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THREE WEEKS' EXPOSURE OF RATS TO DEAROMATIZED WHITE SPIRIT MODIFIES INDICES OF OXIDATIVE STRESS IN BRAIN, KIDNEY, AND LIVER

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Abstract—The present study was undertaken in order to investigate whether dearomatized white spirit induces indices of oxidative stress in subcellular fractions of hemisphere, hippocampus, kidney and liver tissue of rats exposed to 0, 400 and 800 ppm 6 hr/day, 7 days a week for 3 weeks. The results show that white spirit is a strong *in vivo* inducer of oxidative stress in subcellular fractions of brain, kidney and liver. In the liver there was a statistically significant increase in the rate of reactive oxygen species (ROS) generation and a decrease in glutamine synthetase activity. In the kidney there was a statistically significant decrease in the rate of ROS generation. In the hemisphere there was a statistically significant increase in the level of reduced glutathione. In the hippocampus there was a statistically significant increase in the rate of ROS generation. However, *in vitro* addition of dearomatized white spirit had no effect on the rate of cerebrocortical P2 fraction ROS generation. The results suggest that cumulative oxidative damage may be an underlying mechanism of dearomatized white spirit-induced neurotoxicity and that various regions of the brain may respond differently.

Key words: organic solvents; neurotoxicity; synaptosomes; reactive oxygen species; Exxsol D 40

White spirit is widely used as a solvent in paints, printing inks, and varnishes. Various types of white spirit are produced. All are complex mixtures of straight and branched aliphatic, alicyclic, alkyl aromatic, and naphthenic hydrocarbons with boiling points in the range of 150–215°.

Longitudinal epidemiological studies of workers occupationally exposed to solvents show that neuropsychiatric disorders are a frequent cause of early disability pension in this population compared with nonexposed controls [1–3]. In the majority of the studies workers were exposed to mixtures of organic solvents, the principal component being white spirit.

In a cohort study of housepainters (exposed to white spirit) and bricklayers (unexposed) [4, 5], it was shown that signs of brain dysfunction increased significantly with the degree of solvent exposure in the group of painters. In workers from the paint industry [6] it was shown that signs of brain damage in the form of neurasthenic symptoms were significantly more common in the exposed workers, and that the severity of symptoms was dose-related. Identical findings were reported in another study [7]. Triebig [8] performed two epidemiological studies in painters. The results of these two studies do not support the assumption of high-neurotoxic risks in solvent-exposed workers. However, the same author recently published [9] results of a third investigation into spray-painters exposed to solvents for more than 10 years, in which a psychiatric analysis showed that special features of depression and loss of interest and concentration occurred significantly more frequently among spray-painters than among controls.

An important issue is the question of the neurotoxic properties of aromatic versus aliphatic or hydrogen dearomatized hydrocarbons. Industry has replaced aromatic hydrocarbons with aliphatic or hydrogen dearomatized types anticipating reduced neurotoxicity, however, there are no scientific data to support the preference of either type [10]. Consequently, more experimental data are needed before a particular substitution strategy might be recommended.

In animal experiments changes in global, regional and subcellular neurotransmitter content indicate certain acute [11] and/or irreversible [12, 13] effects after exposure to aromatic white spirit. Also, a reduced yield of synaptosomal protein/g brain weight has been found [13], which may reflect reduced synapse density. However, it has not been possible to demonstrate clear-cut irreversible neurobehavioural and neuropathological effects following long-term exposure to aromatic white spirit [12] or toluene [14]. Cognitive deficits, which are characteristic symptoms in solvent-induced encephalopathy in painters, were not induced in the experimental animals. In humans, the development of symptoms of premature aging requires exposure for a number of years.

