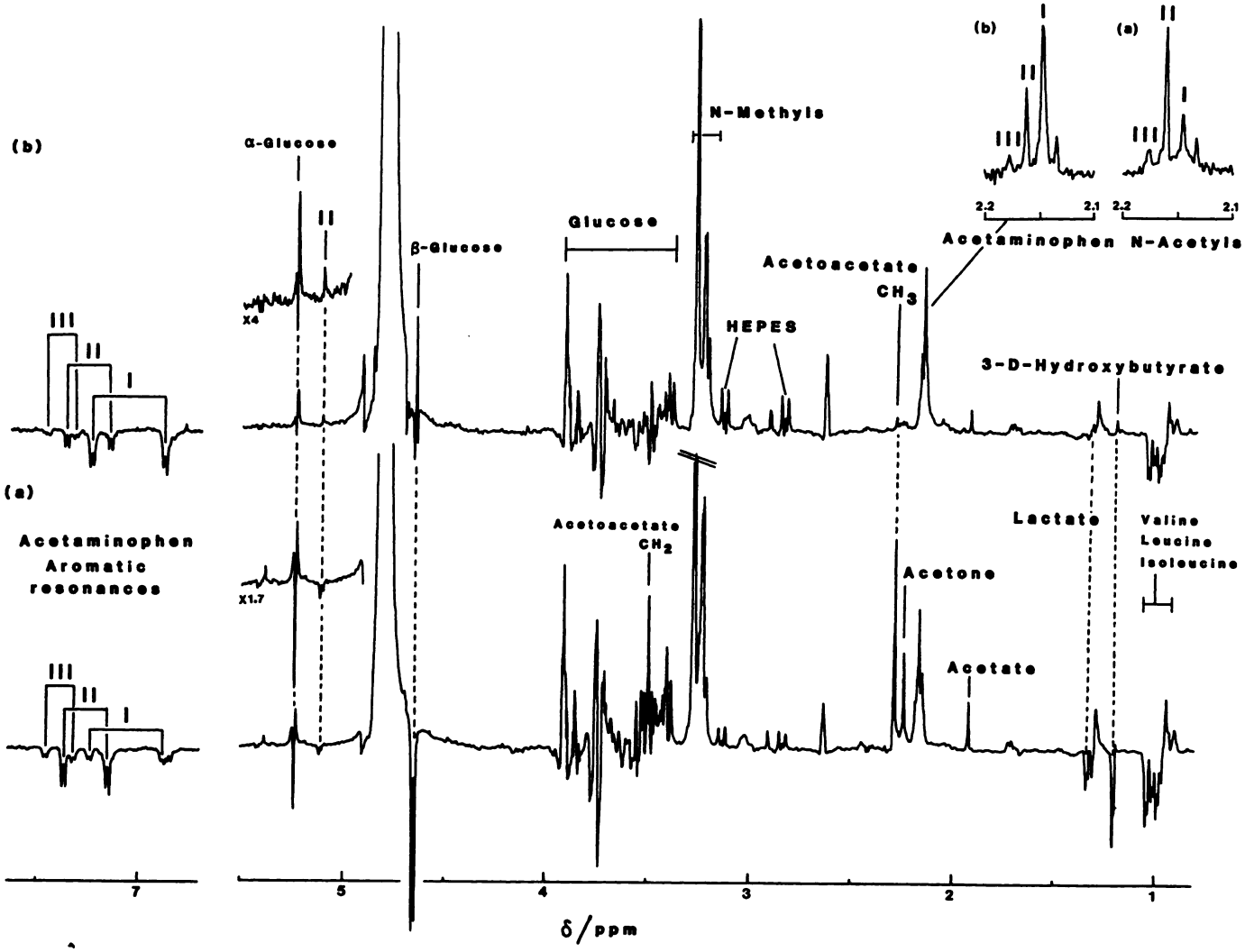

FIG. 4. *500-MHz spin echo (τ = 60 msec) ^1H NMR spectra of lyophilized extracts from hepatocytes redissolved in ^2H_2O*

