Uridine Diphospho Sugars and Related Hexose Phosphates in the Liver of Hexosamine-Treated Rats: Identification Using ^{31}P-{^{1}H} Two-Dimensional NMR with HOHAHA Relay†

Michael E. Perlman, Donald G. Davis,* Scott A. Gabel, and Robert E. London
Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709
Received September 20, 1989; Revised Manuscript Received January 2, 1990

ABSTRACT: The effects of administration of galactosamine (GalN) and glucosamine (GlcN) on the levels of UDP-sugars and hexose monophosphates in rat livers were studied by a variety of ^{31}P NMR methods. The flux of metabolites in the liver was monitored by in vivo NMR and showed elevated levels of UDP-sugars, and even greater increases in resonances at 4.6 ppm for GlcN treatment and at 2.0 ppm for GalN treatment. The individual compounds corresponding to these changes were identified in PCA liver extracts by ^{31}P-{^{1}H} two-dimensional relay spectroscopy with a HOHAHA-type ^{1}H spin-lock. This method of transferring proton magnetization allows for nearly all of the proton chemical shifts to be observed for the hexose moiety of a UDP-sugar present in a complex mixture. The UDP-sugars in the extracts from treated rats were predominantly UDP-hexosamines. Relay spectra were also used to determine that GalN-1-P was the major component (16.0 μmol/g of liver) of the GalN-treated liver, while both α and β anomers of GlcNAc-6-P were readily identified as the major hexose monophosphates in the GlcN experiment. Spectra from the ^{1}H dimension of relay experiments conducted on extracts were nearly superimposable on relay spectra obtained under the same conditions for mixtures of standard compounds of known structure. UDP-GlcN and UDP-GalN were not commercially available, but their presence was established in the extracts after GalN treatment by obtaining relay spectra for a mixture of the compounds produced in situ enzymatically, without purification. These compounds, as well as GalN-1-P, were found to be partially converted to the corresponding carbamic acids by the K_2CO_3 used for PCA neutralization. ^{31}P-{^{1}H} relay spectroscopy should thus be of general utility for the characterization of organophosphates in tissue extracts.

A wide variety of biochemical processes depend on the levels of uridine diphospho sugars, including the glycosylation of proteins (Nikaido & Hassid, 1971) and lipids such as lipid A (Anderson & Raetz, 1987), glycogen synthesis (Leloir, 1964), and the glucuronidation of drugs and toxins (Dutton, 1980). UDP-sugars[1] are normally reported as a collection of weak and poorly resolved signals in ^{31}P NMR studies of tissue and cell extracts, and the more easily observed β-phosphate (hexose) resonances are usually referred to as the diphosphodiester (DPDE) or uridine diphosphoglucose (UDPG) region. Recently, interest in these metabolites has increased since elevated levels have been observed in a number of tumors (Cohen et al., 1986; Corbett et al., 1987) but not in cell lines that are capable of differentiation or greater malignancy (Desmoulin et al., 1986; Fantini et al., 1987). The specific DPDE's that are increased were found by chromatographic analysis of extracts (Wice et al., 1985) to be UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylgalactosamine (UDP-GalNAc), and these UDP-sugars were also shown by ^{31}P NMR to be the dominant DPDE's in melanoma cell extracts (Corbett et al., 1987).

The UDP-hexosamine levels in the liver have also been shown to be elevated after the administration of galactosamine or glucosamine, with accompanying hepatotoxicity (Decker & Keppler, 1974; Chelibonova-Lorer et al., 1983). Glucosamine also exhibits antitumor activity which is accompanied by a similar perturbation in UDP-sugar levels in the tumor (Krug et al., 1984). The uptake of 5-fluorouracil by tumor cells, and thus its antitumor activity, is enhanced by both amino sugars, apparently as a result of uridylate trapping (Keppler et al., 1985). Viral multiplication has also been found to be inhibited by GlcN due to its effects on glycoprotein synthesis (Schwarz & Datema, 1982).

The metabolism of hexosamines in the liver has been studied previously by HPLC analysis of extracts and the use of radiolabeling (Weckbecker & Keppler, 1983; Krug et al., 1984), which requires a series of column systems to separate all of the isomeric UDP-sugars and the corresponding hexose monophosphates. An alternative method is high-resolution ^{31}P NMR, which has permitted the assignment of abundant

† Presented in part at the XIII International Conference on Magnetic Resonance in Biological Systems, Madison, WI, Aug 1988.

[1] Abbreviations: CDTA, 1,2-diaminocyclohexane tetraacetate; 2,3-DPG, 2,3-diphosphoglycerate; FID, free induction decay; GalN, galactosamine; GalN-1-P, galactosamine 1-phosphate; GalNAc-1-P, *N*-acetylgalactosamine 1-phosphate; GlcN, glucosamine; GlcN-1-P, glucosamine 1-phosphate; Glc-6-P, glucose 6-phosphate; GlcNAc-1-P, *N*-acetylglucosamine 1-phosphate; GlcN-6-P, glucosamine 6-phosphate; GlcNAc-6-P, *N*-acetylglucosamine 6-phosphate; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; α-GP, α-glycerophosphate; HOHAHA, homonuclear Hartmann-Hahn cross-polarization; HPLC, high-performance liquid chromatography; NMP, nucleoside monophosphate; NTP, nucleoside triphosphate; P-Chol, phosphocholine; P-E, phosphoethanolamine; P_i, inorganic phosphate; PME, phosphomonoesters; τ_{MIX}, spin-lock or isotropic mixing time; TSP, 3-(trimethylsilyl)-propionic acid; UDP-Gal, uridine 5'-(diphosphogalactose); UDP-GalN, uridine 5'-(diphosphogalactosamine); UDP-GalNAc, uridine 5'-(diphospho-*N*-acetylgalactosamine); UDP-GalNCO_2^-, uridine 5'-(diphospho-*N*-carboxygalactosamine); UDP-Glc, uridine 5'-(diphosphoglucose); UDP-GlcA, uridine 5'-(diphosphoglucuronic acid); UDP-GlcN, uridine 5'-(diphosphoglucosamine); UDP-GlcNAc, uridine 5'-(diphospho-*N*-acetylglucosamine); UDP-GlcNCO_2^-, uridine 5'-(diphospho-*N*-carboxyglucosamine); UDP-sugar, uridine 5'-(diphospho sugar).

UDP-sugars in the DPDE region in the spectra of extracts of animal and plant tissues (Navon et al., 1979; Greiner et al., 1982; Roby et al., 1987). We have therefore investigated the metabolism of hexosamines in the liver of intact rats using in vivo ^{31}P NMR, which reveals the nature and time course of metabolism in the liver. In order to identify and quantitate the resonances observed in vivo, analysis of PCA extracts was performed with high-resolution NMR to completely resolve the UDP-sugar and hexose monophosphate resonances.