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An attractive approach to investigate aging [15] and neurotoxicity [16] is to study the induction of oxidative stress. There is considerable evidence for the induction of excess free radicals and ROS* generation by various neurotoxic aromatic solvents. These compounds include benzene [16], toluene [17–19], xylenes [19–21], and styrene [22]. There is less evidence for the induction of oxidative stress by aliphatic organic solvents. However, it is well-recognised that ethanol possesses this property [23–25], and the potential for epoxide formation exists in some straight chain hydrocarbons [26]. On the basis of available data, an international conference on organic solvent neurotoxicity recommended the investigation of the formation of free radicals as a possible mechanism for solvent neurotoxicity [27].

Many of these studies involve dosing for short periods with purified chemicals in relatively high concentrations, and very little is known about the potential for oxidative damage consequent to more extensive exposures to mixed solvents. The latter conditions may be more relevant for typical industrial settings.

Therefore, the present experiments were undertaken to investigate the potential effects of dearomatized white spirit on subcellular oxidative status following *in vitro* addition and *in vivo* exposure. The dose levels used (0, 400 and 800 ppm) were the same as in our previous long-term studies. The present Danish maximally allowed occupational exposure level is 100 ppm. Specifically, a fluorescence probe was used to provide a direct, unspecific measure of the sum of rates of generation of ROS such as hydroxyl radicals, transition metal-peroxide complexes and lipoperoxyl radicals. Further, the glutamine synthetase activity was used as an index of excess ROS production summated over an extended period as this enzyme has been shown to be unusually susceptible to oxidative damage and previously has been employed in this context [28, 29]. Its depressed level may reflect the consequences of excessive pro-oxidative conditions which may be quantitatively minor but temporally prolonged [30]. Finally, the level of GSH was used as a measure for the functional status of the important defence mechanism: the glutathione system.

MATERIALS AND METHODS

Chemicals. Dearomatized white spirit (Exxsol D 40) was purchased from Exxon Chemical Denmark A/S. The boiling interval was 145–200°, and the aromatic content was <0.4 wt%. The mean molecular mass was 143 g/mol, and the density 0.771 kg/dm^3 (at 15°).

GSH, glutathione-*S*-transferase and aldehyde dehydrogenase, were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). DCF2H-DA and MCB were obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.). DCF was purchased from Polysciences, Inc. (Warrington, PA, U.S.A.). All other chemicals were of analytical grade.

Animals. Male rats (84) (Mol:WIST), mean body weight 350 g, 3 months old, obtained from Møllegaard Breeding Centre Ltd, L1 (Skensved, Denmark) were used. The rats were weight-randomized into three groups each of 28 animals. For the present study 10 animals from each group were randomly selected for the neurochemical studies of hemisphere, hippocampus, liver and left kidney. Eight rats from each group were used for subcellular neurochemical studies in which whole brain weight was determined (only weight of whole brain is presented here). The last 10 rats of each group were used for neurohistopathological examinations (no results presented here).

The rats were housed in stainless steel wire cages, two animals/cage, conventionally in animal rooms with automatic control of temperature (22 ± 1°), relative humidity (55 ± 5%), air exchange (8 times/hr), and fluorescent light (9 p.m. to 9 a.m.), with free access to a commercial pelleted diet (Altromin 1324, Brogåden, Gentofte, Denmark) and acidified tap water in nipple bottles. During daily exposure the food was removed. The animals were inspected daily. Body weight and consumption of food and water was recorded once per week for all 84 animals.

Exposure. The exposure schedule was: one group was sham-exposed (control), the second group was exposed to 400 ppm (2339 mg/m^3), and the third group received 800 ppm (4679 mg/m^3) of dearomatized white spirit in the inhaled air for 6 hr/day, 7 days/week for 3 weeks.

For practical reasons, the exposure period was extended to 4 weeks and 3 days, but each animal was only exposed for 3 weeks. The inhalation exposure conditions and the equipment used have previously been described in detail [31].

