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THE UNIVERSITY OF CALGARY

The Effects of Repeated Exposure to Moderate Concentrations of Hydrogen Sulphide on Learning and Memory in the Rat

by

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Abstract

The effects of repeated exposure to moderate concentrations (125 ppm) of hydrogen sulphide (H_2S) on learning and memory in the rat were investigated. A 16-arm radial maze was used to examine neurobehavioural function in a series of 3 experiments. Experiment 1 involved training animals on a complex spatial maze task, prior to a 5-week period of exposure to H_2S or a control gas mixture. Rats were tested for maze retention after each 5-day exposure period. It was determined that repeated H_2S exposure had no effect on memory for a previously learned spatial task. Experiment 2 was conducted to determine whether H_2S interferes with the acquisition of a novel spatial task. Naive animals received daily maze training and exposure (H_2S or control) sessions over an extended 11-week period (48 sessions). The results indicated that the groups were comparable on 4 of 5 measures of maze performance. However, H_2S animals were impaired in their ability to find all of the reinforcers prior to the end of a trial, relative to normal controls, suggesting that H_2S has a modest, but significant, effect on novel acquisition. Finally, Experiment 3 was conducted to determine what role proactive interference may play in H_2S-related brain impairment. Animals from the preceding experiment were trained on a new reversed-contingency maze task. H_2S animals made more overall arm entries than controls, suggesting that H_2S may impair learning by increasing the animals' susceptibility to interference from irrelevant stimuli. The prefrontal cortex was discussed as a potential target of H_2S. Several pathophysiological mechanisms were hypothesised to account for the effects of H_2S on normal brain function.

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Dedication

To Dwayne and my "Polish" parents, whose support and words of encouragement have always been my greatest source of strength.

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Background

Hydrogen sulphide is a highly toxic colourless gas with an unfavourable reputation, particularly from a physiological standpoint. Exposure to this broad spectrum toxicant can have serious health ramifications, particularly under conditions of acute, high concentration exposure. The deleterious effects of hydrogen sulphide have been recognised in a myriad of natural and industrial settings, including the oil and gas industry, where the vast majority of exposures occur (Arnold, Dufresne, Alleyne, & Stuart, 1985; Burnett, King, Grace, & Hall, 1977).

Under natural conditions, hydrogen sulphide is produced during the decomposition of organic protein (Roth, 1993). Natural sources of hydrogen sulphide include volcanic gases, sulphur springs, crude petroleum, and natural gas. Hydrogen sulphide is also released into the environment as a by-product of certain industrial operations (Beauchamp, Bus, Popp, Boreiko, & Andjelkovich, 1984). Industrial sources of hydrogen sulphide include food processing operations, oil refineries, natural gas plants, sewage treatment facilities, pulp and paper operations, viscose rayon production, manure handling operations, and mining (Milby, 1962).

At least one case of hydrogen sulphide poisoning in a hospital setting (Peters, 1981) and two cases of poisoning in the roofing industry (Hoidal, Hall, Robinson, Kulig, & Rumack, 1986) have been documented. These reports

highlight the evasive presence of this toxic gas in even the most unsuspecting of work environments. While the possibility of a toxicological interaction between hydrogen sulphide and other industrial chemicals may exacerbate workplace health risks, few investigations have been conducted in this regard (Vanhoorne, Van Den Berge, Devreese, Tutgat, Van Poucke, & Van Peteghem, 1991).

The risk of hydrogen sulphide exposure is substantial in the province of Alberta. Here, investigations into the adverse health effects of hydrogen sulphide are particularly relevant, due to the abundance of high sulphur containing oil and gas fields in the region, and the fact that levels of hydrogen sulphide in Alberta's natural gas have been known to reach 90% (900,000 parts per million) in the past (Arnold et al., 1985). The epidemiological implications of these data are significant, since the necessary removal of hydrogen sulphide from natural gas typically results in some release of this hazardous substance into the environment.

Hydrogen sulphide is extremely toxic to humans. The sensitivity of many animal species to hydrogen sulphide has also been documented (Reiffenstein, Hulbert, & Roth, 1992). Symptoms of hydrogen sulphide exposure range from minor irritative effects to coma, and even death. Unfortunately, the exposure response curve for lethality under conditions of hydrogen sulphide exposure is extremely steep, thus reducing one's chances of escape from high concentration exposure conditions (Guidotti, 1996).

In an attempt to reduce hydrogen sulphide-related health problems in the workplace, specific exposure guidelines have been established. The current regulations for workplace exposure to hydrogen sulphide consist of a

time weighted average threshold limit value of 10 parts per million (ppm), based on an eight-hour day, five days a week (ACGIH, 1985) and a time weighted average short-term exposure limit of 15 ppm for 15 minutes. While these regulations were intended to prevent injuries to the eye, they were never properly tested to determine whether they afforded protection from respiratory or nervous system damage.

Some authors have argued that exposure to hydrogen sulphide, in low concentrations, can be tolerated for prolonged periods without adversity (Bhambhani, Burnham, Snydmiller, MacLean, & Martin, 1994). However, conflicting reports have also accrued. Cumulative biochemical effects have been observed under conditions of repeated hydrogen sulphide exposure (Savolainen, Tenhunen, Elovaara, & Tossavainen, 1980). It has also been suggested that hypersusceptibility to hydrogen sulphide may develop under conditions of prolonged or repeated exposure (Ahlborg, 1951). In view of these findings, further investigations into the effects of low level hydrogen sulphide exposure are warranted.

The remainder of this introduction will be dedicated towards a review and critique of the hydrogen sulphide literature. A brief overview of the deleterious physiological consequences of hydrogen sulphide exposure and its proposed mechanisms of toxicity will be followed by a more in-depth perusal of the human neuropsychological literature and neuroimaging data. A review of the animal literature will follow, with a particular emphasis on neurophysiological and neurobehavioural investigations of low level hydrogen sulphide exposure in the rat model. This introduction will conclude with a brief discussion about memory, learning, and the neurobehavioural measurement of

these constructs, followed by a statement outlining the objectives and hypotheses of this dissertation.

Symptoms of Hydrogen Sulphide Toxicity

The deleterious health consequences of hydrogen sulphide vary, depending mainly on concentration and duration of exposure (Guidotti, 1996). Because of its lipid solubility, hydrogen sulphide penetrates biological membranes with considerable ease, and can cause irreparable damage to various organ systems (Reiffenstein et al., 1992).

In extremely low concentrations, hydrogen sulphide is perceptible by its characteristic and offensive "rotten egg" odour (.01-.03 ppm), which becomes increasingly more intense and "sickeningly sweet" at higher concentrations (30 ppm). This malodorous quality serves as a useful warning indicator of its presence in low "subtoxic" concentrations (Beauchamp et al, 1984). However, at increasingly higher concentrations, olfactory perception becomes a less reliable method of detection, due to the onset of olfactory fatigue and olfactory nerve paralysis at concentrations of 100 and 150 ppm, respectively.

Two main effects of hydrogen sulphide exposure on humans have been documented (Beauchamp et al., 1984). The first is its irritant and/or inflammatory effect on moist mucous membranes, such as the eye and the respiratory tract. The second is its ability to induce systemic poisoning with resultant widespread respiratory and nervous system ramifications. Due to its adverse local and systemic effects on various bodily systems, hydrogen sulphide is regarded as both a direct irritant and a chemical asphyxiant (Johnstone & Saunders, as cited in Beauchamp et al., 1984).

Hydrogen sulphide's direct action on moist mucous membranes is typically first experienced in the form of eye irritation, at concentrations ranging from 20-50 ppm (Beauchamp et al., 1984). "Gas eye" refers to inflammation of the cornea and conjunctiva that often occurs in workers exposed to low concentrations of the gas for prolonged periods. Reports of blurred vision, gritty sensations in the eyes, and pain around the eyes have also been documented (Glass, 1990).

Hydrogen sulphide's direct target of moist mucous membranes can also be experienced in the form of inflammation and dryness of the respiratory tract. Because the respiratory tract is unprotected and is in direct contact with the gas, it is particularly vulnerable to hydrogen sulphide's irritative qualities (Guidotti, 1994). Irritation of the respiratory tract has typically been reported following periods of prolonged exposure to concentrations as low as 50 ppm (Beauchamp et al., 1984). However, it is noteworthy that respiratory symptoms have been documented in persons living downwind from industrial sources of hydrogen sulphide. This suggests that hydrogen sulphide, in concentrations below 50 ppm, may be sufficient to induce respiratory problems in otherwise healthy individuals (Dales, Spitzer, Suissa, Schechter, Tousignant, & Steinmetz, 1989).

Pulmonary edema is the most serious consequence of respiratory system irritation and inflammation. It often occurs following prolonged exposure to hydrogen sulphide concentrations above 250 ppm, and is the most notable lesion upon pathological examination of hydrogen sulphide-induced fatalities (Adelson & Sunshine, 1966; Gregorakos, Dimopoulos, Liberi, & Antipas, 1995; Prior, Sharma, Yong, & Lopez, 1988).

Clinical reports of cardiovascular and gastrointestinal disorders resulting from acute hydrogen sulphide exposure have also been documented (NIOSH, 1977; Reiffenstein et al., 1992).

Fortunately, elaborate physiologic mechanisms are in place to remove and metabolise hydrogen sulphide upon entry into the body. The oxidation of hydrogen sulphide to sulphate and subsequent excretion via the kidney represents the primary metabolic and excretory pathway of this toxic gas (Beauchamp et al, 1984). However, if the rate of absorption exceeds elimination or detoxification of the gas, systemic poisoning can result.

The brain is the primary target of hydrogen sulphide under conditions of systemic poisoning. Stimulation and depression of the nervous system have both been reported at concentrations exceeding 200 ppm (Evans, 1967). Systemic hydrogen sulphide poisoning is typically characterised by a preponderance of neurological sequelae concomitant with fewer irritative symptoms (Beauchamp et al., 1984). Symptoms include nervousness, headache, light-headedness, sleep disruption, fatigue, weakness of extremities, spasms, disturbed equilibrium, vertigo, convulsions, and agitation (Beauchamp et al., 1984). Exposure to extremely high concentrations of the gas (500-1000 ppm) can lead to reversible unconsciousness ("knockdown") or death due to paralysis of the brainstem medullary respiratory center (Guidotti, 1996).

Mechanisms of Hydrogen Sulphide Toxicity

Inhalation is the most common route of hydrogen sulphide exposure (Guidotti, 1996). Entry via the pulmonary route facilitates rapid systemic absorption, as hydrogen sulphide enters the circulation directly across the

alveolar capillary barrier, and is eventually distributed to the brain, liver, kidney, pancreas, and small intestine (Voigt & Muller, as cited in Beauchamp et al., 1984). The rapidity of hydrogen sulphide toxicity is presumed to be a consequence of this rapid absorption process.

Following absorption into the bloodstream, most hydrogen sulphide dissociates into sulphide ion, where it interacts with several enzymes and other macromolecules (Guidotti, 1996). The main target of hydrogen sulphide is cytochrome oxidase, a family of related enzymes located within the mitochrondria of the cell (Roth, Skrajny, Bennington, & Brookes, 1997). Sulphide ions selectively bind to cytochrome oxidase and inhibit its activity, thus interfering with cellular respiration. Consequently, molecular oxygen cannot be reduced and oxygen utilisation decreases (Smith & Gosselin, 1979).

Sulphide's inhibition of cytochrome oxidase is synonymous with oxygen deprivation or asphyxiation. Comparisons have been made between hydrogen sulphide and cyanide in this regard, as both agents are recognised for their selective reactivity with cytochrome oxidase and their potent inhibitory effects on this family of enzymes. It is noteworthy that sulphide is regarded as the most potent inhibitor of the two.

Two distinct mechanisms have been proposed to account for the damaging effects of hydrogen sulphide on the brain. A direct mechanism of action, referred to as histotoxic (tissue) hypoxia, describes sulphide-induced brain damage as a consequence of reduced oxygen availability and cellular anoxia (Auer & Benveniste, 1997; Roth, 1993). According to this notion, although oxygen delivery to the tissues is intact, oxygen utilisation is impaired at the tissue level due to sulphide's interference with cytochrome oxidase.

Histotoxic necrosis is the terminal result of this process. Presumably, many systems are affected under these hypoxic conditions, particularly those that rely on mitochondrial metabolism and are involved in neurotransmitter synthesis and metabolism (Kombian, Warenycia, Mele, & Reiffenstein, 1988).

The histotoxic hypoxia argument is widely cited in the literature as a likely cause of brain dysfunction following hydrogen sulphide exposure. The damaging effects of hydrogen sulphide on respiratory and nervous system function can be accounted for by this hypothesis, since both systems require high amounts of oxygen for normal function.

It should be noted that experimental evidence in support of this argument has been challenged in recent years. Cerebral necrosis has proven difficult to produce in ventilated animals treated with sublethal doses of sulphide, at least in the short-term (Baldelli, Green, & Auer, 1993). In view of these findings, an alternate, less direct mechanism of action has been proposed, accounting for hydrogen sulphide-induced brain damage as a consequence of cerebral ischemia due to profound hypotension (Baldelli et al., 1993; Matsuo & Cummins, 1979). A decrease in cardiac output or a reduction in peripheral resistance has been postulated to underlie the severe drop in blood pressure required in such cases. While this argument is compelling, further research is warranted to examine the long-term (e.g. > seven days) histological impact of cerebral hypoxia.

Neuropathological evidence of cerebral ischemia is typically characterised by necrosis in selective regions of the brain. The cerebral cortex, cerebellar purkinje cells, basal ganglia, and hippocampus are regarded as most vulnerable to the damaging effects of ischemia (Auer & Benveniste,

1997). It is noteworthy that abnormalities in these particular brain regions have been observed in cases of hydrogen sulphide poisoning (Matsuo & Cummings, 1979), suggesting that ischemia-related mechanisms may underlie the pathological changes characteristic of hydrogen sulphide poisoning.

However, the ischemia hypothesis of hydrogen sulphide toxicity is challenged by reports of neurobehavioural impairment in persons exposed to low concentrations of hydrogen sulphide. These patients typically experience cognitive difficulties in the absence of other physiological symptoms, such as unconsciousness or profound hypotension. Furthermore, neuroimaging investigations have revealed abnormalities in at least one patient exposed to hydrogen sulphide under conditions of prolonged, low level exposure without unconsciousness (Gaitonde, Sellar, & O'Hare, 1987). In view of these findings, an alternate more direct mechanism of hydrogen sulphide toxicity is suspected, particularly under conditions of prolonged exposure to low concentrations of the gas.

To date, many investigators still attribute hydrogen sulphide-related brain impairment to "hypoxia" without further regard. Hypoxia, or more specifically, hypoxic hypoxia, refers to low blood levels of oxygen in the presence of adequate blood flow (Auer & Benveniste, 1997; Hartman, 1995). However, as noted above, hydrogen sulphide appears to produce chronic neurobehavioural impairment, even in the absence of unconsciousness and overt signs of hypoxia (Kilburn, 1997; Tvedt, Brunstad, & Mathiesen, 1989). Central nervous system hypoxia, therefore, seems an unlikely mechanism of toxicity, particularly under conditions of low level exposure.

At present, the precise mechanisms underlying hydrogen sulphide toxicity and related brain impairment remain to be elucidated. It has recently been proposed that hydrogen sulphide may alter zinc levels in the brain, particularly in the hippocampal region, thus interfering with zinc's modulatory role in nerve cell function (Skrajny, Reiffenstein, Sainsbury, & Roth, 1996). In support of this notion, zinc deficiencies have been associated with disturbances in emotional and affective function, as well as memory and attentional impairments (Lester & Fishbein, 1988). Excessive levels of brain zinc have also been linked with various neuropathological conditions (Cuajungco & Lees, 1997[a]). These findings are intriguing and warrant further investigation before the functional consequences of hydrogen sulphide exposure are well understood.

"Acute" Human Neuropsychological Literature

According to Milby (1962), "acute" hydrogen sulphide exposure refers to episodes of systemic poisoning that are of rapid onset and associated with a preponderance of nervous system sequelae. Unconsciousness can, but does not always, occur in such cases. Neurological and neuropsychological manifestations are a common repercussion of acute exposure to hydrogen sulphide.

It is interesting that patient outcome is often quite variable following acute hydrogen sulphide exposure. In some cases, few or no neurological sequelae have been reported in acutely exposed individuals (Burnett et al., 1977). However, it is important to recognise that many investigators fail to assess or report on the neuropsychological status of their patients at initial

examination or follow-up. This suggests that impairments in neurobehavioural function may often go undetected, thus leading to false conclusions regarding the functional state of the central nervous system.

Neuropsychological assessment of brain function is an integral component of the examination process, due to its ability to reveal widespread, and often subtle, neurobehavioural deficits following acute hydrogen sulphide exposure. Interestingly, neurobehavioural impairments are often detected alongside an otherwise normal physical examination, suggesting that neuropsychological methods may be among the most sensitive for detecting hydrogen sulphide-induced brain dysfunction.

Functional impairments in the areas of motor speed, visuospatial function, and memory have been described in patients who experienced momentary (< five minutes) "knockdown" subsequent to brief hydrogen sulphide exposure (Kilburn, 1997). Intellectual deterioration, agraphia, and acalculia, in association with diffuse electroencephalographic (EEG) slowing in frontal brain regions, have also been documented following acute hydrogen sulphide poisoning and resultant unconsciousness (Hua, Ku, & Huang, 1992). Wasch, Estrin, Yip, Bowler, and Cone (1989) described three patients who demonstrated persistent attention and concentration deficits, in addition to other milder cognitive impairments, following acute hydrogen sulphide exposure. Interestingly, all three patients had abnormally prolonged P-300 event-related potential latencies that coincided well with their level of neurobehavioural impairment.

Prominent neurobehavioural deficits can reportedly persist for years following a brief episode of hydrogen sulphide exposure. Kilburn (1993)

described one survivor of acute hydrogen sulphide poisoning who exhibited profound and widespread neurobehavioural deficits at 39 and 49 months following exposure. Impairments of visual perception, memory, and intellectual function were among the list of deficits reported in this case. Snyder and colleagues (1995) described a patient who, at 18 months post-exposure, continued to show signs of slowed speech, flattened affect, reduced attention span, increased distractibility, memory and learning problems, and decreased insight (Snyder, Safir, Summerville, & Middleberg, 1995). Persistent long-term deficits were also found in six acutely exposed patients 5–10 years following the incident (Tvedt, Skyberg, Aaserud, Hobbesland, & Mathiesen, 1991). Neurological and neuropsychological examinations performed shortly after exposure revealed a broad range of deficits in these patients, however, memory and motor impairments were the most prominent sequelae at both initial examination and follow-up.

