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Diagnosing hydrogen sulfide toxicosis with a silver/sulfide ion-selective electrode

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Diagnosing hydrogen sulfide toxicosis

with a silver/sulfide ion-selective electrode

by

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1993

It was the best of times,

it was the worst of times,

it was the age of wisdom,

it was the age of foolishness,

it was the epoch of belief,

it was the epoch of incredulity,

it was the season of Light,

it was the spring of hope,

it was the winter of despair.

- Charles John Dickens
A Tale of Two Cities

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ABSTRACT

The objective of this project was to develop a rapid, simple and inexpensive quantitative analysis to confirm hydrogen sulfide toxicosis in livestock based on acid extraction of sulfide in a wash-bottle unit and potentiometric determination with an ion-selective electrode (ISE). A silver/sulfide ISE and double-junction reference electrode coupled to a digital pH/mV meter with an automatic temperature compensation probe provided rapid and stable measurement of sulfide down to 0.02 ppm S^{-2} using a liter-beaker calibration technique. The analysis was further facilitated by sealing standardized solutions and extracted unknown samples in serum bottles after flushing with nitrogen gas and storing at 4°C.

The wash-bottle developed during the research provided excellent sulfide recoveries from aqueous samples (87 to 96%), but extraction from animal tissues was plagued by sample coagulation, foaming and poor to excellent percent recoveries. Whole and clotted blood were the worst samples for coagulation and foaming problems, compared to brain, lung, serum or plasma. Diluting the sample prior to extraction, and acidifying with dilute acids prevented coagulation. Foaming was combated with a mineral oil-poloxalene mixture (50:50 v/v), redesigning the wash-bottle, and reducing the nitrogen flow rate. Mean percentage of spike recovered were: blood, 7 to 43; blood clot, 13; serum, 58; plasma, 80; brain, 83 to 101; and lung, 76 to 102.

Poor sulfide recovery and concentrating ability of the extraction unit prevented estimating endogenous blood sulfide concentrations (<0.02 ppm S^{-2}). However, the background sulfide content of whole brain and lungs from Sprague-Dawley rats were 0.69 ± 0.281 ppm (n = 12) and 0.63 ± 0.113 ppm (n = 12), respectively. Endogenous sulfide concentration in cerebrum from pigs was 1.89 ± 0.193 ppm (n = 18).

After fatal exposure of rats by injection of pH corrected sulfide solutions (0, 10 or 20 mg S^{-2}/kg BW IP) or hydrogen sulfide inhalation (0 or 2090 ± 260 ppm H_2S), the analysis detected elevated concentrations of acid-labile sulfide in blood, lung and brain. The lower sulfide salt treatment (n = 6) resulted in elevating the brain sulfide to 0.98 ± 0.170, blood 0.12 ± 0.086, and lung 0.69 ± 0.31 ppm. Concentrations of 1.19 ± 0.101, 0.14 ± 0.48 and 1.7 ± 0.46 ppm S^{-2} (n = 6) were measured in the brains, blood and lungs, respectively, of rats parenterally treated at the higher dosage. The inhalation exposure increased the brain, blood and lung sulfide levels to 1.61 ± 0.198, 0.12 ± 0.105 and 0.85 ± 0.218 ppm (n = 8), respectively. However, these increases in sulfide were often not significantly different ($P > 0.05$) from endogenous levels due to the large variance in the resulting sulfide estimates.

Specimens should be collected as soon as possible, with steps taken to preserve the sulfide content (refrigeration, flash freezing, or zinc precipitation), rapid delivery to the laboratory under preservation, and prompt analysis.

INTRODUCTION

A definitive diagnosis in a suspect hydrogen sulfide poisoning presents a diagnostic dilemma to veterinary practitioners and toxicologists. The history often involves the sudden death of livestock in a confinement facility utilizing a liquid manure pit for the waste management system. Since lethal levels can result in peracute death, often no clinical signs occur, and postmortem and histopathologic lesions are absent. With atmospheric hydrogen sulfide concentrations diluted by the ventilation system or by opening of the facility, air samples are typically not representative. Therefore, diagnosticians are faced with the problem of solving a mystery based on circumstantial evidence.

The objective of this project was to develop a rapid, inexpensive and simple quantitative analysis to confirm hydrogen sulfide toxicosis in livestock based on the ion-selective electrode (ISE) work of McAnalley *et al.*[159] The first step required determining suitability of various cyanide and silver/sulfide ISEs for the analysis, and then developing separation, calibration and measurement techniques. Once established in aqueous solutions, this methodology was applied to various tissue matrices spiked with known amounts of sulfide to determine the recovery rate. After devising techniques for injecting buffered sulfide solutions and generating and delivering hydrogen sulfide atmospheres, the methodologies were applied to whole animals to confirm tissue sulfide levels following an acute exposure and as well as subsequent postmortem changes.

SELECTED REVIEW OF LITERATURE

Hydrogen Sulfide

Properties

Hydrogen sulfide is a highly toxic, colorless, irritating gas, which at low concentrations has a characteristic "rotten egg" odor. Although polysulfides and hydrosulfides exist,[142,172] hydrogen sulfide usually denotes H_2S, or physiologically, the sulfide and hydrosulfide ions.[21] Chemical and physical properties are summarized in Table 1, with other reviews available.[21,142,172,268,272]

At physiologic pH (7.4) hydrogen sulfide exists as H_2S and anionic forms according to the following formulas:[142,172]

$$H_2S \rightleftarrows H^+ + HS^- \qquad pK_a = 7.04 \qquad (1)$$

$$HS^- \rightleftarrows H^+ + S^{-2} \qquad pK_a = 11.96 \qquad (2)$$

Therefore, one-third would exist in the hydrogen sulfide (H_2S) form, two-thirds as hydrosulfide (HS^-), and a small amount as sulfide (S^{-2}). The principles of non-ionic diffusion for transit across phospholipid bilayer membranes dictate that nonpolar molecules (H_2S) will cross more rapidly than ionized forms.[172] Also, hydrogen sulfide is the form believed to be the more active inhibitor of cytochrome oxidase.[12,172,253]

Sources

Hydrogen sulfide can be of natural or industrial origins. Natural sources result from anaerobic degradation of sulfurous organic materials and would include natural gas, petroleum, coal, volcanic gases, sulfur springs, and lakes.[21,172] Estimated annual production from environmental emissions is 90 to 100 million tons/year, with 60 to 80 million tons from terrestrial sources and 30 million tons from aquatic origins.[248] Industrial sources of hydrogen sulfide include any process in which sulfur or sulfurous compounds interact with organic materials at elevated temperatures.[172] Annual industrial emissions are estimated at 3 million tons/year.[248]

The transformation and translocation of sulfur through the environment is described as the "sulfur cycle."[47] In the scheme, which depicts hydrogen sulfide as a major product, sulfur passes from atmospheric, bacterial, plant or animal phases as predominately influenced by microbial metabolism (Figure 1). Details of these reactions have been discussed.[47,172] Vismann[255] adapted the sulfur cycle to a marine water/sediment environment.

