^{1}H-NMR Studies of Metabolic Effects of Endo- and Exogeneous Compounds on Astroglia Cells Cultivated *In Vitro*

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Abstract: A ^{1}H-NMR method is described for the study of metabolic reactions *in-vitro* of neonatal cerebellar rat astroglia cells cultivated on micro carrier beads. In the NMR tube, the formation of metabolic effects on astroglia cells caused by addition of nutrients (pyruvate and fumarate) or neurotoxins can be monitored. Metabolic changes of both endogenous metabolites and toxins, 1,2-epoxybutane and ethyl acrylate are investigated. 1,2,4-Benzenetricarboxylate (BTC) served as an extracellular reference. $Dy(ATP)^{-1}$ was used to depress signals from extracellular compounds. 6-Diazo-5-oxo-L-norleucin was added to cause accumulation of conjugated intracellular metabolites to an extent that permitted detection by ^{1}H-NMR spectroscopy.

1,2-Epoxybutane is shown to be detoxified due to a transferase reaction involving conjugation of reduced glutathione. The sulphur is coupled to carbon-1 or carbon-2 of the epoxide. Addition of ethyl acrylate leads in a similar way to a C1-GSH derivative.

The metabolic status of the astroglia cells were monitored during different exposure conditions.

Keywords: Spin-echo NMR spectroscopy, cell viability, glutathione conjugation, glutathione diethyl ester, glutathione.

INTRODUCTION

The brain is especially vulnerable to xenobiotic compounds particularly for those with lipophilic properties that allow them to cross the blood-brain barrier. When considered as a whole the brain is not a major extra hepatic drug metabolizing organ. Furthermore, there is however a heterogeneity among brains cells as for drug metabolism, glia cells having a higher capacity than neurons to detoxify both endogenous and exogenous reactive species [1, 2] Detoxification of a xenobiotic compound normally is a multiphase process, where the first step involves a family of phase I enzymes, including among others the cytochrome P-450 (CYP) mono-oxygenases superfamily [3, 4] which catalyze the addition of a functional group [5]. The second step in detoxifications involves a multigene family of phase II enzymes which catalyze the conjugation of hydrophilic moiety e.g. glutathione (γ-glutamyl-L-cysteinyl-glycine; GSH) and thereby the xenobiotic compounds are usually less toxic than the parent compound. GSH is the most abundant non-protein thiol-containing molecule and occurs in the brain in the range of 1-3 mM [6], and acts as a major defense mechanism against a variety of agents, including free radicals, reactive oxygen species and cytotoxic drugs [6]. GSH is generally assumed to protect cells against the cytotoxic effect of bifunctional alkylating agents by the formation of non-cytotoxic GSH adducts, a conjugation reaction that may occur spontaneously or be catalyzed by glutathione-*S*-transferase enzymes (GST; E.C 2.5.1.18), one of the phase II enzymes (for reviews see Refs. [7-9]). The present communication evaluates these transformations by studying the metabolic effects of an epoxide, 1,2-epoxybutane and an olefinic derivative, ethyl acrylate, on astroglia cells adhered to micro carrier beads. The metabolic changes were evaluated by non-invasive proton NMR spectroscopy. The ^{1}H-NMR spectroscopy in D_2O was optimized by a combination, where $Dy(ATP)^{-1}$ was used as a shift/relaxation reagent in order to depress signals from extracellular compounds [10, 11] 1,2,4-benzenetricarboxylate was used as an extracellular reference, diethyl GSH was used to load the cells with GSH and finally DAONL was added to the cell culture. DAONL is a glutamate analog and inhibits glutamate binding enzymes e.g. γ-glutamate transpeptidase [12, 13]. DAONL was added in order to hamper the efflux of conjugates of GSH and metabolites, hereby causing intra cellular accumulations of conjugated metabolites to levels detectable by NMR spectroscopy. The present communication demonstrates that conjugates of GSH and added xenobiotics are formed in astroglia cells during *ex-vivo* NMR studies.

MATERIALS AND METHODS

Animals, Chemicals and Glassware

If not otherwise stated the chemicals and glassware were from Merck Ltd, Darmstadt, Germany, and Bie and Berntsen, Copenhagen, Denmark. 7 days old Wistar rats were provided by the Panum Institute, Copenhagen. Materials were purchased from: Tissue culture flasks (75 cm^2, polyethylene terephthalate glycol) *in vitro* scientific products Inc.Ventura, USA; collagen coated micro carrier beads (1.04 g/ml, 150 – 210 microns) Cellon Inc., Luxembourg; fetal calf serum and Dulbecco`s minimal essential modified Eagle`s medium

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#In memory of Professor Jørgen Clausen, who was an ever present source of inspiration.