The cells had been incubated for 3 hr in the presence of 3.3 mM acetaminophen prior to lysis in H_2O buffer (a) and ^2H_2O buffer (b). Peaks for acetaminophen (*I*) and its glucuronide (*II*) and sulfate (*III*) metabolites are seen, in addition to low M_r metabolites seen in Fig. 3. Selective deuteration of glucose, acetaminophen glucuronide (*II*), ketone bodies, and lactate is apparent in b. Inset: expansions of the *N*-acetyl region, 2.1–2.2 ppm, in a and b, Fourier transformed using Gaussian resolution enhancement.

ylene protons of acetoacetate with deuterium from the buffer is also known to be facile (27). The liver is the major site for the production of ketone bodies, which are formed from acetyl-CoA following triglyceride metabolism (24). Their formation would be expected in hepatocytes deprived of glucose, as in fasting of animals. Ketone body production by fasting humans and subjects with uncontrolled diabetes mellitus has been observed in ^1H NMR of intact plasma and urine (12). The methyl proton resonance for lactate was also a singlet in spectra of extracts from cells incubated in ^2H_2O. As above, this is due to deuteration of the CH proton, via NAD^{2}H, resulting in loss of coupling to the methyl protons and a slight isotope shift of the resonance. A similar deuteration has been observed in experiments on red cells (14, 28).

Extracts from acetaminophen-treated cells. In the above experiments on "whole cells," it was possible that resonances due to extracellular acetaminophen only were being detected, and that those for intracellular drug and/or metabolites were broadened. Such broadening could arise from severely restricted intracellular motion, perhaps due to binding to macromolecules or compartmentalization. The major site for drug metabolism is the smooth endoplasmic reticulum (29). Foreign compounds and their metabolites may be compartmentalized within this organelle rather than freely moving in the cytosol.

Fig. 4a shows a spectrum of an extract from cells incubated for 3 hr in H_2O buffer containing 3.3 mM acetaminophen. Now, in contrast to the spectrum from intact cells (Fig. 2), resonances due to free drug, *I*, and also its glucuronide and sulfate metabolites, *II* and *III*, are seen in both the aromatic and *N*-acetyl regions of the NMR spectrum.

Fig. 5 shows the aromatic regions of single-pulse NMR spectra of extracts from cells incubated with acetaminophen, in either H_2O or ^2H_2O, for 1 hr 45 min and 3 hr. By comparison of Fig. 4a with Fig. 5b (spin echo and single-pulse NMR spectra of the same sample, respec-

