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ACUTE HYDROGEN SULFIDE POISONING

DEMONSTRATION OF SELECTIVE UPTAKE OF SULFIDE BY THE BRAINSTEM BY MEASUREMENT OF BRAIN SULFIDE LEVELS

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Abstract—The possibility of measuring sulfide levels in the central nervous system (CNS) opens up many avenues for exploration. In acute hydrogen sulfide (H_2S) poisoning, death results from loss of central respiratory drive. To date, however, measurement of brain sulfide has not been possible. By employing gas dialysis and ion chromatography coupled to electrochemical detection, rat brain sulfide levels could be measured either following inhalation of H_2S or after injection of sodium hydrosulfide (median lethal dose, [LD_{50}] = 14.6 ± 1.00 mg/kg). Accumulation of brain sulfide was linearly proportional to the dose over the range 0.50 LD_{50} to 3.33 LD_{50} units, and was strongly correlated with mortality data ($R = 0.947$). Furthermore, analysis of untreated (control) brain showed an endogenous sulfide level of 1.57 ± 0.04 μg/g (mean ± SE; N = 16). Studies on various rat brain regions (brainstem, cerebellum, hippocampus, striatum and cortex) showed that the endogenous sulfide level of brainstem, 1.23 ± 0.06 μg/g, was significantly lower than that of the other brain regions. Net uptake of sulfide was greatest in the brainstem (3.02 μg/g) compared to the other regions as was the selective accumulation of sulfide as calculated from normalized blood flow rates. The results of subcellular fractionation demonstrated that sulfide was detectable in fractions enriched in myelin, synaptosomes and mitochondria. Approximately one-quarter of the endogenous sulfide content of whole rat brain was found in the mitochondrial fraction. The sulfide content of these fractions increased 2- to 3-fold after 50 mg/kg NaHS, the greatest increases occurring in myelin- and mitochondrial-enriched fractions.

Hydrogen sulfide (H_2S) is used in over seventy industrial processes and is also a major contaminant of certain natural gas deposits [1, 2]. This gas is extremely toxic, and the literature contains many reports of fatal intoxication [3–5]. Acute intoxication with hydrogen sulfide or its alkali salts results in several clinical signs including apnea, hypoxic convulsions, and death [6]. Some of these signs are manifestations of H_2S toxicity in the central nervous system (CNS). The apnea results from the loss of central respiratory drive from brainstem neurons [7–9], and is the major cause of death.

Studies of sulfide toxicity have been hampered by the lack of a suitable method for the detection of sulfide in brain or other tissues. By adapting a technique originally developed for aqueous samples [10], it was possible to detect and quantitate sulfide in sample matrices (homogenates) derived from brain tissue. It thus became possible to study sulfide uptake by whole brain, selected rat brain regions, and subcellular fractions derived from rat brain as well as post-mortem brain material.

MATERIALS AND METHODS

Standardization of sodium hydrosulfide. Sodium hydrosulfide, NaHS, was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and was iodometrically titrated [11] and determined to exist stoichiometrically as the dihydrate, NaHS-$2H_2O$. All subsequent work was carried out taking into account the water of hydration of the salt. Both sodium sulfide (Na_2S) and sodium hydrosulfide (NaHS) have been widely used in H_2S toxicity studies as alkali salts; both, when administered to animals, generate H_2S *in vivo* [1]. At a physiological pH of 7.4, approximately one-third of the sulfide, whether derived from gaseous H_2S, or one of its alkali salts, will exist in the form of H_2S, with the anion, HS^- making up the remainder [1].

Lethality estimates for the NaHS. The LD_{50}, defined as the median lethal dose producing 50% lethality in a population, was determined by probit analysis. Since lethality is the principal human hazard of acute H_2S exposure, the LD_{50} is an important value. Initially, an estimate of the LD_{50} for NaHS (N = 10) was obtained by the method of Reed and Muench [12] and further refined as results from additional animals were added serially during the course of the principal experiments. The LD_{50} (±SD) was found to be 14.6 ± 1.00 mg/kg (N = 75).

Preparation of brain homogenates. Male Sprague–Dawley rats (250–350 g) were injected intraperitoneally with various doses of sodium hydrosulfide ranging in sulfide content from 7.5 to 50 mg/kg as determined by iodometric titration. Controls received comparable volumes of physiological saline.