Table 1. Chemical and physical properties of hydrogen sulfide.[a]

Properties	Value
Formula	H_2S
Molecular weight	34.08
Percent Hydrogen	5.92 %
Percent Sulfur	94.09 %
Specific Gravity (0°C, 760 mm Hg)	1.539 mg/l
Gas density (air = 1.00)	1.19
Melting point	-85.5°C
Boiling point	-60.7°C
Flammability	Pale blue flame
Ignition temperature	260°C
Explosive limits (air mixture)	4.3 - 49 %
Solubility (1 g H_2S in X ml solvent)	
Water (10°C)	187 ml
Water (20°C)	242 ml
Water (30°C)	314 ml
Ethanol (20°C)	94.3 ml
Ether (20°C)	48.5 ml
Glycerol	Soluble
Carbon disulfide	Soluble
Petroleum products	Soluble
Conversion factors (25°C, 760 mm Hg)	
1 mg/l = 717 ppm	
1 ppm = 0.00139 mg/l	
1 ppm = 1.4 mg/m^3	

[a]Sources: Windholz *et al.*,[272] Beauchamp *et al.*,[21] and Lide.[142]

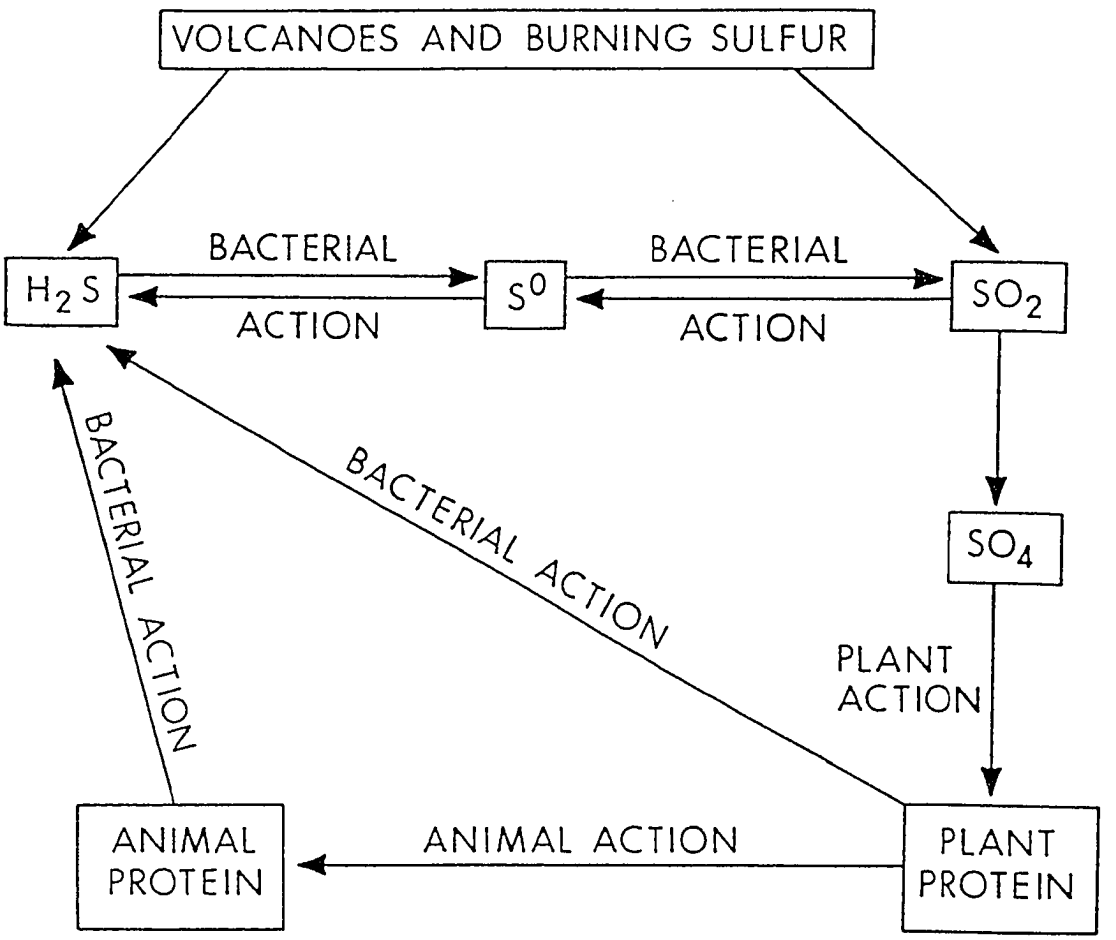


Figure 1. The sulfur cycle illustrating hydrogen sulfide as a major product of microbial metabolism. Reprinted with permission from Cooper RC, Jenkins D, Young L. *Aquatic Microbiology Laboratory Manual.* Austin, Texas:University of Texas, 1976.

Ambient concentrations

Since hydrogen sulfide sources are natural and industrial, background levels will vary with season and location.[268] Estimates of global averages range from 0.1 to 0.2 ppb.[162,204,268] Concentrations in rural areas are about 0.4 ppb, compared with 0.07 to 0.7 ppb and >0.07 ppm for urban and industrial regions, respectively.[21]

Over sixty gases associated with anaerobic digestion of liquid manure have been reported in livestock production units.[54,62,67,171] The most important and potentially toxic gas is hydrogen sulfide.[8,35,54,62,83,144,166,171,178,179,195,208] Peak levels of 0 to 4.5 ppm H_2S are reported under normal operating conditions in swine confinement units, with mean concentrations of 0.09 to 1.7 ppm.[43,66,67,154,208,247] A mathematical model of manure gas production in swine confinement units in the upper midwestern United States estimates 0 to 0.42 ppm H_2S under normal operating conditions.[8] O'Conner *et al.*[179] reported the maximum hydrogen sulfide level in broiler breeder houses in Alberta, Canada, at 40 ppb.

Hydrogen sulfide can reach toxic levels in confinement units of poor design or management, especially when associated with manure pit agitation or inadequate ventilation.[54,66,83,171,195,208] Vigorous agitation of manure pits to facilitate emptying can generate >800 to 1000 ppm H_2S in the animal area.[54,62,83,144,208] Ventilation by itself is a less significant factor.[171,208]

Air quality standards

Air quality standards are established by various national and local regulatory groups, and advisory organizations to protect human and animal health. The difficulty of determining these exposure limits have been discussed.[11,21,172] Some workplace and environmental standards for hydrogen sulfide are listed in Table 2. Since threshold limit values-time weighted averages (TLV-TWA) are designed for occupational exposures in humans (8 hr/d, 5 d/wk), extrapolation to animals continually housed in confinement units for life is erroneous.[8,63,171,208]

Pharmacodynamics

Absorption

Hydrogen sulfide is rapidly and almost exclusively absorbed by the pulmonary route.[21,72,172,253] Some microbial H_2S in ruminant forestomaches is eructated, inhaled and absorbed through the lungs.[27,82,94,119,216] Percutaneous absorption is minimal, if at all existent.[21,72,172,253] Although Yant[275] stated that cutaneous absorption does not occur, toxicoses have been produced under experimental conditions involving large surface areas and high to pure concentrations of hydrogen sulfide.[194,257] Human poisoning has resulted from

Table 2. Human and livestock air quality standards for hydrogen sulfide.

