Biochemical Pharmacology, Vol. 41, No. 9, pp. 1319–1324, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

IDENTIFICATION OF NOVEL HYDRAZINE METABOLITES BY ^{15}N-NMR

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(*Received* 4 *October* 1990; *accepted* 30 *November* 1990)

Abstract—^{15}N-NMR has been used to study the metabolism of hydrazine in rats *in vivo*. Single doses of [$^{15}N_2$]hydrazine (2.0 mmol/kg: 98.6% g atom) were administered to rats and urine collected for 24 hr over ice. A number of metabolites were detected by ^{15}N-NMR analysis of lyophilized urine. Ammonia was detected as a singlet at 0 ppm and unchanged [$^{15}N_2$]hydrazine was present in the urine detectable as a singlet at 32 ppm. Peaks were observed at 107 and 110 ppm which were identified as being due to the hydrazido nitrogen of acetylhydrazine and diacetylhydrazine, respectively. A resonance at 85 ppm was ascribed to carbazic acid, resulting from reaction of hydrazine with carbon dioxide. A singlet detected at 316 ppm was thought to be due to the hydrazono nitrogen of the pyruvate hydrazone. The resonance at 56 ppm was assigned to ^{15}N-enriched urea, this together with the presence of ammonia indicates that the N–N bond of hydrazine is cleaved *in vivo*, possibly by N-oxidation, and the resultant ammonia is incorporated into urea. A doublet centred at 150 ppm and a singlet at 294 ppm were assigned to a metabolite which results from cyclization of the 2-oxoglutarate hydrazone. Therefore ^{15}N-NMR spectroscopic analysis of urine has yielded significant new information on the metabolism of hydrazine.

The simple nitrogen compound hydrazine is used extensively in the preparation of several important industrial products including plastic blowing agents, growth retardants, pharmaceuticals and in rocket propellants. It is also a metabolite of two important drugs, isoniazid [1, 2] and hydralazine [3]. Hydrazine itself has been used in the treatment of weight loss in terminal cancer patients [4]. Hydrazine is a toxic compound causing fatty liver [5–9] and in some cases liver necrosis [10], disturbances of the central nervous system [11] and tumours in various organs [12, 13]. An understanding of the metabolism and toxicity of hydrazine is therefore important.

Some of the urinary metabolites of hydrazine have been partially characterized by various authors [14–16]. Previous metabolic studies have revealed that hydrazine is acetylated first to mono- and then diacetylhydrazine by these metabolites represent only a very small proportion of the dose (<5%), with only about 10% excreted unchanged [14]. A significant proportion (25%) has also been shown to be metabolized to nitrogen detected in the expired air [17, 18]. A large proportion (60%) of the dose has therefore not been accounted for (Fig. 1).

Hydrazine metabolism cannot easily be studied by conventional analytical methodologies because unlike the majority of biologically important elements nitrogen has no moderately stable radioactive isotopes which can be used for labelling. Mass spectrometry can be used to identify non-radioactive

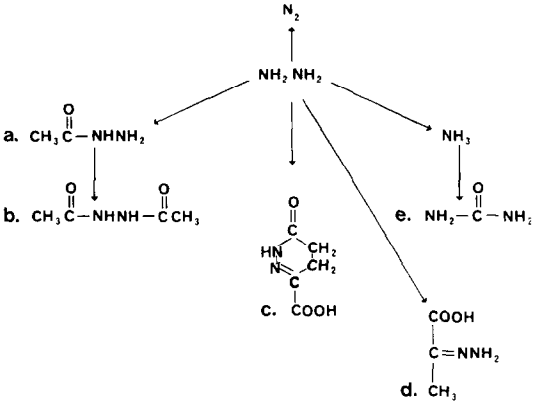

Fig. 1. Metabolic pathways for hydrazine. Metabolites: (a) acetylhydrazine; (b) diacetylhydrazine; (c) 1,4,5,6-tetrahydro-6-oxo-3-pyridazine carboxylic acid (THOPC) formed from 2-oxoglutarate and hydrazine; (d) pyruvate hydrazone; (e) urea.

^{15}N-labelled compounds but such methodology although very sensitive is in practice usually preselective for previously characterized metabolites.