Animals were decapitated 2 min after NaHS injection, and their brains were removed, rinsed in ice-

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cold 0.85% saline, weighed, and placed in 10 vol. of 0.01 M NaOH (pH 12.0 to 12.1). The choice of homogenization conditions was dictated by the dissociation profile of H_2S and HS^-; at a pH of 12.0, HS^- and sulfide ion, S^{2-}, are present in equimolar amounts [13]. Whole brains were homogenized with a Polytron (Brinkmann) at a setting of 5 for 1–2 sec, and the resulting homogenates were centrifuged at 18,800 *g* for 20 min at 4°. Supernatant fractions were decanted and retained while the remaining pellets were resuspended in 20.0 ml of fresh, cold 0.01 M NaOH. To each supernatant and pellet fraction was added 135.0 μl of a 1 M solution of zinc acetate to ensure preservation of sulfide as insoluble ZnS. Samples were then gently mixed, capped and placed on ice until analyzed later the same day. Immediately prior to analysis, 0.08 g ascorbic acid was added to each fraction to prevent sulfide oxidation [14], and the pH was readjusted to 12.

In one experiment designed to demonstrate the toxicological equivalence of injected NaHS and inhaled H_2S, the gas was administered by inhalation using the apparatus and procedure of Lopez *et al.* [15]. Rats received 1650 ± 175 ppm H_2S (as determined by gas chromatography), a concentration approximately twice the EC_{50} for lethality (time to death = 4.9 ± 1.4 min; mean ± SE). Their brains were subsequently taken for analysis and prepared in a manner identical to those from NaHS-injected groups.

In regional experiments, brains were dissected on ice, as outlined, into brainstem (medulla and pons), cerebellum and hippocampus (Kombian *et al.* [16]) and striatum and cortex (Warenycia and McKenzie [17]). Brain regions were then rinsed in ice-cold saline, weighed, and homogenized in 10.0 ml of cold 0.01 M NaOH. The resulting zinc acetate-preserved (67.5 μl of a 1.0 M solution) homogenates were then analyzed directly without an intervening centrifugation step.

Subcellular compartmentalization of sulfide. Subcellular fractions of brain were prepared as described previously [18] with a few modifications. Rats were killed by decapitation, and whole brains were removed and placed immediately into ice-cold 0.32 M sucrose (in 0.01 M NaOH, to trap sulfide). Preliminary experiments demonstrated that the use of regular isotonic 0.32 M sucrose, without 0.01 M NaOH, was undesirable as volatilization of H_2S rapidly took place, leading to a 30% or more loss of sulfide from homogenates. Brains were homogenized in 5–10 vol. of the sucrose solution in a teflon-to-glass homogenizer (1000 rpm, 10 up-and-down strokes), and an aliquot was saved for sulfide analysis. The homogenate was centrifuged at 1000 *g* for 10 min. The pellet (P_1) was saved for analysis, and the supernatant (S_1) (minus a 1.0-ml aliquot for sulfide determination) was subsequently centrifuged at 10,000 *g* for 20 min. The second supernatant (S_2) was saved for analysis and the pellet (P_2) resuspended in 0.32 M sucrose, and layered on top of 0.8 M sucrose (in 0.01 M NaOH) for density gradient centrifugation at 8000 *g* for 30 min. The subcellular fractions collected from the gradient were as follows: myelin-enriched fraction, the interface between 0.32 M and 0.8 M sucrose layers; the synaptosome-enriched fraction, 0.8 M sucrose layer; and the mitochondrial-enriched fraction in the pellet. The pellet was resuspended to a volume of 10.0 ml of fresh cold 0.01 M NaOH. Zinc acetate (67.5 μl of a 1 M solution/10 ml) was then added to each fraction. All three of these fractions were anlayzed without further dilution to determine the sulfide content.

Specificity of the technique for sulfide. Bovine serum albumin (BSA), RIA grade, that was stored at −20° over anhydrous $CaCl_2$, was used exclusively throughout this study. Cysteine, methionine, and taurine were all of analytical grade and were obtained from the Sigma Chemical Co., St. Louis, MO. Thiosulfate was obtained from Fisher Scientific (Fair Lawn, NJ). To further preclude the possibility that protein desulfuration could result in artifactual sulfide levels, control rat brains were flash-frozen with liquid nitrogen and homogenized while still partially frozen. The resulting crude homogenates were then *immediately* analyzed for sulfide.