Standard	Limits	Source
TLV[a]- TWA[b]	10 ppm	ACGIH[1]
STEL[c]	15 ppm	ACGIH[1]
OSHA PEL[d] (transitional limit)		OSHA[186]
PEL - TWA	None	
Ceiling limit	20 ppm	
PEL - STEL (10 min)	50 ppm	
OSHA PEL (final rule limit)		OSHA[186]
PEL - TWA	10 ppm	
Ceiling limit	None	
PEL - STEL	15 ppm	
NIOSH Occupational Exposure Std. (10 min)	15 mg/m^3 (11 ppm)	RTECS[206]
Short Term Inhalation Limits		CHRIS[42]
10 minutes	200 ppm	
30 minutes	100 ppm	
60 minutes	50 ppm	
Livestock Housing		
Cattle barns (Germany)	0.002% (20 ppm)	Haartsen[95]
Farm buildings (Scotland)	5 ppm	O'Connor et al.[179]

[a]TLV = threshold limit value.
[b]TWA = time weighted average.
[c]STEL = short-term exposure limit.
[d]PEL = permissible exposure limit.

the use of sulfurous dermatological and ammonium sulfide permanent wave solutions.[88] Gastrointestinal absorption of enteric hydrogen sulfide or sulfide, or orally and rectally administered exogenous doses have produced systemic effects at elevated levels.[82,172,216] Absorption through the ruminal wall is rapid and a function of sulfide concentration.[216]

Distribution

Radiolabled sulfide salts have been used to evaluate distribution in the whole animal. On the first day post-subcutaneous injection of rats with a $Na_2{}^{35}S$ solution, Gunina[93] reported high specific activity in the kidney and spleen, moderate amounts in the liver, brain and lungs, and low levels in the heart and skeletal muscle. Day six post-injection, the brain, lung, spleen and kidney contained relatively low specific activity; and the liver, heart, and skeletal muscle trace amounts. Intravenous injection of four to six week-old rats with $Na_2{}^{35}S$ and subsequent whole-body autoradiographies at 3 minutes to 6 hours post-injection reveals wide distribution in gastrointestinal and cartilaginous tissues.[53] In adult sheep on a normal ration, one-third of a ruminally administered 3.0 g/d ^{35}S-sulfate dose is microbially converted into sulfide. Some crosses the rumen wall, passes into the abomasum, or is synthesized into microbial proteins.[109,216]

A limited amount of work exists on the fate of inhaled radiolabled H_2S in the body. In an experiment with rats and guinea pigs, histochemistry demonstrated sulfide in the brain, liver, kidney, pancreas and small intestines.[256]

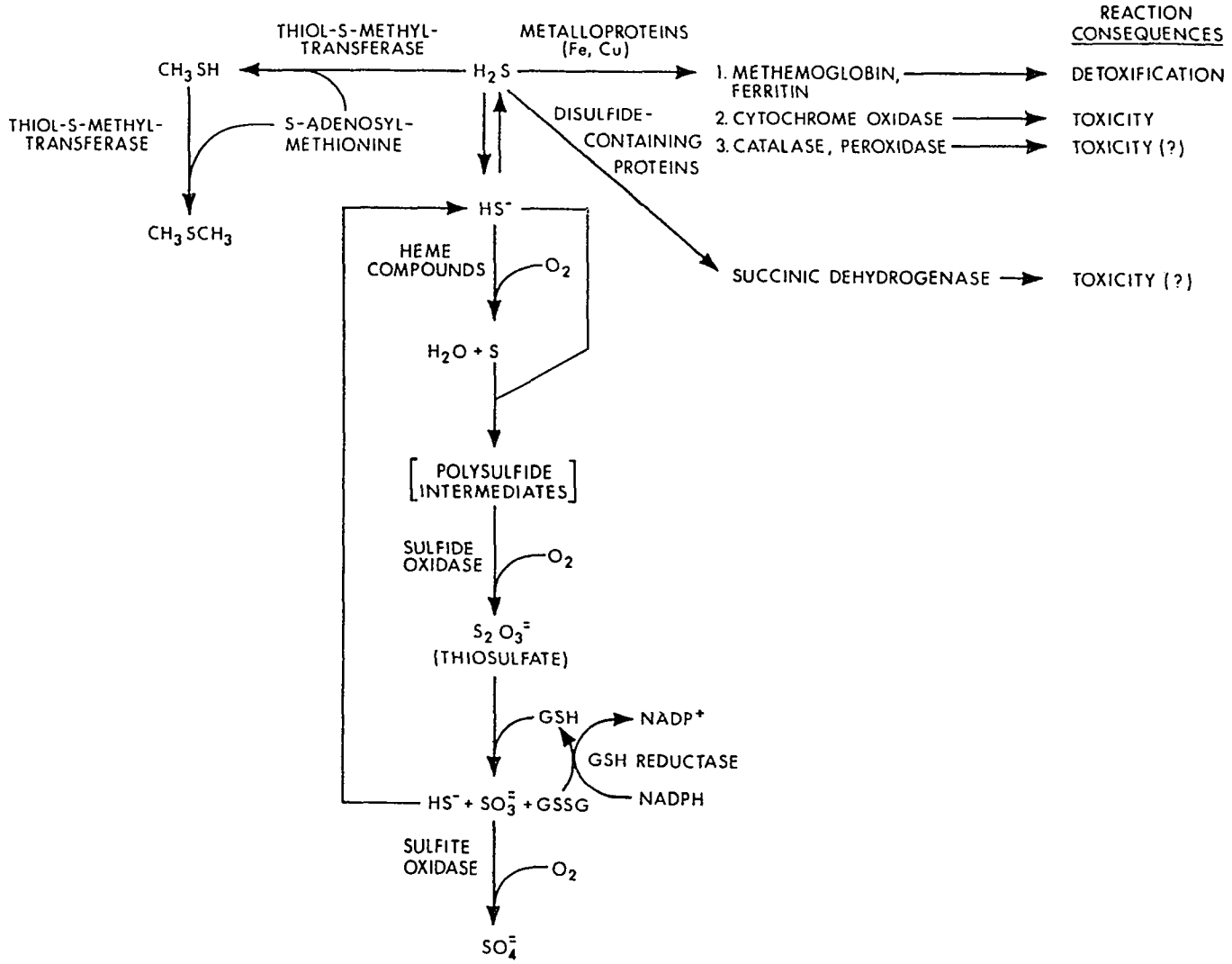
Metabolism

The metabolism of hydrogen sulfide has been reviewed, [21,172,255] with the conclusion that it has not been fully elucidated.[21,133,255] Sulfide metabolism involves sequential enzymatic and non-enzymatic oxidation to sulfate, enzymatic methylation to dimethylsulfide, and reaction with metallo- and disulfide-containing proteins (Figure 2).[21]

Oxidation Oxidation of sulfide to sulfate is the primary detoxification pathway.[15,19,20,53,93,228] A majority of the oxidative capacity is found in the hepatic mitochondrial fraction, although metabolism also occurs in the kidney, lung and blood.[19,20,53,74,228] Intermediates identified include free sulfur, polysulfides, thiosulfate and sulfite (Figure 1).[19,20,228,229]

Methylation Sequential enzymatic methylation of hydrogen sulfide by thiol S-methyltransferase to form dimethylsulfide is a detoxification mechanism for enterally produced H_2S (Figure 1). The highest enzyme activity is found in the large intestines, with lower levels in the stomach, liver, lung and kidney.[265] The capacity and role of this pathway against toxic exogenous H_2S has not been determined.[21]

Figure 2. Proposed metabolic pathways of sulfide in animals. Reprinted with permission from Beauchamp RO, Bus JS, Popp JA, *et al.* A critical review of the literature on hydrogen sulfide. *CRC Crit Rev Toxicol* 1984;13:25-97.