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(DMEM), Gibco/Biocult Lab.Ltd, Scotland and Life Technology, Denmark, respectively. Penicillin (1 mill.i.U./ml) was from Leo Pharmaceutical Comp., Ballerup, Denmark and 5 mm NMR tubes were from Wilmad Glass Corp. Inc., Buena, NJ, USA.

Synthesis of Dysposium-ATP

The relaxation/shift reagent dysprosium-ATP (Dy $(ATP)^{-1}$) was synthesized and used as described originally by Barry *et a l.* [11, 14] and modified by Geraldes and Gupta [15, 16].

Synthesis of Glutathione Diethyl Ester (GSH-DEE)

GSH-DEE was synthesized as described by Levy *et a l.* [17]. GSH-DEE was separated from the mono-ester and from other reaction products by gel filtration on a Sephadex G10 (0.9 x 30 cm) column using a degassed 0.25 M NaCl solution as an eluent. Purity was examined by ^{1}H-NMR on a Bruker 250 MHz apparatus and by RF-HPLC on a Millipore Waters 600E system using a Bondapak ™C18 column (3.9 x 150 mm) using a gradient: 90% water/10% CH_3CN/0.1% trifluoroacetate as initial eluent followed by gradient elution with 10% water/90 % CH_3CN/0.1% trifluoroacetate with an elution time of 45 min. Prior to exposure of the cells for GSH-DEE oxidized GSH-DEE was reduced by addition of 100 µl of 500 mM GSH-DEE solution of 2.5 µl glutathione reductase (60U/ml) and 22.5 µl 0.22 mM NADPH.

Preparation of Primary Astroglia Cell Cultures

Astrocytes were cultured essentially as described by Hertz *et a l.* [18]. Cerebellum was removed from seven days old neonatal rats and placed in DMEM supplemented with 20% fetal calf serum (FCS), 2mM glutamine and 500 U/ml penicillin (AST-DMEM1). The tissue was passed through a Nitex filter (80 µm pore size) in order to separate cells from cellular debris. The cells were resuspended in 10 ml AST-DMEM1 and then diluted to 85 ml in the same medium. The suspension was distributed in seven poly-lysine coated flasks (105 cells/cm^2) and cultivated at 37^{o}C in an incubator with water saturated atmosphere (5% CO_2/95 % air). Medium was changed 2 days after inoculation and then subsequently twice a week. The FCS concentration was decreased to 15% during the second week of culture and to 10% during the third. Micro carrier beads (85 mg/15 ml medium) were added on day 8. The cells reached confluence after 10 - 12 days of growth (Fig. **1**). The DNA content was measure by Hoechst 33258 fluorochrome staining and found to be constant after the second week of growth. In the third week of culture the medium was supplemented with 0.27 mM dibutyl-cyclic AMP (dBcAMP) [19] in order to cause maturation of the astroglia cells [18]. After 3 weeks in culture the primary cell/bead matrix was used for NMR experiments.

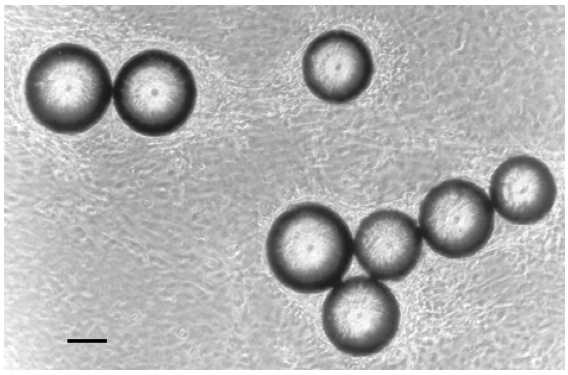

Fig. (1). Confluent cerebellar astroglia on microcarrier beads. Picture taken in-phase in contrast microscope.[a]

[a]1 cm (dark bar) = 56 µm.