Interestingly, one case of delayed sequelae following partial recovery from hydrogen sulphide exposure has also been documented. Tvedt, Edland, Skyberg, and Forberg (1991) described a patient who began to show signs of disorientation, lack of insight, and severe memory deterioration at five days post-exposure. A five year re-examination of the patient revealed good recovery, despite persistent memory and motor deficits.

In summary, acute hydrogen sulphide exposure has been associated with prolonged and widespread neurobehavioural dysfunction. This suggests that hydrogen sulphide may cause permanent and diffuse central nervous system impairment, particularly under conditions of acute exposure with resultant unconsciousness.

"Chronic" Human Neuropsychological Literature

Investigations into the effects of "chronic" hydrogen sulphide poisoning have traditionally focused on cases of repeated or prolonged exposure to low concentrations of the gas. Included among the list of symptoms of chronic hydrogen sulphide exposure are headache, dizziness, memory loss, fatigue, loss of appetite, anxiety, and irritability. Due to the subjective nature of these symptoms, the validity of these complaints has been challenged in past years (Ahlborg, 1951). However, while some investigators dispute the notion that chronic hydrogen sulphide exposure can cause permanent nervous system damage (Ahlborg, 1951), more recent findings suggest that prolonged or repeated exposure to the toxic gas may have an enduring and detrimental impact on the central nervous system.

In a recent study, Kilburn (1997) found evidence of permanent neurobehavioural impairment in five patients who sustained chronic low level hydrogen sulphide exposure in previous years. Neurophysiological measures of balance, reaction time, and visual performance were impaired, while neuropsychological testing revealed an impairment of immediate verbal recall. In this group of patients, the duration of exposure ranged from 11 to 22 years. In three of the five cases, hydrogen sulphide exposure occurred as a consequence of residing downwind from oil wells.

Further evidence of neurobehavioural dysfunction in persons living downwind from industrial sources of hydrogen sulphide comes from a recent study by Kilburn and Warshaw (1995), who demonstrated persistent neurobehavioural dysfunction in a group of residents living in the vicinity of a "sour" crude oil processing plant. Severe affective disturbances, in addition to

deficits in perceptual motor speed, and immediate visual and verbal recall (memory) were found in the residents. Not surprisingly, the severity of impairment was reportedly lower in these subjects than in survivors of acute exposure with resultant unconsciousness (Tvedt, Skyberg et al., 1991). However, while Kilburn and Warshaw's findings are consistent with other studies in the area, caution must be exercised in the interpretation of these data, since participants in the study were involved in a lawsuit against the refinery at the time of assessment. The possibility of symptom feigning and/or exaggeration for personal gain cannot be ignored.

Haahtela and colleagues evaluated the health effects of a strong 48-hour emission of sulphur compounds, released from a local pulp mill, on the residents of a nearby community (Haahtela, Marttila, Vilkka, Jappinen, Jaakkola, & 1992). Hydrogen sulphide was the major component of the emission. A comparison of symptoms reported during the high exposure period and during a low exposure period four months later revealed a significant increase in mental symptoms (defined as depression and anxiety), breathlessness, nausea, eye symptoms, and headache during the high exposure period.

From the preceding studies, it appears that exposure to low concentrations of hydrogen sulphide does have a detrimental impact on human health. However, these results should be considered in light of two factors. First, it is not known to what extent emotional factors may have contributed to the cognitive deficits described in these studies. Depression and/or anxiety, for example, can have a deleterious impact on memory and other effort demanding cognitive processes (Lezak, 1995). In this regard,

emotional dysfunction should be considered a potential cause of the neurobehavioural impairments outlined in the above reports.

Second, malodorous substances, such as hydrogen sulphide, can arouse extreme psychological reactions in people, regardless of their level of toxicity. Negative beliefs about one's own mood, health, and mental performance, have been expressed by individuals exposed to aversive odours (Knasko, 1993). Indeed, in low concentrations, hydrogen sulphide is discernible by an offensive "rotten egg" odour. In this regard, the extent to which odour-induced psychological stressors (e.g. fear of illness) may impact neurobehavioural function under conditions of low level hydrogen sulphide exposure is unclear.

Neuroimaging Studies

Neuroimaging studies have been useful in delineating potential target brain structures of hydrogen sulphide. Bilateral abnormalities in the basal ganglia have been associated with both acute high concentration (Matsuo & Cummings, 1979) and prolonged low concentration (Gaitonde et al., 1987) exposure to hydrogen sulphide.

Unfortunately, structural imaging methods, such as magnetic resonance imaging (MRI) or computerized tomography (CT) have been known to produce negative results in the face of clear neurotoxic sequelae (Callender, Morrow, Subramanian, Duhon, & Ristovv, 1993). In this regard, functional imaging methods (e.g. single photon emission computed tomography: SPECT) have been proposed as an alternate method of investigation, due to their enhanced sensitivity for detecting signs of neurotoxic damage, particularly in patients

with provable neuropsychological impairment (Callender et al., 1993). For instance, Callender and colleagues (1993) found evidence of central nervous system abnormalities, as measured by SPECT, in a group of symptomatic patients previously exposed to hydrogen sulphide. In contrast, MRI abnormalities were detected in only one of the three patients examined via this imaging method. Affected brain areas, as revealed by SPECT, included the cerebellum (n=2), frontal lobe (n=2), temporal lobe (n=2), cerebrum (n=1), and basal ganglia (n=3).

The above findings are consistent with other data which suggest localised involvement of frontal, temporal, and basal ganglia regions in cases of hydrogen sulphide neurotoxicity (e.g. Gaitonde et al., 1987). It is unknown whether or not these regions may be more susceptible to the damaging effects of hydrogen sulphide, although some authors have suggested that entry into the brain via olfactory pathways may leave temporal and frontal brain regions particularly susceptible to hydrogen sulphide toxicity (Callender et al., 1993).

Although additional research is warranted in this area, this notion of temporal and frontal region susceptibility is interesting in light of the various clinical reports describing impairments in memory and other frontal and temporal lobe functions following hydrogen sulphide exposure (Hua et al., 1992).

Individual Susceptibility Factors

At this point, it seems necessary to comment on the widely diverse neuropsychological patterns apparent in the hydrogen sulphide literature. For

example, memory dysfunction is evident in some, but not all, victims of hydrogen sulphide exposure. Variations in hydrogen sulphide concentration and duration of exposure may underlie some of these disparities. Unfortunately, our understanding of the correlation between symptom presentation, concentration, and duration of exposure is incomplete, due to the omission of these data from most published studies.

It has also possible that individual differences in hydrogen sulphide susceptibility may underlie some of the clinical diversities inherent in the literature. Genetic, nutritional, and lifestyle factors, for example, may determine one's sensitivity to hydrogen sulphide. Interestingly, alcohol use has been linked with a decreased tolerance to hydrogen sulphide (Poda & Aiken, 1966). Individuals with psychiatric problems may also be at increased risk of hydrogen sulphide toxicity (Poda & Aiken, 1966). At present, the relationship between psychiatric illness, alcohol use, and increased susceptibility to hydrogen sulphide is not clear.

This notion of individual differences in susceptibility is exemplified in a study which examined serum levels of thiosulfate, a primary metabolite of hydrogen sulphide exposure, in victims of a mass accidental exposure (Snyder et al., 1995). As expected, individuals with higher levels of thiosulfate had a poorer neurological outcome post-exposure. However, the presence of large thiosulfate differences amongst the workers, despite similar exposure conditions, was puzzling.

In view of the above findings, it is evident that differences in hydrogen sulphide susceptibility may exist among the general population, in much the same way that varying degrees of sulphide tolerance exist among other

animal species (Grieshaber & Volkel, 1998). While the precise mechanisms underlying these disparities are unknown, variations in the rate or efficiency of hydrogen sulphide detoxification may play an integral role (Guidotti, 1994).

Animal Literature

Clinical evidence of central nervous system impairment has been substantiated by reports of sulphide-induced neurochemical and morphological alterations in the mammalian central nervous system. Studies have confirmed the sensitivity of both the developing rat brain and the adult rat brain to hydrogen sulphide toxicity.

In an attempt to investigate the impact of prolonged exposure to low concentrations of hydrogen sulphide on amino acid levels in the developing rat brain, Hannah, Hayden and Roth (1989) found a significant reduction in aspartate, glutamate, and gamma-aminobutyrate acid (GABA) levels in the cerebellum and cortex of the rat, at 21 days postnatal. Transient elevations in the amino acid taurine were also observed in the same group of animals.

Subsequent investigations revealed alterations in the growth characteristics of cerebellar Purkinje cells (Hannah & Roth, 1991), and elevated levels of norepinephrine and serotonin in the cerebellum and frontal cortex of the developing rat brain (Skrajny, Hannah, & Roth, 1992) following chronic exposure to low concentrations of hydrogen sulphide. It was later revealed that these alterations in monoamine levels, observed up to 21 days postnatal, were reversible (Roth, Skrajny, Reiffenstein, 1995). On the basis of these findings, it was suggested that exposure to low concentrations of hydrogen sulphide can cause profound, albeit in some cases reversible, neurochemical

and morphological changes in the central nervous system, particularly during critical stages of development.

Kombian and colleagues (1988) looked at the acute effects of brain amino acid levels in the adult rat following the administration of sodium hydrosulphide (NaHS). While no changes in the amino acid levels of the cerebral cortex, striatum, or hippocampus were found, subtle reductions in aspartate and glycine were observed in the cerebellum. Indeed, the most marked change was found in the brainstem, where elevations in aspartate, glutamate, glutamine, glycine, GABA, taurine, and alanine were observed.

In light of the above findings, the authors suggested that amino acid alterations in the brainstem may contribute to the lethality of hydrogen sulphide under conditions of respiratory arrest. Reductions in sodium channel function, concomitant with increased levels of taurine in the brainstem, may also be involved in the loss of respiratory drive associated with hydrogen sulphide exposure (Warenycia, Steele, Karpinski, & Reiffenstein, 1989).

The selective efficiency of various brain regions in the disposal of hydrogen sulphide has been investigated on the basis of reports of altered brainstem neurochemistry subsequent to sulphide treatment. The brainstem has been identified as containing the lowest level of endogenous sulphide in the brain (Warenycia, Goodwin, Benishin, Reiffenstein, Francom, Taylor, & Dieken, 1989). It has also been found to have the highest uptake of sulphide after NaHS treatment, suggesting a selective propensity for sulphide in this particular brain region (Goodwin, Francom, Dieken, Taylor, Warenycia, Reiffenstein, & Dowling, 1989; Warenycia et al., 1989). While the exact function

of endogenous sulphide is not known, it may serve a neuromodulatory function in the brain (Abe & Kimura, 1996).

In addition to alterations in amino acid levels, changes in catecholamine levels have been discovered in the adult rat brain subsequent to high dose administration (2.0 x LD_{50}) of NaHS (Warenycia, Smith, Blashko, Kombian, & Reiffenstein, 1989). Specifically, increases in noradrenaline and adrenaline have been found in the hippocampus, striatum, and brainstem, with the greatest increases occurring in the hippocampus. Elevations in brainstem levels of dopamine and serotonin have also been described.

In summary, experimental animal studies have identified neurochemical and morphological alterations that may underlie the functional manifestations of hydrogen sulphide toxicity. While alterations in brainstem neurochemistry may be involved in the loss of central respiratory drive associated with acute, high concentration exposure, elevated catecholamine levels in the hippocampus may underlie hydrogen sulphide-induced memory impairment, thus implicating the hippocampus as a target structure of hydrogen sulphide.

Indeed, electrophysiological data in support of this notion have been published. Segal (1972) described transient alterations in stimulation driven hippocampal unit responses in rats exposed to hydrogen sulphide via inhalation. Skrajny et al. (1996) described cumulative elevations in the power of hippocampal type 1 theta activity in rats exposed to low concentrations of hydrogen sulphide. Finally, high concentrations of hydrogen sulphide have been associated with the inhibition of synaptic transmission in the hippocampus (Abe & Kimura, 1996). The functional significance of these findings is unclear, although a link between hippocampal suppression and

hydrogen sulphide-induced neurological sequelae has been proposed (Abe & Kimura, 1996; Reiffenstein et al., 1992)

Insufficient animal work has been done to investigate the behavioural impact of hydrogen sulphide, particularly in low concentrations. To date, there has been only one published study looking at the behavioural manifestations of sublethal hydrogen sulphide exposure in the rat. Higuchi and Fukamachi (1977) observed a significant, but transient, inhibition of a previously learned discriminated avoidance response in Wistar rats exposed to hydrogen sulphide concentrations at and exceeding 200 ppm.

McLardy (1970) found no evidence of a learning impairment in rats "sulphide-loaded" via inhalation for 30 minutes. However, these results are difficult to interpret in light of the limited exposure period and constrained learning paradigm used in the study. Clearly, more research is required to investigate the impact of low concentration hydrogen sulphide exposure on neurobehavioural function.

Memory, Learning, and the Radial Arm Maze

Evidently, hydrogen sulphide can have a profound and unforgiving effect on the physiology and functionality of the central nervous system. From a neuropsychological perspective, memory is typically at the forefront of the list of neurobehavioural deficits attributed to hydrogen sulphide toxicity. However, as Reeves and Wedding (1994) have noted, memory is not a single faculty, but can be separated into multiple subsystems, each with its own distinct function (Reeves & Wedding, 1994). Damage to only one of these

subsystems may be sufficient to cause memory loss following hydrogen sulphide poisoning.

Unfortunately, general complaints of "memory loss" without further elaboration on the precise nature of the deficit, are common in human case reports of hydrogen sulphide intoxication. Furthermore, extensive memory testing is typically not performed during the routine assessments of these patients, thus leaving the nature of the deficit and its underlying neuropathology concealed. Consequently, the susceptibility of various memory processes to hydrogen sulphide remains to be elucidated.

Two subsystems of memory that are often discussed in the context of animal learning studies are working and reference memory. Working memory refers to a limited capacity system for the short-term storage of information that is useful in the near future but will change from trial to trial (Reeves & Wedding, 1994). For example, in the case of a rat that is learning the location of food rewards in an eight-arm radial maze, an intact working memory system ensures that the rat does not re-enter a previously visited baited arm during the same trial. In essence, working memory is a form of short-term memory. Because working memory is composed of newly formed representations, it is vulnerable to disruption following damage to specific brain regions (Olton, Becker, & Handelmann, 1980).

Reference (long-term) memory, on the other hand, refers to information about the particular aspects of a task that are consistent from trial to trial and do not change over time (Reeves & Wedding, 1994). It is this form of memory that allows the animal to learn which arms are consistently baited at the outset of each trial. Using the above eight-arm maze example, reference memory is

what the animal requires to remember which of the arms are consistently baited across all trials, and thus, should be entered to obtain a reinforcer. Reference memory is composed of permanent representations, and is relatively immune to disruption (Olton et al., 1980). In this regard, a behavioural dissociation can be made between working and reference memory. While the animal literature is replete with reports of impaired working memory in the face of hippocampal damage and/or cholinergic blockade (Wirsching, Beninger, Jhamandas, Boegman, & El-Defrawy, 1984), reference memory is generally unaffected under the same conditions.

In an attempt to gain better understanding into hydrogen sulphide-induced learning and memory dysfunction, animal models can be utilised to investigate the neurobehavioural impact of this toxic gas. As Overstreet and Bailey (1990) have so eloquently stated, "learning and memory are theoretical constructs that cannot be measured directly (but must be) inferred from observations of behaviour under certain specified conditions". In this regard, it is possible to assess the impact of hydrogen sulphide on memory and learning by employing neurobehavioural measurements of these constructs. Animal models are useful in neurotoxicity research, as they allow the investigator to manipulate important variables (e.g. concentration and/or duration of exposure) that would otherwise not be possible.

The hippocampus is regarded as both a critical brain structure in normal learning and memory and a potential target structure of hydrogen sulphide. The most compelling evidence in favour of this hypothesis has been the presence of memory loss in persons exposed to hydrogen sulphide. Reports of hydrogen sulphide-induced alterations in hippocampal EEG activity (Skrajny

et al., 1996) and increases in hippocampal catecholamine levels (Warenycia et al., 1989) may provide further pathophysiological evidence in support of this hypothesis.

Neurobehavioural tools, such as the radial arm maze, are often employed in these types of investigations to examine memory processes in the face of underlying neuropathology. Under normal conditions, rats solve mazes by learning the relationship between visual cues in the external environment and goal area(s) of the maze. If environmental cues are altered in some way or goal arms are changed, performance decrements are seen. The selective destruction of particular brain regions, such as the hippocampus, can also lead to impaired maze performance.

While the radial arm maze was initially used to study the effects of hippocampal lesions on spatial learning and memory (Olton & Papas, 1979), it has more recently proven valuable for assessing the effects of neurotoxic agents on cognitive processes and for helping to elucidate the susceptibility of various neural structures to a variety of toxins (Olton, 1987; Overstreet & Bailey, 1990; Walsh & Chrobak, 1987), hence its utility in the experiments to follow.

Objectives

Alongside the growing number of cognitive deficiencies reported following episodes of exposure to low concentrations of hydrogen sulphide comes the need for more research to investigate the impact of repeated sublethal hydrogen sulphide exposure on nervous system function. In this regard, the objectives of this dissertation were:

- to determine whether repeated exposure to moderate concentrations of hydrogen sulphide causes quantifiable learning and memory deficits in the adult rat
- to assess whether the neurobehavioural impairments identified in this investigation are consistent with an underlying hippocampal pathology
- to evaluate the usefulness of the radial arm maze for assessing the functional consequences of hydrogen sulphide exposure
- to consider possible mechanisms of action (e.g. zinc hypothesis) underlying hydrogen sulphide's effect on normal brain function

Experimental Rationale

With the above objectives in mind, the goal of Experiment 1 was to examine the effects of repeated exposure to moderate concentrations of hydrogen sulphide on the retention (memory) of a previously learned radial maze task. Experiment 2 was designed to investigate the impact of repeated hydrogen sulphide exposure on the acquisition (learning) of a novel radial maze task. Finally, the goal of Experiment 3 was to examine hydrogen sulphide's effect on radial maze re-acquisition following a prolonged, but discontinued, period of exposure. Findings from the latter experiment were intended to help determine whether previous exposure to hydrogen sulphide would interfere with the rat's ability to re-learn a task with new reward contingencies.