Since the chemical shifts of biological phosphates vary with pH, metal concentration, and ionic strength (Van Wazer & Ditchfield, 1987), standard compounds are added frequently to extracts in order to make definitive assignments of the ^{31}P spectra. A more general approach to extend the information content of the ^{31}P spectra utilizes heteronuclear two-dimensional NMR, which correlates ^{31}P resonances with the chemical shifts of protons to which they are scalar coupled, as recently demonstrated for an intact pancreas (Kushnir et al., 1989). Moreover, by incorporating a relay of ^{1}H magnetization among mutually coupled protons (Bolton, 1982), the ^{31}P-{^{1}H} chemical shift correlation can be further extended from the directly coupled ^{31}P–^{1}H pairs to next-nearest-coupled ^{1}H–^{1}H pairs and beyond. As first demonstrated by Bax et al. (1985) for ^{13}C–{^{1}H} 2-D correlation spectroscopy, and more recently by Zagorski and Norman (1989) for ^{31}P–{^{1}H} 2-D spectra, such extended or multiple relays among the protons are accomplished most efficiently with homonuclear Hartmann–Hahn cross-polarization (Braunschweiler & Ernst, 1983; Davis & Bax, 1985). We have found that this technique can be used to assign unambiguously the UDP-sugars, hexose phosphomonoesters, and other organophosphates present in liver extracts, without further manipulation or purification of the material.

Materials and Methods

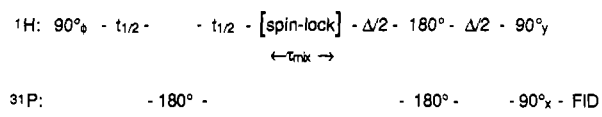
Chemicals. All reference standards, reagents, and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO).

In Vivo NMR Measurements. In vivo ^{31}P NMR spectra were obtained on a Nicolet NT-360 NMR spectrometer at 146.15 MHz using a surface coil placed above the liver after surgical removal of the overlaying skin, as previously described (London et al., 1985). Male, Sprague-Dawley rats weighing between 250 and 375 g were anesthetized with inactin (100 mg/kg of body wt) and metofane. After control spectra were obtained, the rats were injected while in the magnet with the hexosamine solution (1.85 mmol/kg iv). The α-P resonance of NTP at −10.7 ppm was used as a reference.

Preparation of Liver Extracts. Extracts were obtained from livers of animals treated iv with hexosamine either subsequent to an in vivo NMR experiment or 5 h after treatment of animals that did not undergo in vivo NMR analysis. Spectra of the extracts were not substantially different. The liver was removed from the rat 5 h after injection with hexosamine and immediately placed in liquid nitrogen. It was then weighed and powdered in a cold mortar and pestle, and perchloric acid (12%, 1 mL/g of liver) was added to yield a slurry. This was centrifuged at 16500*g* (15 min at 4 °C), and the cold supernatant was treated with 4 M K_2CO_3 until pH 8.0 and then recentrifuged. The extract was stirred with chelating resin (Sigma), the resin washed with water, and the extract evaporated. It was dissolved in 2.5 mL of $^{2}H_2O$ and then filtered into a 10-mm NMR tube, CDTA was added (10 mM), and the pH was adjusted to 8.20 with ^{2}HCl or $NaO^{2}H$.

NMR Spectra of Liver Extracts and Standards. Solution NMR spectra were obtained on a General Electric GN-500 spectrometer (B_0 = 11.75 T), operated at 23 °C with quadrature detection and deuterium lock ($^{2}H_2O$). Proton chemical shifts are reported relative to TMS, TSP being used as an internal shift standard (0.0 ppm). Glycerophosphocholine (GPC) was utilized as an internal chemical shift standard (−0.13 ppm) for ^{31}P spectra (Barany & Glonek, 1984). CDTA (10 mM) was added to all samples, and the pH was adjusted to 8.20 without any correction for the presence of $^{2}H_2O$. One-dimensional ^{31}P spectra were obtained at 202.4 MHz as FIDs with 16K (complex) data points at spectral band widths of 6.5 kHz (32 ppm). The flip angle for the ^{31}P observe pulse was set at 45°, a recycle time of 2.8 s was used, and about 1000 scans were typically accumulated. Two-level, broad-band coherent ^{1}H decoupling was employed. Prior to Fourier transformation, data sets were sequentially multiplied by a 2-Hz positive exponential and a 2-Hz Gaussian filter.

Phase-sensitive, absorption-mode (Muller & Ernst, 1979; States et al., 1982) two-dimensional ^{31}P-{^{1}H} relayed chemical shift correlation spectra were obtained with a pulse sequence adapted from Bax et al. (1985):



(1)

Here the ^{1}H spin-lock is a composite WALTZ-8 sequence (Shaka et al., 1983). For absorption-mode spectra, the phase ϕ of the initial 90° (^{1}H) pulse is cycled through $x,y,-x,-y$, and the FIDS acquired during t_2 are alternately stored in separate blocks of memory with alternate addition and subtraction of the data in each block (i.e., the receiver phase is cycled: +,+,−,−). The delay Δ was set to 60 ms, and the ^{1}H-dimensional spectral width was 2 kHz (centered at 4.5 ppm), while the ^{31}P spectral width was typically 6.5 kHz for spectra of extracts. A delay between scans of 1.0 s was employed. The duration of the spin-lock, τ_{mix}, which determines the extent of the ^{1}H relay, was varied in different experiments from 15 to 210 ms (vide infra). The FID was subjected to Gaussian filtering of 2–4 Hz in the t_2 dimension with one zero filling prior to Fourier transformation. In the t_1 dimension, 15–20-Hz Gaussian filtering, or trapezoidal multiplication (for the more intense phosphomonoester signals), and two zero fillings were employed.

We should note that relay experiments performed on extracts suffer from a loss in sensitivity due to their high salt content, which in turn reduces the coupling of the induced magnetization to the receiver coil and degrades the efficiency of the spin-lock. In order to reduce further loss of signal due to transverse relaxation, and also to compensate for the low levels of certain metabolites in the extracts, we omitted a refocusing step—$\Delta'/2$–180°(^{1}H,^{31}P)–$\Delta'/2$—that is normally included in this sequence between the 90° polarization transfer pulses and acquisition (t_2). Consequently, ^{1}H decoupling during acquisition is inappropriate, and the resonances in the ^{31}P dimension appear as antiphase doublets after Fourier transformation. For samples low in salt, such as commercial standards, conductance loading is less severe, and the complete sequence (Bax et al., 1985) with refocusing and ^{1}H decoupling can be used for sufficiently concentrated samples with Δ' set to 50 ms. In this paper, however, most of the relay spectra were obtained with the truncated sequence (eq 1). When improved resolution in the ^{31}P dimension of spectra of extracts was critical and strong signals were involved, the full sequence with refocusing and ^{1}H decoupling was employed (see Figure 5a,c).