Preparation of the P_2 fractions. On the day after the last exposure the animals were killed by decapitation in CO_2 anaesthesia. Brains were dissected into hemisphere and hippocampus [14, 32, 33]. A part of the liver and the whole left kidney were excised. Tissue was quickly transferred to ice cold 0.32 M sucrose and a 10% (w/v %) homogenate was made [34] and centrifuged at 0–4° at 1500 *g* for 10 min. The decanted supernatant was centrifuged at 20,000 *g* for 10 min. The resulting sediment is the P2 fraction. This crude synaptosomal fraction consists of mitochondria, synaptosomes, and myelin. The P2 pellet was resuspended in HEPES buffer to a concentration of 0.037 g-eq/mL. The composition of the HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; Na_2CO_3, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10; pH 7.4. The samples were frozen in a −80° freezer. A relatively slow freezing rate of the samples was achieved by placing them in a styrofoam box in order to maintain the integrity of synaptosomal structure [35].

Assay for ROS. P2 fractions were further diluted 1:10 with 40 mM Tris (pH 7.4) and loaded with 5 μM DCF2H-DA (0.5 mM in ethanol; stored at −70°) for 15 min at 37°, during which time esteratic activity results in the formation of the non-fluorescent

* Abbreviations: DCF, 2′,7′-dichlorofluorescein; DCF2H, 2′,7′-dichlorodihydrofluorescein; DCF2H-DA, 2′,7′-dichlorodihydrofluorescein diacetate; GSH, reduced glutathione; MCB, monochlorobimane; ROS, reactive oxygen species.

compound, DCF2H [36, 37]. Fluorescence was monitored at excitation wavelength 488 nm (bandwidth 5 nm) and emission wavelength 525 nm (bandwidth 20 nm) on an Aminco-Bowman spectrophotofluorometer. The cuvette holder was maintained thermostatically at 37°. Prior to calculating the rate of formation of DCF, corrections were made for any autofluorescence of fractions. The correction was always less than 6% of values in the presence of DCF2H. DCF formation was quantified from a standard curve over the range 0.05–1.0 μM [36].

In vitro *addition studies.* Cerebrocortical P2 fractions were prepared from nonexposed animals. In brief, tissue was homogenized in 10 vol. of 0.32 M sucrose and centrifuged at 1800 g for 10 min. The resulting supernatant fraction was then centrifuged at 32,500 *g* for 10 min to yield the crude synaptosomal pellet (P2). An initial fluorescence reading was performed after 15 min of pre-incubation. Thereafter, dearomatized white spirit was added in the concentrations 5, 10 and 20 μL/2 mL. Samples were then incubated for an additional 60 min period and the final fluorescence reading was recorded.

In vivo *exposure studies.* An initial fluorescence reading was performed after 15 min of pre-incubation. Following 60 min of further incubation the final recording was made.

Determination of GSH. The method for the determination of GSH levels was a modification of the methods described previously [38, 39]. The principle behind the assay is that MCB, itself a non-fluorescent compound, reacts with GSH to form a fluorescent adduct. It has been showed that there is very little reaction between MCB and protein sulfhydryl groups [39]. MCB was dissolved in ethanol to give a concentration of 5 mM and stored at 0–5° in the dark. MCB was added to 2 mL of a given P2 suspension to a final concentration of 20 μM, after which the suspension was incubated for 15 min at 37° and then centrifuged for 10 min at 32,500 *g*. The fluorescence of the final supernatant was read on an Aminco-Bowman spectrophotofluorometer at excitation wavelength 395 nm and emission wavelength 470 nm. The tissue GSH concentration was determined by reference to a GSH standard curve.

Assay for glutamine synthetase. The enzyme was assayed as γ-glutamyl transferase activity by incubation for 30 min at 37° of P2 fraction together with (mM): L-glutamine, 50; hydroxylamine, 75; Na-ADP, 0.5; $MnCl_2$, 0.2; imidazole-HCl, 50; and sodium arsenate, 25. γ-Glutamyl hydroxylamate formed was quantified after centrifugation (5000 *g*, 5 min) by spectrophotometric assay of the coloured product formed with acidified $FeCl_3$ by use of a standard curve concurrently generated with γ-glutamyl hydroxylamate. The iron complex of 1 mmol/mL of this compound gave an absorbance of 0.340 at 535 nm.