Recently, NMR methods have been used to study the metabolism of several compounds to novel metabolites in biological fluids without purification or prior knowledge of their metabolic disposition [19–22]. We have previously studied the metabolism and biochemical effects of hydrazine using proton NMR [23]. Although ^{15}N-NMR sensitivity is poor compared to many other NMR active nuclei, the chemical shift range is large (900 ppm) and very sensitive to any minor changes in electron density

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Table 1. ^{15}N-NMR chemical shifts of known and possible hydrazine metabolites and related compounds

Metabolite	^{15}N Chemical shift (ppm)	^{15}N-^{1}H Coupling constants (Hz) $^{1}J_{^{15}N,^{1}H}$ and multiplicity
Ammonia	0	Singlet
Methylamine	3	Singlet
Glutamylhydrazine (Amino-N3)	20	Singlet
Hydrazine	32	Singlet
Methylhydrazine (Amino-N2)	34	Singlet
Acetylhydrazine (Amino-N2)	35	Singlet
Glutamylhydrazide (Amino-N2)	35	Singlet
Methylhydrazine (Substituted-N1)	53	Singlet
Urea	56	Triplet (87)
N1-Acetylcarbazate (N2)-COOH	82	Doublet (96)
Carbazic acid (N-COOH)	85	Doublet (97)
Glutamine (Amido-N2)	91	Singlet
Acetamide	93	Triplet (88)
Pyruvate hydrazone (Amino N2)	93	Singlet
Acetylhydrazine (Hydrazido-N1)	107	Doublet (104)
Diacetylhydrazine	110	Singlet
N1-Acetylcarbazate (Hydrazido-N1)	112	Singlet
Glutamylhydrazide (Hydrazido-N1)	113	Singlet
THOPC (Hydrazido-N2)	150	Doublet (99)
THOPC (Hydrazono-N1)	294	Singlet
Pyruvate hydrazone (Hydrazono-N1)	315	Singlet
Pyruvate azine	338	Singlet

at the nitrogen nucleus. Consequently we chose to study the metabolism and hepatotoxicity of doubly-labelled [^{15}N]hydrazine in the rat by NMR.

MATERIALS AND METHODS

[$^{15}N_2$]Hydrazine sulphate (doubly-labelled, 98.6% g atom) and [^{15}N](amido)-glutamine were purchased from MSD Isotopes Ltd (Montreal, Canada). $^{2}H_2O$, [^{15}N] ammonium chloride, sodium pyruvate, urea, urease, hydrazine sulphate, acetamide, methylamine, semicarbazide and methylhydrazine were obtained from the Sigma Chemical Co. (St Louis, MO).

Animal experiments. Male Sprague–Dawley rats (Olac, Bicester, U.K.; 150 g; N = 6) were given doubly labelled [$^{15}N_2$]hydrazine in aqueous solution (64 mg; (2 mmol)/kg; i.p.). Controls (N = 3) received the same volume of 0.9% saline (i.p.). The hydrazine dosing solutions were prepared from a degassed [$^{15}N_2$]hydrazine sulphate solution by titration with equimolar calcium hydroxide to pH 7.4 to precipitate the sulphate.

Animals were housed in metabolism cages arranged for the separate collection of urine and faeces. Urine was collected for 24 hr over ice and frozen prior to analysis during which time the rats were fasted.

In one experiment the rats were killed at 4 hr after dosing and livers removed. Samples of liver were homogenised in methanol/water (50/50; v/v) and after removal of the precipitate by centrifugation, the supernatant was used for NMR analysis.