Viability of Astrocytes

The cell morphology was followed by phase contrast microscopy. The criteria of astroglia cell morphology have been those of McCarthy and Vellis [20]. In order to evaluate the percentage of necrotic cells after xenobiotic exposure (i.e 1,2-epoxybutane and ethyl acrylate) the PI uptake was measured in the astroglia cells. Xenobiotics were added in variable concentration to the culture flasks for 1 hr. The medium was removed before addition of propidium iodide (PI) (5 µg/ml) for 5 min and cells were washed twice in PBS, pH 7.0 after 5 minutes and 1% paraformaldehyde was added for fixation. The red fluorescent exclusion dye propidium iodide (PI) is widely used as a vital dye in tissue culture systems and labels the nucleus of dying cells. The latter lack an intact plasma membrane [21]. Xenobiotics treated cell cultures were examined in a Leica fluorescence microscope equipped with an excitation filter for blue light (488 nm) and an emis-

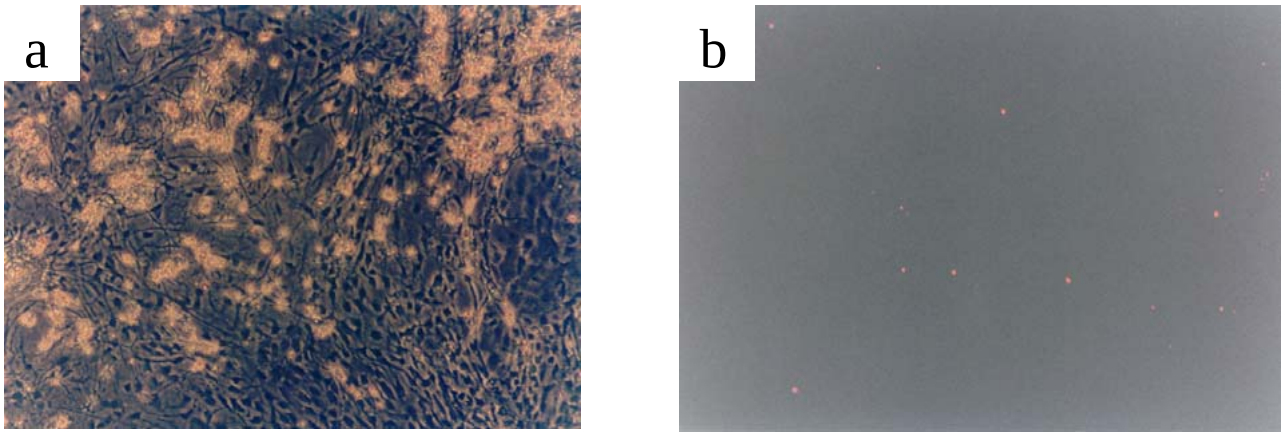

Fig. (2). A cerebellar astroglia cell culture treated with 5 mM 1,2-epoxybutane for one hour (**a**) and subsequently treated with 30 µg/ml propidium iodide for 5 min (**b**).

sion filter (560 nm) (see Fig. **2**). Other biological tests are described in the Supplementary material.

Cell Preparation for NMR Studies

Deuteriated medium was prepared from freeze-dried culture medium without FCS and redissolved in an equal amount of 99.9 % heavy water. BTC was added to the redissolved deuteriated medium to a final concentration of 3 mM and the pD was adjusted to 7.2 with NaOD. Since the astrocyte cells grow on the microcarriers as well on the bottom of the flask cells were liberated with a "rubber police man", transferred to an Eppendorf tube and washed twice with PBS and deuteriated medium with BTC was added to the cell suspension.

The cell suspensions (about 3.5 x 10^6 cells/ml) were pipetted to a 5 mm (diameter) NMR tube. The cells in the NMR tube were confined to the bottom part with a Teflon stopper with a hole.

Ex Vivo ^{1}H Experiments

Proton NMR spectroscopy was performed on Bruker AC 250 or Varian Inova 600 NMR spectrometers using a Hahn spin echo technique [22] with 5 kHz spectral width in 16 K data point. The acquisition time was 1.638 sec with a delay of 1.3 sec between FIDs during which a continuous presaturation of the water resonance occurred. The interpulse delays were set to 68 ms to make signals from singlets and triplets (coupling constants 7 Hz) positively phased. The spin-echo technique allows characterization of the spin systems [23, 24].

Assignments were made by comparison with extensive tabulations of chemical shifts for metabolic intermediates and literature values [25, 26]. The cluster of peaks at 3.20 ppm was assigned to choline containing compounds and was used as intracellular shift reference. To reduce in homogeneities the NMR tube was spun at 18 Hz. Temperature was 27^o C.

A micro capillary pipette was used to place the xenobiotics in the packed cells suspension in the NMR tube.

