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GENERATION OF FORMALDEHYDE BY *N*-DEMETHYLATION OF ANTIPYRINE

DETECTION OF FORMALDEHYDE IN BILE BY ^{13}C-NMR SPECTROSCOPY

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Abstract—The importance of NMR spectroscopy as a tool to investigate metabolic events *in vitro* and *in vivo* becomes more and more evident. Particularly ^{13}C-NMR spectroscopy is able to deliver a wide range of information regarding the chemistry of xenobiotics *in vivo*. We studied the *N*-demethylation of *N*-methyl-^{13}C-labelled antipyrine using an isolated perfused rat liver with a fluorocarbon suspension (FC 43) as oxygen carrier. Bile was collected in different fractions during the experiment. On the vascular side metabolite formation was monitored by continuous flow NMR spectroscopy. In bile the metabolic events were detected by standard NMR techniques. The bile spectra exhibit, among others, a signal at 84.2 ppm, indicating formaldehyde hydrate derived from the *N*-methyl group of antipyrine by an oxidative metabolic pathway. Neither formaldehyde hydrate nor other oxidation products could be detected in the vascular perfusate. The biliary excretion of considerable amounts of formaldehyde during the *N*-demethylation of antipyrine might have toxicological consequences for the intra- and extrahepatic bile ducts.

Isotopic labelling has been used for a long time to detect and quantitate metabolic pathways *in vivo*. During the last few years ^{13}C labelling has been applied increasingly. ^{13}C-NMR provides a suitable method to detect the distribution of the ^{13}C label in intermediates and end-products of metabolic processes [1–6]. In this paper we describe the biliary excretion of formaldehyde derived from *N*-methyl-^{13}C labelled antipyrine in an isolated perfused rat liver system. Antipyrine (phenazone) has been used to evaluate the activity of the hepatic monooxygenase system in man and experimental animal. Although the drug has now been in use for almost 100 years, several aspects of its disposition and metabolism have not been elucidated [7]. Antipyrin plasma half-life or metabolic clearance rates are widely used to assess changes in the activity of hepatic monooxygenases in animals and man induced by diseases, drugs and environmental factors. The drug is completely absorbed from the gastrointestinal tract, distributed evenly in total body water and minimally bound to proteins. The metabolism is rather extensive and only negligible amounts of the drug are excreted unchanged in the urine. The major metabolites are 4-hydroxyantipyrine (AP-4OH), 3-hydroxymethyl-antipyrine (AP-CH_2OH) and *N*-demethylantipyrin (AP-NH_2) [8]. Metabolic biotransformation of *N*-methyl groups of many xenobiotics involves *N*-demethylation to the desmethylamine and formaldehyde. This reaction is catalyzed by a mixed functional oxidase [9]. The oxidation is considered to occur either via direct oxidation of the methyl group to generate a carbinolamine or via *N*-oxide formation and rearrangement to the carbinolamine. The carbinolamines are generally considered to be transient species which decompose to generate formaldehyde. The formaldehyde is rapidly detoxified by oxidation to formate at a rate that exceeds the rate of its production. This oxidation is performed by aldehyde oxidases (EC 1.2.1.3) which have been found in the cytosol, mitochondria and microsomes of hepatocytes [10]. However, there are reports in the literature describing the formation of stable carbinolamines with the suggestion that certain *N*-methyl-group containing xenobiotics like hexamethylamine and pentamethylmelamine may form nascent forms of formaldehyde that might escape the detoxification mechanisms of the liver and perhaps have serious toxicological consequences [11]. When following the metabolism of ^{13}C labelled antipyrine in an isolated, fluorocarbon perfused rat liver using NMR-spectroscopy we were able to demonstrate the biliary excretion of formaldehyde derived from the ^{13}C labelled *N*-methyl group of antipyrine. This suggests that at least part of the formaldehyde formed during the *N*-demethylation of antipyrine bypasses the hepatic detoxification mechanisms with eventually toxicological consequences.