NMR Measurements. Urine samples were lyophilized and reconstituted in order to achieve a four-fold concentration in order to minimize the spectral accumulation time necessary. $^{2}H_2O$ (5–29% v/v for ^{15}N-NMR; 99.9%, v/v for ^{1}H-NMR) was added to each urine sample during reconstruction to provide the internal field frequency lock and to replace the water peak in proton NMR experiments. Samples for proton NMR spectroscopy were spun in 5 mm diameter NMR tubes using a dedicated probe in a 9.4 Tesla instrument (Bruker WH400) with gated decoupling of the residual water peak. Proton chemical shifts were referenced to the internal standard sodium 3-trimethylsilyl-[2,2,3,3,-$^{2}H_4$]-1-propionate (TSP, 2 mM; δ = 0 ppm). Samples for ^{15}N-NMR spectroscopy were analysed in 10 or 15 mm NMR tubes also using dedicated probes in the same instrument. Chemical shifts were referenced to the [^{15}N]ammonium chloride standard (δ = 0 ppm at 40.56 MHz) either externally or present in an internal, concentric 5 mm NMR tube. Typically 25,000 FIDs were collected into 16,000 data points over 16.7 KHz with a total repetition time of 3–9 sec. Exponential multiplication was applied with a 10 Hz line broadening function. Broadband decoupling was found to be of little advantage in improving the sensitivity of novel species and subsequently was not routinely applied.

Chemical syntheses. $^{15}N_2$-Labelled 1,4,5,6-tetrahydro-6-oxo-3-pyridazine carboxylic acid (THOPC). This putative metabolite (Fig. 1) was synthesized by reacting together equimolar concentrations of [$^{15}N_2$]hydrazine sulphate (1 mM) and 2-oxoglutarate (1 mM) in water at pH 3.

The solution was stirred for 4 hr at approximately 50° and left at 0–5°. The THOPC crystallized out and the crystals were filtered off and after drying the synthetic compound was analysed by both proton

NMR spectroscopy and mass spectrometry. EI mass spectrometry revealed a parent ion (*m/e* 144; molecular weight 144) and ions at 126 (M-18) and 116 (M-28). These corresponded to ions at 142, 124 and 114 for the non-labelled THOPC. Proton NMR spectra of the non-labelled synthetic compound in ^2H_2O revealed two triplets (CH_2–CH_2) at 2.55 and 2.84 ppm ($^3J_{^1H-^1H} = 8.6$ Hz). In the spectrum of the ^{15}N-labelled THOPC the three peaks of the downfield triplet were all further split into doublets ($^3J_{^{15}N^1H} = 2.1$ Hz). The ^{15}N-NMR spectrum of THOPC in 90% H_2O/10% D_2O consisted of a singlet at 294 ppm (hydrazono nitrogen = N1-) and a doublet at 150 ppm (amido nitrogen, -N2H-; $^1J_{^1H.^{15}N} = 99$ Hz). A homonuclear nitrogen coupling ($^1J_{^{15}N.^{15}N}$) of 13.2 Hz was also seen. The chemical shifts observed were assigned by comparison with those previously reported for similar compounds in the literature. The purity of the labelled THOPC was estimated as 97.6% g atom ^{15}N from mass spectrometry. Elemental analysis of the synthetic unlabelled compound ($C_5H_6O_3N_2$) found C 41.89% (expected 42.2), H 4.44% (expected 4.22), N 19.61% (19.72). The elemental analysis, mass spectrum and both proton and ^{15}N-NMR are consistent with the structure proposed (Fig. 1c).

^{15}N-*Labelled acetylhydrazine*. This was synthesized in the NMR tube *in situ* by two methods:

(a) [^{15}N]Hydrazine sulphate (1 mmol) was reacted with acetylchloride (1 mmol) in sodium hydroxide solution (20%, D_2O). Two peaks rapidly appeared, at 107 and 110 ppm due to the hydrazono nitrogens of monoacetyl and diacetylhydrazine. In addition a third signal at 35 ppm was observed and was assigned to the amino nitrogen (N2) of acetylhydrazine.

(b) [^{15}N]Hydrazine sulphate (1 mmol) was reacted with a saturated solution of acetamide for several hours. Again ^{15}N signals were observed in the NMR spectrum at 107 and 35 ppm. [^{15}N]Monoacetylhydrazine would be expected to form under these conditions due to the displacement of ammonia from acetamide by the more nucleophilic hydrazine.