RESULTS AND DISCUSSION

Cellular Viability Prior to NMR Experiments

The cell viability following exposure to either 1,2-epoxybutane or ethyl acrylate was analyzed in series of experiments where astroglia cell cultures were exposed to either 1,2-epoxybutane or ethyl acrylate at concentrations ranging from 0.1 mM - 24 mM. An expected and pronounced cell death was observed when cells were exposed to high concentrations (24 mM) of xenobiotics. Up to 5 mM of either 1,2-epoxybutane or ethyl acrylate no significant decrease in cellular viability was observed (Fig. **2**). This picture is similar to one without incubation (data not shown). As an outcome of these experiments a concentration of 3 mM 1,2-epoxybutane or ethyl acrylate was used in all NMR experiments with xenobiotics.

Cellular Viability During NMR Experiments

Due to the extended timescale of the NMR studies of cellular metabolism and a constant exposure to D_2O, it was necessary to evaluate the viability of the cells during and after the experiments. The concentration of GADPH in the medium after the NMR experiment was significantly lower than the concentration in untreated lysed cells (Supplement, Table **1**). The small ratio indicates that only a low level of membrane damage occurs during the NMR experiments (Supplement, Table **1**). The estimation of lactate in the medium and the LDH assay showed about a 50 % increase in specific LDH activity of the medium during 3-4 hrs of incubation (Supplement, Table **1**). When the experiment was extended to six hours no further increase in specific LDH activity was found. Again, these data indicate limited damage to plasma membranes. Furthermore, the amount of membrane damage may also be evaluated from NMR measurements by addition of $Dy(ATP)^{-1}$ (see below).

^{31}P NMR Spectroscopy

The energy status of the cells was evaluated by ^{31}P NMR. The spectra accumulated during a period of 2 hrs showed peaks for ATP and ADP with the γ-phosphate resonance of ATP clearly visible indicating viable cells (data not shown).

Characterization of the NMR-SYSTEM

Addition of the extracellular reference, BTC, is demonstrated in Figs. (**3a**) and (**3b**). Fig. (**3a**) shows the NMR

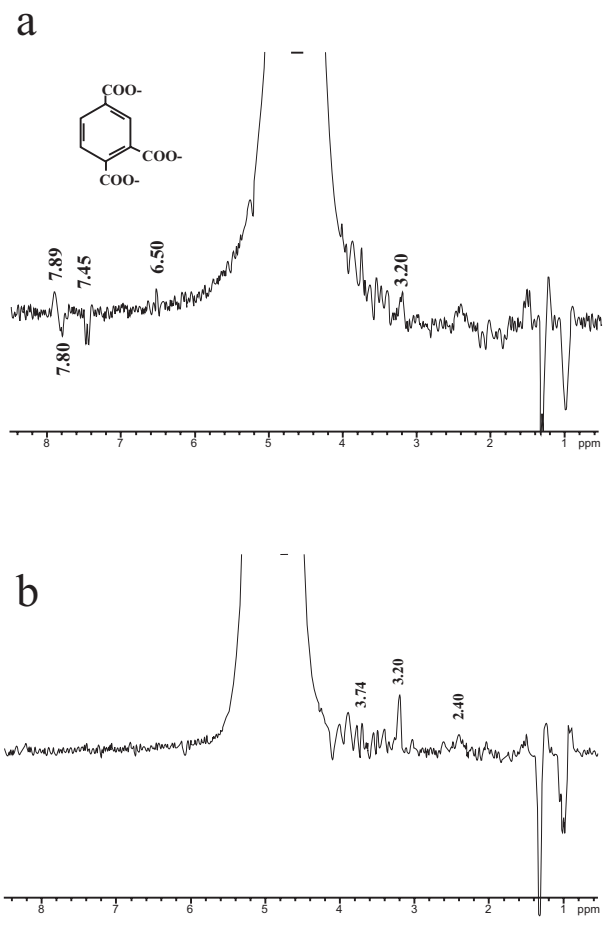

Fig. (3). ^{1}H spin-echo NMR spectrum of matrix bound confluent astroglia in medium/D_2O (~75%), pD= 7.2, 3 mM BTC added.

a) 46 min after transfer to D_2O.

b) 5 mM $Dy(ATP)^{-1}$ added (71 min after transfer).

spectrum of astroglia cell cultures in deuteriated medium to which BTC was added to a final concentration of 3 mM. The chemical shifts of H-3 (7.89 ppm), H-6 (7.80 ppm) and H-5 (7.45 ppm) correspond to those of BTC in the buffer. BTC was chosen as an extracellular marker as it seems to be too charged at pH 7.4 to permeate the plasma membrane of the astroglia cells. Addition of the relaxation agent $Dy(ATP)^{-1}$ (5 mM) caused the signals from BTC and other extracellular signals to disappear (Fig. **3b**). Furthermore, addition of $Dy(ATP)^{-1}$ may also lead to a decrease in the signals from lactate showing that a pronounced part of the lactate at long times is extracellular. The resonance found at 3.20 ppm originates from choline residues most of which are localized in cell membranes. Cell death will expose the inner membrane to $Dy(ATP)^{-1}$ and hence lead to a decrease in this resonance. No such change was observed, which indicated that neither BTC nor any of the other additions cause extensive cellular death.