THIOL-S-METHYL-TRANSFERASE
METALLOPROTEINS (Fe, Cu)
REACTION CONSEQUENCES
CH_3SH
H_2S
1. METHEMOGLOBIN, FERRITIN
DETOXIFICATION
THIOL-S-METHYL-TRANSFERASE
S-ADENOSYL-METHIONINE
DISULFIDE-CONTAINING PROTEINS
2. CYTOCHROME OXIDASE
TOXICITY
3. CATALASE, PEROXIDASE
TOXICITY (?)
CH_3SCH_3
HS^-
HEME COMPOUNDS
O_2
SUCCINIC DEHYDROGENASE
TOXICITY (?)
$H_2O + S$
[POLYSULFIDE INTERMEDIATES]
SULFIDE OXIDASE
O_2
$S_2O_3^=$
(THIOSULFATE)
GSH
$NADP^+$
GSH REDUCTASE
NADPH
$HS^- + SO_3^= + GSSG$
SULFITE OXIDASE
O_2
$SO_4^=$

Protein interactions Interaction of hydrogen sulfide with proteins may represent detoxification or lethal mechanism depending on the essentiality of the molecule, degree of alteration, and resulting biochemical consequences (Figure 1). Ions or metabolites of H_2S can react with metallo-proteins and disulfide groups within the secondary and tertiary structure of proteins.[21,255] In general, these interactions may reduce biological activity due to alteration in active site, receptor, conformation, or ion channels.

Metallo-proteins known to interact with hydrogen sulfide include: catalase,[231] cytochrome oxidase,[12,125,175,176,211,241,270] horseradish peroxidase,[269] potato polyphenol oxidase,[122] tyrosinase,[122] free heme,[18,20,228,229] hemoglobin,[74,228] methemoglobin,[224-226,232] myoglobin,[174,192] and ferritin.[18] Inhibition of mitochondrial cytochrome oxidase and subsequent disruption of oxidative metabolism is considered the primary biochemical lesion in hydrogen sulfide toxicosis.[74,125,172,175,224] Heme compounds, such as hemoglobin, heme and hemin, are also associated with sulfide oxidation.[20,74,228] Formation of sulfmethemoglobin from hydrosulfide and methemoglobin is considered a minor detoxification pathway for endogenous sources of H_2S.[225] The rapid oxidation of sulfide to thiosulfate catalyzed by ferritin has been demonstrated.[18]

Reaction of the hydrosulfide anions with disulfide bridges or sulfhydryl groups in proteins is an additional metabolic pathway with potential for significant biochemical consequences.[53,223,258,259] Enzymes implicated in this mechanism include cytochrome oxidase,[223] succinic dehydrogenase,[25] and monoamine oxidase.[259] Reaction with disulfide bonds or sulfhydryl groups in structural or nonessential proteins may represent an important sequestering pool for sulfide.[21] Curtis *et al.*[53] reported that sulfide binding to blood protein is rapid and significant, and available for subsequent oxidation to sulfate.

Others An additional route of hydrogen sulfide detoxification of unknown significance is its oxidation catalyzed by cations of calcium, cobalt, copper, iron, magnesium, manganese, and nickel in the presence of oxygen.[41]

Excretion

Hydrogen sulfide is primarily oxidized to sulfate and excreted by the urinary system.[53,71,93] Dziewiatkoski[71] orally dosed rats with ^{35}S-barium sulfide and recovered 50% of the label as sulfate within 24 hours post-administration. Similarly, rats administered ^{35}S-sodium sulfide intraperitoneally or orally excrete 84 to 93% and 52 to 69%, respectively, over 48 hours. The major portion of the label is eliminated within the first 24 hours, with forty-five percent collected in the urine within six hours.[53]

Biliary elimination of oral and parenteral sulfide is minimal. Fecal recovery of labelled ^{35}S from rats orally dosed with barium sulfide is 8 to 10% in 24 hours.[71] Rats with cannulated bile ducts excrete 4.7 to 5.0% of the label from ^{35}S-sodium sulfide in the bile within six hours.[53]

The occurrence of pulmonary excretion is controversial, but insignificant if existent. Intravenous administration of ^{35}S-sodium sulfide in dogs results in <0.5% being eliminated by respiration, with most appearing within the first minute.[93] Evans[74] rapidly injected sulfide into the jugular, femoral or ear vein of rats and rabbits and reported detection of hydrogen sulfide on the breaths in 1 to 10 seconds, with none after one minute. Similar results are noted for large, rapid intra-arterial injections, but none is detected if large doses are slowly administered. Conversely, no hydrogen sulfide or volatile sulfur metabolites are exhaled from mice injected intraperitoneally with up to LD_{50} doses, with the conclusion that pulmonary elimination is insignificant (<0.1%).[235]

With ruminal microflora potentially producing large amounts of hydrogen sulfide from dietary sources,[82,216] eructation of the gas in ruminant animals may be a major excretion route. Hydrogen sulfide is reported on the breaths of animals in cases of sulfur toxicosis in cattle.[45,94,216] Experimentally, Bird[27] noted the gas on the breaths of sheep intraruminally infused with sodium sulfide solution to produce clinical poisoning. Similarly, calves fed diets elevated in sulfate to produce polioencephalomalacia frequently had H_2S on their breaths.[89]

Toxicity

Hydrogen sulfide is a highly toxic gas as characterized by its steep slope of the dose-response curve and small difference between LD_0 and LD_{50} doses. The range between 1 and 100% mortality is about twice the threshold level.[236] Lethality results at less than a two-fold increase of sulfide concentration over endogenous levels in the brain.[258] In general, exposure to concentrations >500 to 700 ppm H_2S will likely produce clinical signs compatible with acute toxicosis.[21,72,96,172]

Lethal concentrations and doses

Relative toxicity of a compound can be expressed as the percentage of a population under defined exposure and observation conditions that exhibit a given qualitative effect, as extrapolated from the cumulative dose-effect curve (percentage versus $\log_{10}$ dose). Population, exposure and observation variables may include: species, strain, age, sex, body weight, reproductive status; dose, route, location, duration; and toxic effect, duration post-exposure, physical conditions. With death as the end-point, the statistic calculated is often the

Table 3. Published lethal concentrations for hydrogen sulfide.

Species	Dosage	Criteria	Source
Mice	100 ppm 7hr	LC_{50}, n/a	Hays[101]
	50 ppm 15hr	LC_{50}, n/a	
	30 ppm 18.5hr	LC_{50}, n/a	
	10 ppm >5day	LC_{50}, n/a	
Rat	444 ppm 4hr	LC_{50}, SD[a], ♂&♀, 90-100g BW, 14d observation	Tansy *et al.*[236]
	549 & 587 ppm 2hr	LC_{10} & LC_{50}, LE[b]+SD+F344[c], ♂&♀, 14d observation	Prior *et al.*[198]
	422 & 501 ppm 4hr	LC_{10} & LC_{50}, LE+SD+F344, ♂&♀, 14d observation	
	299 & 335 ppm 6hr	LC_{10} & LC_{50}, LE+SD+F344, ♂&♀, 14d observation	
	301 & 628 ppm 2-6hr	LC_{10} & LC_{50}, LE, ♂&♀, 14d observation	
	244 & 713 ppm 2-6hr	LC_{10} & LC_{50}, SD, ♂&♀, 14d observation	
	344 & 608 ppm 2-6hr	LC_{10} & LC_{50}, F344, ♂&♀, 14d observation	
	235 & 617 ppm 2-6hr	LC_{10} & LC_{50}, LE+SD+F344, ♂, 14d observation	
	364 & 641 ppm 2-6hr	LC_{10} & LC_{50}, LE+SD+F344, ♀, 14d observation	
	298 & 644 ppm 2-6hr	LC_{10} & LC_{50}, LE+SD+F344, ♂&♀, 14d observation (pooled)	

[a]SD = Sprague-Dawley rat.
[b]LE = Long Evans rat.
[c]F344 = Fischer-344 rat.