[^{15}N]*Pyruvate hydrazone and pyruvate azine*. [^{15}N]Hydrazine sulphate (1 mmol) was mixed with sodium pyruvate (1 mmol) in water (20% D_2O) at 25° *in situ* and the reaction followed by ^{15}N-NMR. The resonance due to hydrazine at 33 ppm was attenuated while peaks of approximately equal intensity rapidly appeared at 93, 315 and 338 ppm. These presumably correspond to the amino nitrogen (N2) and the hydrazono nitrogen (N1) of the pyruvate hydrazone and the equivalent nitrogens of the pyruvate azine, respectively. Following addition of a further 1 mmol of sodium pyruvate the hydrazine peak was further attenuated while the peak at 338 ppm due to the azine increased at the expense of the two peaks at 93 and 315 ppm due to the hydrazone.

RESULTS

It was possible to observe several ^{15}N-NMR signals from metabolites present in freshly collected urine after several hours of signal accumulation (>10,000 scans). However these signals could be more easily observed with improved signal to noise ratios following lyophilization and reconstitution in a reduced volume of water. There were no ^{15}N-NMR signals detectable in urine from untreated rats. The peaks observed have been assigned on the basis of their chemical shifts, pH dependencies, multiplicity and the magnitude of any coupling constants. ^{15}N-NMR chemical shifts are particularly susceptible to large changes on protonation and changes of conformation of the molecule. The assignments were ultimately confirmed by the addition of ^{15}N-labelled standards where available, otherwise concentrated non-labelled standards were used and the natural abundance of ^{15}N was utilized (Table 1).

The ^{15}N-NMR spectrum of lyophilized urine is shown in Fig. 2. It can be seen that there are a number of resonances. The small singlet observed at 0 ppm was assigned to [^{15}N]ammonia as it was co-resonant with a reference standard when this was present in an internal concentric tube.

It should be noted that observation of [^{15}N]-ammonium is problematic because of its increased longitudinal relaxation (T_1) time compared to the other labelled species. Optimization of the acquisition parameters from ammonium impairs observation of new metabolites, which was the object of this study. However, in other spectra of urine we have obtained, the presence of a resonance at 0 ppm, consistent with [^{15}N] ammonia, is unequivocal. The singlet observed at 32.6 ppm was due to unchanged ^{15}N-labelled hydrazine being excreted into the urine. This assignment was verified by spiking the urine with a ^{15}N-labelled hydrazine standard solution. The signal observed at 85 ppm was assigned to carbazic acid, the carbon dioxide adduct of hydrazine.* It was found that in the ^{15}N-NMR spectrum of a solution of [^{15}N]hydrazine, the resonance from hydrazine at 32 ppm moved to 85 ppm on addition of sodium bicarbonate and this could be reversed by the addition of hydrochloric acid.

The ^{15}N resonances observed at 107.2 and 110.1 ppm in urine sample were coincidental (±1 ppm) with those observed in saturated aqueous solutions of acetylhydrazine and 1,2-diacetylhydrazine respectively. In addition a third signal at 34.5 ppm was also seen in some spectra. This resonance was assigned as the amino nitrogen (N2) of acetylhydrazine. The variability in the appearance of this resonance may be due to T_1 relaxation effects and the urinary pH and composition we suspect are critical factors for the adequate longitudinal relaxation of this nitrogen. Peaks with the same chemical shift were also seen when ^{15}N-labelled acetylhydrazine was synthesized in the NMR tube *in situ* by the two independent methods described (see Methods section).

The triplet centred at 56 ppm which is characteristic of a diprotonated [^{15}N]nitrogen nucleus collapsed

* Since acceptance of this manuscript a publication on the use of ^{15}N-NMR to study labelled ammonia metabolism has been published [26]. A finding of relevance to our studies of hydrazine metabolism is that labelled hippurate was present in the urine of ammonia treated rats with a similar resonance to the one we have assigned to carbazic acid.