The feasibility of using NMR for studying the conversion of xenobiotics was demonstrated by the oxidation of acetaldehyde to acetate (spectra not shown).

Assignment of Spin-Echo Spectra

The spin-echo spectra [22] show both positive and negative resonances depending on the number of neighboring protons and the magnitude of coupling constants as demonstrated for the methyl resonance of lactic acid (1.33 ppm) (Fig. **3a**). The signs of resonances are therefore a good tool in the assignment. Furthermore, for molecules biosynthesized during the reaction deuterium incorporation may take place. As couplings between deuterium and protium is 1/6.5 times that of the corresponding between two protons, the former couplings are normally not seen. This will lead to a change in sign of the relevant resonances in the spin-echo spectrum of the deuteriated species compared to that of the protio species. This can be illustrated by alanine. In alanine the methyl resonance will normally be negative due to coupling to the α-proton. However, if the incorporation takes place in D_2O you may expect a positive methyl resonance (however this is not found as described below).

Metabolites Found in the Astroglia System by NMR

The proton NMR spectroscopy of astroglia cells with/without addition of BTC and Dy(ATP)-1 revealed resonances from a number of metabolites. In several cases the peaks were identified by co-addition of known metabolites. An example is the singlet resonance at 6.50 ppm observed in several spectra (see Fig. **3a**). Addition of 2 mM fumarate confirmed the assignment. This peak persisted after addition of $Dy(ATP)^{-1}$ and BTC showing that fumarate in cytosol. The signal disappeared after 145 min and the disappearance was associated with appearance of new negative signals at 2.15 ppm, positive signals at 2.40 and 3.74 ppm, corresponding to glutamate.

A similar scenario was observed by addition of sodium pyruvate (2 mM) to the cell/bead matrix. Addition of pyruvate was associated with a resonance peak at 2.37 ppm due to the methyl group of pyruvate. This peak decreased in intensity and disappeared after 300 min. Simultaneously, a new peak appeared at 1.50 ppm due to alanine. This alanine peak is a negative doublet indicating that protium not deuterium is incorporated at the α-position (see previously). During this experiment a growing acetate peak is seen at 1.91 ppm. The ratio between the alanine and acetate peaks seemed to vary according to the amount of glucose present. The addition of fumarate and pyruvate demonstrates transamination reactions of intermediate α-keto acids of the tricarboxylic acid cycle with the formation of alanine and glutamate in in-vitro cultivated astroglia, respectively. The data concerning glutamate are in agreement with the data of Sonnewald *et al*. [27], who found that glutamate added to astrocyte culture medium was taken up in the cells by the glutamate transporter and metabolically transformed by transamination reactions.

Enrichment of GSH in Astroglia Cells

Addition of DAONL to the astroglia cell culture caused appearance of new NMR resonances at 2.05 ppm (negative doublet), 2.34 ppm (positive multiplet), 3.75 ppm (positive triplet) all belonging to glutamine. This is in agreement with DAONL being an inhibitory glutamate analogue which inhibits the membrane bound γ-glutamyl-transpeptidase (EC 2.3.2.2) and thereby preventing the transportation of glutamine out of the cells [12, 13].

A reference NMR spectrum of 5 mM GSH-DEE in PBS/D_2O (pD 7.4) showed resonances from both the oxidized and reduced form (Fig. **4**). The reduced form is seen as a characteristic pattern of two positive and overlapping triplets at 1.27 ppm (CH_3), overlapping negative quartets at 4.23 ppm (CH_2 of ethyl group), a negative resonance at 2.14 ppm (H-β of Glu), a positive resonance at 2.50 ppm (H-γ of Glu), a negative multiplet at 2.92 ppm (H-β of Cys), a positive singlet at 3.75 ppm (H-α of Gly) and finally 4.50 ppm (H-α of Cys). The majority of the resonances for the oxidized-form are coinciding with those of the reduced-form. For the oxidized-form, different positions are seen for H-γ of Glu (2.98 and 3.21 ppm both negative) and for H-α of Gly, positive singlet at 3.99 ppm. The oxidized form may serve as a model for the conjugated GSH.