Statistical analysis. For food and water consumption, body weight, weights of the whole brain, brain parts and kidney, the SAS PC version software package [40] was used. The results were analysed by analysis of variance followed by Dunnett's test where indicated. For neurochemical results; differences between groups were assessed by one-way analysis of variance followed by Fisher's Least Significant Difference Test using a two-tailed distribution.

The general acceptance level for statistical significance was $P < 0.05$. No specific P values are given.

RESULTS

Clinical signs during exposure

During the first week, the white spirit-exposed rats showed signs of irritation of mucous membranes, and appeared to be sedated. Both types of effect gradually diminished during the second week.

Food and water consumption

During the last week of exposure the food consumption of the 800 ppm group was significantly decreased compared to the control group (control: 209.4 ± 6.6, 800 ppm: 190.6 ± 10.4 g/cage/week). The water consumption of the 800 ppm group was significantly increased during the same week (control 229 ± 26.2 and 800 ppm: 278 ± 39.8 g/cage/week; N = 14).

Body weight

After 3 weeks of exposure, the body weights of the dosed groups were higher than the body weights in the control group. The difference was statistically significant in the 400 ppm group. (Control: 383.9 ± 24.0 g; 400 ppm: 399.0 ± 19.5 g; 800 ppm: 394.0 ± 29.5 g; N = 28).

Weights of whole brain, hemisphere and hippocampus

No differences between groups were found (results not shown).

Kidney weight

The kidney weights of the white spirit-exposed rats were statistically significantly increased compared to the control (control: 1.07 ± 0.07 g; 400 ppm: 1.30 ± 0.10 g; 800 ppm: 1.38 ± 0.17 g; N = 10). The relative kidney weights (kidney weight/body weight in per cent) of the white spirit-exposed rats were statistically significantly increased compared to the controls. Further, there was a statistically significant difference between the relative kidney weights of the two groups of exposed animals in a dose dependent manner. The relative kidney weights were: control: 0.280 ± 0.017; 400 ppm: 0.317 ± 0.028; 800 ppm: 0.351 ± 0.036; N = 10.

Indices of oxidative stress

In vitro *addition studies. In vitro* addition of dearomatized white spirit had no effect on the rate of cerebrocortical P2 fraction ROS generation (5 μL/2 mL: 105 ± 4; 10 μL/2 mL: 101 ± 5; 20 μL/2 mL: 94 ± 6 as per cent of control).

In vivo *studies.* Hemisphere: in the P2 fraction of hemisphere the only effect was a statistically significant increase in the levels of GSH (Fig. 1A) at exposure to 400 and 800 ppm of dearomatized white spirit. This elevated GSH may indicate over compensation to pro-oxidant events as also reflected by the downward trend in ROS production, because of the evidence that glutathione levels exhibit a