Enzymatic Synthesis of UDP-GlcN and UDP-GalN. A 2 mM solution of UDP-GlcN was prepared by incubation of

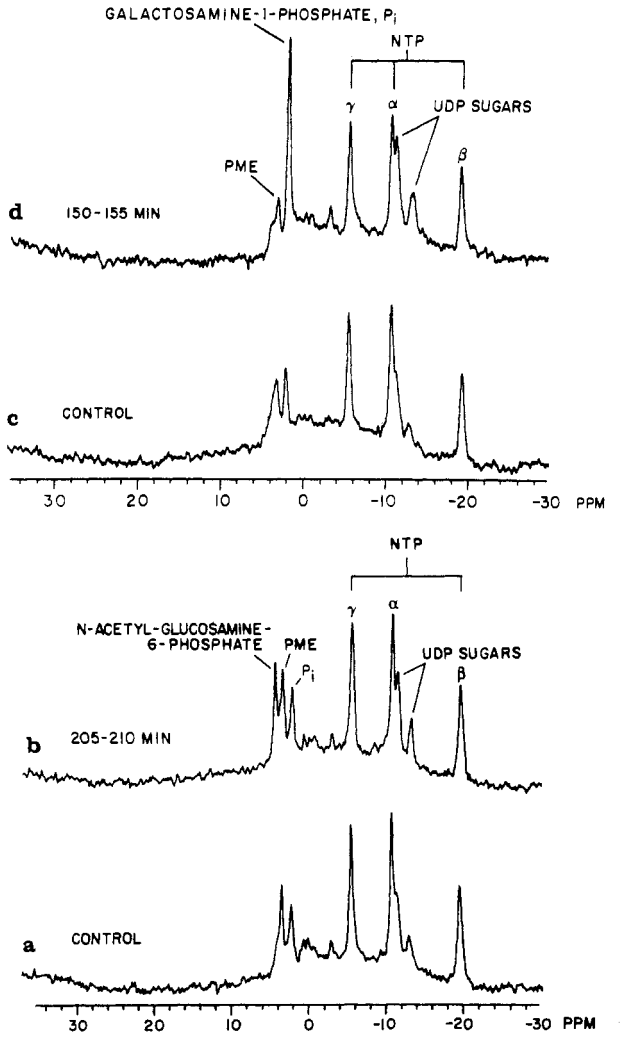


FIGURE 1: In vivo ^{31}P NMR spectra of the liver of an anesthetized rat recorded just prior to (control), and at specified times after, the injection with (a and b) GlcN and (c and d) GalN as described under Materials and Methods. Each spectrum corresponds to 252 accumulations over 5 min (10-kHz spectral width, 8K data points, 1.2-s interpulse delay). Data were subjected to an exponential multiplication of 30 Hz prior to Fourier transformation.

UTP and GlcN-1-P with UDP-glucose pyrophosphorylase, inorganic pyrophosphatase, $Mg(OAc)_2$, and cysteine in 4 mL of Tris buffer at pH 8.5 (75 mM) with 10% ^2H_2O at 37 °C (Weckbecker & Keppler, 1982). After this reaction had reached completion, it was then treated with UDP-glucose 4′-epimerase and incubated at pH 8.30 at 37 °C for 2 h to yield a 3:1 isomer ratio of UDP-GlcN:UDP-GalN (Weckbecker & Keppler, 1983). Both reactions were monitored by ^{31}P NMR. The volume was reduced to 1.5 mL, CDTA (66 mM) was added, and the pH was adjusted to 8.20 prior to acquisition of the ^{31}P and 2-D spectra at 23 °C.

Results

In Vivo ^{31}P Nuclear Magnetic Resonance. The metabolic response to the administration of the amino sugars can be monitored by both in vivo and in vitro NMR techniques. Changes in the ^{31}P NMR spectra of the liver of anesthetized rats, which had been surgically modified as described previously (London et al., 1985) and had received either GlcN or GalN (1.85 mmol/kg), were observed over a period of 5 h with surface coils. Spectra obtained both before treatment and after the majority of changes had occurred are shown in Figure 1. Glucosamine treatment results in the appearance of a phosphomonoester resonance at 4.6 ppm and, over a longer time scale, an increase in the intensity of the resonance corresponding to the hexose phosphate moiety of the UDP-sugars at −13.0 ppm. On the basis of the change in intensity of the latter resonance, the total UDP-sugar content of the liver is approximately doubled after a period of 200 min. Galactosamine treatment produces a much more rapid increase in intensity of a resonance at 2.0 ppm that overlaps the P_i resonance, as well as a larger increase in the UDP-sugar resonance. The latter had increased by a factor of 4 after 200 min. Difference spectra (not shown) reveal an early decrease in the nucleoside triphosphate resonances. Since this decrease coincides with the increase in phosphomonoesters rather than with the slower increase in UDP-sugar resonances, it appears to reflect a small decrease in the hepatic ATP level associated with phosphorylation of the amino sugars. More quantitative in vivo analyses are described elsewhere (London et al., 1990). The identification of the phosphomonoesters discussed below supports this interpretation.

^{31}P NMR Studies of Extracts. In order to determine the identity of the UDP-sugars and the phosphomonoesters that increased during hexosamine metabolism, PCA extracts of the livers were prepared and examined first by conventional 202-MHz ^{31}P NMR. In the UDP-sugar region (Figure 2), tentative assignment of some of the doublets (ca. 20-Hz splitting due to ^{31}P–^{31}P coupling) could be arrived at on the basis of the ^{31}P spectra of pure standard compounds. Instead of sequentially adding standards for complete identification, we chose to use two-dimensional ^{31}P–$\{^1H\}$ relayed coherence transfer spectroscopy for assignment purposes. In this experiment (Bax et al., 1985), ^1H chemical shift information developed during the evolution period, t_1, is "mixed" or relayed under the influence of the ^1H spin-lock (τ_{mix}) to those protons that are part of the same spin-coupled network. This shift information is then transferred to ^{31}P via the protons directly coupled to phosphorus. Fourier transformation with respect to t_2 and t_1 yields in the F_2 dimension conventional ^{31}P spectra and in F_1 the subspectra of the protons that are directly coupled to ^{31}P, as well as those of protons that are passively connected via their homonuclear couplings. The extent of the relay beyond the proton directly coupled to ^{31}P depends upon the magnitudes of the ^1H–^1H coupling constants and the duration of the spin-lock.

Examples of such relayed spectra for the hexose phosphate resonances of the UDP-sugars found in liver extracts of normal and GlcN-treated rats are shown as two-dimensional contour plots in Figure 3. In the ^{31}P dimension each phosphate resonance consists of a pair of antiphase doublets. The large splitting (20 Hz) arises from the homonuclear coupling to ribose–^{31}P, and the smaller, antiphase splitting (7 Hz) is from the heteronuclear coupling to the hexose anomeric proton (H-1). Since isotropic mixing of the protons involves a net transfer of magnetization and these spectra are recorded in the absorption mode, the relayed cross-peaks in the ^1H dimension are in phase with the primary or direct cross-peaks. Thus, where resolution permits, slices taken parallel to the ^1H dimension through the four ^{31}P lines may be combined by addition or subtraction of the appropriate files, thereby compensating for the loss of sensitivity due to the ^{31}P–^{31}P splitting, as well as the absence of ^1H decoupling during acquisition.