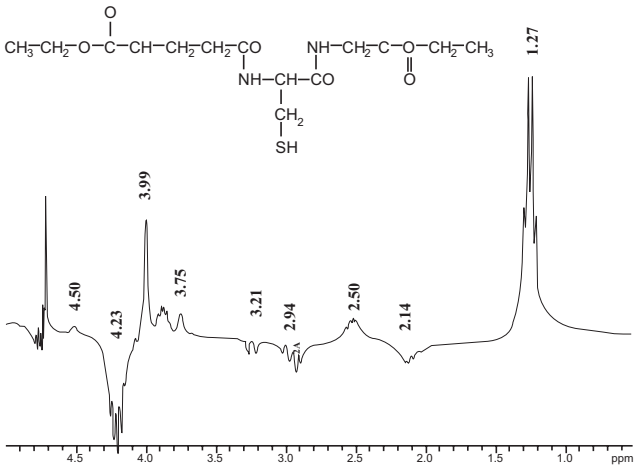

Fig. (4). ^1H NMR spin-echo spectrum of the diethyl ester of GSH (mixture of ox- and red-form).

To use GSH-DEE instead of GSH leads to uptake into the cells [17]. Addition of GSH-DEE to the cell culture gave rise to new peaks at 4.21, 4.02, 3.75, 2.94, 2.54, 2.12 and

1.26 ppm, which all are due to GSH-DEE (Fig. **5a**). GSH-DEE is metabolized by carboxyl esterase (E.C. 3.1.1.1.) to GSH and ethanol and in the period between 20 min and 110 min after GSH-DEE addition a rise in the ethanol content was traced since an growing peak due to free ethanol's methylene group can be localized as a negative quartet at 3.64 ppm (Fig. **5b**). Addition of DAONL to the ester enriched astroglia cell culture caused appearance of new NMR signals at 2.14, 2.43 and 3.75 ppm plus signals from the ethyl group hidden underneath signals from the GSH diethyl ester. The signals are due to glutamine ethyl ester, see above. In addition, at time 110 min the cleavage product glycine or glycine ethyl ester liberated from GSH/GSH diethyl ester can be seen at 3.55 ppm (Figs. **5a** and **b**).

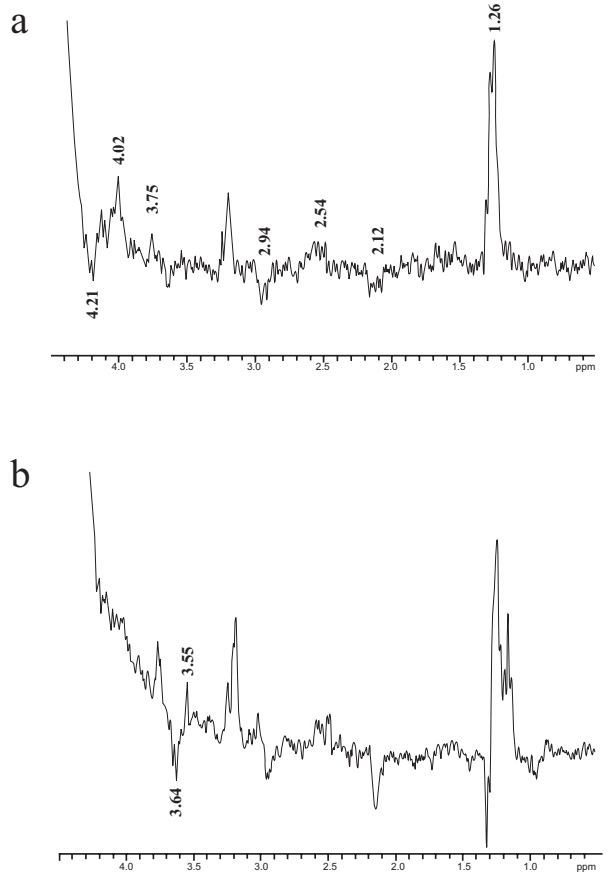

Fig. (5). ^{1}H spin-echo NMR spectrum of matrix bound confluent astroglia in medium/D_2O (~75%), pD= 7.2.

a) 3 mM BTC and 5m M GSH-DEE (reduced)(20 min after transfer).

b) Addition of of 100 μM DAONL (110 min after transfer).