Sulfide analysis. Analysis of samples was carried out according to the method of Goodwin *et al.* [10]. Ten milliliters of each sample (either supernatant, resuspended pellet, homogenate, fraction, or solution of test compound) was processed by a continuous flow procedure utilizing a gas dialysis membrane unit (Technicon 157-B-129, path length of 15 cm) and 6 M HCl was added (0.1 ml/min) to the sample stream (1.2 ml/min) to release H_2S from the zinc sulfide precipitate present in each sample. H_2S that had passed across the dialysis membrane was then trapped in a 0.01 M NaOH receiving solution flowing (0.80 ml/min) on the other side of the membrane. Two milliliters of this solution was then applied to a Dionex System 12 ion chromatograph (IC) having a Dionex AS3 fast run anion separator column (200 μl sample loop). The IC eluent consisted of 20 mM NaOH, 40 mM H_3BO_3 and 14.7 mM ethylene diamine. A flow-through electrochemical detector that monitored the reaction:

$$2\,Ag + S^{2-} \rightarrow Ag_2S + 2e^-$$

was then used to detect and quantitate sulfide present in column effluents. In this study, optimal quantitation (peak height as well as near full-scale linearity) was found to be maximal at an applied potential of 0.00 V for the silver working electrode versus an Ag/AgCl reference electrode, which also corresponds to the applied potential recommended by others for purely aqueous samples [19]. Using this IC method and coupling it to electrochemical detection resulted in the lower limit of detection for sulfide of 0.02 μg/g S^{2-} in brain tissue, or 0.002 μg/ml S^{2-} of homogenate or solution at a detector sensitivity of 300 nA/V. To obtain an estimate of total brain sulfide levels, the contents of supernatant and pellet fractions derived from each brain were combined. Since subsequent determinations showed that addition of fraction sulfide contents was, in fact, equivalent to sulfide content of whole brain homogenates, various rat brain region homogenates were analyzed directly. Standards containing known amounts of sulfide (sodium sulfide, Na_2S) were processed in parallel throughout the sulfide determinations on experimental samples. Detector sensitivity for these experiments was 1 μA/V.

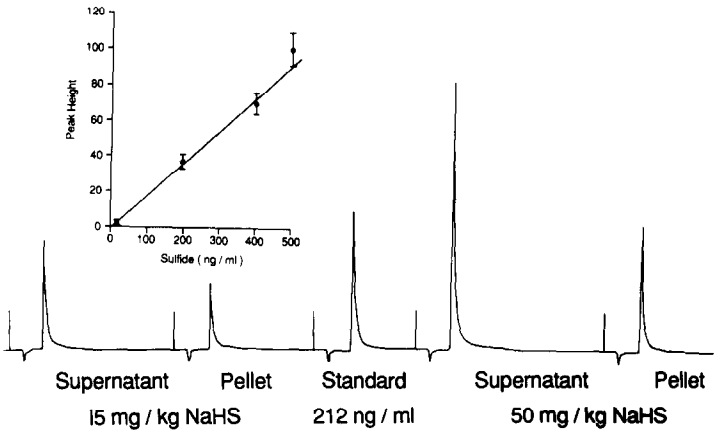

Fig. 1. Rat brain sulfide levels after 15 or 50 mg/kg NaHS in supernatant and pellet fractions analyzed by gas dialysis and ion chromatography. The vertical axis is the output (mV) of the electrochemical detector. Authentic standards of sodium sulfide (Na_2S) were analyzed concomitantly; one such standard is included for purposes of graphic interpolation. The calibration curve for sulfide standards (triplicate determinations) is reproduced in the inset; in these experiments detector sensitivity was 1 μA/V.

The effectiveness of the entire analytical procedure was evaluated in three experiments by addition of exogenous sulfide (1.0-ml aliquot of a 0.05 mg/ml solution) to samples prior to homogenization of brain tissue. Subsequent quantitation after the gas dialysis step showed recovery of exogenous sulfide from whole brain homogenates added to control brain samples to be 81.3 ± 1.35% (mean ± SE, N = 3). No further losses throughout the column separation step were evident.