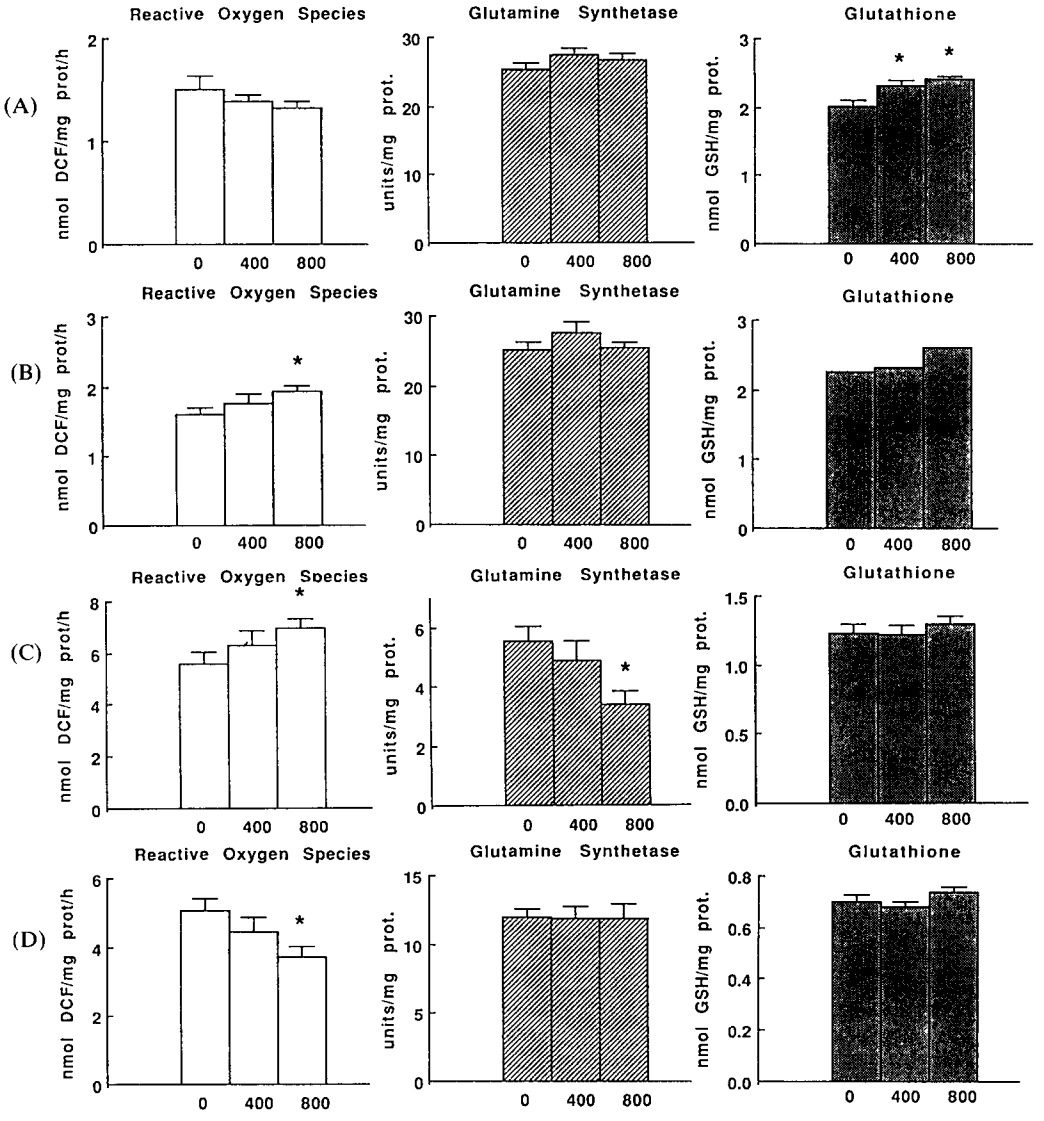

Fig. 1. Effects of 3 weeks inhalation exposure 6 hr/day, 7 days/week to 0, 400 or 800 ppm of dearomatized white spirit on the rates of generation of ROS, glutamine synthetase activities and levels of GSH in P2 fraction of hemispheres (A), hippocampus (B), liver (C) and left kidney (D). Values are the means ± SEM from 10 animals. *= value differs significantly from the control level ($P < 0.05$, two-tailed test).

biphasic response to pro-oxidant conditions, and that, following oxidative stress, GSH levels can initially be depressed and subsequently elevated.

Hippocampus: in the P2 fraction of hippocampus the effect was a statistically significant increase in the rate of ROS generation (Fig. 1B) at exposure to 800 ppm of dearomatized white spirit.

Liver: in the P2 fraction of liver there was a statistically significant increase in the rate of ROS generation and a decrease in glutamine synthetase activity (Fig. 1C) at exposure to 800 ppm of dearomatized white spirit. Glutamine synthetase was depressed in the liver, implying oxidative stress. This is confirmed by the elevated rate of ROS production.

Kidney: in the P2 fraction of the kidney the only effect was a statistically significant decrease in the rate of ROS generation (Fig. 1D) at exposure to 800 ppm of dearomatized white spirit. Depressed ROS in kidney, accompanied by no change in glutamine synthetase activity or GSH level suggests a successful over-reaction to pro-oxidant events.