Effects of A ddition of 1 ,2-epoxybutane an d Ethyl A crylate

The NMR reference spectrum of 0.5 M 1,2-epoxybutane in PBS/D_2O was assigned as 0.99 ppm (CH_3), 1.56 (H-3), 2.93 ppm (methine proton) and 2.73 ppm (methylene proton H-2) (Fig. **6c**). After supplementation of the cell culture with GSH-DEE and DAONL addition of 1,2-epoxybutane (3 mM final concentration) caused peaks with chemical shifts of 1.41 ppm (positive), 2.52 ppm (positive) and 3.52 ppm (negative) and 3.65 ppm (negative doublet)(Fig. **6a**). In addition, negative resonances at ~2.98 ppm and 3.21 ppm corresponding to conjugated GSH (see above) are likely falling underneath the creatine and choline resonances leading to a diminishing of these resonances (see Figs. **6a**,**b**). The observed chemical shifts correspond to a mixture of conjugate type 1 and 2 (Fig. **7**). No hydrolysis product was observed.

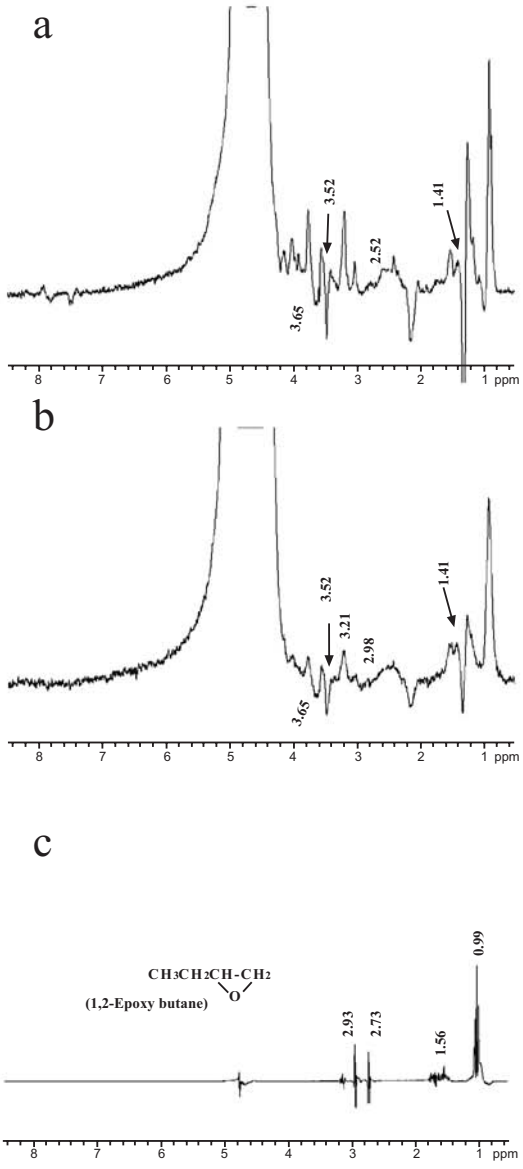

Fig. (6). ^{1}H spin-echo NMR spectrum of matrix bound confluent astroglia in medium/D_2O (~75%), pD= 7.2.

a) 3 mM BTC and 3m M GSH-DEE (reduced) added followed by 100 μM DAONL.

b) 2 mM 1,2-epoxybutane added to a) (76 min after transfer).

c) 5 mM $Dy(ATP)^{-1}$ added to cells (126 min after transfer).

d) ^{1}H NMR spin-echo reference spectrum of 0.5 M 1,2-epoxybutane in PBS/D_2O.

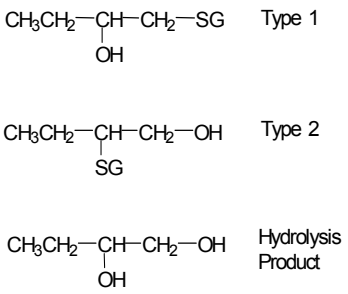

Fig. (7). Possible transformation products of 1,2-epoxybutane.