Examples of such combined slices, plotted as one-dimensional subspectra for three different isotropic mixing times, τ_{mix}, are shown in Figure 4. The progressive development of proton resonances beyond the direct cross-peak arising from H-1 is clearly seen as τ_{mix} is increased from 15 to 161 ms. We note also that the rate at which relay occurs between a pair

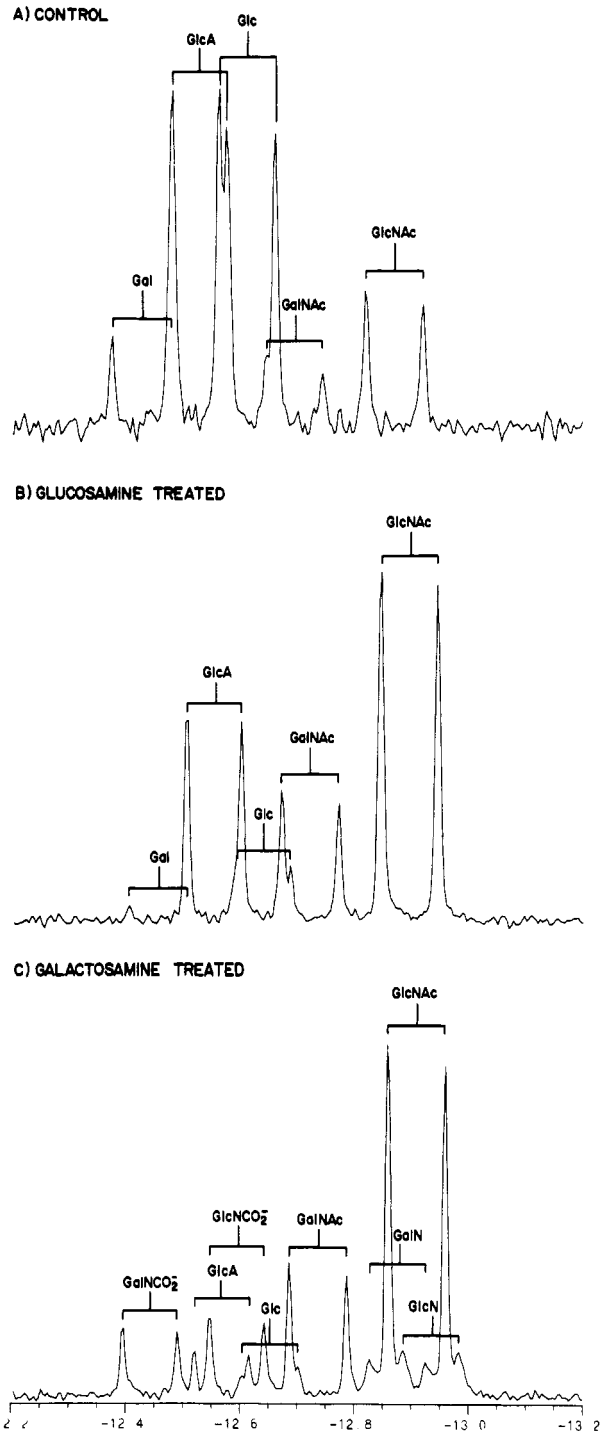


FIGURE 2: ^{31}P NMR spectra of liver extracts from rats treated as follows: (A) fed control; (B) 5 h after treatment with GlcN; (C) 5 h after treatment with GalN (K_2CO_3 neutralization of PCA). Assignments were based on relay spectra, except for UDP-Gal in (B) and UDP-Glc in (C) which were identified by ^{31}P shift.

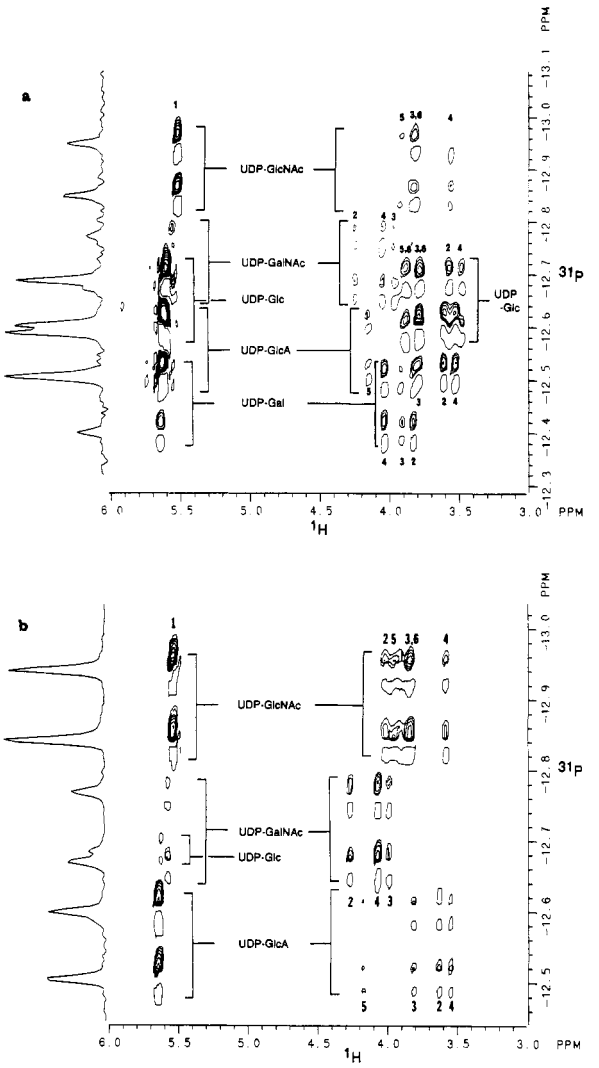


FIGURE 3: UDP-sugar (β-phosphate) region of ^{31}P–{^1H} relayed coherence transfer spectrum of liver extract obtained from (a) control rat at 164 ms and (b) rat treated with GlcN at 161-ms isotropic mixing time. On the y axis is the corresponding ^{31}P spectrum from Figure 2. Filled and empty cross-peaks indicate opposite phase relationships. Data were acquired with the pulse sequence (eq 1) and the conditions described under Materials and Methods. Further parameters are an F_2 spectral width of 6.5 kHz and the following: (a) 2 × 64 × 4096 (complex) time domain matrix, 256 × 4096 after transformation, scans/t_1 increment = 650, and run time = 36.5 h; (b) 2 × 64 × 2048 time domain matrix, 256 × 2048 after transformation, scans/t_1 increment = 360, and run time = 18.0 h. When τ_{mix} = 85 ms, weak cross-peaks for H-2 and H-5 are visible for UDP-Glc.

of protons H′ and H″ depends upon the magnitude of the coupling constant, $J_{H'H''}$. Thus for the galactose derivatives, such as UDP-GalNAc and -Gal in Figure 3, relay essentially stops at H-4 due to the weak coupling (<1 Hz) to H-5. The high degree of correspondence between ^1H-relayed spectra from the extracts and those obtained for authentic compounds can be judged by a comparison of spectra c and d of Figure 4, where the latter is taken from a data set obtained at the same τ_{mix} for a mixture of commercially obtained UDP-sugars. The proton chemical shifts obtained for the mixture of known UDP-sugars by the relay experiment under these conditions are provided in Table I. By comparison of the ^1H chemical shift relay patterns corresponding to each ^{31}P doublet in the extract spectra with those of known compounds, it is possible to identify all but the weakest of the components on this basis. Very high convergence of the ^1H shifts for UDP-sugars in extracts and reference mixtures is achieved when both systems include CDTA to sequester metal ions.