Collectively, these experiments were designed to help elucidate the effects of repeated exposure to moderate concentrations of hydrogen sulphide on specific elements of learning and memory.

Hypotheses

It is hypothesised that exposure to moderate levels of hydrogen sulphide will interfere with normal learning and/or memory in the adult rat. Unfortunately, there exists a paucity of well controlled studies from which to generate this a priori hypothesis, thus a more precise hypothesis cannot be made at this time.

EXPERIMENT ONE:

Objectives

Experiment 1 was designed to investigate the effect(s) of repeated exposure to hydrogen sulphide (125 ppm) on memory. Animals were tested for their retention of a previously learned complex radial arm maze task. A detailed analysis of two memory subsystems, namely working (short-term) and reference (long-term) memory, was included in this investigation.

Memory loss is a well known repercussion of acute exposure to high concentrations of hydrogen sulphide, particularly when loss of consciousness has occurred. Complaints of memory dysfunction following prolonged or repeated exposure to low concentrations of hydrogen sulphide have also been made. Unfortunately, insufficient research has been conducted to test the validity of these reports. While hypoxia has often been hypothesised as the underlying cause of memory dysfunction in survivors of high concentration exposure, this argument is weakened by the presence of memory loss in patients who did not lose consciousness at the time of exposure.

This first experiment was intended to test whether repeated exposure to moderate levels of hydrogen sulphide is sufficient to cause memory dysfunction in rats, despite no loss of consciousness during the exposure period. The radial arm maze is regarded as a useful tool for examining the effects of suspected neurotoxicants on memory processes, particularly when damage to the hippocampus and/or its cholinergic input has occurred (Walsh & Chrobak, 1987).

An underlying premise in the hydrogen sulphide literature has been that hippocampal dysfunction, secondary to hypoxic brain damage, is the

underlying cause of memory loss following high concentration exposure. However, whether hydrogen sulphide, in lower concentrations, exerts a direct neurotoxic effect on the hippocampus is presently unknown. Due to the sensitivity of the radial arm maze for evidencing the behavioural manifestations of hippocampal damage, results from this first experiment were intended to provide insight into this issue.

Method

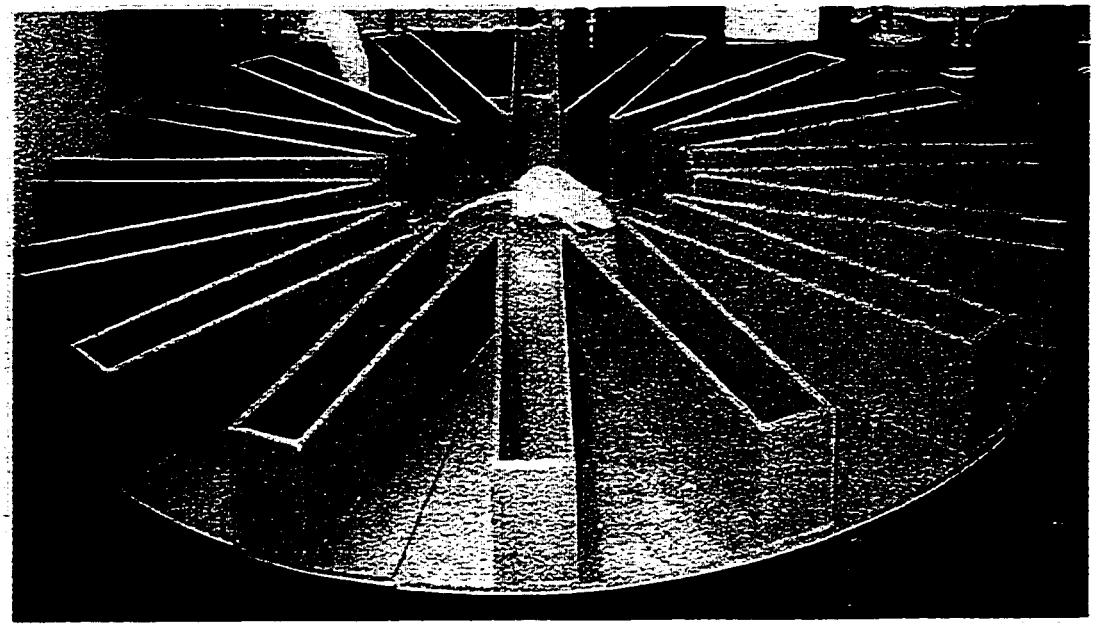
Animals

Twenty-four male Sprague Dawley rats, weighing in the range of 300-350 grams, were obtained from Charles River Laboratories. Animals were individually housed in plastic cages with wood chip bedding, and placed in a Maxi-Miser® ventilated Caging System to maintain an optimal standard of air quality at all times. Water was provided ad libitum, except during behaviour training and exposure. Animals were maintained on a 12-hour light/dark cycle. All behaviour training, exposure, and testing sessions were carried out during the light phase of this cycle.

Apparatus

A 16-arm radial arm maze, constructed from plywood, was designed for the purposes of this experiment (see Figure 1). The center platform of the maze measured 66 centimetres in diameter. Sixteen arms (70 centimetres long x 13 centimetres wide x 16 centimetres high) extended from the center platform of the maze. Removable doors were positioned between the center platform and the entrance of each arm. Circular food cups were drilled into the floor of the maze, at the distant end of each arm. The maze was painted dark grey and placed on a table in the testing room, approximately

Figure 1. Sixteen-arm radial maze constructed for use in the present investigation.



45 centimetres above the floor. The room was well illuminated with ceiling lights. A variety of extramaze markers were placed around the testing room, including tables, chairs, cereal boxes, experimental equipment, and other inanimate objects. The placement of these objects, the radial arm maze, and the experimenter remained constant throughout the duration of the experiment.

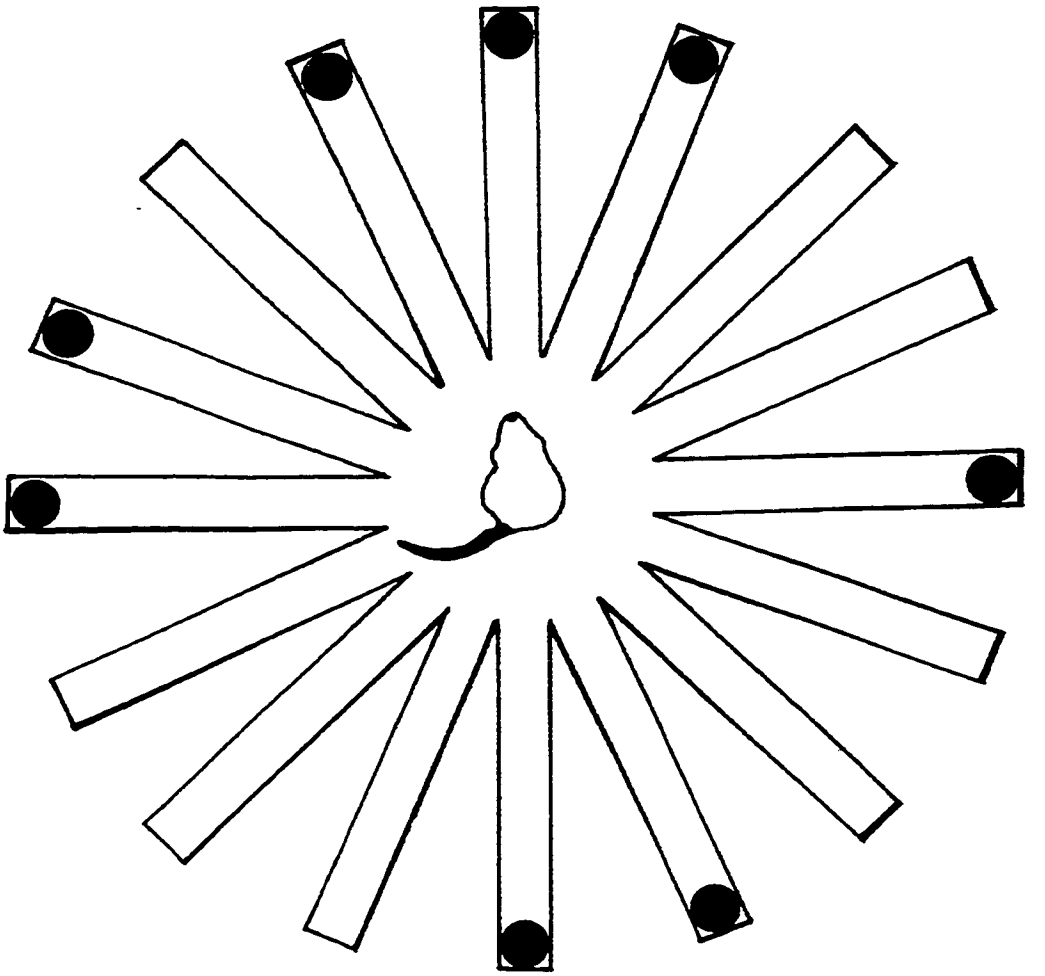
Design and Procedure

Food Deprivation. During the first eight days following arrival from Charles River laboratories, animals were provided food ad libitum. Subsequently, all animals were placed on a food deprivation schedule to reduce their body weights to 85% of their pre-deprivation weight. Animals were fed and weighed daily by the experimenter. Upon reaching their 85% target weight, animals were kept on a food deprivation schedule and maintained at this weight for the remainder of the experiment.

Acquisition Phase. Behaviour training commenced upon all animals reaching target weight. Initially, animals were shaped to run through the maze by locating reinforcers (Fruit Loops®) placed at the entrance of 8 of the 16 maze arms (Day 1). After 10 minutes, animals were removed from the maze, returned to their home cages, and fed.

Throughout the experiment, arms containing reinforcers were referred to as "baited" arms. A distinct pattern of eight baited and eight non-baited arms, such as the pattern depicted in Figure 2, was randomly assigned to each animal prior to the onset of training. This pattern remained constant for the remainder of the experiment. Baited and non-baited arms were assigned in such a way that rats adhering to a fixed pattern of responding (e.g. entering

Figure 2. Sample pattern of baited and non-baited arms assigned to each animal prior to training. Black circles correspond to the location of reinforcers in "baited" arms.



each adjacent arm) would be unable to obtain all food rewards during their first eight arm entries. Attempts were made to discourage stereotypical responding of this type, as the underlying premise of this experiment depended on the animals' attending to extramaze stimuli to learn spatial locations within the maze.

The purpose of baiting a different pattern of arms for each rat was to eliminate the remote chance of animals utilising odour trails left by their counterparts in their search for reinforcers. While it is highly unlikely that rats rely on odour cues or other intramaze markers during arm selection (Olton & Samuelson, 1976), precautions were nevertheless taken in this experiment.

Animals were trained for a total of 56 days (one training session per day) over a four-month period. Beginning on the second day of training, reinforcers were placed in food cups located at the end of each arm. Training sessions involved placing each animal in the center of the maze in a consistent orientation (facing the top arm). Animals were allowed to run freely through the maze until all eight reinforcers were consumed or 10 minutes had elapsed. Reinforcers were not replaced, so a maximum of eight reinforcers could be located and consumed during any one trial.

Performance across learning trials was measured by the: (1) number of correct (non-repeated) arm entries made within the first eight choices (CAE: maximum=8), (2) total number of correct arm entries made during the trial (TC: maximum=8), and (3) total number of arm entries required to collect all eight reinforcers (TAE). In other words, a score of eight on all of these measures was indicative of perfect performance during that trial. An entry was defined as the animal placing all four paws inside the maze arm, although once an

animal was in this position, it almost inevitably ran to the end of the arm. Training was discontinued when asymptotic levels of performance were reached on the above dependent measures.

At the end of each training session, the maze was thoroughly cleaned with an ammonia based cleaning agent and rotated to ensure none of the arms faced the same direction on two consecutive days. Every attempt was made to discourage animals from relying on intramaze markers (e.g. subtle differences between maze arms) to locate food rewards. The orientation of baited and non-baited arms in relation to extramaze markers remained constant throughout the experiment.

Immediately following the completion of 56 training sessions, four animals were removed from the study due to poor performance during the acquisition phase of the experiment. The remaining 20 animals were divided into two groups and matched across groups based on task performance. Random assignment of animals in both groups to either the experimental (n=10) or control (n=10) condition was carried out.

Exposure. Hydrogen sulphide exposure took place in a 90-litre acrylic environmental chamber, designed to house and permit observation of 12 animals. A vacuum pump was used to deliver filtered room air, mixed with certified hydrogen sulphide (2000 ppm of H2S balanced with nitrogen; supplied by Praxair Products Inc., Alberta Region) through the chamber to achieve a final hydrogen sulphide concentration of 125 ppm. The concentration of gas in the chamber was monitored using a digital hydrogen sulphide monitor (Gastech Model Safe T Net - 2000, Calgary, Alberta). The mixture was passed through a diffuser on the top of the chamber. Air flow through the chamber

was 30 litres/minute, allowing for a complete chamber replacement every three minutes.

Since the hydrogen sulphide used in this experiment was balanced with nitrogen gas, animals in the control condition were exposed to a nitrogen/air mixture to control for the effects of nitrogen gas on maze performance. The chamber characteristics and flow rate (30 litres/minute) in this condition were identical to those described in the hydrogen sulphide condition. Consequently, animals in the control group were exposed to equivalent amounts of nitrogen as the experimental group.

Experimental Design. Baseline data was collected on the sixth and seventh day following the completion of behaviour training. On both days, animals were placed in the center of the maze, as previously described, and permitted to run through the maze until all reinforcers had been consumed or 10 minutes had elapsed.

The following dependent measures were recorded by the examiner: (1) number of correct (non-repeated) arm entries made within the first eight choices (CAE), (2) total number of arm entries required to obtain all eight reinforcers (TAE), (3) number of working memory errors made within the first 12 arm entries (WME), and (4) number of reference memory errors made within the first 12 arm entries (RME: Olton & Samuelson, 1976). A working memory error was committed when the animal re-entered an arm it had already visited during the same trial. In contrast, a reference memory error occurred when the animal entered a non-baited arm during the trial. Subject means for each dependent measure were calculated from the data.

The exposure phase of the experiment began eight days following the completion of behaviour training, and was carried out over five consecutive weeks. Animals were exposed to either hydrogen sulphide (experimental group) or a nitrogen/air mixture (control group) four hours a day, five days a week (Monday to Friday). Animals were not provided access to the maze during any of the five-day exposure periods.

Post-exposure data were collected on days 6 and 7 of each week, following each exposure period. During these days, animals were placed in the center of the maze and permitted to run throughout the maze and collect all reinforcers, as described above. Dependent measures were the same as those collected during baseline. Subject means for each dependent measure were calculated from each two-day data set.

Data Analysis. A multivariate analysis of variance (MANOVA) was conducted on data collected during the acquisition (learning) phase to ensure no group differences in maze performance existed prior to exposure. Specifically, a MANOVA (Wilk's criteria) was conducted on the following two measures of performance: (1) the number of correct (non-repeated) arm entries made within the first eight choices (CAE), and (2) the total number of arm entries required to collect all eight reinforcers (TAE). Data were divided into four 14-day blocks, and means for each dependent variable were calculated. Each level of the time variable represented data collected across 14 days of the experiment.

Statistical analyses of baseline and post-exposure data were carried out by way of MANOVAs across all weeks of the experiment (baseline + five weeks exposure). Means for all four dependent outcome measures were

calculated from weeks 1 and 2 post-exposure data and weeks 3 and 4 post-exposure data. Consequently, levels 1 through 4 of the within subjects time variable were represented as follows: time 1 (baseline), time 2 (mean of weeks 1 and 2 post-exposure), time 3 (mean of weeks 3 and 4 post-exposure), time 4 (week 5 post-exposure).

Two independent data sets were generated from the four dependent outcome measures described earlier. MANOVAs were conducted on each of these data sets, across all four levels of the time variable. The first data set consisted of the: (1) number of correct (non-repeated) arm entries made within the first eight choices (CAE) and (2) total number of arm entries required to obtain all eight reinforcers (TAE). The second data set consisted of the: (1) number of working memory errors (WME) and (2) reference memory errors (RME) made within the first 12 entries.

Each data set was analysed independently for group, time, and group x time interaction effects. Statistically significant Bonferroni corrected MANOVAs ($p=.05/2=.025$) were followed with repeated measures analyses of variance (ANOVA) using time as the within subjects (repeated) factor. The experimentwise error rate was maintained at .05.

Results

Acquisition Phase

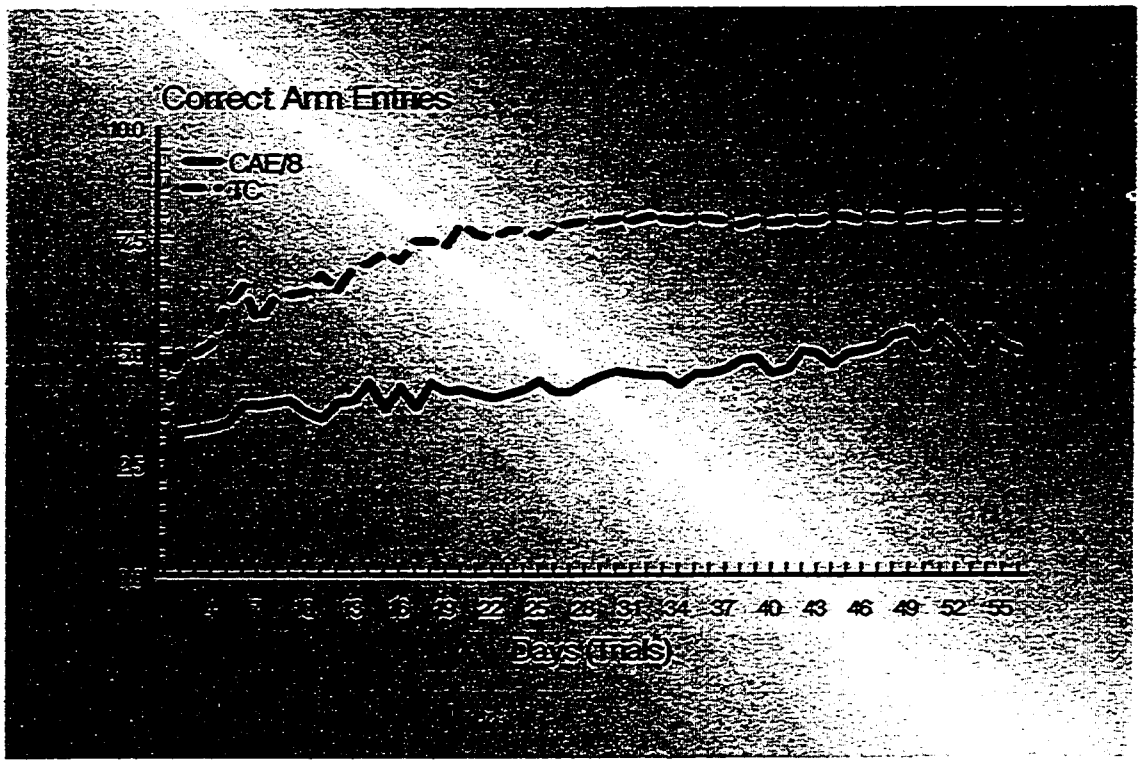
On the initial day of training, animals spent the majority of their time in the center platform of the maze, and would enter a few of the maze arms only after considerable hesitation. Within a few days of training, nearly all of the animals had started to locate and consume the reinforcers located in the maze arms.