Except for the hemisphere exposure to 400 ppm of dearomatized white spirit, there was no statistically significant effects on any *in vivo* parameter but values were intermediate between the control values and 800 ppm values when these latter were significantly changed.

DISCUSSION

Dearomatized white spirit is a very complex mixture typically composed of more than 200

different saturated aliphatic and alicyclic C_7–C_{12} hydrocarbon components, of which the C_9–C_{12} fraction predominates. The content of aromatics in the present batch was <0.4 wt%. The components are highly reduced, lipophilic compounds with high octanol:water partition coefficients. Hydrocarbons are metabolized by oxidative conversion to alcohols by cytochrome P450 dependent monooxygenases located in the endoplasmic reticulum. Oxidation of *n*-alkanes $\leq C_7$ results in secondary mono- or dialcohols. The potential for epoxide formation exists in straight chain hydrocarbons [26]. Higher *n*-alkanes are oxidized at the terminal carbon atoms. Branched aliphatic components are predominantly oxidized to secondary or tertiary alcohols. Monocyclic and polycyclic alkanes are mainly oxidized at the CH_2-groups in the ring structures. Subsequently, conjugation of the hydroxy groups with glucuronic acid or sulphate may occur. Alternatively, some metabolites undergo further oxidation to aldehydes/ketones or carboxylic acid. The fatty acids generated from the *n*-alkanes may enter β-oxidation.

Thus, oxidative processes are characteristic features of the catabolism/detoxification of dearomatized white spirit components. These oxidative metabolic pathways constitute a number of potential intracellular sources for ROS generation: dehydrogenases, mixed function oxidases, various soluble oxidases, mitochondrial and endoplasmatic reticulum located electron transport systems, and free metal ions (iron) [41]. Various defence systems, e.g. vitamins E and C, superoxide dismutases, catalase, the glutathione system, lipases and proteases, compete to minimize oxidative reactions and to degrade damaged lipids and proteins in the cells [41].

The resultant of this symphony determines the potential for oxidative damage of cells. Possible mechanisms and targets for ROS toxicity include lipid peroxidation, protein oxidation, DNA and elevated levels of free intracellular calcium. However, it is also likely that ROS generation in some cases is secondary to primary tissue damage [41].

The brain has an extremely high rate of oxidative metabolism, and low levels of activity of protective systems. Furthermore, the brain contains large amounts of targets for ROS, including polyunsaturated fatty acids, proteins and catecholamines. Together with the lack of ability to generate new neurons, these facts emphasize that the brain is an especially susceptible target for ROS induced cell damage.

The precise nature of the oxygen radicals generated in the brain is not known. The use of the fluorescence probe (DCF2H) provides a direct, unspecific measure of the sum of rates of generation of ROS such as hydroxyl radicals, transition metal-peroxide complexes, and lipoperoxyl radicals [42].

In the P2 fraction of the liver there was a statistically significant increase in the rate of ROS generation at 800 ppm of exposure despite the GSH concentration being maintained. The effect on ROS generation rate may reflect potential for ROS induced hepatotoxicity. This is supported by a significant decrease in the activity of glutamine synthetase, an index of oxidative protein damage. This reflects that oxidative metabolism of dearomatized white spirit to a great extent takes place in the liver.

The effects induced on water consumption and absolute and relative kidney weights suggest nephrotoxicity of the exposure to dearomatized white spirit. This is further investigated in an ongoing six months exposure study using clinical biochemical, pathological and histological approaches. The possible nephrotoxicity of dearomatized white spirit agrees with earlier findings of a slight nephrotoxicity of aromatic white spirit in male rats, and with compromised kidney function caused by another type of white spirit solvent as reported [43].

In the P2 fraction of the kidney the only effect observed was a statistically significant decrease in the rate of ROS generation (Fig. 1D) at exposure to 800 ppm of dearomatized white spirit. This may reflect a reduced need for oxidative processes in this organ as the processes mainly take place in other organs, especially the liver.