In the ^{31}P NMR spectra of extracts from GalN-treated rats (Figure 2C), it was possible to identify UDP-GlcNAc, UDP-GalNAc, and UDP-GlcA through their relay spectra, and the weakest signal was assigned to UDP-Glc on the basis of the ^{31}P chemical shift, leaving four resonances unassigned. On the basis of a report by Weckbecker and Keppler (1982), two of the unknowns were likely to be UDP-GlcN and UDP-GalN. These compunds were not commercially available, but could be prepared in situ. A solution of UTP and GlcN-1-P, in the presence of UDP-glucose pyrophosphorylase and inorganic pyrophosphatase, provided UDP-GlcN, 25% of which was converted to UDP-GalN upon addition of a 4′-

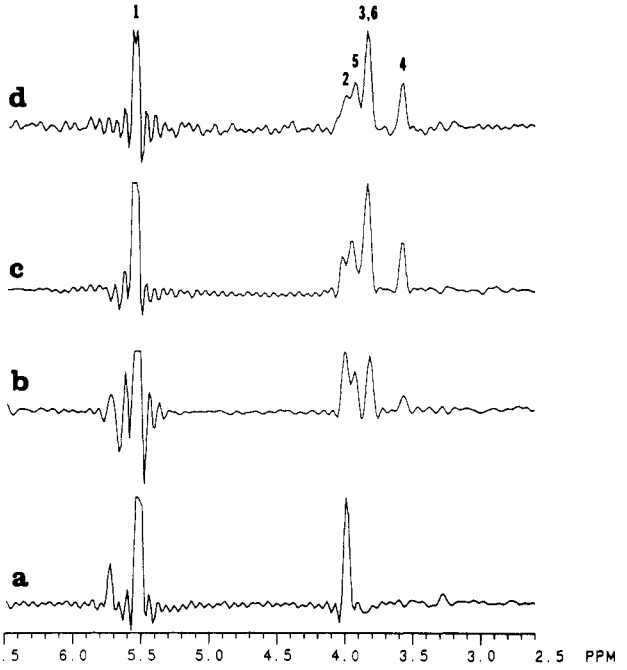


FIGURE 4: Proton-dimension slices (combined) for UDP-GlcNAc in ^{31}P–$\{^1H\}$ relay spectra of a liver extract from a rat treated with GalN as in Figure 2C. The τ_{mix} for these spectra was (a) 15, (b) 85, and (c) 161 ms. Spectrum d is from a prepared mixture of commercially available UDP-sugars with a mixing time of 164 ms. The spectra were acquired (eq 1) as described under Materials and Methods. Additional parameters for spectra a–c: F_2 spectral width = 6.5 kHz; data matrix = 2 × 64 × 2048 and after Fourier transformation = 256 × 2048. Scans per t_1 increment and run time (h): (a) 360, 16.5; (b) 360, 17.5; (c) 650, 33.0. For spectrum d, see Table I (footnote *a*).

Table I: Proton Chemical Shifts of UDP-sugars from Relay Spectra[a]

hexose	H-1	H-2	H-3	H-4	H-5	H-6,6′
GlcN[b]	5.652	2.986	3.725	3.503	3.929	3.725
						3.829
GlcNAc	5.539	4.012	3.839	3.583	3.940	3.839
						3.940
GalN[b]	5.712	3.277	3.916	4.025		
GalNAc	5.572	4.262	3.991	4.071		
Glc	5.618	3.573	3.798	3.505	3.897	3.798
						3.897
GlcNCOO$^-$[c]	5.530	3.651	3.740	3.571	3.934	3.847
GlcA	5.645	3.616	3.810	3.538	4.167	
Gal	5.661	3.838	3.929	4.056		
GalNCOO$^-$[c]	5.569	3.903	3.903	4.053		

[a] Obtained as a mixture of the five commercially available compounds in aqueous 10–20 mM solutions in ^2H_2O (10 mM CDTA, 0.2 M sodium phosphate buffer, pH 8.2, 23 °C) by 2-D relay spectroscopy at τ_{mix} = 164 ms as described under Materials and Methods (eq 1). Other conditions: F_2 spectral width = 812 Hz; data matrix = 2 × 64 × 512 (complex); transformed data set = 256 × 512; scans per t_1 value = 16; run time = 55 min. Assignments were based on ^1H double-resonance experiments and literature determinations (Lee & Sarma, 1976). No entry for chemical shift indicates that a proton does not appear in the relay at the τ_{mix} used (except for UDP-GlcA which lacks H-6,6′). [b] Relay data from enzymatic synthesis (see Figure 5). [c] Relay data from extracts: assignments are based on variation of τ_{mix} and are tentative for H-2, -3, and -4 of UDP-GalNCOO$^-$ due to signal overlap.

epimerase. Both reactions were conducted in an NMR tube, and the progress was monitored by ^{31}P NMR. In Figure 5, the ^1H-dimension slices from the relay spectrum of this mixture are compared with the relayed ^1H spectra of the two unassigned upfield ^{31}P signals in the GalN extract. The close correspondence of the spectra from the extracts and from the UDP-GlcN and -GalN in the enzyme reaction is clear. Here, the assignments of resonances in the ^1H spectra were made by running the relay experiment at different mixing times.

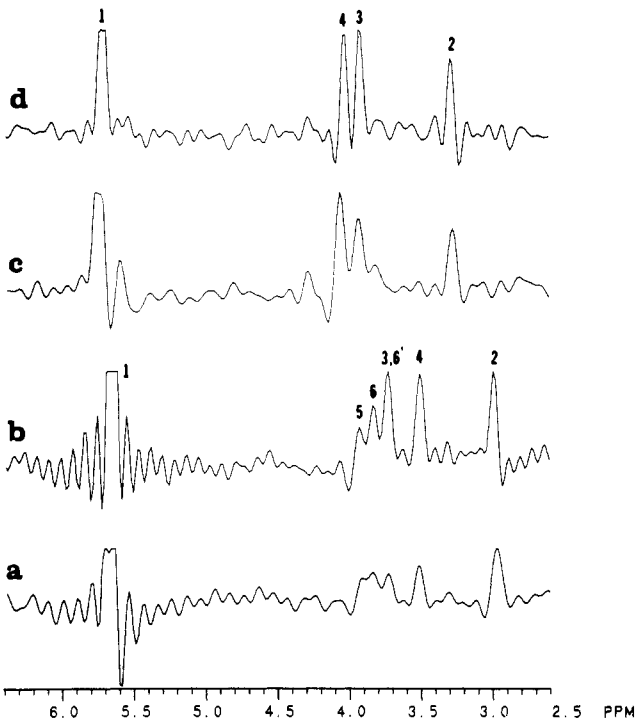


FIGURE 5: ^1H-dimensional slices from ^{31}P–$\{^1H\}$ relay spectra of UDP-GlcN (a and b) and UDP-GalN (c and d). Spectra a and c (τ_{mix} = 164 ms) are from a liver extract from a GalN-treated rat; the sequence including refocusing and ^1H decoupling was used due to the overlap of UDP-GlcN and -GalN with UDP-GlcNAc. Spectra b and d are from the enzymatic synthesis conducted in situ (161 ms) (see Materials and Methods). For the extract, the following conditions were utilized: spectral width in F_2 of 811 Hz; delay between scans of 0.7 s; 1280 scans per t_1 value; time domain matrix = 2 × 40 × 512, after processing 160 × 512; run time = 37.5 h. Parameters for the enzyme reaction were a spectral width = 710 Hz, time domain matrix = 2 × 48 × 1024 which after processing became 192 × 1024, 240 scans/t_1 increment, and run time = 13.5 h.