Performance across learning trials was variable, as presented in Figure 3. An average of 2.3 (range = 0 - 5) and 5 (range = 3 - 7) correct (non-repeated) arm entries within the first 8 choices were made by the animals on the first and last day of training, respectively. The average number of correct arm entries in total ranged from 3.5 (range = 0 - 8) on the first day of training, to 8 (range = 8 - 8) on the final day. These data indicate that by the end of training, all animals were entering the entire set of baited arms, despite having made some errors during their first 8 entries. However, while some animals reached near-perfect performance by the end of training (e.g. making 7 correct entries within their first 8 entries), other animals failed to reach this level of performance. These variable results were attributed to the complexity of the task, at least for this particular strain of rats.

A MANOVA of the data collected during behaviour training failed to reveal a significant group difference in maze performance prior to exposure [$F(2,17)=1.10$, $p=.36$] or a significant group by time interaction [$F(6,13)=2.31$, $p=.10$]. These data demonstrate that animals in both groups exhibited a similar rate and level of learning during acquisition, and thus were well matched across groups prior to exposure.

In contrast, a significant time effect was revealed upon analysis [$F(6,13)=24.68$, $p=<.01$], indicating that maze performance for both groups of animals improved across time (see Figure 3). Follow-up analyses demonstrated a significant increase in the number of correct (non-repeated) arm entries made within the first eight choices [$F(1,18)=77.61$, $p=<.01$] over time, but not in the total number of arm entries required to collect all eight reinforcers [$F(1,18)=.62$, $p=.44$].

Figure 3. Performance of animals during maze acquisition. A modest increase in the number of correct arm entries made within the animals' first eight choices (purple line) was demonstrated across trials. In contrast, the total number of correct arm entries made during a trial (black line) reached a ceiling earlier in the course of training.


Correct Arm Entries
CAE/8
3C
100
75
50
25
0
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
Days (Trials)

Baseline and Post-Exposure Phase

No signs of eye irritation, respiratory distress, or altered consciousness were evident in either group of animals during any of the four-hour exposure periods, nor did any of the animals die as a consequence of exposure. This is in agreement with the chronic nature of the present experiment, as animals were exposed to hydrogen sulphide concentrations far below the median lethal concentration (LC_{50}), which in this particular strain of rats is 444 ppm, based on a four-hour exposure (Tansy, Kendall, Fantasia, Landin, & Oberly, 1981). While animals in the experimental group were more subdued (e.g. motionless) than control animals during the exposure period, the groups resumed comparable levels of activity shortly following removal from the exposure chamber. Behavioural observations were otherwise unremarkable.

The effect of exposure on all four dependent variables is presented in Table 1. A MANOVA of the first data set failed to reveal a significant group difference, [$F(2,17)=.20$, $p=.82$], group x time interaction [$F(6,13)=.19$, $p=.98$], or time effect [$F(6,13)=1.91$, $p=.15$] in maze performance, as measured by the number of correct (non-repeated) arm entries within the first eight choices (CAE) and the total number of arm entries (TAE). Similarly, an analysis of the second data set also failed to reveal a significant group difference [$F(2,17)=.46$, $p=.64$], group x time interaction [$F(6,13)=1.33$, $p=.31$], or time effect [$F(6,13)=2.70$, $p=.06$] in maze performance, as measured by the number of working memory errors (WME) and reference memory errors (RME).

These data demonstrate that during baseline and following hydrogen sulphide exposure, animals were comparable on all measures of maze performance (see Table 1). Hydrogen sulphide failed to impair maze

Table 1

Effects of Repeated Exposure to Hydrogen Sulphide on Four Measures of Radial Arm Maze Retention

	Time			
CAE/8[a]				
H_2S	5.45 (0.90)	5.23 (0.87)	5.23 (1.30)	5.05 (1.04)
Control	5.45 (0.64)	5.10 (0.89)	4.83 (0.83)	4.75 (0.83)
TAE[b]				
H_2S	17.00 (4.28)	17.05 (5.65)	17.80 (7.69)	18.70 (5.52)
Control	17.05 (4.03)	17.75 (5.36)	19.08 (6.38)	19.25 (4.43)
WME/12[c]				
H_2S	1.05 (0.73)	0.98 (0.79)	1.00 (0.78)	1.25 (0.83)
Control	1.30 (0.89)	0.95 (0.55)	1.35 (0.78)	0.85 (0.63)
RME/12[d]				
H_2S	4.10 (0.94)	3.75 (1.02)	3.88 (1.56)	4.40 (0.91)
Control	3.80 (1.09)	4.53 (1.27)	4.48 (1.18)	4.80 (0.75)

Note. The values represent means and standard deviations for the experimental (H_2S) and control groups. [a]Number of correct arm choices within first 8 arm entries. [b]Total number of arm entries required to collect all 8 reinforcers. [c]Number of working memory errors within first 12 arm entries. [d]Number of reference memory errors within first 12 arm entries.

performance, even after five weeks of repeated exposure. The lack of a significant time effect indicates that maze performance did not improve beyond baseline. These data suggest that performance had reached asymptotic levels at the time of baseline.

Discussion

Results from the present experiment suggest that repeated exposure to moderate concentrations of hydrogen sulphide has little effect on the performance of a previously learned complex spatial memory task. Animals exposed to hydrogen sulphide did not commit a greater number of errors during their first eight arm entries, nor did they require a greater number of arm entries to complete the task. Furthermore, experimental animals did not commit a greater number of working or reference memory errors than their control counterparts. In other words, hydrogen sulphide did not impair the animals' ability to perform either the short-term working memory or long-term reference memory components of the task.

Several arguments could be made on the basis of these findings. First, animals in the present study were not exposed to high concentrations of hydrogen sulphide, nor did they show overt signs of respiratory distress, cardiac dysfunction, or reduced consciousness at any time during the exposure period. One could argue that, in the absence of reduced blood oxygen levels (hypoxic hypoxia) or reduced blood flow to the brain (cerebral ischemia), hydrogen sulphide does not impair brain function. However, this hypothesis is challenged by reports of chronic neurobehavioural impairment in humans following hydrogen sulphide exposure without concomitant unconsciousness (Kilburn, 1997).

An alternate argument concerns the hippocampus and its presupposed susceptibility to hydrogen sulphide. Despite the sensitivity of the radial arm maze for detecting behavioural manifestations of hippocampal damage, performance decrements were not seen in the present experiment, suggesting that the hippocampus may not be a primary target structure of hydrogen sulphide. In other words, hydrogen sulphide-induced memory loss may not be related to alterations in hippocampal function. Indeed, other potential target structures of hydrogen sulphide have been revealed via neuroimaging methods (Callender et al., 1993). The functional implications of these findings remain to be elucidated, however, it is tempting to speculate that hydrogen sulphide's effect on these non-hippocampal structures may underlie the cognitive impairments associated with low level hydrogen sulphide exposure.

Unfortunately, many of the animals in the present study had difficulty learning the radial arm maze task during behaviour training. While strain dependent performance differences on tests of learning and memory have been discussed elsewhere (Andrews, Jansen, Linders, Princen, & Broekkamp, 1995), many of the animals in this experiment still performed below expected levels, based on previous reports (Levin, Kaplan, & Boardman, 1977). These findings suggest that Sprague Dawley rats have difficulty learning complex spatial tasks. Consequently, hydrogen sulphide-induced performance decrements may have been difficult to detect against a background of modest learning, as exhibited by many of the animals.

Arguments have surfaced as to the overall utility of the rat model in hydrogen sulphide research. It has been suggested that rats, and other organisms that routinely encounter hydrogen sulphide in their habitats, may

have advanced biochemical mechanisms that help protect the organism from the toxic repercussions of hydrogen sulphide (Vetter & Bararinao, 1989). In light of this viewpoint, it is interesting that animals in the present study did not show signs of eye irritation, despite such claims being made by humans exposed to significantly lower concentrations of the gas. Rats may be more resilient to the effects of hydrogen sulphide, and if so, the modest concentrations of hydrogen sulphide used in the present study may have been too low to induce behavioural deficits in this species of animal.

In summary, Experiment 1 demonstrated that repeated exposure to moderate concentrations of hydrogen sulphide does not impair the rat's memory for a previously learned radial arm maze task. Whether or not newly acquired information is more susceptible to the damaging effects of hydrogen sulphide is not known, and will be the focus of Experiment 2.

EXPERIMENT TWO:

Objectives

Experiment 2 was designed to investigate the effect(s) of repeated hydrogen sulphide exposure (125 ppm) on novel learning. Experimentally naive animals received daily behaviour training and exposure sessions, thus allowing for an evaluation of novel learning under conditions of repeated exposure to hydrogen sulphide.

In light of the results from the preceding experiment, exposure to moderate concentrations of hydrogen sulphide evidently had no effect on the animal's ability to remember a previously learned spatial task. In other words, hydrogen sulphide did not impair memory for material that was learned prior to the exposure period. However, the effect(s) of hydrogen sulphide on the acquisition of new material remains to be elucidated, and thus was the focus of this experiment.

Method

Animals

Thirty male Sprague Dawley rats, weighing between 300 and 350 grams, were obtained from Charles River Breeding Laboratories. Animals were individually housed in clear plastic cages with wood chip bedding and maintained in a Maxi-Miser® ventilated Caging System, as described in Experiment 1. Water was provided ad libitum, except during behaviour training and exposure. Animals were maintained on a 12-hour light/dark cycle. All behaviour and exposure sessions were conducted during the light phase of this cycle.

Apparatus

The apparatus was the same as described in Experiment 1. The location of the maze within the testing room, and the characteristics of the room itself were also consistent with Experiment 1.

Design and Procedure

Food Deprivation. During the initial 63 days following arrival from Charles River laboratories, animals were fed ad libitum to ensure optimal growth. Subsequently, all animals were placed on a food deprivation schedule to reduce their body weights to 85% of their pre-deprivation weight. Animals were fed and weighed daily by the experimenter. Upon reaching their target weight, animals were kept on a food deprivation schedule and maintained at this weight for the remainder of the experiment.

Preliminary Testing. During Experiment 1, it became apparent that a positive relationship existed between future maze performance and the initial level of exploratory behaviour exhibited by a animal. In most cases, animals that were most active at the outset of behaviour training tended to learn the task better than their less active counterparts. The goal of preliminary testing, therefore, was to identify and remove six inactive animals from the experiment outset, presuming these animals would not learn the task as quickly or efficiently as more active animals.

Preliminary testing began 10 days following the start of food deprivation, after all animals had reached their pre-specified target body weight. A distinct pattern of 10 baited (Fruit Loops® cereal) and 6 non-baited arms was randomly assigned to each animal prior to the onset of preliminary

testing. This pattern, assigned in a similar manner as described in Experiment 1, remained constant for the remainder of the experiment.

The decision to bait ten arms was based on results from Experiment 1, demonstrating that the animals had difficulty learning a 16-arm radial maze task with equal numbers of baited and non-baited arms. An alternative approach was to bait 12 of the 16 arms, however, it has been demonstrated that under such conditions, animals can learn the task in as few as 12 trials (Levin, 1977). Since the goal of the present experiment was to examine the effects of hydrogen sulphide over a prolonged acquisition period, a more difficult paradigm, requiring a longer period of learning, was warranted. The importance of task complexity in the detection of learning and memory impairments has been discussed elsewhere (Olton & Markowska, 1994).

Preliminary testing took place over two consecutive days. On both days, each animal was placed in the center of the maze and allowed to run through the maze until five minutes had elapsed. Animals were evaluated for their activity level by examining the number of arm entries made during each trial. Six animals that made few or no arm entries during the preliminary trials were removed from the experiment at this time.

Exposure. Hydrogen sulphide (experimental) and nitrogen/air (control) exposure was conducted in the same manner as described in Experiment 1.

Experimental Design. Behaviour training and exposure began three days following the completion of preliminary testing. Two groups, comprised of 12 animals each, were formed by way of a quasi-randomized assignment method. Each animal was evaluated on the basis of data obtained during preliminary testing, and paired with an animal demonstrating a comparable

level of activity and exploratory behaviour. Animals from each pair were randomly assigned to either the control or experimental condition.

Beginning on the first day of the experiment, animals were exposed to hydrogen sulphide (125 ppm) or a nitrogen/air mixture for a four-hour period beginning at 8:00 in the morning. Upon completion of the four-hour exposure period, animals were removed from the exposure apparatus and returned to their home cages. The exposure equipment was subsequently cleaned, and animals were provided with a minimum half hour "recovery" period prior to the commencement of behaviour training.

Behaviour training involved placing each animal in the center of the maze in a consistent orientation (facing the top arm). Animals were allowed to run freely through the maze until all 10 reinforcers were consumed or 10 minutes had elapsed. Upon completion of the trial, animals were removed from the maze, returned to their home cages, and fed.

Maze performance was evaluated on the basis of the following five dependent outcome variables: (1) number of correct (non-repeated) arm entries made within the first 10 choices (CAE: maximum=10), (2) total number of correct arm entries made during the trial (TC: maximum=10), (3) number of working memory errors made within the first 12 arm entries (WME), (4) number of reference memory errors made within the first 12 entries (RME), and (5) total number of arm entries required to collect all 10 reinforcers (TAE). As described in Experiment 1, a working memory error was defined as a re-entry into a previously baited arm. In contrast, a reference memory error was defined as an entry into an arm from the non-baited set.

At the end of each day, the radial arm maze was thoroughly cleaned and rotated to ensure none of the arms faced the same direction on two consecutive days. Consistent with Experiment 1, the orientation of baited and non-baited arms in relation to extramaze cues remained constant. Daily exposure and behaviour training sessions (one training session per day) were conducted for 48 days over a period of 11 weeks. Animals were trained and exposed between four and five days a week.

Data Analysis. Subject means for the two-day preliminary data (total number of arm entries) were obtained for each animal. An ANOVA was conducted on these data to ensure no group differences in maze exploration or activity level existed prior to the onset of behaviour training and exposure.

Statistical analyses of Experiment 2 data were carried out by way of MANOVAs across all 48 days of the experiment. Data were divided into four consecutive 12 day blocks; a mean for each block was calculated for each of the dependent outcome measures described above. In effect, five dependent measures were examined at four levels of the within subjects (repeated) time variable. Each level of the time variable represented data collected across 12 days of the experiment.

Three independent data sets were generated for the five dependent outcome measures described earlier. A MANOVA was conducted on each of these data sets, across all four levels of time. The first data set was comprised of the: (1) number of correct (non-repeated) arm entries made within the first 10 choices (CAE) and (2) total number of correct arm entries (TC). The second data set consisted of the: (1) number of working memory errors made within the first 12 entries (WME), and (2) number of reference memory errors made

within the first 12 entries (RME). The third data set was comprised solely of the total number of arm entries required to collect all 10 reinforcers (TAE).

Each data set was analyzed independently for group, time, and group x time interaction effects. Statistically significant Bonferroni-corrected MANOVAs ($p=.05/3=.017$) were followed with repeated measures ANOVAs with time as the within subjects factor. The experimentwise error rate was maintained at .05.

Results

Preliminary Testing

An ANOVA of the data obtained during preliminary testing failed to reveal a significant group difference in activity level prior to the onset of the experiment [$F(1,22)=1.15$, $p=.29$]. These data demonstrate that the animals were well matched across groups on the basis of an early performance marker (total number of arm entries) prior to the start of behaviour training and exposure. The rationale behind the use of this measure to ensure well balanced groups was described earlier.

Behaviour Training and Exposure

None of the animals showed overt signs of eye irritation, breathing difficulties, or altered consciousness at any time during the experiment, nor did hydrogen sulphide exposure prove lethal for any of the animals. As noted in the previous experiment, a 125 ppm concentration of hydrogen sulphide is far below the median LC_{50} of 444 ppm for Sprague Dawley rats, thus death due to exposure was highly unexpected in this experiment.

The only notable behavioural difference between the two groups was the level of activity exhibited by the animals during exposure. While

experimental animals were typically subdued (e.g. motionless) during this period, control animals were more active, often engaging in grooming and aggressive behaviours towards each other. Nonetheless, comparable levels of activity were resumed soon after the animals were removed from the exposure apparatus and returned to their home cages.

The effect of exposure on all five dependent outcome measures is demonstrated in Table 2. Analyses of the first data set, which included the: (1) number of correct (non-repeated) arm entries made within the first 10 choices (CAE) and (2) total number of correct arm entries (TC) revealed a statistically significant group effect [$F(2,21)=5.29$, $p=.01$], and a significant time effect [$F(6,17)=8.50$, $p=<.01$], but no significant group x time interaction [$F(6,17)=.98$, $p=.47$].

Subsequent analyses of the group effect revealed a statistically significant difference in the TC variable [$F(1,22)=9.78$, $p=.01$], demonstrating that experimental animals were less likely than control animals to locate all reinforcers and complete the task prior to the end of the ten minute trial (see Figure 4). A closer examination of the CAE variable revealed that although control animals, when compared to the experimental group, did made a greater number of correct (non-repeated) arm entries within their first 10 choices at times 1, 2, and 4, this difference did not reach statistical significance [$F(1,22)=2.65$, $p=.12$].