Amino acid analysis. Brain tissue was prepared for amino acid analysis as described by Kombian *et al.* [16], based on the method of Reiffenstein and Neal [20]. Suitable dilutions were then analyzed by high performance liquid chromotography (HPLC) for taurine and methionine using a pre-column derivatization method [21].

Statistics and calculations. Dose data were evaluated by analysis of variance (ANOVA) as well as Duncan's New Multiple Range Test, adjusted for unequal groups [22]. Sulfide contents of the various brain regions were assessed by Duncan's Multiple Range Test [22]; a P value of 0.05 or less was considered significant. Net uptake for a given brain region was calculated according to the following formula: Net sulfide uptake = (sulfide level after 50 mg/kg NaHS) − (endogenous sulfide level). Linear regression and correlation were performed according to standard statistical procedures [22] utilizing commercially available software. Data describing the sulfide content of subcellular fractions were evaluated using the two-tailed Student's *t*-test for unpaired samples.

RESULTS

Sulfide contents of control and poisoned rat brains. Representative chart recordings as well as the calibration curve for sulfide standards (Fig. 1) depict the relationship between administered dose and the amount of sulfide detectable in whole rat brain fractions (supernatants and pellets) after doses of 15 (the LD_{50}) and 50 mg/kg of NaHS. For comparison purposes a sulfide standard (212 ng/ml) has also been depicted. The equivalent of the sulfide content of a whole brain homogenate could be obtained by addition of supernatant and pellet sulfide contents and was identical to the sulfide content of a whole brain homogenate. The dose relationships obtained by the present method are reproduced in Fig. 2B, which shows that the relationship between the administered dose of NaHS and resulting brain sulfide levels was linear and significant ($P < 0.001$, ANOVA). Brain sulfide levels after NaHS differed significantly ($P < 0.05$ or less) from control (i.e. untreated brain) levels for doses of NaHS at the LD_{50} equivalent or higher. Correlation of lethality data (Fig. 2A) with brain sulfide levels at corresponding doses of NaHS revealed an extremely high degree of correlation (r value = 0.947). It was particularly noteworthy that untreated control brains unexpectedly contained an "endogenous" level of sulfide of 1.57 ± 0.04 μg/g (mean ± SE; N = 16). Regression analysis of all the data points from NaHS-injected animals yielded a y-intercept of 1.54 μg/g.

Specificity of the analytical technique for sulfide. In no instance could appreciable sulfide release be detected. The lack of protein desulfuration or significant alkaline hydrolysis of brain tissue (zero-time homogenates), BSA or sulfur-containing amino acids, particularly cysteine and methionine, may be deduced from Table 1. These results are particularly important as they demonstrate that it was unlikely that the endogenous levels of sulfide were an artifact resulting from either the method or the conditions of homogenization.

Net uptake of sulfide by rat brain regions. Endogenous sulfide levels, as well as net uptake of sulfide of various brain regions after a dose of 50 mg/kg NaHS, are given in Table 2. The brainstem region had an endogenous sulfide level of 1.23 ± 0.06 μg/g (mean ± SE; N = 10) that was significantly lower (22–31%) than the mean values of the other regions. The net uptake of sulfide, on the other hand, was