The two other unidentified doublets in the GalN-extract ^{31}P spectrum (Figure 2C), having chemical shifts at −12.422 and −12.576 ppm (average values), were similar to UDP-Gal and UDP-Glc, respectively, both in ^{31}P shifts and in their ^1H spectra, except for the shifts of the H-1 and H-2 protons. They also slowly disappeared from the ^{31}P spectra on standing, while increases in the levels of UDP-GlcN and UDP-GalN were observed. This suggested that the unknowns were labile derivatives of UDP-GalN and -GlcN. The most likely candidates were carbamic acids formed during neutralization of the PCA extract with K_2CO_3. Therefore, an extract from a GalN-treated rat was neutralized with KOH instead. The resulting ^{31}P spectrum, which was assigned by a relay experiment, did not contain these signals. Furthermore, when the mixture of UDP-GlcN and -GalN produced enzymatically was treated with CO_2 and the pH maintained above 8 with K_2CO_3, a 60% conversion was observed to compounds with similar ^{31}P shifts and identical ^1H relay spectra with those of the unknowns in the extract. These compounds are therefore carbamic acids arising from reaction of the amino groups of UDP-GlcN and -GalN with CO_2.

Since the experiments run on the extracts include the entire ^{31}P spectral width, assignments of resonances throughout the spectrum can be obtained. The phosphomonoester region, in which significant changes occurred during the in vivo studies and is therefore of particular interest, is shown in Figure 6. Assignments were made, as in the case of the UDP-sugars, on the basis of comparison of the relay spectra with those of authentic sample mixtures. Of note is our ability to identify the α and β anomers of the hexose 6-phosphates, which in the

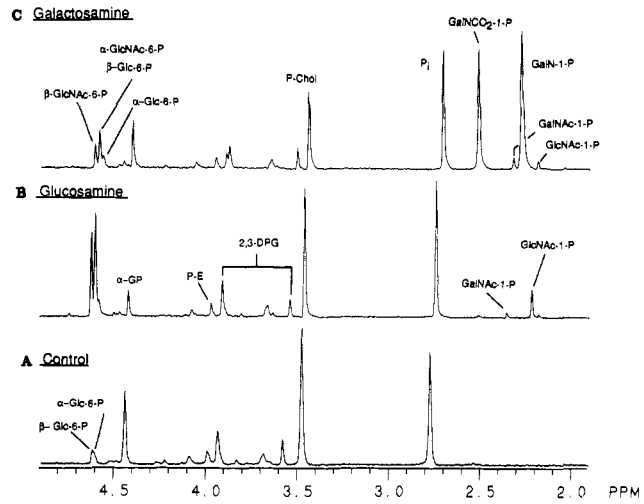


FIGURE 6: Phosphomonoester region of the ^{31}P NMR spectra of liver extracts from rats that were (A) fed, (B) GlcN treated, and (C) GalN treated with K_2CO_3 neutralization. For experimental conditions, see Figure 2. Assignments were based on relay spectra. GPC and GPE could also be identified and appear at −0.13 and 0.44 ppm, respectively.

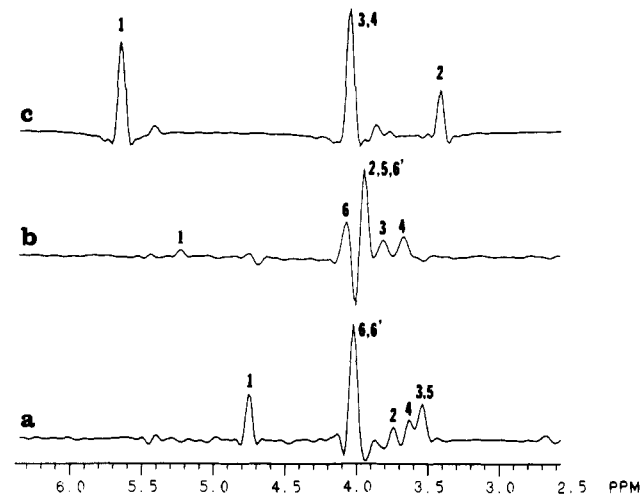


FIGURE 7: ^{1}H-dimension slices from ^{31}P–{^{1}H} relay spectra of liver extracts: spectra of (a) β and (b) α anomers of GlcNAc-6-P from GlcN treatment and (c) GalN-1-P from GalN treatment. See Figures 3b and 4c, respectively, for conditions. τ_{mix} = 161 ms.

^{31}P dimension differ in chemical shift by as little as 0.01 ppm but which have significantly different and characteristic spectra in the ^{1}H dimension (Figure 7). The phosphomonoesters that increase substantially in the in vivo studies (Figure 1) were found on the basis of the relay spectra to be α- and β-GlcNAc-6-P for GlcN metabolism and GalN-1-P for the GalN study. Nearly half of the latter compound appeared in the K_2CO_3-neutralized extract as the carbamic acid at 2.53 ppm. As in the case of UDP-GalN, only a trace of the carbamate could be detected in extracts neutralized with KOH. In addition, treatment of GalN-1-P with CO_2 in K_2CO_3 (0.1 M) results in 20% conversion to a compound with an identical ^{31}P NMR shift and relay spectrum. As indicated in Figure 6, many other common phosphomonoesters and phosphodiesters were identified by relay spectra.

The amounts of individual UDP-sugars and the major hexose monophosphates found by ^{31}P NMR in the extracts are shown in Table II. The dominance of a hexose 1-phosphate in the GalN study and a hexose 6-phosphate in the GlcN study was consistent with the in vivo spectra. The UDP-sugar pool increased 4.3- and 1.8-fold, respectively, in the GalN and GlcN experiments, which is consistent with the 4- and 2-fold

Table II: Levels of Hexose Phosphates in Liver[a]

compd	^{31}P shift[b]	treatment		
		control[c]	GlcN[c]	GalN[d]
UDP-GlcN	−12.960			1.87 ● 0.65[e]
	−12.863			
UDP-GlcNAc	−12.945	0.34 ± 0.09	1.75 ± 0.60	2.96 ± 0.96
	−12.845			
UDP-GalN	−12.904			1.34 ± 0.48[e]
	−12.805			
UDP-GalNAc	−12.753	0.14 ± 0.03	0.63 ● 0.22	1.05 ● 0.32
	−12.653			
UDP-Glc	−12.671	0.63 ± 0.16	0.19 ± 0.07	0.21 ± 0.08
	−12.574			
UDP-GlcA	−12.586	0.53 ± 0.12	0.57 ± 0.29	0.31 ± 0.10
	−12.490			
UDP-Gal	−12.474	0.14 ± 0.04	0.03 ± 0.01	
	−12.374			
GlcNAc-1-P	2.201		0.56 ± 0.16	0.36 ● 0.03
GalN-1-P	2.285			15.98 ± 6.42[e]
GalNAc-1-P	2.347		0.07 ± 0.02	0.35 ± 0.26
α-Glc-6-P	4.584	0.30 ± 0.04	0.48 ± 0.20	0.34 ± 0.19
β-Glc-6-P	4.594	0.35 ± 0.05	0.58 ± 0.25[f]	0.41 ± 0.23[f]
α-GlcNAc-6-P	4.599		2.26 ± 0.83	1.68 ± 0.80
β-GlcNAc-6-P	4.622		1.72 ± 0.64	0.92 ± 0.37