MATERIALS AND METHODS

Chemicals. D_2O (99.7 at. %; MSD Isotopes Ltd. Montreal, Canada), ^{13}C-methyl iodide (90 atom% ^{13}C; Amersham-Buchler, Braunschweig, F.R.G.) and the artificial oxygen carrier FC 43 (Pfrimmer &

Co., Erlangen, F.R.G.) are commercially available. (1-methyl-^{13}C)-antipyrine (1-methyl-^{13}C)-1,2-dihydro-1,5-dimethyl-2-phenyl- 3H-pyrazol-3-on) has been prepared by *N*-methylation of norantipyrine [11]. For the synthesis of the ^{13}C-labelled compound the following modifications of the original procedure are essential. The reaction is performed in a sealed tube to suppress the escape of labelled methyl iodide. The usual solvent methanol cannot be applied since it yields unlabelled methyl iodide by reaction with hydrogen iodide formed in the reaction course. Methanol is therefore substituted by diglyme. To avoid ether cleavage of this solvent the hydrogen iodide is buffered by addition of solid sodium bicarbonate. Norantipyrine (2.44 g, 14 mmol), ^{13}C-methyl iodide (2.0 g, 14 mmol), sodium bicarbonate (1.2 g, 14 mol) and diglyme (14 ml) in a thick-wall glass tube are cooled in liquid nitrogen and the tube is sealed under vacuum (0.005 torr). After heating for 24 hr at 110° the usual work-up of the reaction mixture yields the crude product. The (1-methyl-^{13}C) antipyrine has been extensively purified by recrystallisation from ligroin and the purity records (>99%) have been established by analytical and spectroscopic methods. Yield: 2.46 g (92%); m.p. 113°

Reference formaldehyde. A solution of monomeric formaldehyde has been prepared by thermal depolimerization of paraldehyde. The gaseous monomer was absorbed in an ice-cooled FC 43/D_2O mixture.

Biological system–liver perfusion. Isolated perfused rat liver with a fluorocarbon as artificial oxygen carrier (perfluorotributylamine, 20% w/v; FC 43R, Green Cross Corp., Japan) has been used as a biological system that is well controlled for more than three hours and allows investigation of hepatic drug metabolism [12]. 250–300 g liver donor rats (male Sprague–Dawley) pretreated for three days with phenobarbital (80 μg/g b.w. i.p.) were housed with free access to food and water. The isolation procedure was started between 8 and 10:00 a.m. under ether anesthesia. After cannulation of the portal vein, perfusion was started immediately. With the bile duct cannulated the liver was excised and transferred to the perfusion chamber. The perfusion with the synthetic medium (FC 43) has been performed in a flow controlled recirculating system. The perfluorotributylamine suspension was equilibrated with 95% O_2/5% CO_2 in a film oxygenator. Venous flow, arterial pressure, temperature, pH, O_2 consumption, lactate/pyruvate ratio and bile flow were continuously monitored and shown to be stable for a period of at least 3 hr. After completion of the isolation procedure the preparation was allowed to equilibrate for 30 min (−30–0 min). Then ^{13}C labelled antipyrine was added in a final concentration of 7.5×10^{-4} Mol/l.

Sample preparation. From the bile duct cannulated rat liver, bile was collected in time intervals of 30 min during the experiment. The bile flow was determined by weighing. Samples were kept at −20°. For ^{13}C-NMR measurements the bile fractions were transferred to a 5 mm NMR sample tube. After addition of an internal reference (dioxane) the samples were diluted with D_2O to 400 μl.

NMR spectra. ^{13}C NMR spectra were recorded at 100.62 MHz on a Bruker WM 400 instrument controlled by a Bruker Aspect 2000 computer system. For data storage a CDC disc (80 Mbyte) was employed. Continuous flow ^{13}C NMR spectra [13] were recorded before and after the addition of ^{13}C labelled antipyrine to the perfusion medium (total volume 150 ml) from −30 min to 180 min perfusion time.

RESULTS

Figure 1 exhibits the continuous flow ^{13}C spectra of the vascular perfusate with and without the addition of *N*-methyl-^{13}C labelled antipyrine. The signal at 35.6 ppm has to be assigned to the *N*-methyl-^{13}C of the antipyrine molecule. Other signals at 18.4, 64, 72–81, 100–128 ppm are due to constituents of the artificial oxygen carrier (FC 43). During the experiment the resonance of antipyrine at 35.6 ppm decreased almost linearly up to 180 min perfusion time ($r^2 = 0.973$) (Fig. 2).

The rate of decline in the NMR signals arising from antipyrine on the vascular side was accompanied by increasing signals from enriched ^{13}C positions in the bile (Fig. 3).