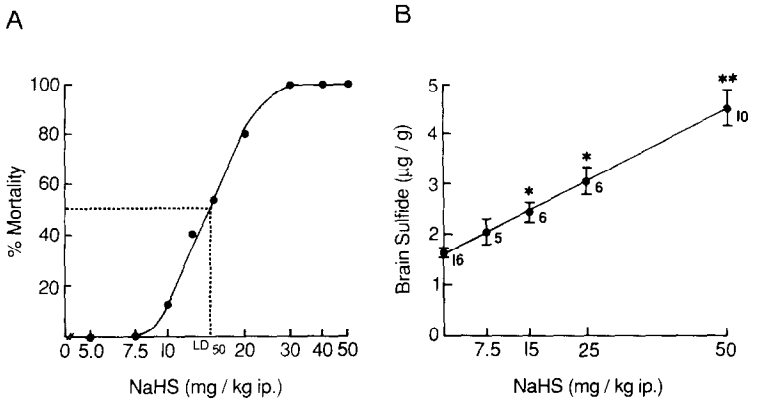

Fig. 2.(A) Dose–response curve for lethality of NaHS in Sprague–Dawley rats. Dashed lines demonstrate the LD_{50} which was found by probit analysis to be approximately 15 mg/kg. Each point represents a mortality estimate derived from five or more animals. (B) Dose-level relationship between brain sulfide levels and the administered dose of NaHS. The dose effect was highly significant ($P < 0.001$, ANOVA); single asterisks (*) denote levels different from the endogenous (i.e. control) as determined by Duncan's Multiple Range Test with $P < 0.05$ as the significance level. The double asterisk (**) indicates that the brain sulfide level at the highest dose tested was significantly different ($P < 0.05$) not only from controls but also from the two preceding doses of NaHS. The correlation of lethality and resulting brain sulfide level for NaHS yielded an r value of 0.947. The brain sulfide level in rats that received 1650 ± 175 ppm gaseous H_2S, a lethal concentration (2.0 to 2.5 × EC_{50}), was 5.00 ± 0.89 μg/g (mean ± SE; N = 5) and did not differ significantly from that of NaHS-injected animals.

Table 1. Specificity of brain sulfide determination

Test compound or tissue*	Sulfide (μg/ml)
Whole rat brain, *immediately* frozen in liquid N_2, homogenized in 20.0 ml of 0.01 M NaOH while still partially frozen, and then *immediately analyzed*	0.174 ± 0.11†
Fresh BSA, 20 mg/ml‡	0.004–0.008
Cysteine, 4 mg/ml§	ND‖
Methionine, 4 mg/ml§	ND
Taurine, 4 mg/ml§	ND
Thiosulfate, 1.6 mg/ml	ND

* Brain tissue and test compounds were all dissolved in 0.01 M NaOH. N = 3 in all cases.

† The sulfide content of this experimental group, expressed in terms of μg/g wet weight, was 1.75 ± 0.10 and did not differ from "non-zero" time analyses where the sulfide content ranged from 1.25 to 1.95 μg/g (mean ± SE 1.57 ± 0.04; N = 16).

‡ BSA was chosen as a suitable model protein for detection of possible sulfide liberation from disulfide bridges as this protein contains 17 disulfide bridges [23]. The concentration of 20 mg/ml BSA protein is approximately twice the protein content of a rat brain homogenate, assuming that protein constitutes 10% of the wet weight of approximately 2 g.

§ The concentrations of these amino acids represent *at least* a 5000-fold excess of the concentrations found in whole rat brain.

‖ The threshold limit of detection for sulfide of the assay at a detector setting of 300 nA/V was 0.002 μg/ml.

ND = not detectable.

highest in the brainstem (3.02 μg/g) and was from 8 to 102% higher than in the other brain regions. In contrast, the striatum had the highest endogenous level of sulfide and showed the lowest uptake of sulfide after NaHS as well as the lowest net uptake. After normalizing to comparable blood flow rates [24], calculation of normalized sulfide uptake for all brain regions showed that the brainstem selectively accumulated sulfide (Table 3). Compared to cortex, which has the next highest net uptake but also the highest blood flow rate, the brainstem accumulated almost 2.5 times more sulfide.

Regional levels of sulfide were correlated with those of methionine, a sulfur containing amino acid, and taurine, an amino acid derived from cysteine (Table 4). A higher correlation of endogenous sulfide level could be demonstrated with taurine ($r = 0.968$) than with methionine ($r = 0.728$).

Table 2. Regional distribution of sulfide in rat brain after i.p. injection of 50 mg/kg NaHS

	Sulfide (μg/g)			
	Endogenous	After 50 mg/kg NaHS	Net uptake*	% of Endogenous†
Brainstem	1.23 ± 0.06‡	4.25 ± 0.34§	3.02	246
Cerebellum	1.58 ± 0.17	3.61 ± 0.26	2.03	128
Hippocampus	1.76 ± 0.13	3.82 ± 0.21	2.06	117
Striatum	1.85 ± 0.13	3.34 ± 0.28	1.49	81
Cortex	1.77 ± 0.08	4.56 ± 0.32‖	2.79	158

For estimation of regional distribution and uptake of sulfide, N = 10 for each brain region in both controls (endogenous sulfide) and animals receiving 50 mg/kg i.p. NaHS. Sulfide was determined as described in Materials and Methods. Values are given as the means ± SE. Endogenous sulfide as well as sulfide levels after 50 mg/kg i.p. in the remaining brain portions (thalamus, hypothalamus, mesencephalon, etc.) were 1.59 ± 0.10 and 3.92 ± 0.15 μg/g respectively.