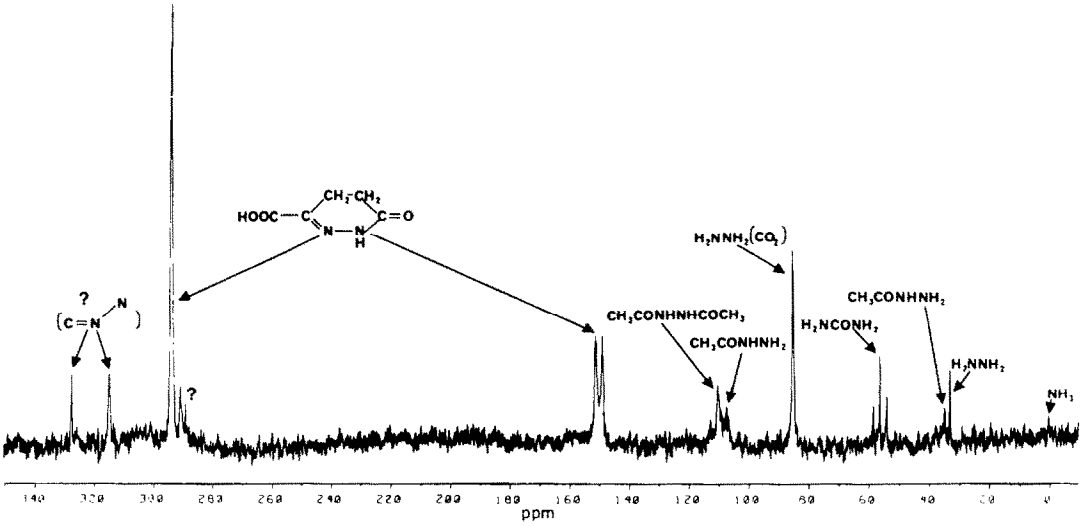

Fig. 2. ^{15}N NMR spectrum (40.56 MHz) of lyophilized rat urine, collected 0–24 hr after a single dose of [^{15}N]hydrazine/kg (i.p.). Urine was reconstituted in water (25% of original volume; 95% H_2O/5% D_2O). Sweep width 16.7 kHz, total acquisition time 62 hr.

into a singlet of approximately three-fold the original intensity on inverse gated broad-band irradiation of the proton frequency. Furthermore when the heteronuclear coupling was maintained, the signal was attenuated when incubated at 37° in the presence of the enzyme urease and the [^{15}N]ammonia signal increased in intensity. During the incubation the intensity of the singlet at 0 ppm, assigned to [^{15}N]ammonia, increased. ^{15}N-NMR of a saturated standard solution of urea similarly gave a triplet at 56 ppm, which also collapsed into a singlet on broad-band proton irradiation and gave rise to an increase in intensity of the peak at 0 ppm when incubated with urease. The triplet at 56 ppm was therefore unequivocally assigned to [^{15}N]urea. The doublet centred at 150 ppm also collapsed into a singlet on broad-band proton irradiation indicative of a mono-protonated nitrogen nucleus. This resonance was associated with an intense singlet at 293.8 ppm. Both these resonances were indistinguishable from those due to ^{15}N-labelled THOPC standard when this was added to urine.

The magnitude of the $^1J_{^{15}N,^1H}$ coupling constants were consistent with the bond hydridisation expected of a ^{15}N-substituted position (N2, amido nitrogen) of THOPC (99 Hz) and for ^{15}N-substituted urea (87 Hz).

Consistent with the observations of a stable coupling at the original urinary pH, the two signals (56 and 150 ppm) were the only clear signals also to be seen in a refocussed INEPT ^{15}N-NMR spectrum of the reconstituted urine. The singlet at 293-8 ppm was also just detectable in homogenates of liver from rats dosed with [^{15}N]hydrazine 4 hr previously, however the signal to noise ratio was very poor. A resonance was also detected at 314.8 ppm which was coincidental with that assigned to the hydrazono nitrogen (N1) of the pyruvate hydrazone when the hydrazone was formed *in situ* (see Methods). However a singlet from the amino nitrogen (N2) of the pyruvate hydrazone observed at 93 ppm was not observed in any of the urine samples. Addition of excess pyruvate to the urine did not give the resonance assigned to the nitrogens of the pyruvate azine (338 ppm). Also the methyl group of the pyruvate hydrazone (1.97 ppm) was not seen in ^1H-NMR studies of the urine of hydrazine dose animals. Therefore the resonances observed at 289, 314.8 and 327.4 cannot be assigned to nitrogen nuclei of specific metabolites with confidence.