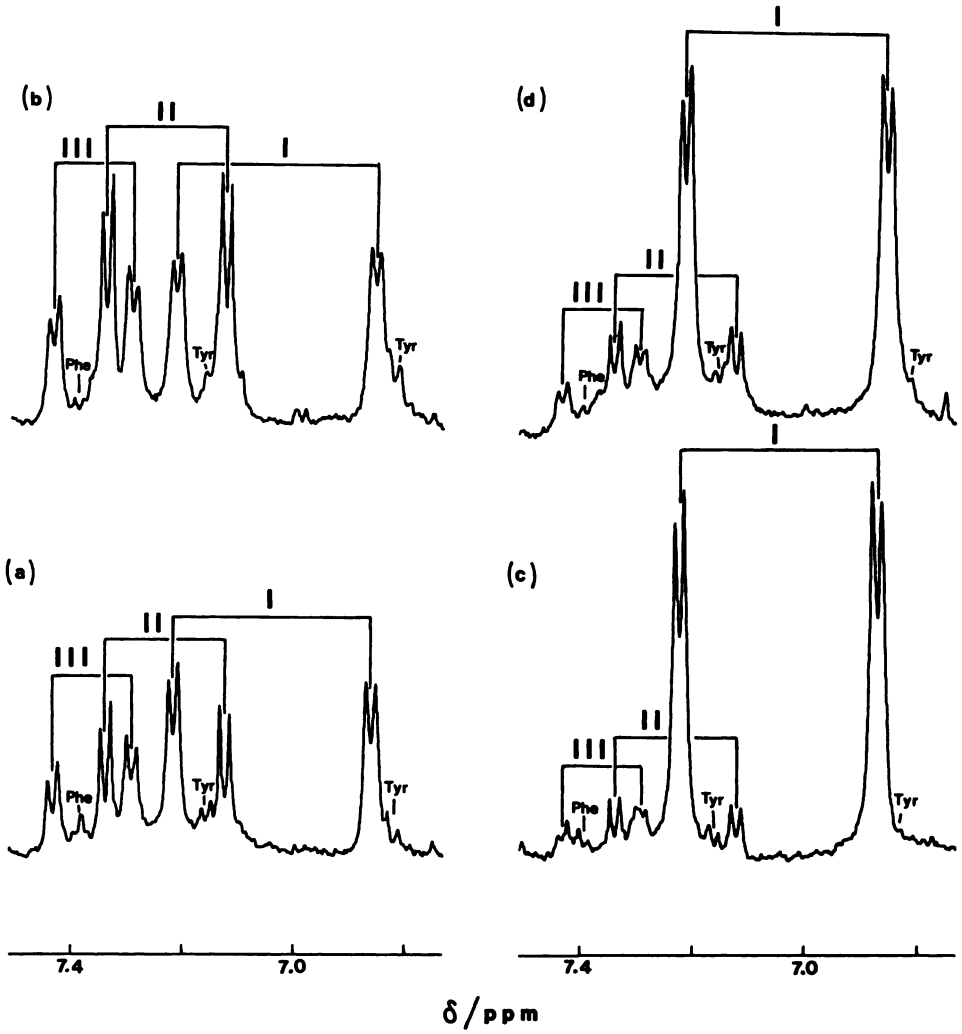

FIG. 5. *500-MHz single-pulse ^{1}H NMR spectra of lyophilized extracts from hepatocytes redissolved in ^2H_2O*

Cells were incubated in the presence of 3.3 mM acetaminophen in H_2O buffer for 1 hr 45 min (a) and 3 hr (b), and ^2H_2O buffer for 1 hr 45 min (c) and 3 hr (d) prior to cell lysis. The aromatic resonances of acetaminophen (*I*) and the extent of metabolism of acetaminophen to its glucuronide (*II*) and sulfate (*III*) conjugates can be seen from changes in peak heights. Peaks *Tyr* and *Phe* are due to tyrosine and phenylalanine, i.e., normal metabolites that are present in controls (see Fig. 3a).

tively), it can be seen that the relative peak intensities in the spin echo spectrum are severely distorted: the peaks due to the free drug and sulfate are suppressed relative to the glucuronide due to relaxation effects.

Acetaminophen was found to be metabolized approximately three times more slowly by hepatocytes in deuterated buffers (see also Table 1), suggesting that several (possibly three) secondary isotope effects are involved in the rate-limiting steps. The glucuronide/sulfate ratios (*II*/*III*) observed in this experiment, 1.5, agrees well with those reported for hepatocytes previously by Moldeus (5) using high performance liquid chromatography analytic methods. He observed rates of 38.8, 24.0, and 3.9 nmol/10^6 cells/30 min for metabolism of acetaminophen to the glucuronide, sulfate, and glutathionyl conjugates, respectively: a glucuronide/sulfate ratio of 1.6. No ^{1}H NMR resonances due to glutathionyl conjugates of acetaminophen were observed. If the intracellular GSH concentration in the hepatocytes used here is, in fact, quite low, then this would account for both the lack of observation

TABLE 1

Percentages of acetaminophen (I) and its glucuronide (II) and sulfate (III) conjugates in hepatocyte extracts, as determined by ^{1}H NMR spectroscopy from peak heights of aromatic resonances in single-pulse experiments

Ratio (*II* + *III*) in H_2O/(*II* + *III*) in ^2H_2O = 2.85 (at 105 min) and 2.88 (at 180 min).

	Incubation times/solvent			
	105 min		180 min	
	H_2O	^2H_2O	H_2O	^2H_2O
	%		%	
Acetaminophen, *I*	43	80	25	74
Glucuronide, *II*	34	12	44	16
Sulfate, *III*	23	8	29	10
II/*III* ratio	1.5	1.5	1.5	1.6

of resonances from GSH itself and from GSH conjugates of acetaminophen.