Figure 4 exhibits the ^{13}C NMR spectra of bile collected during a 30 min perfusion period from 90 to 120 min after the addition of ^{13}C labelled antipyrine to the vascular perfusion. The signals are exclusively derived from ^{13}C labelled carbon atoms or from internal reference (dioxane) and FC 43 constituents. In the lower trace the ^{13}C resonances are separated into methylene (positive signals) and methyl groups (negative signals) by the DEPT technique [14]. Signals within 30–60 ppm have to be assigned to the *N*-methyl groups of antipyrine and its metabolites. The assignment has been achieved by comparing the spectral data generated during the experiment with data from chemically synthesized compounds. Interestingly a signal at 84.6 ppm with increasing intensity has been documented in the bile of the isolated perfused rat liver. According to the DEPT measurement this signal originates from a methylene group. The chemical shift value is found in the range expected for formaldehyde hydrate (dihydroxymethane) [14]. Unequivocal assignment as formaldehyde hydrate has been achieved by comparison of ^{13}C NMR data in a solution of formaldehyde in FC 43. An estimate by reference to the internal standard dioxane of antipyrine transformed via demethylation to formaldehyde amounts to *ca.* 10% of total metabolism. Neither formic acid nor $^{13}CO_2$, the oxidation products of formaldehyde hydrate could be detected, which might be due to concentrations that are below the sensitivity level of ^{13}C-NMR spectroscopy.

DISCUSSION

The hepatic metabolism of endogenous [15, 16] and exogenous [13, 17] compounds can be estimated in intact livers by ^{13}C NMR spectroscopy. Radiofrequency surface coils [18] or continuous flow ^{13}C NMR spectroscopy [12] are tools to follow the metabolic activity of organs without disturbing the

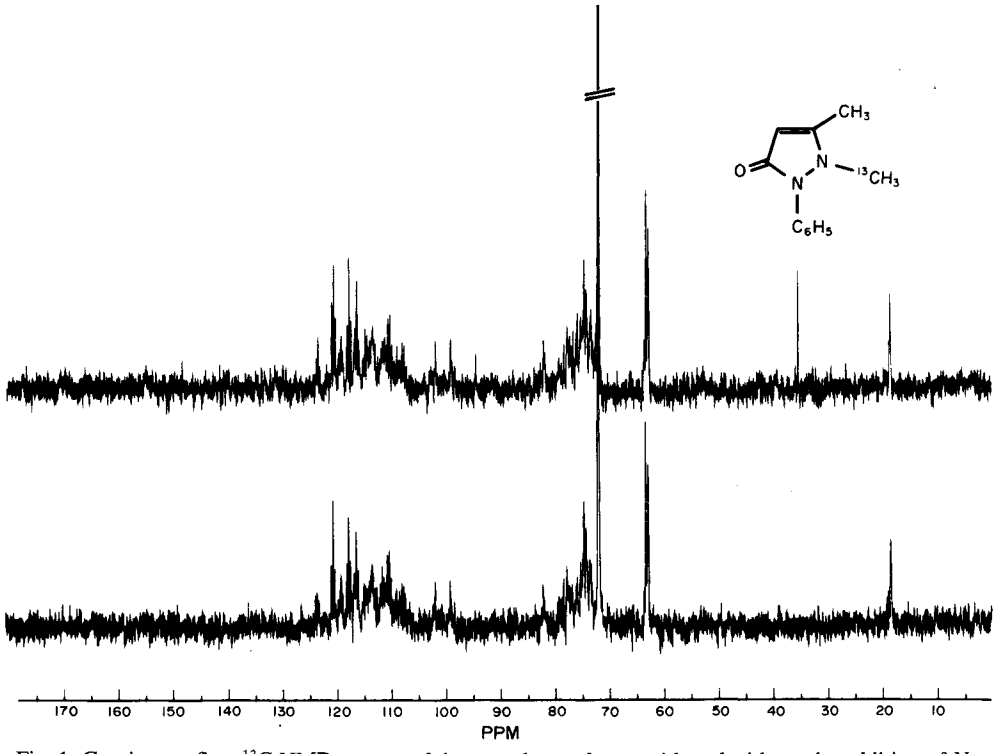

Fig. 1. Continuous flow ^{13}C-NMR spectra of the vascular perfusate with and without the addition of *N*-methyl-^{13}C labelled antipyrine.

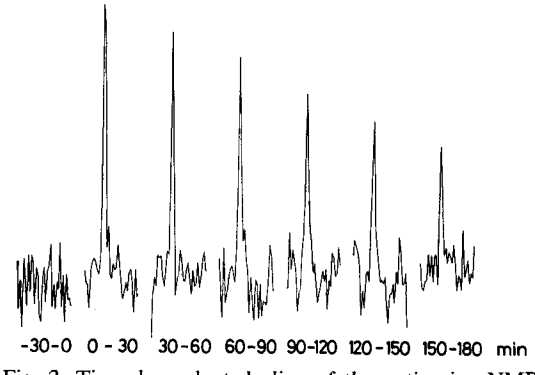

Fig. 2. Time-dependent decline of the antipyrine NMR-signal in the vascular perfusate.