The *in vitro* addition of dearomatized white spirit had no effect on the rate of ROS generation in the cerebrocortical P2 fraction despite the maximum concentration used (20 μL/2 mL, corresponding to 9638 mg/L) being very high. This is 2060 times greater than the 800 ppm in the exposure air (4.679 mg/L). The *in vivo* brain concentration of dearomatized white spirit is not known, but in a previous study with aromatic white spirit [11] an *in vivo* whole brain:air concentration ratio of 2.23 was found at 800 ppm exposure. The main fraction was aliphatic. The brain:air concentration ratio in the present experiment was probably not much different from this.

In the P2 fraction of the hemisphere the only effect was a statistically significant increase in the level of GSH (Fig. 1A) at exposure to 400 and 800 ppm of dearomatized white spirit indicating a response to oxidative stress. Glutamine synthetase was unharmed and ROS production unchanged possibly because of the rise in GSH level.

The effect in the hippocampal P2 fraction was a statistically significant increase in the rate of ROS generation (Fig. 1B) at exposure to 800 ppm. GSH levels were not depressed suggesting successful defence in this region. In contrast to the hemisphere, no compensatory increase in GSH levels was observed in the hippocampal tissue.

The lack of effect on ROS generation of *in vitro* added dearomatized white spirit to cerebrocortical synaptosomes and to hippocampal synaptosomes (results not given) isolated from unexposed animals suggests that the effects on the hippocampus after *in vivo* exposure are caused by systemic factors.

Exposure to 400 ppm of dearomatized white spirit had no effects on any *in vivo* parameter but values were intermediate between the control values and 800 ppm values when these latter were significantly changed. This stresses exposure-related effects and further suggests a dose relationship.

The P2 fractions of the hemisphere and hippocampus are crude subcellular fractions composed of myelin fragments, pinched off nerve terminals (synaptosomes), and extrasynaptosomal mito-

chondria. The myelin fragments are inert with regard to the indices of oxidative stress and the extrasynaptosomal mitochondria are not likely to be active in the incubation buffers used.

Synaptosomes contain mitochondria, microperoxisomes, and neurotransmitter systems. They are autonomous unnucleated resealed presynaptic terminals surrounded by a well-functioning membrane and they constitute a well-documented *ex situ* model system for the nerve terminal *in vivo*. Therefore, the use of the crude synaptosomal preparation in the study of indices for oxidative stress provides an indication of the overall status in the presynaptic nerve terminal *in vivo*.

The observed increases in ROS production are relatively minor, and generally, not all of the parameters reflecting oxidative stress assayed, are responsive to treatment. However, a quantitatively minor imbalance of intracellular redox status that is continued over a long period, is likely to significantly impair metabolic processes and thus adversely affect tissue health.

The use of an occupationally relevant complex mixture of solvents in this study necessarily complicates mechanistic understanding of the results obtained. One compound may be inducer of an enzyme with the potential for ROS generation while another compound is subsequently broken down by the induced ROS-generation pathway. This possibility of interaction of some of the constituents precludes the extrapolation of data obtained from single solvents, to the evaluation of the hazard posed by a heterogenous collection of organic solvents. No data is available on such potential interaction of compounds. For several aromatic solvents, and some chlorinated aliphatics and ethanol, there is considerable evidence of oxidative effects underlying or accompanying neurotoxicity. Therefore, measurement of ROS formation by this very sensitive direct measure of overall oxidative events may be a good indicator of neurotoxic cell damage [42]. Recent results suggest that cumulative oxidative damage plays a role in Alzheimer's disease [44] and aging [15].

The present results suggest that cumulative oxidative damage may also be a/the mechanism behind white spirit-induced neurotoxicity in humans. The results also indicate that various regions of the brain may respond differently to oxidative stress. The differences observed between *in vitro* and *in vivo* results may be related to the more extensive and diverse routes of catabolism of the solvent species that are likely to occur only in the intact animal.

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