Table 4. Published lethal dosages for sulfide salts.

Species	Dosage	Criteria	Source
Mice	0.53 mM S^{-2}/kg BW IP (17 mg S^{-2}/kg)	LD_{50}, ♂ Swiss-Webster, 25-30g BW	Smith[221]
	0.25 mM Na_2S/kg BW IP (8 mg S^{-2}/kg)	LD_{50}, ♂, 25-30g BW	Smith *et al.*[227]
	0.32 mM Na_2S/kg BW IP (10 mg S^{-2}/kg)	LD_{50}, ♀ CD1, 25-30g BW, 2hr observation	Susman *et al.*[235]
Rat	55 mg Na_2S/kg BW IP (7 mg S^{-2}/kg)	LD_{75}, ♂ Charles River, 210-260g BW, 5 min observation	Bitterman *et al.*[28]
	14.45 mg NaHS/kg BW IP (5 mg S^{-2}/kg)	LD_{50}, ♂ Sprague-Dawley, 250-350g BW, ? observation	Kombian *et al.*[131]
	14.6 mg NaHS/kg BW IP (5 mg S^{-2}/kg)	LD_{50}, ♂ Sprague-Dawley, 250-350g BW, ? observation	Warenycia *et al.*[258]

median lethal dose (LD_{50}) or lethal concentration (LC_{50}), but other percentages can be estimated. Published lethal concentrations and dosages for hydrogen sulfide or sulfide salts are listed (Table 3 and 4).

Toxicity factors

Dosage The dosage of hydrogen sulfide or sulfide salt is a primary factor in determining toxicity; this would include concentration, duration, frequency, route and site of administration, and rate. Gas concentration is more important than length of exposure.[29,266] O'Donoghue[29] demonstrated that when the gas concentration is increased slowly, swine can adapt to higher (250 to 700 ppm) and longer exposures (2 to 3 hr), and still recover upon termination of the trial. Rapid parenteral injection of large doses of sulfide produced toxicity, versus no effect with slower administration; the latter being attributed to detoxification.[74] Dosage and concentration are also related to differences in physiological responses associated with gas levels. At acutely toxic concentrations, apnea results at a lower dose than subacute levels that produce hyperpnea prior to respiratory arrest.[7,241] In addition to rate and concentration injected, site of administration is important in determining metabolism and net physiological results. The difference between lethal subcutaneous and intravenous doses is 10 to 35 fold.[93] Injection site distance from target nerve centers influences the effects.[74]

Animal factors Species differences in toxic concentrations and responses to toxicity are small.[172,212,268] Sayer[219] found the following for acutely toxic hydrogen sulfide exposures (ppm): goats(900) > human(600-1000) > guinea pig(750) > dog(600) > rat(500) > canaries(200). Mice are more sensitive than rats with regard to dose and clinical course.[227] In studying concentration-time interactions in rats exposed to H_2S, Prior *et al.*[198] reported no difference between Sprague-Dawley, Long Evans and Fischer-344 strains of rats. Klentz and Fedde[129] found chickens less sensitive to hydrogen sulfide than mammals, with exposures of 4000 ppm not resulting in immediate death. However, water sulfide levels of 0.5 ppm produces toxicity in catfish, with a clinical course compatible to mammalian H_2S poisoning.[2,241]

Individual factors in hydrogen sulfide susceptibility have been reported. One study involving male and female rats of three strains reports a difference in mortality between the sexes, 30% in the males versus 20%.[198] An age-condition factor has been identified in which neonates are more resistant than juvenile or adult mice, and the heavier body weight animals survive longer.[219,224] Increased sensitivity with repeated exposure is controversial.[5,21,172]

Environmental factors Water pH, temperature and oxygenation are factors influencing sulfide toxicity in fish.[2,241] Beck *et al.*[22] pretreated male Wistar rats with 0.33 or 0.66 mg ethanol/kg BW IP prior to exposure to 800 ppm H_2S and noted a dose response in reducing the mean time to unconsciousness. This could be a factor in ruminants on ensiled feedstuffs or on distillery by-product based rations and elevated dietary sulfur.

Classification

Definitions The progression of clinical and pathological processes are often summarized as acute, subacute or chronic, as applied to hydrogen sulfide toxicosis to describe the onset and duration and thus related clinical signs and concentration of exposure. For unity the NRC proposed the following definitions.[172]

Acute The effects of a short, single, massive exposure (>1000 ppm) that rapidly produces systemic intoxication mediated by free, unoxidized H_2S in circulation acting directly on the nervous system and resulting in respiratory distress. The toxic mechanism is believed to be inhibition of mitochondrial cytochrome oxidase.

Subacute Continuous exposure to moderate concentrations (100 to 1000 ppm) that produce irritative ocular and pulmonary lesions. Eye irritation is most common, but the resulting pulmonary edema is potentially fatal.

Chronic Controversial and often subjective "lingering neurasthenic symptoms" caused by intermittent exposure to low levels (50 to 100 ppm).

As with most classification schemes there is clinical overlap between the subdivisions. Local irritative lesions which are typical of subacute exposures, but are often noted in acute cases.[172] Also, the residual deficits reported after acute toxicosis are sometimes referred to as chronic.[21]

Cumulative toxin or chronic toxicosis ? Since hydrogen sulfide or resulting anions are rapidly detoxified and excreted, it is generally considered a noncumulative toxin[4,96,129,172,222,241,275] without long-term effects.[10,16,36,129] However some feel that insufficient evidence exists to prove or disprove chronic toxicosis.[4,7]

Swine exposed to 1000 ppm H_2S, can recover immediately upon termination of the gas, and appear clinically normal.[180] Torrans and Clemens[241] reported that catfish cytochrome oxidase activity inhibited 50% of normal returned to control levels in six hours. Cows continuously exposed for three weeks at 20 ppm did not develop alterations in heart rate, feed consumption, or lactation.[102] Treatment of male Sprague-Dawley rats with 50 ppm

H_2S 5 d/wk for 25 weeks failed to show significant changes ($P > 0.05$) in sensory or motor nerve conduction velocity, or light and electron microscopic histology.[79] Repeated gas exposures do not cause chronic ocular lesions.[4]

More recent experimental and epidemiological works support the existence of cumulative effects and various forms of chronic toxicosis. Brain total lipid, phospholipid and cholesterol content are reduced in male guinea pigs treated for 1 hr with 20 ppm H_2S for 11 days, with significant differences ($P < 0.05$) in cerebral and brain stem total lipids and phospholipids. Cerebral lipid peroxidation is also significantly increased.[98] NMRI mice exposed for 2 hours per treatment at 4 day intervals to 100 ppm for 4 treatments develop reduced cerebral RNA content and orotic acid uptake into RNA, and increased cytochrome oxidase inhibition.[211] This suggests only partial resolution of depressed activities between gas treatments. Hannah and Roth[99] reported abnormal cerebellar Purkinje cell development in the pups from rats exposed to 20 or 50 ppm 7 h/d from day 5 postcoitus through day 21 postnatal. Periodontal disease induced by microbially produced volatile sulfur compounds, 90% H_2S and CH_3SH, increasing oral mucosal permeability due to altered collagen maturation and gingival fibroblast protein synthesis is a nonconventional, chronic focal toxicosis.[173] Elevated sulfate diets induced polioencephalomalacia in calves.[89] Similarly, nervous signs that develop while infusing sheep intraruminally with sodium sulfide are not immediately reversible and accumulate over time.[27]