* Net uptake was calculated as the difference between sulfide levels at 50 mg/kg i.p. minus the endogenous sulfide level.

† Net uptake/endogenous sulfide level × 100.

‡ $P < 0.05$ compared to the other brain regions as determined by multiple comparison using Duncan's Multiple Range Test.

§ $P < 0.05$ compared to striatum.

‖ $P < 0.05$ compared to cerebellum, hippocampus and striatum.

Table 3. Regional blood flow and the net uptake of sulfide in rat brain

	Net uptake (μg/g)	Blood flow* (ml/min/g)	Normalized uptake† (μg/g)	Accumulation ratio‡
Brainstem	3.02	0.60	1.29	2.34
Cerebellum	2.03	0.71	1.52	1.34
Hippocampus	2.06	0.61	1.31	1.57
Striatum	1.49	1.10	2.36	1.58
Cortex	2.79	1.30	2.79	1.00

* Values for regional blood flow were adapted from Siesjo [24].

† All values were normalized to the sulfide uptake of cortex; as the cortex has the highest blood flow of all the regions studied, cortical net uptake was arbitrarily chosen as the normalization standard. The other values were obtained using the formula: Net uptake (cortex) $\times \frac{\text{Blood flow (region)}}{\text{Blood flow (cortex)}}$.

‡ Accumulation ratios were calculated as: $\frac{\text{Measured net uptake}}{\text{Normalized uptake}}$ for a given brain region.

Table 4. Correlation of endogenous sulfide level of rat brain regions and levels of sulfur-containing amino acids

	Endogenous sulfide* (μg/g)	Methionine† (μmol/g)	Taurine† (μmol/g)
Brainstem	1.23 ± 0.06	0.088 ± 0.020	1.81 ± 0.23
Cerebellum	1.58 ± 0.17	0.049 ± 0.010	4.95 ± 0.40
Hippocampus	1.76 ± 0.13	0.068 ± 0.014	5.88 ± 0.76
Striatum	1.85 ± 0.13	0.041 ± 0.005	8.49 ± 0.53
Cortex	1.77 ± 0.08	0.065 ± 0.017	7.21 ± 0.74
		$r = 0.728$	$r = 0.968$

* Analysis was carried out as described in Materials and Methods; N = 10 for each brain region.

† Amino acid analyses were conducted as described under Materials and Methods. Values are means ± SE; for methionine, N = 9 for each brain region; for taurine, N = 13 for each brain region.

Subcellular distribution of sulfide in rat brain. Approximately 27% of the endogenous sulfide content of whole rat brain homogenates was found in the fraction enriched in mitochondria; synaptosomal and myelin fractions contained 5.0 and 2.5%, respectively, of the homogenate sulfide content (Table 5). After poisoning with 50 mg/kg of NaHS, the fraction with the greatest increase in sulfide was the one enriched in myelin; compared to the myelin fraction from control animals, the increase in the myelin fraction from sulfide-poisoned brain was almost 3-fold. The increase in mitochondrial sulfide

Table 5. Sulfide distribution in rat brain subcellular fractions from control and NaHS-treated animals

	Homogenate	S_1	P_2	Myelin	Synaptosomes	Mitochondria
Controls N = 4	6.33 ± 0.58	3.56 ± 0.73 (56.2)	2.81 ± 0.30 (44.4)	0.17 ± 0.06 [6.7]	0.32 ± 0.01 [12.6]	1.68 ± 0.06 [65.9]
NaHS, 50 mg/kg i.p. N = 4	21.68 ± 5.19	8.45 ± 1.32 (39.0)	6.10 ± 1.31 (28.0)	0.48 ± 0.09* [8.6]	0.68 ± 0.09† [12.3]	3.87 ± 0.35‡ [69.7]
% Increase (NaHS/controls × 100)				282.4	212.5	230.4

Fractionation as well as sulfide analyses were carried out as described in Materials and Methods. Sulfide content of fractions is given in μg/fraction and expressed as means ± SE. Figures in parentheses represent recovery as percent of respective homogenates. Although apparent recoveries in S_1 and P_2 fractions from sulfide-treated brain appear lower than those of controls, statistical comparison of the respective recoveries did not reveal any significant differences. The figures in square brackets represent the percent recovery from the corresponding P_2 fractions. The removal of a 1.0-ml aliquot for analysis of P_2 sulfide content was taken into account in calculating the percent recovery of sulfide in myelin, synaptosomal and mitochondrial fractions.