[a] μmol/g of wet liver. [b] Average of chemical shifts (ppm) determined for several extracts. Values quoted in the text are also reported as averages. [c] n = 3. [d] n = 3; for K_2CO_3 neutralization. [e] Includes the corresponding carbamic acid derivatives. [f] Hidden. Amount estimated on the basis of a 1.2:1 β:α ratio.

increases observed in vivo (London et al., 1990). In the control extracts, 27% of the UDP-sugars were amino sugars, while the corresponding levels in the GlcN and GalN extracts were 75% and 93%, respectively, as a result of the almost complete disappearance of UDP-Glc and -Gal. Even more extreme effects occurred for the hexose monophosphates, none of which were amino sugars in the control extracts, while of those detected in the livers from treated rats all were amino sugars. In the case of GalN administration, 80% of hexose monophosphates exist as GalN-1-P, which is present at 16.0 μmol/g.

Discussion

By use of a combination of in vivo and in vitro ^{31}P NMR and heteronuclear relay spectroscopy, the effects of hexosamines on the hepatic metabolism of hexose phosphates can be examined. The methods complement each other well in terms of the type of information they provide. The in vivo spectra indicate the time-dependent changes in the levels of specific classes of organic phosphates in the liver, while the in vitro studies on the extracts reveal the detailed changes within each class. ^{31}P–{^{1}H} spin-locked relay experiments were found to be particularly useful for analysis of the liver extracts and have several advantages over the more traditional one-dimensional methods that rely primarily on ^{31}P chemical shifts and their pH dependence for the identification of individual components of extracts. The relay experiments provide a correlation of the ^{31}P chemical shift with the chemical shifts of all of the protons that are part of the same spin-coupled network, thus giving a unique signature or fingerprint for each organophosphate moiety. Because the ^{31}P and ^{1}H spectral information is dispersed over two dimensions, spectra of complex mixtures can still be readily analyzed even if there are partial degeneracies or overlaps in either dimension. In many cases, the identification of extract constituents can be made immediately and unambiguously by visual comparison of the spectra from the ^{1}H dimension of 2-D relay spectra with those of mixtures of known compounds run under similar conditions. These proton spectra are particularly diagnostic since the proton chemical shifts are in general less sensitive

to medium effects than the ^{31}P shifts and the relative peak intensities are characteristic of the structure. Since the ^{31}P shifts are no longer the major basis for identification, the practice of peak identification based on the careful, sequential addition of small amounts of standard compounds to the extract can be avoided, resulting in much less sample manipulation and preventing adulteration of the extract.

The spin-locked relay experiment is sensitive enough to characterize UDP-sugars and other hexose phosphates present in extracts of a single rat liver at concentrations as low as 0.3 mM. In our hands, such an experiment requires about 32–36 h of data acquisition when complete ^1H chemical shift information is desired (τ_{mix} = 160 ms). For many hexose 1-phosphates and UDP-sugars a less extensive relay will suffice for identification of the carbohydrate structure, giving somewhat better sensitivity due to less signal decay during the shorter spin-lock period and thus decreasing the run time to about 16–18 h (τ_{mix} = 85 ms; see Figure 4b). The run time can also be shortened by the use of fewer increments in the t_1 dimension, with lower, yet acceptable, resolution for the ^1H relay spectra (see Figure 5). The ability to obtain relay spectra of mixtures allows several known standards to be run together; when samples are commercially available and high concentrations (10–20 mM) can be used, 2-D spectra with full relay data can be obtained in 1 h or less.

Even if pure standards are not commercially available, relay spectra can also be obtained from a crude enzymatic synthesis without any separation from the enzymes or other reagents. The identity of UDP-GlcN and -GalN in extracts was established by comparing ^1H chemical shifts from relay spectra of extracts and from spectra of an enzymatic reaction mixture that only required concentration before the experiment was run.

In retrospect, the formation of carbamic acids from UDP-GlcN, UDP-GalN, and GalN-1-P in the presence of K_2CO_3/CO_2 was not surprising, for many precedents exist for reaction of CO_2 with amino groups in peptides and in hemoglobin (Roughton & Rossi-Bernardi, 1966; Imaizumi et al., 1982). The high sensitivity of UDP-GlcN and -GalN ^{31}P shifts to pH in the range of 8.2 used in these studies implies that the amino group is weakly basic, which would favor carbamate formation. Although there are several advantages to the use of potassium carbonate for neutralization of acid extracts, the potential for generation of carbamic acids under these conditions from certain amines must be considered.

Relay spectroscopy is particularly well suited for analysis of hexose phosphates due to their generally large ^1H–^1H coupling constants, although glycerol derivatives such as GPC, GPE, and α-GP and monoesters such as P-E and P-Chol are also amenable to identification, as shown in Figure 6. The nucleotide portion of the UDP-sugars shows weaker relay patterns than the hexose moiety, due to smaller ^{31}P–^1H and ^1H–^1H coupling constants. Although they can have very similar ^{31}P chemical shifts, anomers of hexose 6-phosphates can be distinguished by relay spectra because of the significant difference between their proton spectra. The shift of the anomeric proton is particularly diagnostic, and although far removed from the phosphate group, it can be observed via isotropic mixing provided by the ^1H spin-lock. The anomeric ratio (β:α) was found to be in favor of the α anomer in the case of GlcNAc-6-P (0.6:1), whereas for Glc-6-P it was 1.2:1. The α anomer has also been observed to be the major form of GlcN in solution (Walker et al., 1978).

The dramatic perturbations in the UDP-sugar composition in the liver caused by hexosamine treatment are consistent with the conclusions derived by Keppler and co-workers from conventional biochemical studies (Decker & Keppler, 1974; Weckbecker & Keppler, 1982). Entry of GalN into the metabolic pool occurs via phosphorylation by galactokinase to yield GalN-1-P, which accumulates to high levels, as seen in Table II. Administration of GlcN was observed to cause an increase in UDP-GlcNAc and UDP-GalNAc without any generation of free UDP-hexosamines and is consistent with N-acetylation occurring prior to UDP-sugar formation (Chelibonova-Lorer et al., 1983; Bates et al., 1966; Kornfeld et al., 1964). The ^{31}P–{^1H} relay spectra showed that liver extracts also contain both GlcNAc-1-P and GlcNAc-6-P, but neither GlcN-1-P or GlcN-6-P was detected. The hexose phosphomonoester distribution resulting from GlcN treatment provides additional support for N-acetylation early in GlcN metabolism (McGarrahan & Maley, 1962). The significantly greater increase in UDP-sugars produced by GalN compared with GlcN treatment is consistent with the more effective uridylate trapping and thus the greater hepatotoxicity produced by GalN (Decker & Keppler, 1974). It was also possible to distinguish Glc-6-P from GlcNAc-6-P, despite very similar ^{31}P chemical shifts, but levels were little changed from those of the controls.