A case report involving cattle housed in a slatted floor barn and one human case associated with an industrial source suggest chronic toxicosis.[24,80] In the human report, a 20 month-old neonate living near a coal mine burning high sulfur wastes developed an encephalopathy. The maximum recorded H_2S level in the house over a four month monitoring period was 0.6 ppm.[80] Paired epidemiological studies of occupations with chronic low level exposures (shale oil and pulp mills) generally conclude that the frequency of neurasthenic symptoms increases with dose and length of employment.[4,120]

Residual sequelae of acute hydrogen sulfide toxicosis are typically long-term or permanent cardiovascular, neurological and psychiatric problems attributed to the hypoxia associated with apnea, pulmonary edema and inhibition of oxidative metabolism.[107,157,246,250,263] Although low in frequency,[10,36] the long duration of these problems unfortunately lends to an association with the word "chronic."

Acute and Subacute Effects on Selected Systems

Major effects of hydrogen sulfide in the organ systems are local irritation and systemic toxicity.[21,72,150,172,268] Local irritative responses are limited to moist epithelia, especially the respiratory and ocular membranes. Mechanism of action is uncertain but has been attributed to direct action via sulfuric acid formation[150] and local cytotoxic hypoxia from cytochrome oxidase inhibition.[72] Systemic toxicity occurs when absorption exceeds detoxification, resulting in micromolar levels of H_2S in circulation and nanomolar concentration inhibiting mitochondrial cytochrome oxidase in target organs.[12,21] Based on the inhibition of aerobic metabolism, tissues most sensitive would be those with high oxygen requirements and low capacity to utilize alternate pathways.[7] This especially includes nervous system tissues, in addition to cardiovascular and pulmonary systems.[7,21,172,253,268] In general, neurologic symptoms appear first, most frequently, and with greater severity than the local irritative effects, although both are potentially fatal.[10,36,74,96,128] Physiological and pathological effects of hydrogen sulfide exposure are summarized in Table 5.[21,172]

Nervous system

Fatalities associated with hydrogen sulfide are generally due to irreversible cellular changes in the nervous system that result in acute respiratory paralysis.[21,74,96,253,268] Depression of cytochrome oxidase has long been considered the mechanism of systemic toxicosis, although alternate or additional routes causing respiratory distress have recently been proposed. At <200 ppm the gas depresses the central nervous system, >200 ppm can stimulate the CNS, and >700 ppm results in paralysis (Table 5).[4]

Development Recently, Hannah and Roth[99] exposed pregnant Sprague-Dawley rats to 0.0, 2.0, 20, and 50 ppm H_2S for 7 hr/d from day five postcoitus until day twenty-one postpartum. They demonstrated abnormal Purkinje cell development in the pups at 20 and 50 ppm ($P < 0.05$). Alterations in architecture and growth characteristics of the Purkinje cell dendritic fields included longer branches, increased vertex path length, variability in branch numbers, and asymmetrical growth pattern when random terminal branching is normal. Reduced capacity for potential conduction is suggested.

Function Sciatic nerve bundles from *Rana pipiens* (frog) exposed to 5,300 to 987,000 ppm H_2S results in reversible blockage of axonal compound action-potentials, which is not due to metabolic inhibition but an "anaesthetic" effect. Action potentials are higher and have variations in conduction velocity after recovery. Interaction of the gas or its anions with nerve membrane proteins (disulfide linkage) is postulated to alter membrane structure and therefore function.[23] Utilizing a patch clamp technique, Warenycia[262] applied sodium sulfide

Table 5. Clinical signs and symptoms of human hydrogen sulfide exposure.[a]

Concentration (ppm)	Physiologic Effects
0.02 - 0.77	Odor threshold
3 - 100	Offensive odor
10 - 50	Ocular irritation, keratoconjunctivitis
50 - 100	Respiratory irritation
50 - 200	Marked keratoconjunctivitis
100 - 200	Olfactory fatigue and paralysis
250 - 500	Pulmonary edema
500 - 1000	Unconsciousness
500 - 1000	Respiratory paralysis
500 - 1000	Nervous system stimulation
> 900	Peracute death
1000	Nervous system paralysis

[a]Adapted and reprinted with permission from Beauchamp RO, Bus JS, Popp JA, *et al.*, 1984. A critical review of the literature on hydrogen sulfide toxicity. *CRC Crit Rev Toxicol* 13:25-97.

(160 to 320 ppm S^{-2}) and taurine or cysteic acid solutions to undifferentiated murine neuroblastoma cells (NIE-115) and demonstrated disruption of sodium channel function. Subsequent treatment of the inhibited sodium channels with reducing agents (ß-mercaptoethanol or dithiothretiol) reverses the blockade, thus supporting the theory of disulfide bond reduction. However, chronic exposure of male Sprague-Dawley rats to 50 ppm H_2S for 5 d/wk for 25 weeks has no effect ($P > 0.05$) on sensory and motor tail nerve conduction velocity.[79]

Hydrogen sulfide also has an anesthetic effect on mucous membranes,[7] and ocular (corneal)[135,207] and olfactory [21,253,268] nerve receptors. The threshold of olfactory perception varies with the individual, but estimations range from 0.0005 to 0.13 ppm.[21,268] Initially hydrogen sulfide has a "rotten egg" odor that increases in intensity up to 20 to 30 ppm, and from 30 to 150 ppm is described as "sickly sweet."[253,268] At ~100 ppm H_2S olfactory fatigue occurs, and complete sensory paralysis develops at ~150 ppm.[4,21,74,268] Olfactory fatigue or paralysis will occur gradually with longer exposure to lower concentrations.[5]

Carotid chemoreceptors Haggard *et al.*[97] reported a paradoxical dose-respiratory response while exposing dogs to hydrogen sulfide. At 500 ppm, slight respiratory depression results and animals die from pulmonary edema after hours of exposure. Twice this exposure level produces hyperpnea of greater rate and depth, followed by apnea and death in 15 to 20 minutes. Exposure to 3000 ppm causes immediate respiratory paralysis. Heymans *et al.*[104,105] demonstrated a role of the carotid chemoreceptor, is affected by the composition of the blood flowing to the brain. By injecting sulfide into the common carotid arteries of dogs he induced the reported hyperpnea. In addition, similar hypertensive but less consistent bradycardic effects are shown to be mediated by the carotid body. Ammann[7] proposes that reduced oxidative metabolism within the chemoreceptors induced by H_2S inhibited cytochrome oxidase has the same net result as reduced pO_2 in carotid blood, therefore stimulating CNS respiratory nuclei to produce hyperpnea. A similar hyperpnea mediated by intrapulmonary CO_2-sensitive receptors has been found in avians.[129]

Other researchers have confirmed these transient, carotid body mediated, dose-effects of sulfide.[74,187,271] Although the reflex is primarily chemoreceptor mediated, some direct or central mediation may exist.[74] All agree the resulting fatal apnea results from central nervous system depression.[21,172,271]