biological system. The results presented in this paper describe a combination of continuous flow ^{13}C NMR spectroscopy in the vascular perfusate of an isolated perfused rat liver to detect the metabolism of ^{13}C labelled antipyrine with conventional NMR spectroscopical measurements in bile samples of the perfused organ. While a continuous decrease of the labelled substrate on the vascular side with a half-life of 150 min could be demonstrated, a simultaneous increase in oxidation products and, most interestingly, formaldehyde hydrate on the biliary side could be seen. As has been demonstrated in the past the metabolism of antipyrine is rather extensive and only negligible amounts of the drug remain unchanged by phase I or phase II metabolic pathways in humans as well as in experimental animals [18]. In the rat 96.92% of an orally administered dose are recovered within 72 hr in the urine, 3.05% in the faeces and only 0.2% in the air [19]. Approximately 93% of an administered dose of 3-^{14}C antipyrine could be identified within 24 hr in rat urine mainly as 5-hydroxymethylantipyrine (21.7%), norantipyrine sulfate (20%), 4-hydroxyantipyrine sulfate (14.0%), 4,4-dihydroxyantipyrinesulfate (15%), 4-hydroxyantipyrine-glucuronide (9.2%) and 5-hydroxymethylantipyrine-glucuronide (12.8%). Similar results have been demonstrated for humans. In contrast to the metabolism of the rat roughly 75% of the label recovered were metabolites conjugated with UDP-glucuronic acid [20]. These results point to a very extensive phase II metabolism of antipyrine in different species. The intermediary metabolism of antipyrine is less well understood. Metabolic biotransformation of *N*-methyl groups, however involves *N*-demethylation to the desmethylamine and formaldehyde. This reaction is known to be catalyzed by one or several cytochrome P-450-dependent mixed functional oxidases. The oxidation generates carbinolamines, a transient species which decompose to form formaldehyde. Formaldehyde either enters the one-carbon pool as N5, N10-methylenetetrahydrofolate or binds to plasma and tissue proteins with further oxidation to formate and CO_2 [21]. The rate of oxidation usually exceeds the rate of formaldehyde formation. There are, however, reports in the literature describing the formation of stable carbinolamines with the suggestion, that the metabolism of certain xenobiotics generates a nascent form of formaldehyde which might escape

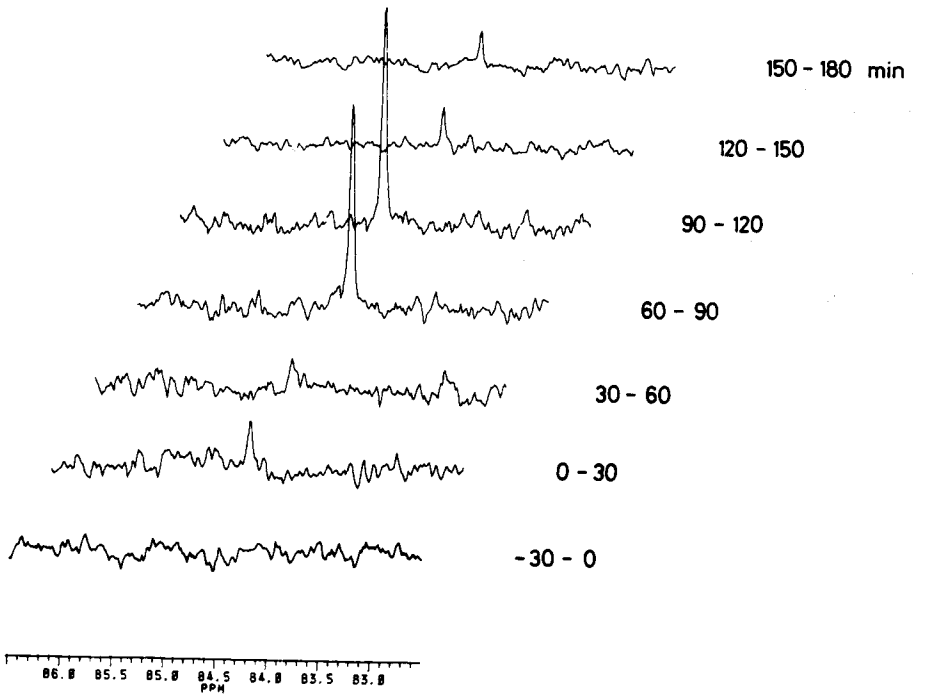

Fig. 3. Time-dependent increase of signals from enriched ^{13}C positions in the bile. The signal at 84.6 ppm could be assigned to formaldehyde hydrate.