Follow-up analyses of the significant time effect demonstrated that performance on both the CAE [$F(1,22)=33.52$, $p=<.01$] and TC [$F(1,22)=43.52$, $p=<.01$] dependent variables improved over time, indicating that animals were learning the task, as expected.

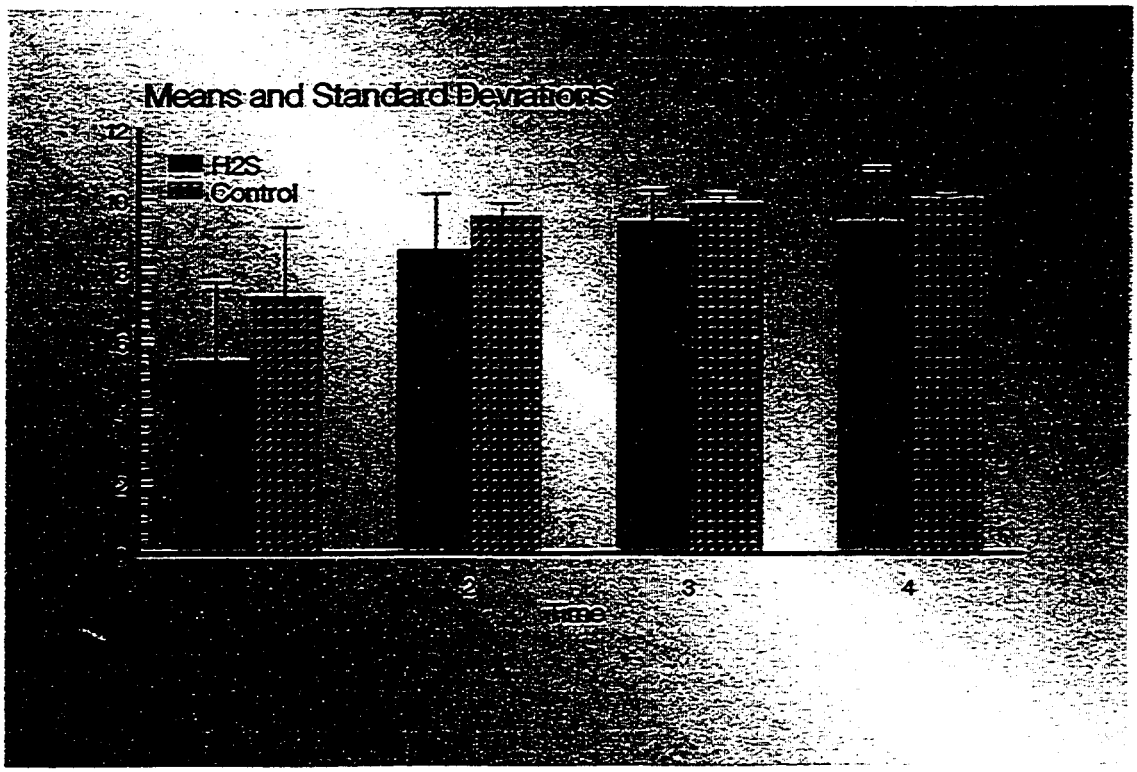
Table 2

Effects of Repeated Exposure to Hydrogen Sulphide on Five Measures of Radial Arm Maze Acquisition

	Time			
CAE/10[a]				
H_2S	3.98 (1.19)	5.63 (0.68)	6.02 (0.51)	5.93 (0.54)
Control	4.85 (1.23)	5.78 (0.64)	5.99 (0.44)	6.19 (0.84)
TC/10[b]				
H_2S	5.43 (2.11)	8.56 (1.52)	9.35 (0.91)	9.33 (1.49)
Control	7.25 (2.02)	9.53 (0.34)	9.86 (0.23)	9.99 (0.03)
WME/12[c]				
H_2S	0.99 (0.47)	1.53 (0.46)	1.49 (0.55)	1.28 (0.40)
Control	1.18 (0.58)	1.62 (0.40)	1.23 (0.43)	1.01 (0.49)
RME/12[d]				
H_2S	3.01 (1.20)	4.04 (0.70)	3.80 (0.56)	3.74 (0.54)
Control	3.55 (0.98)	4.01 (0.58)	4.03 (0.52)	3.56 (0.74)
TAE[e]				
H_2S	14.25 (7.78)	24.34 (6.99)	26.48 (4.71)	28.11 (2.99)
Control	20.00 (7.27)	27.43 (3.46)	25.88 (3.83)	23.78 (4.89)

Note. The values represent means and standard deviations for the experimental (H_2S) and control groups. [a]Number of correct arm choices within first 10 arm entries. [b]Total number of correct arm entries. [c]Number of working memory errors within first 12 arm entries. [d]Number of reference memory errors within first 12 arm entries. [e]Total number of arm entries required to find all 10 reinforcers.

Figure 4. Total number of correct arm entries (TC). A statistically significant difference between the groups on this measure indicates that the experimental group made fewer correct arm entries than the control group during this experiment ($p=.01$).

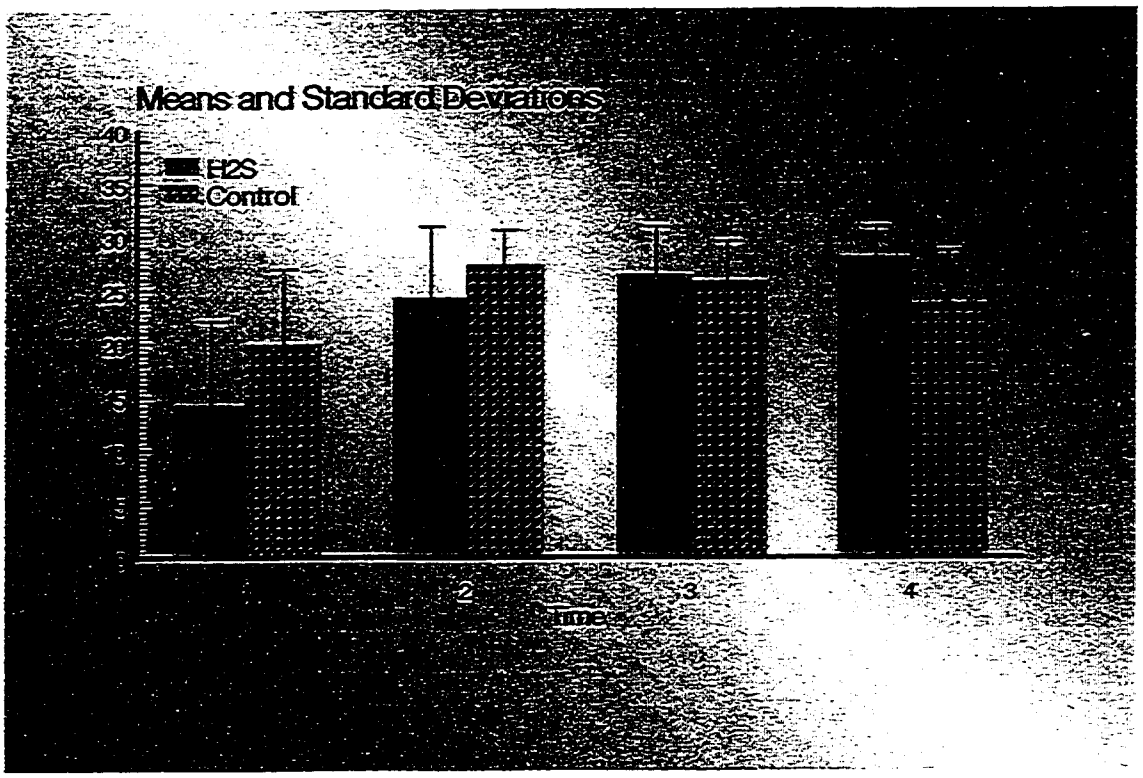

Means and Standard Deviations
H2S
Control
2
3
4
Time

Analyses of the second data set, which included the: (1) number of working memory errors made within the first 12 entries (WME), and (2) number of reference memory errors made within the first 12 entries (RME) failed to reveal a significant group effect [$F(2,21)=.35$, $p=.71$] or significant group x time interaction [$F(6,17)=1.06$, $p=.42$]. These data suggest that animals in both groups were comparable with respect to the number of working and memory errors made during the course of the experiment.

While a significant time effect was revealed upon analysis of this data set, [$F(6,17)=7.77$, $p=<.01$], subsequent analyses failed to confirm this effect as being due to a selective decrease in the number of working memory errors [$F(1,22)=.15$, $p=.70$] or reference memory errors [$F(1,22)=2.38$, $p=.14$] made by the groups over time (see Table 2). In effect, the WME and RME variables, unlike the TC variable, proved to be a poor measure of maze acquisition across time.

A MANOVA of the third data set (total number of arm entries required to collect all 10 reinforcers: TAE) failed to reveal a significant group effect [$F(1,22)=.38$, $p=.54$]. The group x time interaction approached, but did not reach significance when Bonferroni corrections were applied [$F(3,20)=3.61$, $p=.03$]. However, follow-up analyses were still conducted, revealing a statistically significance difference in this variable between time 1 and time 4. Upon closer examination, it was revealed that while control animals made a greater number of arm entries than their experimental counterparts at time 1, the reverse was true at time 4 (see Figure 5). In other words, experimental animals made more arm entries at time 4, despite making less correct arm choices (TC) than the control group.

Figure 5. Total number of arm entries required to complete task (TAE). This figure demonstrates that although the experimental group made fewer arm entries at times 1 and 2 than controls, an opposite pattern was seen at times 3 and 4. Thus, experimental animals made a greater number of arm entries later in the study, and yet were less likely to locate all reinforcers than controls, as demonstrated in the preceding figure.


Means and Standard Deviations
H2S
Control
40
35
30
25
20
2
3
4
Time

A likely explanation for the performance differences at time 1 is that the control animals made more arm entries in total, and thus, had a greater likelihood of locating more reinforcers than the experimental animals by chance alone. It is noteworthy that although disparities in the TC variable were slight at time 4, experimental animals made more arm entries than their control counterparts, and yet were still slightly less effective at locating all 10 reinforcers prior to completion of the trial (see Table 2).

Finally, analyses of the third data set revealed a significant time effect [$F(3,20)=15.28$, $p=<.01$], suggesting that the total number of arm entries (TAE) changed over time. However, this effect was mainly due to an increase in the number of entries made by both groups beyond time 1.

To test the hypothesis that group differences in the number of working and reference memory errors may have surfaced later in the trial, beyond the animals' 12th arm entry, a post-hoc statistical analysis (MANOVA) of the working memory and reference memory errors made during arm entries 13 through 20 was conducted. Data were divided into four consecutive 12-day blocks and analyzed across all four measures of the time factor, as depicted in Table 3. The analysis revealed a significant group x time interaction [$F(6,17)=4.03$, $p=.01$] and time effect [$F(6,17)=4.59$, $p=.01$], but no significant group effect [$F(2,21)=.46$, $p=.64$]. Further analyses of the data demonstrated that the significant group x time interaction was due to differences in both the number of working memory errors [$F(1,22)=7.51$, $p=.01$] and reference memory errors [$F(1,22)=9.94$, $p=.01$] made by the groups across time, as demonstrated in Figures 6 and 7, respectively.

Table 3

Working and Reference Memory Errors Made During Late Arm Entries, as Calculated for Post-hoc Analysis

	Time			
Post-WME[a]				
H_2S	1.48 (1.14)	2.73 (1.16)	3.23 (0.55)	3.09 (0.61)
Control	2.22 (1.01)	3.13 (0.48)	2.73 (0.39)	2.54 (0.76)
Post-RME[b]				
H_2S	1.30 (0.81)	2.14 (0.83)	2.13 (0.46)	2.66 (0.45)
Control	1.88 (0.94)	2.48 (0.48)	2.25 (0.52)	2.08 (0.43)

Note. The values represent means and standard deviations for the experimental (H_2S) and control groups. [a]Number of working memory errors made during arm entries 13 through 20. [b]Number of reference memory errors made during arm entries 13 through 20.

Figure 6. Working memory errors calculated for post-hoc analysis. A significant group x time interaction reflected group differences on this measure at different levels of the time variable ($p=.01$). In particular, a decrease in the number of errors made by the control group was apparent after time 2, while the number of errors made by the experimental group continued to rise until time 3, after which a modest decline was seen.

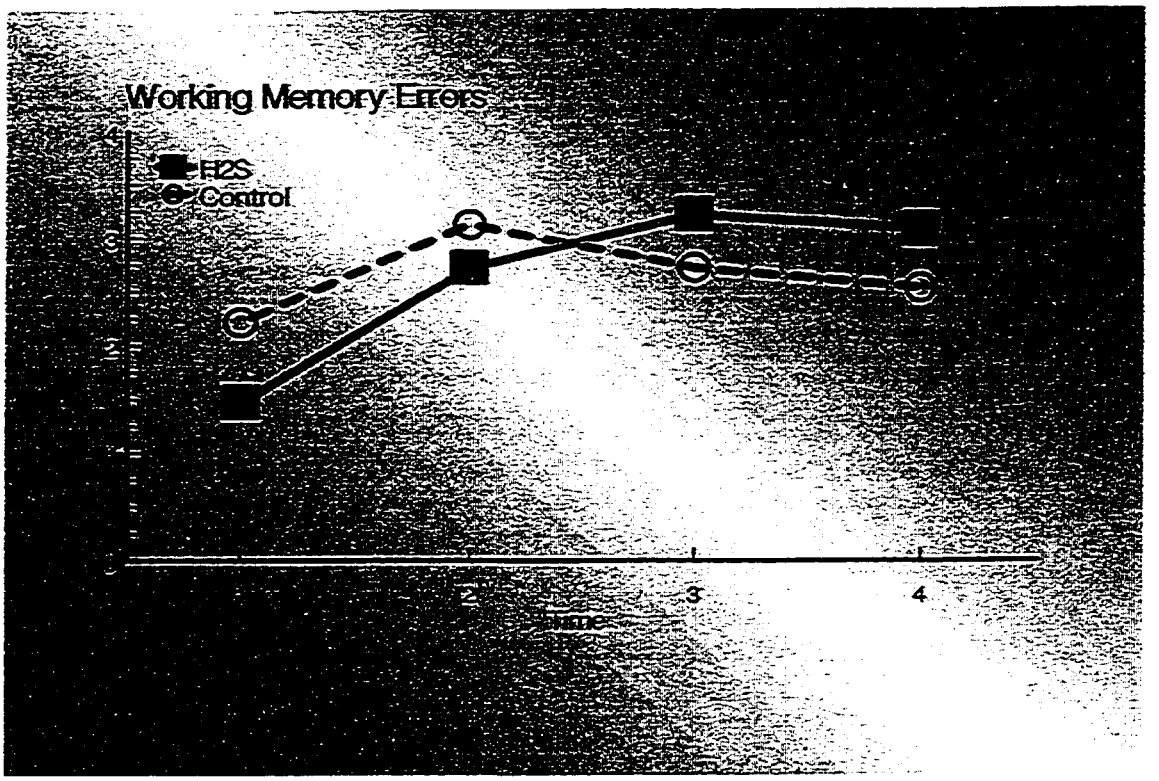
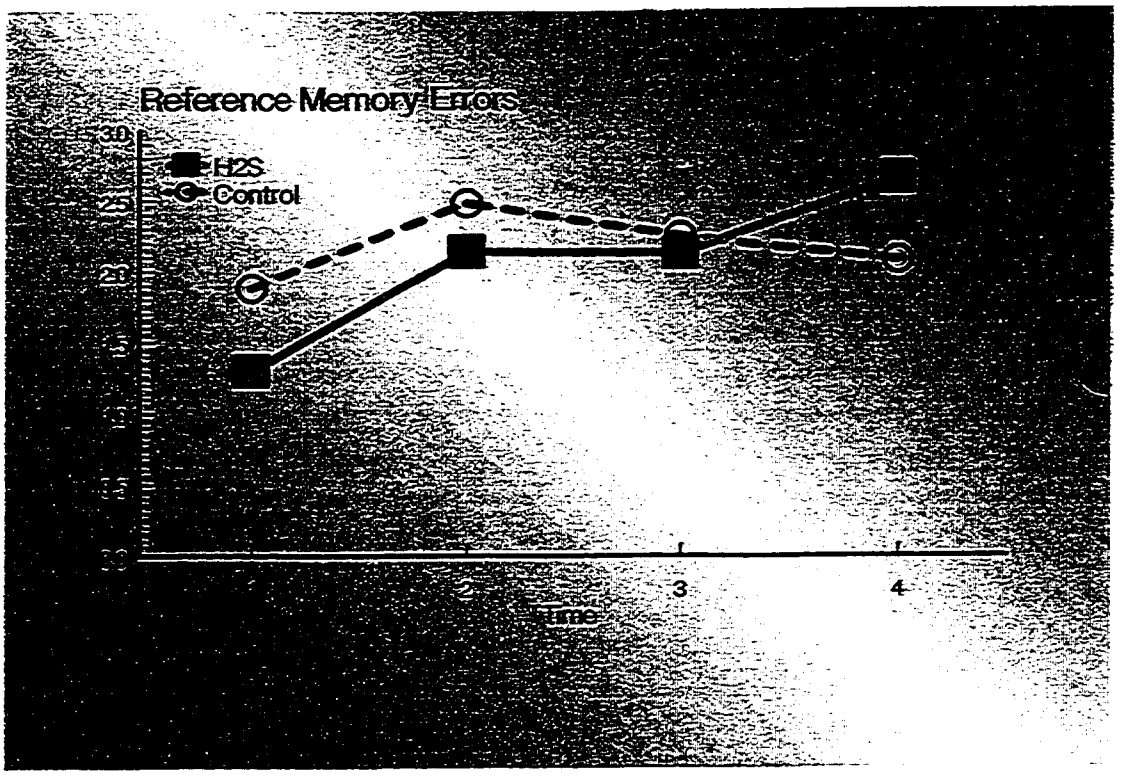

Working Memory Errors
H2S
Control
2
3
4
Time

Figure 7. Reference memory errors calculated for post-hoc analysis. A significant group x time interaction reflected group differences on this measure over time ($p=.01$). Specifically, a decrease in the number of errors made by the control group was seen after time 2, while the number of errors made by the experimental group increased across all levels of time.