Biochemistry CB-20 adult female mice were injected with radiolabled leucine, exposed to 100 ppm H_2S for 2 hr, and the cerebrums collected at 6, 24, 48 or 72 hr post-inhalation. A significant reduction of 21 to 26% ($P < 0.05$) in leucine uptake is reported at 24

and 48 hr post-exposure in brain homogenates, as well as a 31% decrease in the cerebral myelin at 48 hr treatment. Acid proteinase in the homogenates is significantly reduced at all sampling periods. No significant effects ($P > 0.05$) are noted for homogenate protein and RNA content, or myelin protein level. These results suggest decreased protein synthesis and proteolysis, likely related to impeded energy metabolism mediated through the inhibition of cytochrome oxidase.[73]

Repeated exposures to 100 ppm H_2S for 2 hr at 4 day intervals in female NMRI mice to evaluate cumulative effects reveal that cerebral protein content is not significantly altered ($P > 0.05$) and acid proteinase activity is decreased ($P < 0.05$).[211] However, RNA content ($P < 0.01$), uptake of radiolabled orotic acid into RNA ($P < 0.001$), cytochrome oxidase activity and glutathione ($P < 0.05$) are reduced after four treatments. The enzyme 2',3'-cyclic nucleotide-3'- phosphohydrolase, employed as a marker for myelin forming glia, significantly increases ($P < 0.05$), thus suggesting demyelination associated with exposure. No change is reported in acetylcholine esterase or superoxide dismutase activities ($P > 0.05$). These alterations are attributed secondarily to impaired oxidative metabolism.

Haider *et al.*[98] studied the effects of hydrogen sulfide exposure on the brain lipid content by exposing guinea pigs to 20 ppm for 1 hr/day for 11 days and measuring total lipid, phospholipid and cholesterol levels. Total lipids and phospholipids decrease in the cerebrum, cerebellum and brain stem, with significant reductions in the cerebrum and brain stem ($P < 0.05$). No change in the cholesterol concentration is reported. Lipid peroxidation in the cerebrum is significantly increased ($P < 0.05$). The authors do not speculate on the mechanism for the altered lipid metabolism.

Selective accumulation of sulfide in various brain regions has been demonstrated.[258] Adult, male Sprague-Dawley rats were injected with 17 mg S^{-2}/kg BW IP to produce acute death, after which the regions of the brain were collected and analyzed for sulfide by gas dialysis-ion chromatography.[87] Results show that brain stem has the lowest endogenous sulfide but the highest net uptake (Table 6). After normalization to account for regional blood flow, the authors conclude that the brain stem selectively accumulates sulfide. Selectivity is attributed to the high solubility of hydrogen sulfide in lipophilic materials, which corresponds with the high white matter content of the brain stem.

Examination of the subcellular distribution of endogenous sulfide reveals 27% located in the mitochondrial enriched fraction, 5% in the synaptosomal fraction, and 2.5% in the myelin portion.[258] After exogenous dosing of rats, myelin shows a 282% increase in sulfide content, while the mitochondrial and synaptosomal portions increase 230 and 212%,

Table 6. Distribution of endogenous and exogenous[a] sulfide concentrations in Sprague-Dawley rat brain.[b]

Brain regions	Sulfide (ppm)		% of Endogenous
	Endogenous	Net uptake	
Brain stem	1.23 ± 0.06[c]	3.02	246
Cerebellum	1.58 ± 0.17	2.03	128
Hippocampus	1.76 ± 0.13	2.06	117
Striatum	1.85 ± 0.13	1.49	81
Cortex	1.77 ± 0.08	2.79	158
Thalamus + hypothalamus	1.59 ± 0.10	n/a	n/a

[a]50 ppm NaHS/kg BW IP (17 mg S^{-2}/kg).
[b]From Warenycia *et al.*[258]
[c]Mean ± SE; n = 10.

respectively. Speculating on the biological role of endogenous sulfide, they suggest it may result from enzymatic processes or be associated with control of neuronal excitability. Although regional distribution correlates with methionine ($r = 0.728$) and taurine ($r = 0.968$) content in the brain, endogenous sulfide is unlikely artifactual.

Neurotransmitters Alterations in cerebral synaptosome metabolism and transmitter kinetics have been demonstrated in adult male Wistar rats injected with 4.8 mg S^{-2}/kg BW IP.[200] A 15% reduction in synaptosome respiration is attributed to altered oxidative metabolism either due to structural modifications in synaptosomal or mitochondrial membranes, or inhibition of local cytochrome oxidase. Functionally, synaptosomal release and uptake of γ-aminobutyric acid and dopamine are decreased, as are intervening changes in transmembrane potential. Compared to inhibition with tetrodotoxin (sodium channel blocker), the decreased release of transmitters by sulfide is thought to result from reduced sensitivity of sodium channels and diminished permeability to sodium. Decreased transmitter uptake is related to altered membrane permeability or partial inhibition of presynaptic receptors, likely due to membrane peroxidation.

Brains of acutely and sublethally poisoned adult male Sprague-Dawley rats (0, 3 or 7 mg S^{-2}/kg BW IP) were evaluated for changes in neurotransmitter amino acid levels in the cortex, cerebellum, striatum, hippocampus, and brain stem.[131] No significant alterations ($P > 0.05$) are reported in aspartate, glutamine, glutamate, glycine, taurine, alanine, and γ-aminobutyric acid content of the cortex, striatum or hippocampus. Cerebellar aspartate and glycine are significantly decreased only in the sublethal treatment. However, all amino acids in the brain stem are dramatically elevated in both groups, suggesting that apnea associated with hydrogen sulfide toxicosis may result from imbalances in amino acid neurotransmitter levels or metabolism in the respiratory centers. Again, the underlying mechanism is felt to be histotoxic hypoxia, especially altered tricarboxylic acid cycle metabolism of amino acid.

Catecholaminergic innervation from the brain stem and norepinephrine, epinephrine, dopamine and 5-hydroxytryptamine influence respiration. Warenycia *et al.*[261] investigated the role of these monoamine neurotransmitters in hydrogen sulfide toxicosis. Adult male Sprague-Dawley rats were administered sublethal (3 mg S^{-2}/kg BW IP) or lethal (7 mg S^{-2}/kg BW IP) sulfide doses and cerebrum, cerebellum, striatum, hippocampus, and brain stem collected for analysis. Cerebral and cerebellar catecholamine levels did not change, however corpus striatum, hippocampus and brain stem increase significantly ($P < 0.05$) in norepinephrine and epinephrine concentrations. In addition, dopamine and 5-hydroxytryptamine are significantly elevated in brain stem. The rapid increases in neurotransmitter levels during hydrogen sulfide

toxicosis is attributed to inhibition of catabolic enzymes such as monoamine oxidase, likely through sulfhydryl group interactions. *In vitro* work demonstrates a dose-response inhibition of monoamine oxidase to sulfide over the previously injected concentration range, but this is not confirmed in the above *in vivo* study except at 35 mg S^{-2}/kg IP (47% inhibition). Rapid *in vivo* metabolism of sulfide likely accounts for this discrepancy.