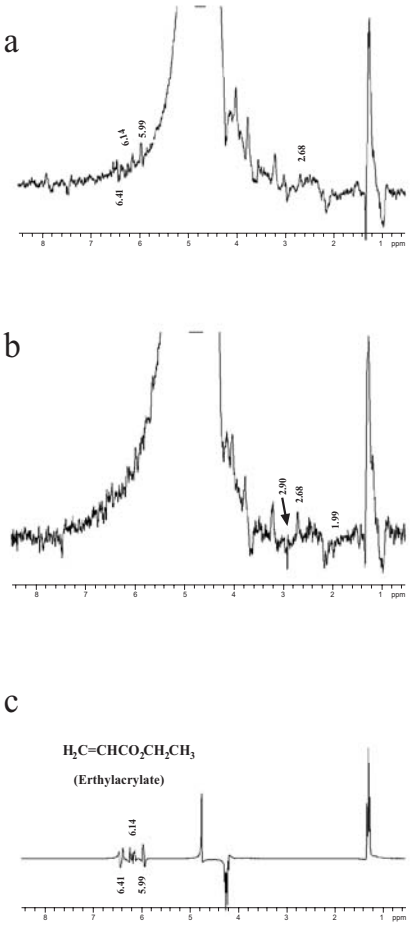

Fig. (8). ^{1}H spin-echo NMR spectrum of matrix bound confluent astroglia in medium/D_2O (~75%), pD= 7.2.

a) 3 mM BTC and 3mM GSH-DEE (reduced) added followed by 100 µM DAONL.

added to a) (60 min after transfer).

b) 3 mM ethyl acrylate aded to cells shown in a. (90 min after transfer).

c) 5 mM $Dy(ATP)^{-1}$ added to cells shown in b (159 min after transfer).

d) ^{1}H NMR spin-echo reference spectrum of 0.5 M ethyl acrylate in PBS/D_2O.

After addition of 3 mM ethyl acrylate to the astroglia cell culture new peaks appear at 6.41 ppm, 6.14 ppm and 5.99 ppm (Fig. **8a**). A new positive signal appears at 2.68 ppm (Fig. **8a**). This may be related to the H-α protons in conjugate type 1 (Fig. **9**).

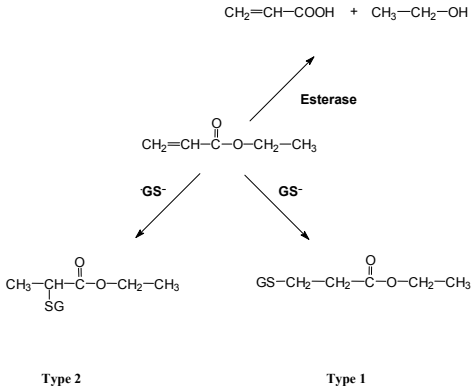

Fig. (9). Possible transformation products of ethyl acrylate.

During the incubation deformations of the peak at 2.90 ppm (cysteinyl protons of GSH) and a new negative signal at 1.99 ppm appears (Fig. **8b**). Furthermore, five resonances belonging to γ–glutaminyl cysteinyl and other cysteinyl components can be observed. These signals may be related to GSH resonances (for comparison see Fig. **6a**). Our data indicate the formation of type 1 conjugates in the astroglia cells. This process is probably enzyme catalysed as attempts to cause a coupling under *in vitro* conditions without enzyme was unsuccessful. A possible epoxidation by a CYP-enzyme can be ruled out as formation of an epoxide followed by conjugation as seen previously leads to a product containing a hydroxy group. The chemical shifts of the conjugates do not support this.

CONCLUSIONS

The present communication demonstrates that spin-echo NMR spectroscopy is suitable for the study of an ex-vivo cell/matrix system, in this case the detoxifications of xenobiotics in astrocytes. We have demonstrated that it is possible to detect conjugates of glutathione of epoxybutane and ethyl acrylate by NMR spectroscopy and thereby illustrate different alternative conjugation reactions with GSH. A vehicle for this is the addition of glutathione diester to cells. The conjugation for 1,2-epoxybutane leads to a mixture of the products, type 1 and 2, see Fig. (**7**). For ethyl acrylate the addition product is that expected based on chemical reactivity.

The NMR technique is very suitable as it allows a constant monitoring of all major metabolites and demonstrates the presence of not only conjugation reactions but also esterase activity and amination/deamination reactions.

ACKNOWLEDGEMENTS

The authors wish to thank the Lundbeck foundation and Pharmabiotek for support.

ABRREVIATIONS

BTC = 1,2,4-Benzenetricarboxylate

$Dy(ATP)^{-1}$ = dysprosium-ATP

DAONL = 6-Diazo-5-oxo-L-norleucin

GSH = glutathione

GST = glutathione transferase

SUPPLEMENTARY MATERIAL

Supplementary material can be viewed at www.bentham.org/open/tomrj

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