Reference Memory Errors
H2S
Control
30
25
20
15
10
5
0
2
3
4
Time

Discussion

These data indicate that repeated exposure to moderate concentrations of hydrogen sulphide leads to subtle impairments in the acquisition of a novel 16-arm radial maze task. Specifically, animals exposed to hydrogen sulphide were less efficient at locating all reinforcers (TC) subsequent to the end of a trial, than animals in the control condition.

In contrast, experimental animals did not commit more incorrect arm entries during their first 10 choices (CAE) or require more arm entries to complete the task (TAE). Moreover, experimental animals did not commit a greater number of working (WME) or reference memory errors (RME) during their first 12 entries than their control counterparts. The presence of a significant group difference on only one of the five dependent outcome measures suggests that repeated exposure to moderate concentrations of hydrogen sulphide has a measurable, albeit subtle, effect on novel learning.

The most marked finding from these results is that animals exposed to hydrogen sulphide made fewer correct arm choices and, consequently, were less efficient at completing the task within the 10-minute time limit than the control animals. By the end of a trial, control animals were more likely to have entered all of the correct arms, and located all 10 reinforcers, than experimental animals.

Based on these results, it was postulated that hydrogen sulphide may have a deleterious effect on acquisition beyond the conservative limits of working and reference memory initially examined in this experiment. While the maximum capacity of a rat's working memory is not known, estimates as high as 25-30 have been made on the basis of experimental findings (Olton,

Collison, & Werz, 1977). This suggests that, under normal conditions, maze performance may not reach chance levels until the number of arm entries approaches this range. Therefore, although both groups of animals in this experiment made a comparable number of working and reference memory errors during their first 12 choices, performance discrepancies may not have been evident until later in the trial, following the animals' 12th arm entry. A post-hoc MANOVA was conducted to test this hypothesis.

Results of the post-hoc analysis revealed a significant group x time interaction, indicating that the number of working and reference memory errors differed between groups across levels of the time factor. While the control group made more working and reference memory errors than the experimental group at time 1 (presumably because control animals were more active than their experimental counterparts), the experimental group made more errors at time 4. The groups did not differ with respect to the type of errors being made. This suggests that the experimental group's impaired performance on the TC variable was not due to a selective deficit in either short-term or long-term memory, as experimental animals did not make a disproportionate number of working or reference memory errors relative to their control counterparts.

One explanation for the present results is that hydrogen sulphide may reduce an animal's general memory span, thus moving back the boundaries of chance performance and interfering with novel learning. This could explain why some experimental animals had difficulty locating the last one or two reinforcers in this experiment. Although this deficit cannot be accounted for by a simple impairment in either working or reference memory, as measured in this

investigation, it suggests that hydrogen sulphide may cause an impairment in the animal's ability to discriminate between arms it has previously entered (both baited and non-baited) and arms it has not yet visited.

This notion could also explain why animals in the experimental group often appeared as though they were not utilising any particular strategy to locate the last reinforcer(s) during the final few minutes of a trial. On more than one occasion, experimental animals re-entered the same arm several times in close succession. For example, as demonstrated in Table 4, one animal entered the same arm eight times prior to the end of the trial, at which time only eight of the ten reinforcers had been located. It is important to note that while this animal made several entries into an arm from the baited set, other animals made similar numbers of re-entries into arms from the non-baited set. Overall, the animals seemed unaware of their errors, despite making several re-entries into the same arm. This behaviour seems somewhat synonymous with the "rule-breaking" behaviour seen in human patients with prefrontal cortex damage (Milner, 1965). On the basis of these findings, it appears that the animals' ability to remember recent visits to the same arm (either baited or non-baited) was reduced as a consequence of hydrogen sulphide exposure. Whether or not prefrontal structures may be involved remains to be elucidated.

The susceptibility of memory to proactive interference has been repeatedly demonstrated in a radial arm maze paradigm (Olton & Samuelson, 1976). Results from these studies have confirmed that as the overall number

Table 4

Individual Arm Selection Data Obtained From One Experimental Animal During Trial 40 of Task Acquisition

	Arms[a]													
		2					7		9	10		12		14		
Order of Arm Entries[b]	32	7	26	1	27	9	2	10	3	15	4	16		5		6
	55	9	30	8	31	11	22	14	40	23	39	24		17		18
		42	49	12	41	13		28		47		34		35		29
		45		20	46	21		53		54		38		37		36
				44	50	25		56								43
						33										51
						48										
						52										

Note. Despite making 56 arm entries during this trial, the animal was unable to find reinforcers located in arms 13 and 15. In light of the number of re-entries into arm 6, it appears that the animal had difficulty remembering recent entries into the same arm, particularly during later arm entries (>12). [a]Baited arms are highlighted in green. [b]Numbers in the table correspond to the order of entries made by the animal (e.g. the animal's first entry was into arm 4). Arm numbering is arbitrary and used for clarification purposes only.

of arm entries increases, the probability of making a correct arm entry decreases, a finding that has been attributed to interference among items stored in working memory (Olton & Papas, 1979). In light of the present findings, it is possible that hydrogen sulphide exacerbated the effects of proactive interference, thus attenuating the animal's memory span under conditions of novel learning. Additional research is warranted to investigate the concomitant impact of hydrogen sulphide and proactive interference on learning.

Despite the presence of a group difference on the TC variable in this experiment, the magnitude of this difference decreased over time. Thus it appears that the experimental animals were learning the task, albeit at a much slower rate than the control animals. Unfortunately, this trend is difficult to interpret in light of a potential ceiling effect that would have made it impossible to see performance improvements in the control group, had they accrued. In other words, performance gains were no longer measurable in the control animals, since all animals had reached perfect or near perfect performance on the TC variable at the end of the experiment.

Hydrogen sulphide may have a detrimental effect on mental processing speed, thus explaining why animals in the experimental group exhibited a slower rate of learning than the control group. Indeed, exposure to other neurotoxins, such as lead (Anger, 1990) and manganese (Hua & Huang, 1991) has been associated with impairments in this cognitive domain. However, group differences in activity level could also explain these results, since experimental animals were less active at the start of behaviour training, and consequently got off to a slower start than their control counterparts.

It is also important to keep in mind that while a significant group difference was seen on the TC variable in this experiment, the difference was subtle, and may be partly due to group differences in activity level. As demonstrated in Figure 5, control animals entered more maze arms (both baited and non-baited) than their experimental counterparts at times 1 and 2. This difference may help explain why animals in the control group were more efficient at locating the reinforcers and completing the task prior to the end of a trial, at least during the initial stages of the experiment. In other words, animals exhibiting a higher level of locomotor activity have a greater chance of wandering into more arms (including more "correct" arms) than a group of less active animals. Indeed, a positive relationship between the total number of arm entries (TAE) and the total number of correct arm entries (TC) made by the experimental group over time was observed (see Table 2).

While non-cognitive factors (e.g. motivation, loss of appetite) may have contributed to the present results, this is unlikely. To rule out the possibility that experimental animals were less hungry than the control animals, and consequently, less motivated to find all ten reinforcers, animals in the experimental group were given an additional reinforcer (Fruit Loops® cereal) immediately following removal from the maze. Presumably, animals would not eat the piece of cereal if they were no longer hungry, or had acquired an aversive reaction towards the reinforcer. As expected, the reinforcer was immediately consumed in all cases, suggesting that the present results were not due to appetite suppression or a reduced incentive value of the food reward.

It is also unlikely that experimental animals were merely confused or disoriented in this experiment, as confused animals would have had difficulties discriminating between baited and non-baited arms from the outset of the experiment. However, it could be argued that the experimental animals were not allowed to recover from the acute toxic effects of hydrogen sulphide prior to the start of behaviour training. Indeed, animals in the experimental condition were less active during exposure than the control animals, suggesting that they may have been experiencing some acute physiological effects of the gas.

In summary, subtle performance decrements on a complex radial arm maze task were associated with repeated exposure to moderate concentrations of hydrogen sulphide in the present experiment. This suggests that hydrogen sulphide may impair the acquisition (learning) of new material, as opposed to the retention (memory) of previously learned material, as investigated in Experiment 1. To the extent that a sound relationship between normal hippocampal function and the working memory component of a complex maze task exists (Olton & Papas, 1979) these data are in no way suggestive of a selective hippocampal deficit. The goal of Experiment 3 will be to examine whether hydrogen sulphide interferes with an animal's ability to learn a new task, despite interference from a preceding one (proactive interference).

EXPERIMENT THREE:

Objectives

The goal of Experiment 3 was to study the effects of prior exposure to moderate concentrations of hydrogen sulphide (125 ppm) on maze "re-learning". Animals from the preceding experiment were trained to locate reinforcers in the arms opposite to those baited in Experiment 2. Acquisition of the present task, therefore, required the suppression of previously correct arm entries, hence, the ability to release from proactive interference.

As described in Experiment 2, proactive interference refers to the interfering effects of performing one task on the performance of a subsequent task (Pinel, 1993). Based on the preceding results, it was postulated that hydrogen sulphide exposure may impair the acquisition of new material by interfering with the animal's ability to release from proactive interference. In other words, animals may have difficulty learning the new task because of a tendency to enter arms that were baited during the previous task, but are no longer baited in the present one.

Experiment 3 was specifically designed to investigate the role of proactive interference on acquisition of a new task following an extended, but discontinued period of hydrogen sulphide exposure. Animals were not exposed to hydrogen sulphide at any time during this experiment. The advantage of this paradigm was twofold. First, the impact of hydrogen sulphide on acquisition could be evaluated in the absence of potentially confounding acute effects of the gas. Second, insight into the persistence of hydrogen sulphide-related impairments could be obtained by examining radial

arm maze performance for an extended period following exposure termination.

Method

Animals

Sixteen animals from Experiment 2 were used in the present experiment. Subsequent to the completion of Experiment 2, a performance evaluation for each animal was conducted. Animals were subjectively evaluated according to their performance on the CAE variable during the last 10 days of the previous experiment. The eight best animals from each group were chosen for inclusion in the present experiment on the basis of these data.

Animals were individually housed in clear plastic cages with wood chip bedding, as described in the preceding experiment. Water was provided ad libitum, except during behaviour training. Animals were maintained on a 12-hour light/dark cycle. All behaviour training was conducted during the light phase of this cycle.

Apparatus

The same apparatus, as described in Experiments 1 and 2, was used for the purposes of this experiment. The location of the maze within the testing room, and the room characteristics were also consistent with those previously described.

Design and Procedure

Food Deprivation. Animals were kept on a food deprivation schedule and maintained at their reduced body weight, as achieved during Experiment 2. Following daily training sessions, animals were weighed, and subsequently fed by the examiner.

Experimental Design. Behaviour training for the present experiment began three days following the completion of Experiment 2. Group assignment remained consistent with the preceding experiment. In other words, equal numbers of control and experimental animals from Experiment 2 were respectively assigned to the control (n=8) and experimental (n=8) conditions in the present experiment.

Animals were trained to locate reinforcers (Fruit Loops cereal®) in the arms opposite to those baited in Experiment 2. Specifically, the six previously non-baited arms were baited for the purposes of this experiment, while the 10 previously baited arms were not baited. Therefore, optimal performance on the current task depended on the animal's ability to inhibit previously correct (reinforced) responses, while at the same time, disinhibiting previously incorrect (non-reinforced) responses.

At the start of each trial, animals were placed in the center of the maze and allowed to run through the maze until all six reinforcers were located or 10 minutes had elapsed. Upon completion of the trail, animals were removed from the maze, weighed, returned to their home cages, and fed.

Maze performance was evaluated on the basis of the following five dependent outcome measures: (1) number of correct (non-repeated) arm entries made within the first six choices (CAE: maximum=6), (2) total number of correct arm entries made during the trail (TC: maximum=6), (3) number of working memory errors made within the first 12 entries (WME), (4) number of reference memory errors made within the first 12 entries (RME), and (5) total number of arm entries required to collect all six reinforcers (TAE). As described in the preceding two experiments, a working memory error was committed

when the animal re-entered a previously baited arm. A reference memory error occurred when the animal entered an arm from the non-baited set.

A total of 20 behaviour training sessions was conducted over a period of four weeks. Animals were trained once a day, five days a week. At the end of each day, the maze was thoroughly cleaned and rotated to ensure none of the arms faced the same direction on two consecutive days. The orientation of baited and non-baited arms remained constant.

Data Analysis. Statistical analyses of these data were conducted by way of MANOVAs. Data for all 20 days of the experiment were divided into two 10-day blocks; a mean for each block was calculated for each of the dependent outcome measures described above. Therefore, five dependent measures were examined at two levels of the within subjects time variable. Each level of the time variable represented data collected across 10 days of the experiment.

Three independent data sets were formed from the five dependent outcome measures described above. A MANOVA was conducted on each of these data sets, across both levels of the time variable. The first data set consisted of the: (1) number of correct (non-repeated) arm entries made within the first six choices (CAE), and (2) total number of correct arm entries (TC). The second data set consisted of the: (1) number of working memory errors made within the first 12 entries, and (2) number of reference memory errors made within the first 12 entries (RME). The third data set consisted solely of the total number of arm entries required to collect all six reinforcers (TAE).

Each data set was analyzed independently for group, time, and group x time interaction effects. Statistically significant Bonferroni-corrected

MANOVAs (p=.05/3=.017) were followed with repeated measures ANOVAs with time as the within subjects (repeated) factor. The experimentwise error rate was maintained at .05.

Results

No behavioural differences in the two groups were noted at any time during the experiment. Moreover, no signs of respiratory distress or other related health problems were evident during the course of the experiment.

A MANOVA of the first data set, which included the (1) number of correct arm entries within the first six choices (CAE) and (2) total number of correct arm entries (TC) failed to reveal a significant group effect [F(2,13)=1.29, p=.31] or group x time interaction [F(2,13)=.14, p=.87]. In contrast, a significant time effect was found [F(2,13)=7.76, p=.01], reflecting experimental and control group improvements in the CAE [F(1,14)=11.15, p=.01] and TC [F(1,14)=9.43, p=.01] variables over time. Table 5 presents group data for each of the dependent measures examined in the present experiment.

Analyses of the second data set, which included the (1) number of working memory errors within the first 12 entries (WME), and (2) number of reference memory errors within the first 12 entries (RME) failed to reveal a significant group effect [F(2,13)=2.78, p=.10] or group x time interaction [F(2,13)=1.22, p=.33]. Nevertheless, the experimental group committed slightly more working memory errors at both levels of the time factor than the control group, as depicted in Table 5. The lack of a statistically significant group difference on the WME variable was likely due to the modest number of subjects available for the experiment, and the resultant lack of statistical power.

Table 5

Effects of Previous Exposure to Hydrogen Sulphide on Five Measures of Radial Arm Maze Re-acquisition

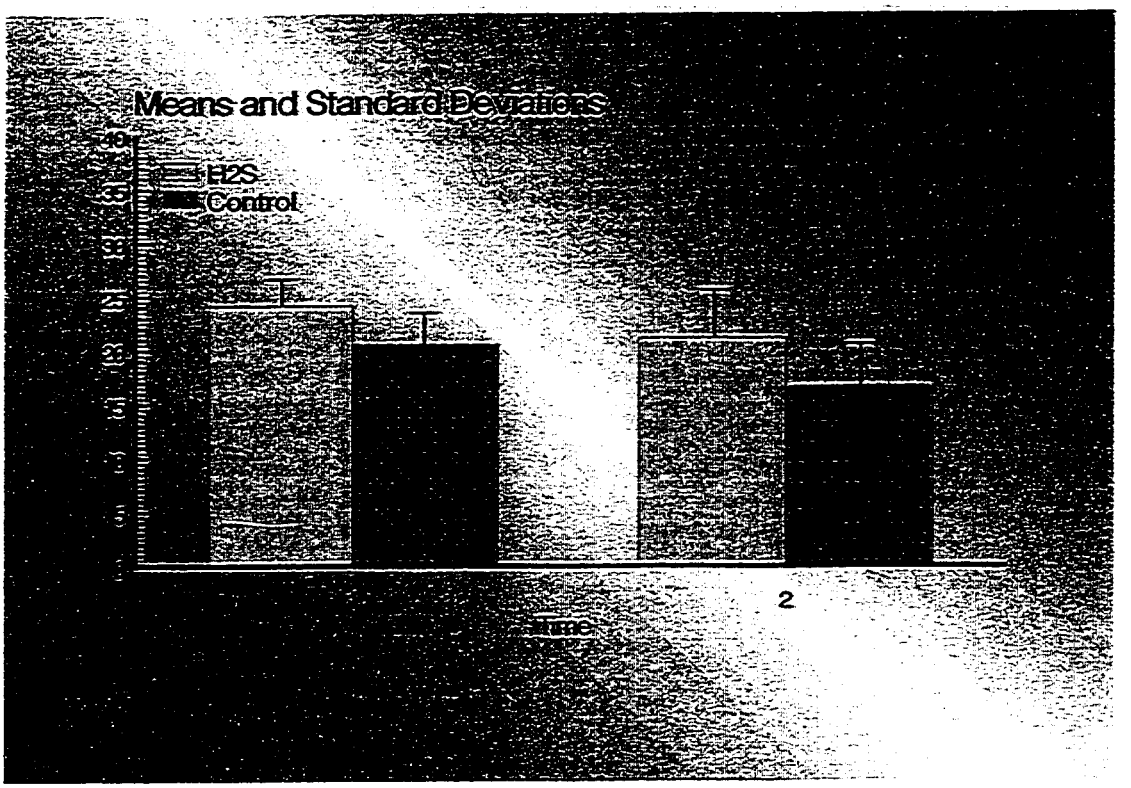
	Time	
	1	2
CAE/6[a]		
H_2S	2.36 (0.33)	2.65 (0.46)
Control	2.08 (0.25)	2.45 (0.38)
TC/6[b]		
H_2S	5.81 (0.21)	5.91 (0.25)
Control	5.91 (0.08)	6.00 (0.00)
WME/12[c]		
H_2S	0.80 (0.45)	0.86 (0.55)
Control	0.38 (0.29)	0.35 (0.29)
RME/12[d]		
H_2S	7.05 (0.48)	6.61 (0.57)
Control	7.39 (0.36)	6.54 (0.92)
TAE[e]		
H_2S	24.19 (2.79)	21.21 (4.57)
Control	20.58 (2.51)	16.71 (3.86)

Note. The values represent means and standard deviations for the experimental (H_2S) and control groups. [a]Number of correct arm choices within first 6 arm entries. [b]Total number of correct arm entries. [c]Number of working memory errors within first 12 arm entries. [d]Number of reference memory errors within first 12 arm entries. [e]Total number of arm entries required to find all 6 reinforcers.