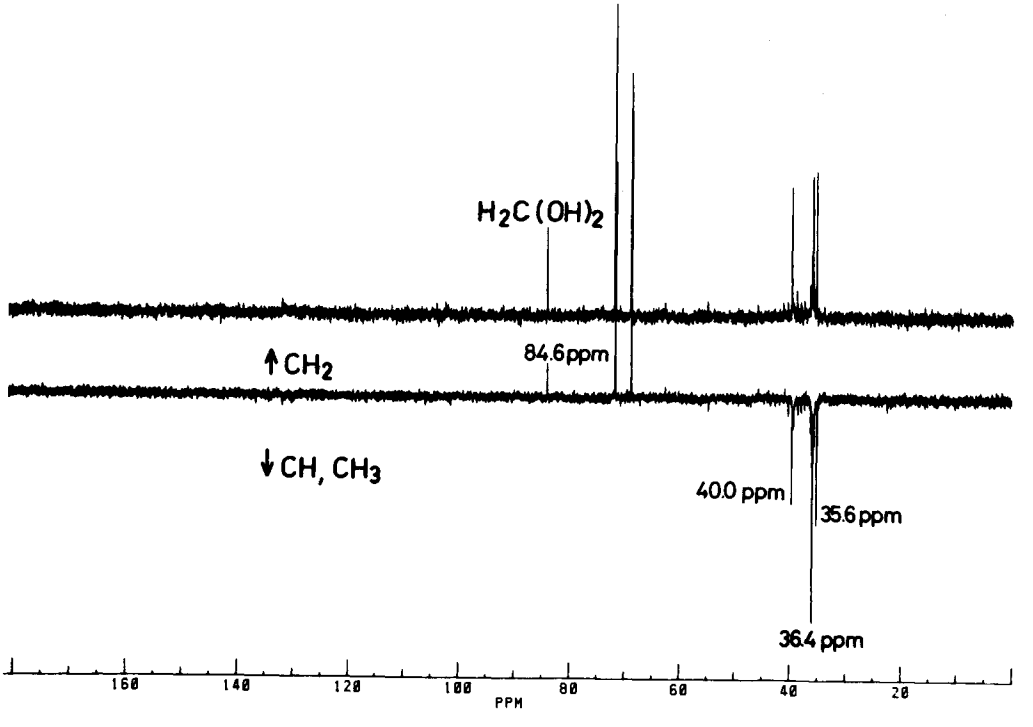

Fig. 4. ^{13}C NMR spectra of bile collected from 90 to 120 min after the addition of ^{13}C labelled antipyrine to the vascular perfusate (upper trace). In the lower trace the same spectrum obtained by the DEPT technique is shown. According to authentic metabolites the assignments of the N-^{13}C resonances are as follows: 35.6 ppm, antipyrine; 36.32 ppm, sulfate conjugate of 4-OH-antipyrine; 36.47 ppm, glucuronide of 4-OH-antipyrine; 37.5 ppm, 4-OH-antipyrine; 36.39 ppm, 5-OH-antipyrine; 36.1 ppm, sulfate conjugate of 5-OH-antipyrine; 40.0 ppm Ca^{2+} complex of 4-OH-antipyrine.

the hepatic detoxification mechanisms [11]. Investigating the hepatic metabolism of antipyrine, the substrate and its main metabolites were detected in the bile with the *N*-methyl carbon used as a tracer for ^{13}C NMR spectroscopy. Most interestingly, formaldehyde hydrate could be detected in considerable amounts. Neither formaldehyde nor other oxidation products were seen on the vascular side which might be due to the still low sensitivity of continuous flow ^{13}C NMR spectroscopy. Our results do confirm the assumption that the *N*-methyl group of antipyrine is hydroxylated in a first step to form the transient species of 1-hydroxymethyl antipyrine which is then cleaved to norantipyrine and formaldehyde. In the aqueous solution the formaldehyde molecule is finally detected as its hydrate. No signal that might be assigned to such an intermediate species like 1-hydroxymethylantipyrine as proposed by others [22] could be detected. The detection of rather high concentrations of formaldehyde formed during *N*-demethylation of antipyrine that apparently bypasses hepatic detoxification mechanisms and is excreted via the bile might have important toxicological consequences for the intra- and extrahepatic bile ducts and the intestine especially since the concentration of antipyrine used is higher but in the same order of magnitude as the therapeutical one. On the other hand, the metabolism we observed occurs in an isolated organ, the oxidation capacity of which might be different from the *in vivo* situation. Our investigation confirms the concept that ^{13}C NMR spectroscopy is a powerful tool to observe the intermediary metabolism of endogenous as well as exogenous compounds in intact organs or organisms. Further studies will have to prove if *N*-demethylation of other xenobiotics also results in biliary excretion of formaldehyde hydrate.

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