Prior studies of hepatic hexosamine metabolism have very often not included determinations of the ratio of 4′-epimers of the UDP-sugars since effective chromatographic resolution is not straightforward, and even use of HPLC requires multiple steps (Weckbecker & Keppler, 1983). Due to the significant upfield shift of glucose relative to galactose isomers, ^{31}P NMR is a convenient alternative method. With this approach, the ratios of UDP-GlcN/UDP-GalN and UDP-GlcNAc/UDP-GalNAc in the liver extracts of GalN-treated rats were found to be 1.4 and 2.8, respectively, as compared with 1.9 and 3.8 found by HPLC by Weckbecker and Keppler (1983). Treatment with GlcN was found by NMR also to give a ratio of 2.8 for N-acetylated UDP-hexosamines, while the value for control animals was 2.4. This indicates that the 5-fold increase in N-acetylated UDP-hexosamine levels caused by GlcN administration does not significantly alter the equilibrium created by UDP-acetylglucosamine 4′-epimerase. This was previously observed for GalN treatment (Weckbecker & Keppler, 1983), and the ability of the epimerase to maintain a constant epimer ratio in the liver is further supported by the identical UDP-GlcNAc/UDP-GalNAc values found by ^{31}P NMR for GlcN and GalN treatment.

The current study appears to be the first application of $^{31}P/^1H$ relay spectroscopy to the identification of constituents of complex mixtures in biological fluids. It is anticipated that this method should be of value for future studies of phosphate metabolism due to the structural details that can be obtained without prior separation. The extensive qualitative and quantitative information on the phosphorylated metabolites of the hexosamines in the liver that was obtained by combining one- and two-dimensional heteronuclear NMR techniques is indicative of the potential of this approach.

Acknowledgments

We express our appreciation to C. Tyler Burt for his helpful suggestions.

References

Anderson, M. S., & Raetz, C. R. H. (1987) *J. Biol. Chem. 262*, 5159–5169.

Barany, M., & Glonek, T. (1984) in *Phosphorus-31 NMR, Principles and Applications* (Gorenstein, P. G., Ed.) pp

511–545, Academic Press, Orlando, FL.
Bates, C. J., Adams, W. R., & Handschumacher, R. E. (1966) *J. Biol. Chem. 241*, 1705–1712.
Bax, A., Davis, D. G., & Sarkar, S. K. (1985) *J. Magn. Reson. 63*, 230–234.
Bolton, P. H. (1982) *J. Magn. Reson. 48*, 336–340.
Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521–528.
Chelibonova-Lorer, H., Gavazova, E., Ivanov, S., & Antonova, M. (1983) *Int. J. Biochem. 15*, 559–564.
Cohen, J. S., Lyon, R. C., Chen, C., Faustino, P. J., Batist, G., Shoemaker, M., Rubalcaba, E., & Cowan, K. H. (1986) *Cancer Res. 46*, 4087–4090.
Corbett, R. J. T., Nunnally, R. L., Giovanella, B. C., & Antich, P. P. (1987) *Cancer Res. 47*, 5065–5069.
Davis, D. G., & Bax, A. (1985) *J. Am. Chem. Soc. 107*, 2820–2821.
Decker, K., & Keppler, D. (1974) *Rev. Physiol. Biochem. Pharmacol. 71*, 78–106.
Desmoulin, F., Galons, J.-P., Canioni, P., Marvaldi, J., & Cozzone, P. J. (1986) *Cancer Res. 46*, 3768–3774.
Dutton, G. J. (1980) *Glucuronidation of Drugs and Other Compounds*, CRC Press, Boca Raton, FL.
Fantini, J., Galons, J.-P., Marvaldi, J., Cozzone, P. J., & Canioni, P. (1987) *Int. J. Cancer 39*, 255–260.
Greiner, J. V., Kopp, S. J., & Glonek, T. (1982) *Invest. Ophthalmol. Vis. Sci. 23*, 14–22.
Imaizumi, K., Imai, K., & Tyuma, I. (1982) *J. Mol. Biol. 159*, 703–719.
Keppler, D. O., Holstege, A., Weckbecker, G., Fauler, J., & Gasser, T. (1985) *Adv. Enzyme Regul. 24*, 417–427.
Kornfeld, S., Kornfeld, R., Neufeld, E. F., & O'Brien, P. J. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 371–379.
Krug, E., Zweibaum, A., Schultz-Holstege, C., & Keppler, D. (1984) *Biochem. J. 217*, 701–708.
Kushnir, T., Kaplan, O., Askenasy, N., & Navon, G. (1989) *Magn. Reson. Med. 10*, 119–124.
Lee, C.-H., & Sarma, R. H. (1976) *Biochemistry 15*, 697–704.
Leloir, L. F. (1964) *Biochemistry 3*, 1.
London, R. E., Galvin, M. J., Thompson, M., Jeffreys, L., & Mester, T. (1985) *J. Biochem. Biophys. Methods 11*, 21–29.
London, R. E., Gabel, S. A., & Perlman, M. E. (1990) in *Applications of NMR in Agriculture and Biochemistry* (Finley, J. W., Schmidt, S., & Serianni, A., Eds.) Plenum Press, New York (in press).
Maley, F., Tarentino, A. L., McGarrahan, J. F., & DelGiacco, R. (1968) *Biochem. J. 107*, 637–644.
McGarrahan, J. F., & Maley, F. (1962) *J. Biol. Chem. 237*, 2458–2465.
Muller, L., & Ernst, R. R. (1979) *Mol. Phys. 38*, 963.
Navon, G., Shulman, R. G., Yamane, T., Eccleshall, T. R., Lam, K.-B., Baronofsky, J. J., & Marmur, J. (1979) *Biochemistry 18*, 4487–4499.
Nikaido, H., & Hassid, W. Z. (1971) *Adv. Carbohydr. Chem. Biochem. 26*, 351.
Roby, C., Martin, J.-B., Bligny, R., & Douce, R. (1987) *J. Biol. Chem.* 262, 5000–5007.
Roughton, F. J. W., & Rossi-Bernardi, L. (1966) *Proc. R. Soc. London, B 164*, 381–400.
Schwarz, R. T., & Datema, R. (1982) *The Glycoconjugates* (Horowitz, M. I., Ed.) Vol. 3, pp 47–79, Academic Press, New York.
Shaka, A. J., Keeler, J., Frenkiel, T., & Freeman, R. (1983) *J. Magn. Reson. 52*, 335–338.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.
Van Wazer, J. R., & Ditchfield, R. (1987) in *Phosphorus NMR in Biology* (Burt, C. T., Ed.) pp 1–24, CRC Press, Boca Raton, FL.
Walker, T. E., London, R. E., Barker, R., & Matwiyoff, N. A. (1978) *Carbohydr. Res. 60*, 9–18.
Weckbecker, G., & Keppler, D. O. R. (1982) *Eur. J. Biochem. 128*, 163–168.
Weckbecker, G., & Keppler, D. O. R. (1983) *Anal. Biochem. 132*, 405–412.
Wice, B. M., Trugnan, G., Pinto, M., Rousset, M., Chevalier, G., Dussaulx, E., Lacroix, B., & Zweibaum, A. (1985) *J. Biol. Chem. 260*, 139–146.
Zagorski, M. G., & Norman, D. G. (1989) *J. Magn. Reson. 83*, 167–172.