*–‡ Significantly different from control as evaluated by Student's *t*-test with 6 degrees of freedom: * $P < 0.05$, † $P < 0.005$, and ‡ $P < 0.01$.

after poisoning with NaHS was marginally less, with a 2-fold increase (230%). Synaptosomal increases after poisoning were 212% compared to similar fractions for control brains. Table 5 summarizes the findings for sulfide distribution of the various subcellular fractions.

DISCUSSION

This report demonstrates for the first time detection and measurement of sulfide in the CNS. The technique appears to be specific, reproducible and highly sensitive with a lower detection limit in the 2–5 ng/ml range. Although protein desulfuration has been described previously [25, 26], the possibility that alkaline hydrolysis of disulfide linkages of CNS proteins led to spurious sulfide readings was rejected after extensive studies on BSA, a protein with 17 disulfide residues [23]. Under our conditions of homogenization and assay, a solution of 20 mg/ml of BSA released negligible amounts of sulfide. This is consistent with recent measurements of cystine hydrolysis in alkaline media where it was shown that liberation of sulfide is minimal at pH 11–12 [14], a conclusion in agreement with the earlier polarographic measurements of Stricks and Kolthoff [27]. Analysis of zero-time controls also further attest to the validity of the method for brain sulfide quantitation since resulting sulfide levels in these samples were consistent with all previous observations in untreated brains. Thus, it is also unlikely that observed measurements were of artifactual origin due to the breakdown of a naturally occurring constituent of brain tissue such as a protein or an amino acid.

The presence of detectable sulfide (1.57 ± 0.04 μg/g) in all untreated animals indicates that there is an endogenous level of sulfide in the brain. Furthermore, this endogenous level of sulfide could also be demonstrated in the various brain regions. Although the exact function of this sulfide is unknown, there are enzymatic processes that lead to the formation of H_2S and to its rapid metabolism *in vivo* [28, 29]. Therefore, it remains to be seen whether endogenous levels are of physiologic significance with respect to mechanisms underlying the control of neuronal excitability. Furthermore, it will be of interest to determine whether elevation of intraneuronal or CNS sulfide levels correlations with known pathophysiological states characterized by failure of central respiratory drive [30].

It was somewhat surprising that lethality was reflected in sulfide levels less than 2-fold greater than endogenous values. Closer examination of the mortality data, however, showed that a similar phenomenon was apparent in that the dose–response relationship was very steep and, in fact, correlation of mortality–dose data with brain level–dose data provided excellent corroboration ($r = 0.947$) of the mortality–brain level relationship. Brain sulfide levels following H_2S intoxication were approximately 10% of those expected from the dose administered (assuming uniform tissue distribution), and increased in a dose-dependent manner. The low amounts present reflect either the extreme lethality of H_2S in the CNS or the probable metabolism and possible

formation of non-labile species of sulfide [29]. Extrapolation from sulfide values of poisoned brains (corresponding to the various doses of NaHS used) led to a sulfide value of 1.54 μg/g as the y-intercept, or predicted endogenous sulfide level of rat brain. This value easily falls within the range of endogenous sulfide levels measured in untreated (control) animals (1.57 ± 0.04 μg/g expressed as the mean ± SE, N = 16). Such a close correspondence between the calculated and experimentally measured control levels further reflects the high degree of reproducibility of this method in quantitating brain sulfide.

Studies on the endogenous sulfide level of various brain regions revealed that the brainstem, containing the medullary respiratory centers [31], had the lowest endogenous level of sulfide. After treatment with 50 mg/kg NaHS, the brainstem also displayed the greatest uptake of sulfide. Upon comparison of blood flow rates to the various brain regions studied, it became apparent that this region selectively accumulated sulfide. A partial explanation for this observation may lie in that the composition of the lower brainstem (medulla + pons) is approximately 50% grey matter and 50% white matter. White matter contains more lipids than grey matter [32], and the 5-fold greater solubility of H_2S in lipophilic solvents as compared to water [33] suggests that H_2S may be preferentially concentrated in membranes characterized by a high lipid content. To some extent the experimental data support this view. The cortex, which consists entirely of grey matter, has an accumulation ratio for sulfide of one. Brainstem, composed of equal parts grey and white matter, has an accumulation ratio of 2.34. If the brainstem were composed exclusively of white matter, then the expected accumulation ratio could be calculated to be 4.68, or very close to the theoretical value of 5.