In contrast, a significant time effect was revealed upon analysis of the data [$F(2,13)=7.59$, $p=.01$]. Subsequent analyses revealed that this effect was due to a decrease in the number of reference memory errors made by the groups over time [$F(1,14)=16.35$, $p=<.01$], as opposed to any change in the number of working memory errors made by the animals [$F(1,14)=.14$, $p=.72$]. These data appear to demonstrate that although the animals were learning to avoid the 10 non-baited arms during acquisition of the new task, their tendency to re-visit previously baited arms did not change over time. This result is not surprising, since the average number of WMEs made by the experimental and control groups was .8 and .38, at time 1, respectively. This suggests that the animals were making few re-entries into previously baited arms from experiment outset.

Finally, a MANOVA of the third data set, comprised solely of the total number of arm entries required to complete the task (TAE), revealed a significant group effect [$F(1,14)=7.90$, $p=.01$], but no group x time interaction [$F(1,14)=.19$, $p=.67$]. This suggests that the groups differed with respect to the total number of arm entries required to collect all six reinforcers. Although the experimental group made more arm entries at both times 1 and 2, improvements were demonstrated by both groups over time. In other words, the number of TAEs made by both groups decreased, as demonstrated by the significant time effect [$F(1,14)=11.30$, $p=.01$]. In light of these findings, it appears that experimental animals were less efficient at locating the six reinforcers, and as a result, made more incorrect arm entries prior to task completion, as demonstrated in Figure 8.

Figure 8. Total number of arm entries required to complete the task (TAE). A statistically significant group difference on this measure demonstrated that the experimental animals made more arm entries during their attempts to locate the reinforcers than control animals ($p=.01$) .


Means and Standard Deviations
H2S
Control
40
35
30
25
20
15
2
Time

To test the hypothesis that group differences in the number of working and reference memory errors may have surfaced later in the trial, beyond the animals' 12th arm entry, a post-hoc statistical analysis (MANOVA) of the working memory and reference memory errors made during arm entries 13 through 20 was conducted. Data were divided into two consecutive 10-day blocks and analyzed across two measures of time, as described in the Data Analysis section of this experiment, as demonstrated in Table 6.

The post-hoc analysis revealed a significant group effect [$F(2,13)=4.35$, $p=.04$], but no significant time effect [$F(2,13)=3.54$, $p=.06$] or group x time interaction [$F(2,13)=3.42$, $p=.06$]. Further analyses of the group effect revealed a significant group difference in the number of working memory errors [$F(1,14)=7.37$, $p=.02$] and reference memory errors [$F(1,14)=5.19$, $p=.04$], although the latter difference was not significant when Bonferroni corrections were applied. Figures 9 and 10 demonstrate the results of this post-hoc analysis.

Discussion

These data lend further support to previous findings of impaired learning in animals repeatedly exposed to moderate concentrations of hydrogen sulphide. Acute hydrogen sulphide toxicity could be ruled out as a potential cause of behavioural impairment on the basis of these findings, since animals in the present experiment were not exposed to hydrogen sulphide at any point during the training period.

These findings indicate that repeated exposure to moderate concentrations of hydrogen sulphide has an detrimental effect on the rat's

Table 6

Working and Reference Memory Errors Made During Late Arm Entries, as Calculated for Post-hoc Analysis

	Time	
	1	2
Post-WME[a]		
H_2S	1.36 (0.46)	1.50 (0.77)
Control	1.03 (0.58)	0.50 (0.46)
Post-RME[b]		
H_2S	3.56 (0.68)	2.80 (1.07)
Control	2.90 (0.71)	1.93 (1.20)

Note. The values represent means and standard deviations for the experimental (H_2S) and control groups. [a]Number of working memory errors made during arm entries 13 through 20. [b]Number of reference memory errors made during arm entries 13 through 20.

Figure 9. Working memory errors calculated for post-hoc analysis. Experimental animals made a significantly greater number of working memory errors during later arm entries (13 through 20) than normal controls ($p=.02$).

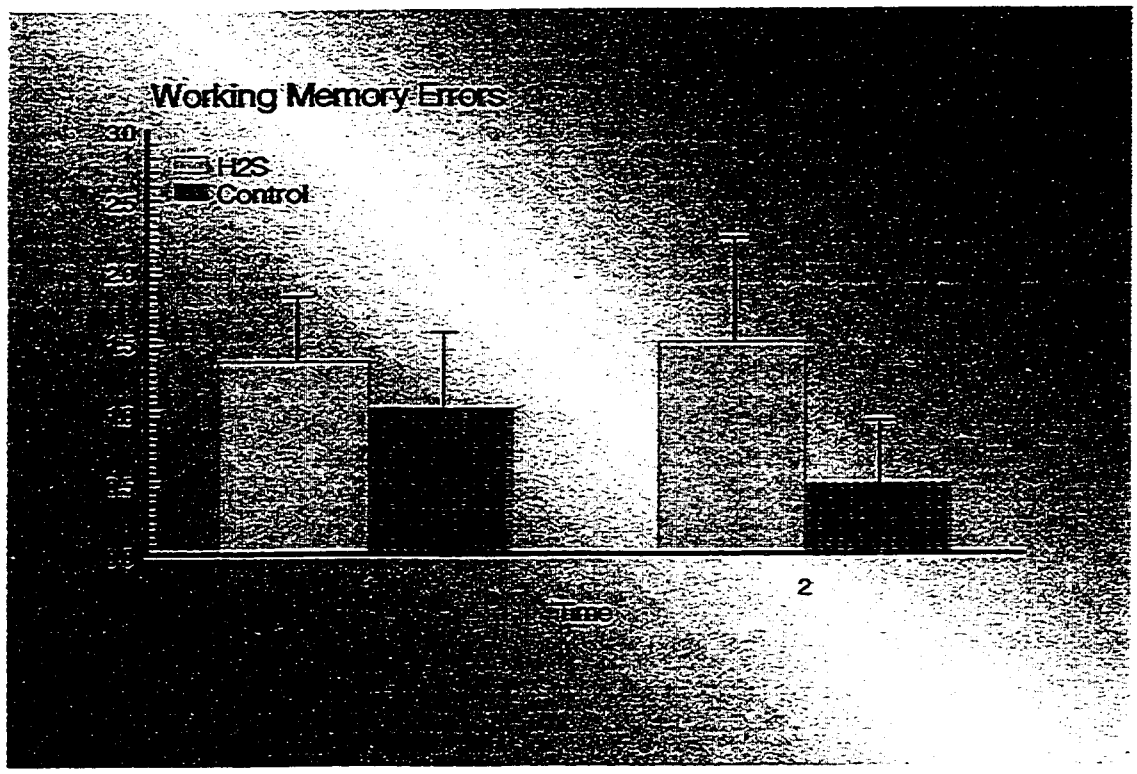
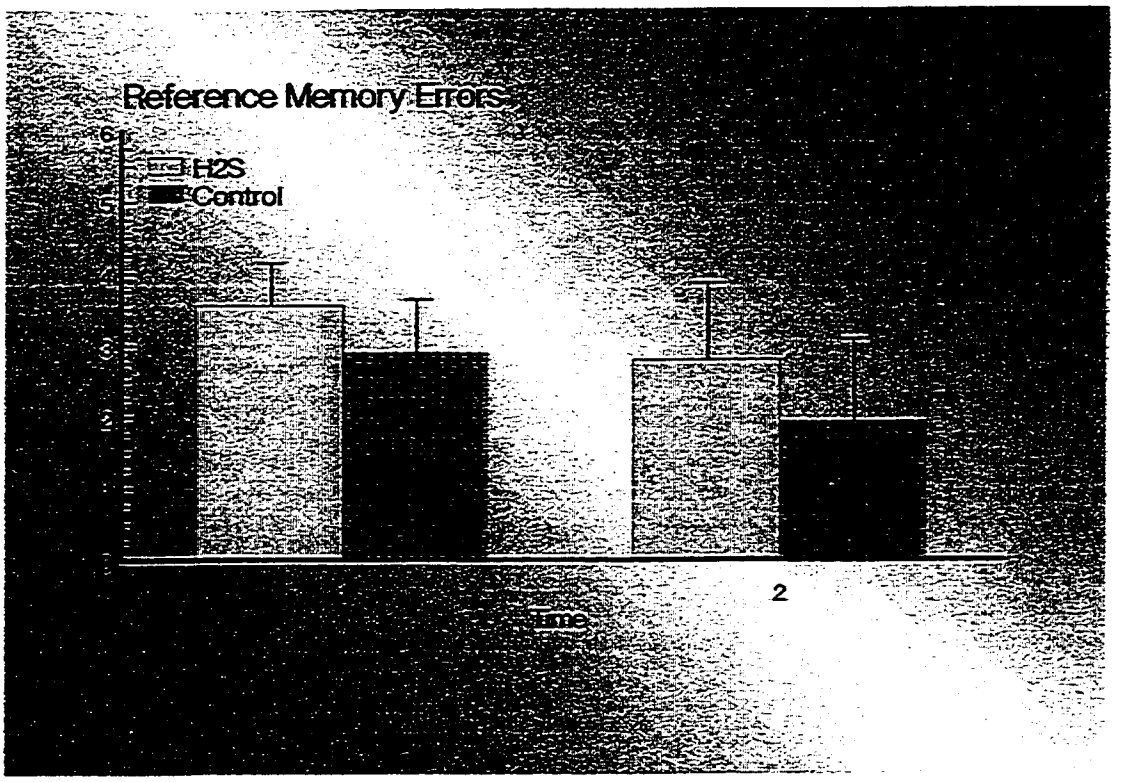

Working Memory Errors
H2S
Control
Time
2

Figure 10. Reference memory errors calculated for post-hoc analysis. Experimental animals made a greater number of reference memory errors during later arm entries than controls ($p=.04$). It should be noted that this difference was not statistically significant when Bonferroni corrections were applied.


Reference Memory Errors
H2S
Control
6
5
4
3
2
1
2
Time

ability to "re-learn" a complex 16-arm radial maze task. Specifically, experimental animals required more arm entries to locate all reinforcers (TAE) than their control counterparts. The experimental group also made more working memory errors within their first 12 entries (WME) than the control group, although this difference failed to reach statistical significance.

In contrast, experimental animals did not make more incorrect arm entries during their first six choices (CAE) or locate fewer reinforcers within the 10-minute time limit than the control animals (TC). Moreover, they did not commit a greater number of reference memory errors within their first 12 arm entries (RME) than their control counterparts.

The learning task presented to the animals in this experiment was particularly challenging, in that it not only required animals to discriminate between reinforced and non-reinforced locations during acquisition of a new maze task, but also required them to suppress previously correct responses from an earlier task. In this regard, success was dependent on the animal's ability to release from proactive interference.

There is little direct evidence from these data to confirm proactive interference as a major underlying factor in the results. In other words, if hydrogen sulphide interferes with learning via mechanisms that inhibit an organism's ability to release from proactive interference, it was presumed that experimental animals would have made more arm entries into the 10 non-baited arms, since these arms constituted the "baited" set in the preceding experiment. Thus, experimental animals should have committed a greater number of reference memory errors within their first 12 entries than the control group. Statistical analyses of the data failed to support this notion. However,

proactive interference cannot be dismissed as a potential source of influence in the present results, since other less obvious sources of interference, related to prefrontal dysfunction, could have been contributory.

Despite the unquestionable difficulty of the present learning paradigm, animals did have a greater likelihood of locating all reinforcers in this experiment, compared to the preceding experiment, since there were fewer reinforcers to find in this task. This could account for the fact that the groups did not differ on the TC variable, and were equally likely to locate all six reinforcers prior to the end of a trial. A re-examination of the data from Experiment 2 confirmed that all animals located a minimum of six reinforcers during each trial. Difficulty did not arise until later in the trial, when animals were searching for the last one or two reinforcer(s), thus accounting for the significant group difference on the TC variable. This suggests that had the number of reinforcers been greater in this experiment, a significant group disparity on the TC variable might have been found.

As noted above, the most marked finding from these data was that experimental animals required more arm entries to locate the reinforcers than their control counterparts. A notable, albeit non-significant, group effect on the WME variable was revealed, suggesting that experimental animals were making slightly more re-entries into previously baited arms (as opposed to non-baited arms) than control animals. This suggests that animals previously exposed to hydrogen sulphide were less effective at remembering which arms they had already entered in search of food rewards. A post-hoc MANOVA was subsequently conducted to see whether group differences in the number

of working or reference memory errors were evident after the animals' 12th arm entry.

Results of the post-hoc analysis revealed that the experimental animals made a greater number of both working and reference memory errors during later arm entries than control animals, although the group difference in reference memory errors was not significant once Bonferroni corrections were applied. Consistent with Experiment 2, these data imply that the performance decrements exhibited by experimental animals are not the result of a selective deficit in either short-term or long-term memory. However, experimental animals do appear to have difficulties in the task. These difficulties become more apparent during later arm entries in a trial.

Thus, the question remains, what neuropathological mechanisms underlie the subtle, yet significant, acquisition impairments found in animals currently or previously exposed to hydrogen sulphide? In light of these behavioural data, it is difficult to argue that hydrogen sulphide selectively targets hippocampal structures in the rat brain. To date, a plethora of studies have demonstrated a marked relationship between hippocampal pathology and impaired performance on the working memory (short-term) component of a radial arm maze task (Olton, 1983; Walsh, Miller, & Dyer, 1982). However, while the experimental group in this investigation did not make a significantly greater number of working memory errors than normal controls or show blatant difficulties releasing from proactive interference, it cannot be assumed that the hippocampus and/or prefrontal cortex are resilient to the damaging effects of hydrogen sulphide. As Olton and Markowska (1994) have suggested, large functional consequences do not necessarily coincide with

large insults to the nervous system. Thus, further behavioural investigations and histological examinations of brain tissue from exposed animals would help elucidate the site(s) and magnitude of pathology underlying hydrogen sulphide-induced behavioural dysfunction.

This experiment was unique in that it offered some insight into the functional ramifications of hydrogen sulphide beyond exposure termination. On the basis of these data, it is evident that hydrogen sulphide has a deleterious impact on neurobehavioural function that cannot be accounted for by the acute physiological effects of the gas. Whether or not repeated hydrogen sulphide exposure exerts transient or more permanent effects on neurobehavioural function is a question that awaits future investigation.

FINAL DISCUSSION

In light of our meagre understanding of the neurobehavioural effects of repeated hydrogen sulphide exposure, the current investigation was undertaken to examine the effects of repeated exposure to moderate concentrations of hydrogen sulphide on learning and memory in the rat.

The main findings from this investigation were as follows: (1) repeated exposure to hydrogen sulphide had no effect on the retention of a well rehearsed spatial task, (2) experimental animals were impaired relative to normal controls on one measure of performance during the re-acquisition of a reversed contingency maze task, and (3) the deleterious effects of hydrogen sulphide persisted well beyond the period of exposure. In summary, these data provide evidence of mild brain dysfunction following a prolonged period of repeated hydrogen sulphide exposure.

The deficits noted in this investigation were not suggestive of a memory deficit. Experimental animals demonstrated a preserved retention of well rehearsed material in addition to a relatively intact acquisition of new material. Deficits appeared when task demands became increasingly difficult for the animals. Consequently, animals appeared confused and disoriented as they struggled (and often failed) to remember which arms to enter during the last few minutes of a trial.

One plausible explanation for the current findings is that hydrogen sulphide elicits a general defect in acquisition, thus interfering with the animals' ability to learn new material. Hydrogen sulphide may be a cumulative poison, which could help explain why experimental and control animals were comparable in their acquisition of a novel maze task during Experiment 2.

Conceivably, the accumulation of hydrogen sulphide in brain tissue had not yet reached the point required for neurobehavioural disruption until the end of the experiment, at which time the task had already been reasonably well learned.

The plausibility of this hypothesis awaits further investigation, since the cumulative effects of hydrogen sulphide have not been experimentally validated (Beauchamp et al., 1984). It has been argued that low concentrations of this gas can be tolerated for extended periods without harm, although more recent investigations have challenged this notion (Kilburn, 1997).

An alternate explanation is that hydrogen sulphide selectively interferes with the acquisition of more complex tasks, such as those that require the suppression of previously learned responses. In other words, hydrogen sulphide may cause a disorder of interference. This could account for the impaired performance of animals in Experiment 3.

Although evidence of proactive interference was not immediately apparent in this investigation, the possibility of an interference deficit and/or involvement of prefrontal brain structures cannot be dismissed. It is conceivable that increased distractibility, reminiscent of prefrontal dysfunction, impaired the animals' ability to resist interference from irrelevant stimuli and discriminate between baited and non-baited arms (Fuster, 1997). Presumably, interference could have originated from the animal's earlier responses, either during the same trial or a previous one. This could help explain why experimental animals made more total arm entries during the final experiment

than normal controls, assuming they were distracted and not attending to relevant stimuli in the environment during arm selection.

Indeed, behavioural similarities between animals in this study and human prefrontal patients are apparent. Human patients with prefrontal damage generally have little difficulty engaging in old, well rehearsed activities (Fuster, 1997). In comparison, the rats in this investigation were not impaired in their ability to remember a spatial task learned prior to exposure. Moreover, human prefrontal patients are capable of forming new memories, as were the animals in this investigation.

As noted above, prefrontal patients have little difficulty performing everyday, routine activities (Fuster, 1997). In contrast, they typically encounter difficulties when faced with highly challenging and/or novel situations, presumably due to their inability to organise and initiate the required behaviours to reach a desired goal. Fuster (1997) has suggested that these patients experience a failure in temporal integration, such that they are unable to organise temporally discrete actions into meaningful goal-directed behaviours. This same explanation has been used to account for the deficits exhibited by rats with prefrontal damage, and could arguably account for the deficits observed in the present investigation.

Animals exposed to hydrogen sulphide in this study did not experience difficulties during the performance of a well rehearsed spatial task, as demonstrated in Experiment 1. Performance decrements were not evident until the animals were placed in a highly challenging situation, such as the one imposed on them during Experiment 3. It could be argued that the subtle impairment seen in Experiment 2 was also a consequence of the same

prefrontal disruption, since animals did not experience difficulties until the task demands were most challenging (e.g. during the last few minutes of a trial).