Deuteration of sugars. The ^1H NMR resonance for the C_1-α anomeric proton of acetaminophen glucuronide (*II*), at 5.2 ppm, produced in cells incubated in a ^2H_2O buffer is a simple singlet (Fig. 4b). This is phased upright in spin echo spectra whereas the 4-Hz doublet usually seen for this proton, through coupling to the C_2H proton, appears to be phase modulated (Fig. 4a). The loss of this coupling suggests that there is complete deuteration at the C_2 position. A similar effect was observed for glucose itself: loss of coupling to the C_1-β and C_1-α anomeric protons at 4.65 and 5.25 ppm, respectively, i.e., partial collapse to upright singlets in the spin echo spectra (Fig. 4b). There were also small deuteration shifts. Therefore, it appeared that a partial deuteration of the C_2H proton of glucose was also occurring in experiments carried out in ^2H_2O buffer. It seems likely that the deuteration of glucose occurs via conversion of G6P (*IV*) to F6P (*V*).

The interconversion of G6P and F6P involves the transfer of the C_2-proton to the C_1-position by the enzyme phosphoglucoisomerase (EC 5.3.1.9), and proceeds via a *cis* "ene-diol" intermediate (30). It has been shown previously, using tritium-labeling experiments (31), that the hydrogen atom is transferred largely via an intramolecular rearrangement, but that exchange with solvent also occurs to a lesser extent. We have investigated this enzymic reaction with respect to the deuteration reaction seen in the above NMR experiments.[2] When either G6P or F6P was used as initial substrate, incorporation of deuterium was observed by the time equilibrium had been reached. However, the initial rate of deuteration was dependent on the direction of the reaction: incorporation of deuterium was faster when G6P was converted to F6P than vice versa.

For this enzyme to be responsible for the deuteration reactions seen in hepatocytes, there must be futile cycling of glucose down the glycolytic pathway to the level of at least fructose 6-phosphate. A similar futile cycling has been reported by Ugurbil *et al.* (32). They showed that a ^{13}C label in glucose at C_6 was transferred to the C_1 position by triosephosphate isomerase, and thus required cycling at least to triosephosphate.

CONCLUSIONS

Proton NMR spectra of intact hepatocytes contain intense signals from glucose and mobile triglycerides, including the N-CH_3 headgroup of fatty acylphosphatidylcholine. However, ^1H spin echo NMR spectra of extracts, obtained from similar cells under mild conditions, contain many more well resolved resonances from low M_r species. This suggests that resonances for many low M_r metabolites in intact cells are broadened beyond detection. Such broadening may arise from diffusion within regions of inhomogeneous magnetic fields created by the different cellular compartments, restricted molecular motion due either to localization within small intracellular compartments or to binding to macromolecules, or from interaction with paramagnetic ions, e.g., the cytochrome P-450 system.

Under conditions in which intact hepatocytes were known to metabolize acetaminophen, no new NMR signals for drug metabolites were seen in intact cells. This is important in the wider field of *in vivo* NMR studies where many molecules may be "NMR-invisible," particularly in proton NMR spectra. Consequently, the biochemical information obtainable from NMR studies of intact organs or cells may be misleading. However, an increase in intensity of sugar resonances which can be related to glycogen breakdown was seen.

Wherever possible, it is important to examine cell extracts also. Glucuronide and sulfate conjugates of acetaminophen were readily detected and quantified in ^1H NMR spectra of extracts from hepatocytes and the pattern of metabolism agreed well with previous high performance liquid chromatography studies (5).

A major advantage of ^1H NMR studies is that both drug metabolites and the normal cellular metabolites can be monitored simultaneously. Therefore, biochemical reactions associated with drug metabolism can be followed. In this study, a time-dependent production of ketone bodies was observed, although this was not affected by acetaminophen. The presence of ^2H_2O was found to reduce both the rate of acetaminophen metabolism and the rate of ketone body production. Also, selective incorporation of deuterium into the ketone bodies, lactate, glucose, and acetaminophen glucuronide was observed. Such deuteration studies provide considerable insight into the biochemical reactions occurring within the cell (33, 34), in the present case suggesting that a futile cycling of glucose down the glycolytic pathway to at least the level of fructose 6-phosphate.

Proton NMR studies of the type described here appear to have considerable potential in molecular pharmacological studies. Many classes of drugs are known to be metabolized by hepatocytes and both the products of drug metabolism and the effect on the drug and/or normal cell metabolism can be studied together.

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