No attempt has been made to quantitate the metabolites detected as this is inaccurate with unrelaxed species present and with spectra having poor signal to noise ratios. However, from other studies using proton NMR* and gas chromatography [14] the concentrations of metabolites in the urine can be estimated to be approximately the following: Hydrazine: 3 mM; acetylhydrazine 0.3 mM; diacetylhydrazine 0.9 mM; THOPC 1.28 mM.

DISCUSSION

The work described herein is to our knowledge the first study by ^{15}N-NMR of the *in vivo* metabolism of a ^{15}N-labelled xenobiotic in the rat, although several elegant studies have previously been performed using ^{15}N-NMR in biology [24, 26]. Doubly-labelled [^{15}N]hydrazine represents an excellent model compound for such studies because it is readily available and its chemical reactivity ensures it has several metabolites of which the asymmetrical ones have two distinct and often observable nitrogen centres. However with the increasing availability of high field NMR-spectrometers and ^{15}N-labelled compounds ^{15}N-NMR urinalysis should be accessible to a number of similar metabolic applications.

* Sanins SM, Nicholson JK and Timbrell JA, unpublished observations.

The use of ^{15}N-NMR has allowed the identification of new metabolites of hydrazine. It has shown that free hydrazine is excreted into the urine unchanged and not only as acid labile hydrazones. The finding that unchanged hydrazine is excreted suggests that it is not as rapidly metabolized or as chemically unstable as might have been expected. The use of NMR has allowed the separate identification of the cyclised oxoglutarate hydrazone, THOPC. Hydrazones of acetylhydrazine had previously been shown to be metabolites after dosing with [^{14}C]acetylhydrazine, [14], but such methodology is clearly not an option in the case of hydrazine. The spectra have also confirmed the presence of acetyl and diacetylhydrazine, as metabolites; both compounds were previously only detected after derivatization [14]. Clearly ^{15}N derived from labelled hydrazine is incorporated into urea and ammonia. This indicates that the N–N bond is cleaved *in vivo*, although whether chemically or enzymically is currently unknown. Presumably the liberated ammonia is incorporated into urea via the urea cycle. Alternatively hydrazine may form a carbamoyl phosphate analogue, *N*-amino carbamoyl phosphate, as hydrazine, like ammonia, is a substrate for carbamoyl phosphate synthetase. The hydrazine analogue is reported to be less stable than carbamyl phosphate [25]. *N*-Amino carbamyl phosphate is not a substrate for ornithine transcarbamylase and therefore does not enter the urea cycle, therefore it is unlikely that labelled urea is derived from this source. In summary the presence of hydrazine may inhibit the production of carbamyl phosphate from ammonia and this could contribute to the toxic effects of hydrazine. Any labelled ammonia produced from hydrazine could be incorporated into glutamine or alternatively might result from N–N bond cleavage of glutamyl hydrazine [15]. Consequently we attempted to detect glutamine labelled with ^{15}N in the amido nitrogen in urine and liver extracts. However no ^{15}N-labelled glutamine could be detected. ^{15}N-Labelled acetamide might result from N–N bond cleavage in acetylhydrazine but no labelled acetamide was detected by ^{15}N-NMR either.

An alternative source of labelled urea could be autoxidation of semicarbazide formed from urea and hydrazine. No semicarbazide was detected in urine samples however using NMR and other methods.

The synthesis of the pyruvate hydrazone was followed by NMR *in situ* because once formed it competes with hydrazine for available pyruvate. Whereas the 2-oxoglutarate hydrazone immediately undergoes an intramolecular cyclisation to THOPC which stops any further reaction with 2-oxoglutarate. Both of these hydrazones may form in the liver, as THOPC has certainly been detected in liver extracts from rats dosed with hydrazine both by proton and ^{15}N-NMR.* Keto acid levels are likely to be high after hydrazine administration as pyridoxal phosphate dependent transaminases are inhibited by hydrazine. The formation of these hydrazones will occur chemically but whether an enzyme mediated reaction also occurs is unknown. The toxicological significance of these hydrazones is currently unknown although depletion of ketoacids is a possible consequence of hydrazine administration.

Acknowledgements—The authors are grateful to the Wellcome Trust for their support of Dr Preece and this research.

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