It is important to keep in mind that while behavioural measures of brain dysfunction in rats are generally limited to tests of spatial function, tests of human spatial function are often susceptible to verbal coding (Milner, 1965). Thus, it is uncertain to what degree the deficits revealed in this investigation compare with the human prefrontal syndrome. In this regard, a more compelling argument for prefrontal involvement in the present study is viable from behavioural investigations of rats with damage to prefrontal structures.

Radial arm maze performance is typically impaired in animals with prefrontal damage. Kolb, Sutherland, and Whishaw (1983) found that rats with prefrontal lesions made more incorrect arm entries during radial maze acquisition than normal controls. Other reports have emerged in support of these findings (Chiba, Kesner, & Gibson, 1997; Kesner, DiMattia, & Crutcher, 1987).

While deficits in both working and reference memory are notable after prefrontal damage, a more enduring reference memory deficit is common (Kesner, et al., 1987). This finding is not well understood, given the widely accepted notion of prefrontal involvement in working memory. It is possible that the working memory component of a radial maze task can be mastered by way of different neural systems (e.g. the hippocampus) that are presumably left intact following frontal damage.

Animals in the present experiment demonstrated a comparable, albeit more subtle, deficit to that described in previous prefrontal animal work. In particular, animals from the experimental group made more working and

reference memory errors during later arm entries relative to normal controls. This difference was not apparent during the animals' initial 12 entries, suggesting that only when an animal is highly challenged (e.g. required to find the last reinforcer before the end of the trial) does performance suffer as a result of hydrogen sulphide exposure.

As noted earlier, animals may be impaired in their ability to discriminate between baited and non-baited arms during highly challenging situations. The relative subtlety of this deficit may be a reflection of mild pathophysiological changes to prefrontal structures following hydrogen sulphide exposure, compared to the dramatic behavioural alterations expected following surgical ablation of brain tissue (Kesner et al., 1987; Kolb et al., 1983).

Another clue that hydrogen sulphide may target prefrontal structures comes from the human neuroimaging and neuropsychological research. In particular, functional imaging has revealed evidence of frontal lobe pathology in human exposure victims (Callender et al., 1993). Neuropsychological deficits on measures of frontal lobe function have also been reported. In at least one case, diffuse EEG slowing in frontal brain regions was correlated with poor performance on tests sensitive to frontal lobe pathology (Hua et al., 1992). Of course, this does not include the myriad of memory and attention complaints from human exposure victims, many of which could be attributed to frontal lobe pathology.

Overall, these data suggest that rats exposed to hydrogen sulphide are behaviourally comparable to human prefrontal patients and rats with prefrontal lesions. Repeated hydrogen sulphide exposure appears to interfere with the normal learning of animals under highly challenging conditions.

Increased distractibility and/or reduced attention, both of which have been reported in association with prefrontal cortex damage (Fuster, 1997) may interfere with the animals' ability to block out irrelevant information during high task demands. Thus, the importance of the prefrontal cortex for the suppression of task irrelevant information seems particularly relevant in this investigation.

It could further be argued that the terminal consequence of hydrogen sulphide's effect on brain function is a reduction in the normal memory capacity of a rat, since there was some indication from this study that rats experienced the most difficulty during later arm entries. Due to factors such as increased distractibility and/or reduced attention, animals may have suffered a premature breakdown in active short-term (working) memory, thus leading to an increased number of incorrect arm entries and a diminished capacity for learning as the task continued and the demands increased. At this point, animals could no longer rely on active short-term memory storage to remember preceding events or plan future ones.

It is noteworthy that the deficits revealed in this experiment were not typical of those found in "hippocampal" rats. This finding was somewhat unexpected in light of the importance of hippocampal structures in learning and memory and recent electrophysiological evidence of hippocampal susceptibility to repeated hydrogen sulphide exposure (Skrajny, et al., 1996). However, other reports of abnormal hippocampal electrophysiology in the absence of characteristic behavioural deficits have been published (Hesse, 1979). On the basis of these findings, it appears that there may be a large functional compensatory mechanism inherent in the hippocampal system.

Innumerable reports of a selective working memory impairment in rats with hippocampal damage and/or destruction of its afferent cholinergic pathways have been documented (Olton & Papas, 1979; Walsh & Chrobak, 1987; Wirsching et al., 1984). Animals in the present investigation did not exhibit a selective working memory impairment, suggesting that hydrogen sulphide does not selectively target hippocampal structures as previously suspected.

While alterations in hippocampal EEG activity have been reported under conditions of repeated hydrogen sulphide exposure, these data are not inconsistent with the present results. Reciprocal connections between prefrontal and hippocampal structures have been identified in rats and primates (Ferino Thierry, & Glowinski, 1987; Goldman-Rakic, Selemon, & Schwartz, 1984). It is possible that alterations in hippocampal EEG activity may be a secondary effect of pathophysiological changes in prefrontal regions, which in turn, send projections to the hippocampus and alter the firing pattern of cells in this region. In other words, hippocampal electrophysiological changes may be a consequence of disabled input from prefrontal areas. A more direct target of hippocampal structures may be required to produce the distinguishing behavioural characteristics of hippocampal damage.

One notable feature of the current findings was the appearance of a marked performance deficit following exposure termination. It was during Experiment 3 that the most profound impairment appeared, and yet exposure had been terminated several weeks earlier. This suggests that the functional brain impairments associated with hydrogen sulphide are enduring and are not due to acute physiological effects of the gas.

Depending on factors such as lesion site and severity, the magnitude of deficits associated with prefrontal damage is highly variable (Fuster, 1997; Milner, 1965). In light of this understanding, I would argue that the deficits noted in the present investigation were likely a reflection of mild pathophysiological changes to prefrontal brain structures. At present, the precise mechanisms underlying hydrogen sulphide neurotoxicity are not known.

One potential mechanism of action concerns zinc and the possibility of zinc-mediated neuronal disruption under conditions of hydrogen sulphide exposure. The importance of zinc for normal brain function has been well established (Golub, Keen, Gershwin, & Henrickx, 1995; Sandstead, Penland, Alcock, Dayal, Chen, Li, Zhao, & Yang, 1998). A link between elevated or depleted levels of zinc in the brain and various neuropathological conditions has been suggested on the basis of recent experimental and clinical findings. In particular, alterations in mood (e.g. depression) and cognitive function have been associated with zinc depletion (Nowak, 1998), while significant zinc elevations have been associated with neuropathological conditions such as global ischemia (Choi & Koh, 1998), Alzheimer's disease (Cuajungco & Lees, 1997[a]), and other neurodegenerative disorders (Cuajungco & Lees, 1997[b]).

Approximately 15% of brain zinc concentrations are contained within the synaptic vesicles of glutamatergic neurons in the telencephalon (Frederickson, 1989; Haug, 1984). This suggests that most, if not all, zinc enriched neurons in the brain are excitatory. The hippocampus contains the highest concentrations of zinc in the brain; other high zinc regions include the

neocortex (layers I-III and V), subiculum, amygdala, thalamus, and striatum (Choi & Koh, 1998).

The chemical binding of zinc ions to sulphide has been demonstrated by in vitro exposure of brain tissue to hydrogen sulphide (Danscher, Juhl, Stoltenberg, Krunderup, Schroder, & Andreasen, 1997) and in vivo sodium sulphide exposure (Perez-Clausell & Danscher, 1986). This method has proven valuable for the visualization of sulphide-bound zinc ions in brain tissue under normal and pathological conditions (Timm, 1958).

It is possible that zinc alterations may be involved in the neurobehavioural disruption associated with hydrogen sulphide toxicity. According to the results of at least one study, sulphide may alter the normal physiology of neuronal boutons and/or vesicles. Perez-Clausell and Danscher (1986) traced the location of sulphide-bound zinc ions in the rat brain at various survival times following intracerebral injections of sodium sulphide. Their findings demonstrated the diffusion of zinc-sulphide accumulations from the synaptic vesicles into the extracellular space (synaptic cleft).

In light of the above findings, it is plausible that an excessive accumulation of extracellular zinc may occur in response to sulphide-induced changes in the physiology of zinc enriched neurons, thus accounting for the deleterious cognitive effects of repeated hydrogen sulphide exposure. The aberrant release of zinc from its cellular storage sites, and resultant increase in extracellular zinc, can damage neurons and participate in other pathophysiological processes (Cuajungco & Lees, 1997[b]; Koh, Suh, Gwag, He, Hsu, & Choi, 1996).

The mechanisms by which this action occurs are uncertain. However, they may involve zinc's interaction with N-methyl-D-aspartate (NMDA) glutamate receptors. Choi and Koh (1998) have suggested that the co-release of zinc and glutamate may attenuate the activation of NMDA receptors, relative to another class of glutamate receptors. Since NMDA receptor channels have special properties (e.g. high calcium permeability), the reduced activation of NMDA receptor gated channels may interfere with the normal excitatory response of glutamatergic neurons, thus modifying the nature of excitatory transmission.

It is also possible that synaptically released zinc may move outside excitatory synaptic clefts and modulate nearby GABA synapses (Choi & Koh, 1998). Consistent with earlier reports, an interference in normal synaptic transmission would be a likely consequence of this activity (von Euler, as cited in McLardy, 1970).

Koh et al. (1996) have suggested that zinc accumulation in post-synaptic neurons precedes neuronal degeneration. In light of these findings, it has been proposed that ischemia induced neuronal death may be mediated by the transynaptic movement of zinc from pre-synaptic terminals into post-synaptic neurons. Whether or not a similar mechanism may underlie hydrogen sulphide induced changes in brain function is uncertain.

It is noteworthy that high concentrations of zinc have been associated with an inhibition of cytochrome oxidase (Nicholls & Malviya, 1968; Skulachev, Chistyakov, Jasaitis, & Smirnova, 1967) since a similar mode of action is thought to underlie hydrogen sulphide toxicity (Smith & Gosselin, 1979). Sulphide ions bind to cytochrome oxidase and inhibit its activity, thus

interfering with cellular respiration. A role for zinc in this process has not yet been established. However, sulphide-bound zinc may interfere with cytochrome oxidase upon being released into the synaptic cleft. Further research is needed to investigate a potential role for zinc in the sulphide mediated inhibition of cytochrome oxidase.

Cuajungco and Lees (1997[b]) have suggested that both abnormally high and low levels of vesicular (chelatable) zinc may play a role in neuronal death, albeit by different modes of action. It is possible that attenuated, as opposed to elevated, levels of zinc may underlie the neurobehavioural deficits associated with hydrogen sulphide exposure. Zinc deficiency has been associated with abnormal electrophysiological function of the hippocampus (Hesse, 1979) and a reduction in the number of glutamate NMDA receptors (Browning & O'Dell, 1995).

Alternatively, hydrogen sulphide may interfere with the vascular physiology of the brain. This premise is based on preliminary findings of dilated blood vessels in the brains of experimental rats included in this investigation (unpublished observations). These alterations were noted several months following exposure termination, suggesting that the vascular changes were enduring and potentially irreversible.

The functional relevance of these findings is not understood. It is plausible that long term alterations in cerebral blood flow may underlie this vessel pathology (Kandel, Schwartz, & Jessell, 1991). During hydrogen sulphide exposure, cerebral hyperperfusion may function as a compensatory mechanism for reduced brain oxygen levels secondary to depressed respiratory function. Thus, although the animals used in this study did not lose

consciousness or show overt signs of respiratory distress during exposure, a hypoxia-induced mode of action cannot be completely dismissed.

Increased cerebral perfusion is a compensatory mechanism involved in the preservation of brain oxygen under various pathological states (Auer & Benveniste, 1997; Johannsson & Siesjo, 1975). Conditions that directly interfere with blood oxygen levels (e.g. hypoxic hypoxia) or decrease the oxygen carrying capacity of the blood (e.g. anemia) inspire an increase in cerebral blood flow to preserve brain oxygenation (Auer & Benveniste, 1997). Alterations in brain vascularity, such as those noted at present, are plausible under these conditions, since vasodilation is a noted consequence of hypoxia (Joseph, 1996).

Conceivably, a decrease in respiratory rate and/or an impairment in pulmonary function could cause hypoxia under conditions of hydrogen sulphide exposure. Since decreased respiration interferes with blood re-oxygenation (Joseph, 1996), it is possible that experimental animals in this study experienced reduced brain oxygen levels due to a subtle depression in respiratory rate.

Alternatively, a disruption in pulmonary function may have led to hypoxia during the present investigation. Reports of compromised respiratory function under conditions of acute and/or prolonged hydrogen sulphide exposure are not uncommon (Dales et al., 1989; Parra, Monso, Gallego, & Morera, 1991). A slow progressive deprivation of brain oxygen levels could be expected if the normal functions of the pulmonary system were disturbed (e.g. CO_2 - O_2 gas exchange). A similar mode of action has been proposed to

account for the presence of subtle neuropsychological disturbances in asthmatic children (Prigatano & Levin, 1988).

One difficulty in applying this theory to the present behavioural data is that experimental animals in this study did not show "classic" hypoxia-induced deficits in brain function. Due to the selective vulnerability of certain brain regions (e.g. hippocampus) to reduced oxygen supply, memory loss is a common repercussion of cerebral hypoxia. Animals in this study did not demonstrate a memory deficit per se, nor did they display evidence of profound hippocampal involvement. The disparity of these data with the preliminary neuropathological findings described above is puzzling and in need of further investigation.

We are currently in the process of examining brain zinc concentrations in rats exposed to hydrogen sulphide during this investigation. To the best of my knowledge, no previous attempts have been made to look at sulphide-zinc binding in animals exposed to hydrogen sulphide in vivo, nor has it been possible to make immediate comparisons between neuropathological and behavioural manifestations of hydrogen sulphide exposure. It is anticipated that these data will provide insight into the pathophysiological mechanism(s) underlying hydrogen sulphide-related brain dysfunction.

The results of this investigation are enlightening when applied to our current understanding of the human hydrogen sulphide literature. Clinical reports in this field are typically replete with vague descriptions of learning and memory problems in human exposure victims. Descriptions of patients experiencing "increased forgetfulness" or "problems remembering" are not uncommon, and yet have been of limited benefit when attempting to elucidate

the precise nature of the neurobehavioural dysfunction or pathological mechanisms underlying hydrogen sulphide toxicity. In light of the present findings, it is now clear that hydrogen sulphide exerts a subtle, yet significant, effect on normal brain function that interferes with memory and learning in a way that may be difficult to operationalize in human patients.

Of course, this investigation was not without its limitations. First, while the radial arm maze proved to be a sensitive tool for examining learning and memory dysfunction in the present study, use of this tool proved very time consuming. Animals required a prolonged period of daily training sessions to ensure adequate maze learning. However, even with such a rigorous training regimen, many animals failed to reach near-perfect performance by the end of training. Thus, the second limitation of this study concerned the demands of the task, which may have been too great for the animals.

The importance of task demand in an investigation of learning and memory cannot be understated, since the magnitude of an impairment can vary considerably. As discussed by Olton and Markowska (1994), subtle deficits can go undetected if task demands are too small ("ceiling" effect). In contrast, when task demands are too great, performance may be so poor that further impairment becomes difficult to detect ("floor" effect). It could be argued that the task demands in this study were too high, since only the "brightest" animals seemed able to reach criterion performance during training. Although attempts were made to lower the demands in Experiment 2, a marked changed in performance was not found. This suggests that the demands of the task remained too high, at least for this particular strain of rat.

A third limitation of this study was its limited sample size, particular in the case of Experiment 3 (n=16). Low statistical power may have interfered with the detection of small effects in this study (Howell, 1987). Unfortunately, sample size was limited by the use of a small, fixed capacity exposure apparatus and the removal of rats from Experiment 3 for the purposes of brain analysis, neither of which could be avoided.

A fourth limitation concerned the non-blind nature of this study. The importance of an experimenter blind procedure in neurotoxicology research has been well established (Benignus, 1992). Unfortunately, investigator blinding was not possible in the present study, since rats were removed from the exposure apparatus, returned to their home cages, and trained by the same experimenter. Although no attempt was made to memorise the group assignment of individual rats, the identity of certain animals became apparent over time.

A final limitation of this study concerns probable dose-response disparities between rats and humans. Certain aquatic species of animals have developed adaptive detoxification mechanisms which allow them to survive in high sulphide environments (Grieshaber & Volkel, 1998; Vetter et al, 1989). It is still not certain to what degree rats may have developed a similar, yet less advanced, mechanism of survival, considering their evolutionary roots. For hundreds of years, rats have been recognised for their impeccable ability to survive amidst the sewers, despite the concomitant presence of hydrogen sulphide in the same environment. To what degree rats may be more resistant to the effects of hydrogen sulphide than humans is not known. Rats may be more biologically equipped to tolerate high concentrations of hydrogen

sulphide than primates. If so, more marked neurobehavioural effects would be expected in humans exposed to the same hydrogen sulphide concentrations as used in this study. At present, interspecies differences in size and metabolic rate, as well as possible detoxification mechanisms, impair our ability to extrapolate from one species to the next.

The relevance of the current findings to the human condition is apparent. Despite complaints of ill health and cognitive decline in persons living and/or working under conditions of perpetual hydrogen sulphide exposure, the etiology of such complaints has been poorly understood. This investigation was the first of its type to investigate the legitimacy of such complaints. Based on the present findings, it appears that repeated exposure to sublethal concentrations of hydrogen sulphide has deleterious effects on brain function that may be comparable to those reported by human exposure victims.

In closing, I would like to propose some ideas for future investigation in this area. First, in light of the present discoveries, it seems imperative that the neurobehavioural ramifications of exposure to lower hydrogen sulphide concentrations be investigated. Ideally, this would involve an investigation of variable concentrations and exposure durations, in light of how little we know about long-term exposure to low concentrations of the gas.

Second, more behavioural research is needed to investigate the cumulative potential of hydrogen sulphide. One way to investigate whether or not hydrogen sulphide impairs acquisition following a cumulative build-up in brain tissue would be to train naive rats to learn a complex maze task following a prolonged, but terminated period of exposure. Negative findings

would challenge this notion, while lending support to the interference theory described earlier.

Lastly, neurobehavioural testing should go beyond the domains of learning and memory examined in this investigation. Impaired motor function is a common sequelae of hydrogen sulphide exposure, the nature or cause of which is not understood. Behavioural tests of motor function in the rat could help elucidate the nature of the motor dysfunction associated with hydrogen sulphide exposure. With these endeavours in mind and practice, we are well on our way towards an enhanced understanding of hydrogen sulphide's impact on the human nervous system.

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