Correlation of methionine and taurine levels with endogenous sulfide levels showed only a strong relationship with the latter in the various brain regions. This finding is not surprising since cysteine, the precursor of H_2S *in vivo* [28], also functions as a biosynthetic precursor for taurine [34]. Low levels of taurine may then presumably reflect the corresponding low levels of cysteine and, therefore, lower endogenous sulfide levels. The low levels of taurine in the brainstem as compared to the taurine levels of other brain regions are in agreement with other recent work [35], and may be noteworthy in light of a recent report that the combination of H_2S and taurine totally abolishes sodium currents in patch-clamped neuronal cells [36]. The lesser correlation between regional endogenous sulfide levels and methionine content corroborates the evidence that this amino acid does not function as an H_2S precursor *in vivo*. Thus, it may not be surprising that the highest methionine level is found in the brainstem.

Subcellular fractionation studies clearly demonstrated that H_2S intoxication results in sulfide uptake into nerve cells since the sulfide content of synaptosomes increased. In addition, the sulfide contents of the mitochondrial and myelin fractions also increased. The failure of an earlier study [37] to detect intramitochondrial sulfide in all likelihood reflects the greater sensitivity of detection for sulfide in the present method. The relative increases (compared to controls) in sulfide content of the individual fractions derived from the P_2 fraction of 50 mg/kg NaHS-treated brains were similar to those of whole brain homogenates and were 2- to 3-fold.

The sulfide content of mitochondrial fractions from poisoned brains was 3.87 ± 0.35 μg. Since the total volume of sample was 10.0 ml, the sulfide concentration can be calculated to be in the range 9.9 to 14.3 μM. In this regard, Powell and Somero [38] found that ATP production of rat liver mitochondria is inhibited completely by 20.0 μM sulfide, a concentration not far from that encountered in the present study. Furthermore, over one-quarter of the endogenous sulfide content of whole brain, and over half of the sulfide content of the P_2 fraction could be accounted for within fractions enriched by mitochondria. This finding may indicate that H_2S formed by metabolism *in vivo* selectively accumulates in mitochondria for purposes of detoxification through oxidation. All previous evidence indicates that the primary site of sulfide oxidation is mitochondrial, where sulfate and thiosulfate are the primary oxidation products [39, 40]. Since H_2S has been traditionally considered a mitochondrial poison [1], the extent that biochemical indices of lethality may be projected from subcellular fractions to the whole brain level further testifies to the reliability of the present method.

Sulfide toxicity in the CNS may be explained, at least in part, by the action of H_2S and HS^- on cytochrome aa_3 [41]. Indeed, H_2S is a more potent inhibitor of this cytochrome than cyanide [42]. However, there may be other explanations for H_2S toxicity such as the formation of persulfides (general formula: RSSH). Sulfhydryl modification leading to altered enzymatic function has been shown for over a dozen enzymes after persulfide generation [29]. Similarly, receptor and ion channel function as well as signal transduction could also change under such conditions. Studies of calcium channel antagonist binding have already demonstrated sulfhydryl modification of receptor protein by mercaptans acting as sulfide donors [43].

The possibility of quantitating sulfide in the CNS opens new vistas for toxicology and neuropharmacology. Measurement of sulfide in brain, as opposed to blood [44], may lend itself to post-mortem analysis of suspected H_2S toxicity. Preliminary results with rats poisoned with 50 mg/kg NaHS and stored at 4° suggest that the method is useful for at least 36 hr post-mortem. Control brains contained 1.55 ± 0.17 μg/g sulfide, whereas sulfide-poisoned brains had 2.45 ± 0.04 μg/g ($P < 0.01$, N = 3 in both cases). This highly reproducible and sensitive method has already been used in this laboratory to detect endogenous sulfide levels in samples of normal human post-mortem brainstem, where the endogenous sulfide level was 0.67 ± 0.05 μg/g (mean ± SE, N = 5).

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