Clinical signs and symptoms Overt effects of H_2S on the nervous system are governed by factors of toxicity and exposure (Tables 5 and 7). Clinical signs, symptoms and epidemiology of acute and subacute exposures are published.[10,36,128,197,275]

Pathology Few lesions other then those compatible with asphyxia are present on gross and histologic examination since hydrogen sulfide is an acute toxin with a hypoxic mechanism. Postmortem evaluation is often omitted in experimental and accident deaths because many authors state that the toxicant has no distinct pathology.[5,96,180]

Lund and Wieland[153] reported extensive necrosis of the parietal and occipital cerebral cortex, reduced numbers of cerebellar Purkinje cells, and focal gliosis after inhalation of 500 ppm H_2S for 22 minutes. In an adult male human that presented unconscious after acute poisoning, computerized tomography of the brain revealed bilateral symmetrical lucent lesions in the cerebral hemispheres which are interpreted as necrosis of the lentiform nucleus.[157] In a similar case in which the victim died 34 days post-exposure, the neurological pathology is summarized as "marked cerebral edema" and "focal brain necrosis."[36] Other fatal human exposures that included autopsy data are also limited to single phrase descriptions such as "small petechial brain hemorrhage"[170] or "meningeal congestion and cerebral edema."[38]

In a triple, acute fatality of sewer works, Adelson and Sunshine[5] noted greenish-purple discoloration in the cerebral cortices and nuclear masses, which dissipated after 96 hours of preservation in 10% buffered formalin. No histopathology is described. A similar colored pigment is reported in the lungs, liver and kidney, but not the brain, from a gassed truck driver.[38] However, most clinical and experimental papers do not report this pigmentation. *In vitro* reaction of reduced hemoglobin with hydrogen sulfide yields a green hematin-like compound that is likely sulfhemoglobin.[74]

Chronic exposure of male Sprague-Dawley rats at 50 ppm H_2S 5 d/wk for 25 wk failed to produce histologic lesions detectable on light or electron microscopic examination.[79] Circumstantially, an acute encephalopathy developed in a 20 month-old male living adjacent to a coal mine emitting low levels of H_2S. Computerized tomography revealed lesions in the basal ganglia and associated white matter.[80]

Table 7. Some acute and subacute effects reported in human hydrogen sulfide toxicosis cases.[a]

Clinical sign or symptom	Epidemiological survey			
	Kleinfeld *et al.*[128]	Burnett *et al.*[36]	Arnold *et al.*[10]	% Total
Unconsciousness	11	163	135	59.1
Agitation, confusion, dizziness, vertigo	39	50	54	27.3
Headache	11	27	65	19.7
Nausea or vomiting	12	38	62	21.4
Dyspnea	5	6	57	13.0
Convulsions	1	16	5	4.2
Pulmonary edema	2	34	14	9.6
Cyanosis	5	19	3	5.2
Conjunctivitis	2	19	46	12.8
Sore throat, cough	5	24	41	13.4
Weakness	13	8	19	7.6
Hemoptysis	n/a	2	1	0.6
Death	4	10	7	4.0
Miscellaneous	34[b]	n/a	79[c]	21.6
Total cases in survey	52	221	250	523

[a]Adapted from Vicas and Whitcraft.[253]
[b]Hoarseness, tachycardia, numbness, chest burning, tremors, hyperreflexia.
[c]Malaise, chest pain, bradycardia, neuropsychologic syndrome.

Superficial ocular lesions consist primarily of keratoconjunctivitis resulting from direct irritation.[4,10,36,96,128,152] To histologically characterize the corneal aspects, Michal[163] exposed rats to 1300 ppm for 10 minutes or 54 ppm for 3 hours and described the resulting lesion as superficial corneal edema with sloughing epithelia. Cytological evaluation of the ocular cells from male Fischer-344 rats reveals predominately corneal and conjunctival epithelia, and a few neutrophils.[140]

Experimental and field cases of hydrogen sulfide toxicosis in ruminants have demonstrated a relationship with polioencephalomalacia. Gould *et al.*[89] fed diets with elevated sulfate to produce the disease in Holstein calves. Pathological changes include cortical autofluorescence under UV light, cerebrocortical neuronal necrosis, cerebrocortical poliomalacia, and cavitation. Clinical and histological evidence for the association is also reported in cattle that developed CNS signs after surviving manure gas exposures. Lesions are described as severe toxic necrosis of nerve cells in the cerebral cortex and hippocampus, with milder involvement in the corpus geniculatum, nucleus ruber and terminal vagal nucleus.[56,59]

Respiratory system

In addition to the systemic respiratory effects mediated through the nervous system, hydrogen sulfide produces local uniform irritative lesions and an often fatal pulmonary edema.[91,101,172,198,268] Although edema is traditionally the criterion for differentiating acute and subacute toxicosis,[172,275] it can also develop in acute cases.[5,185,198] Severity of lesions and edema are dose dependent.[91,149] Pulmonary edema is reported in all species after H_2S exposure.[172]

Parenteral administration of sulfide salt solutions has the same physiological and pharmacological effects as hydrogen sulfide inhalation.[21,74,86,258] However, Lopez *et al.*[149] demonstrated that injection of adult male Sprague-Dawley rats with 7 mg S^{-2}/kg BW IP did not produce the marked pulmonary edema, foam in trachea, severe lung congestion, or lungs that failed to collapse postmortem, when compared to a corresponding treatment of 1655 ppm H_2S for 5 minutes.

Pulmonary edema Inhalation of hydrogen sulfide may result in respiratory tract injury and pulmonary edema. Lopez *et al.*[151] noted that this response appears to have a poor dose-effect relationship in that it develops only after a minimal exposure level is surpassed. This threshold is estimated at between 200 to 300 ppm H_2S.[91,150,165,198]

The pathophysiology of pulmonary edema is speculative. Various proposed mechanisms include: hydrostatic pressure,[21,151] altered cellular metabolism secondary to neurogenic hypoxia or cardiac arrhythmias,[21,22,133,172] direct damage to respiratory capillaries or

epithelia,[150,172] and mast cell degranulation.[148] In determining the biochemical and cytological alterations in the respiratory tract of male Fischer-344 rats exposed to 0, 10, 200 or 400 ppm H_2S for 4 hours and killed 1, 20 or 44 hours post-treatment, Lopez *et al.*[151] concluded that transient changes in protein ($P < 0.01$) and lactate dehydrogenase ($P < 0.05$) levels in the bronchoalveolar lavage fluid over the various sampling periods at 400 ppm indicates immediate increases in vascular permeability and cytotoxicity. In a similar experiment following the histological development of hydrogen sulfide induced edema, fluid accumulates initially around vessels, then interstitium, and finally alveoli. A mild perivascular edema with normal alveoli in sub-threshold treatments suggests reabsorption of interstitial fluid preventing subsequent accumulation as bronchoalveolar edema.[148]

Function Green *et al.*[91] exposed male Fischer-344 rats to sub-threshold (194 ppm) and threshold (290 ppm) H_2S concentrations to explore relationships between onset of pulmonary edema and alterations in surfactant properties. Although both treatments significantly increase protein and lactate dehydrogenase levels in the bronchoalveolar lavage fluid ($P < 0.001$), only the threshold dose significantly altered the surface tension properties as assessed with a captive bubble surface tensiometer ($P < 0.01$). Mechanistically, increased capillary permeability permits leakage of plasma proteins or membrane lipids of blood components, which function as surfactant inhibitors by competing with surfactant phospholipids for space at the air to liquid interface. *In vitro* experimentation with normal rat surfactant and serum proteins demonstrates a dose-response and threshold relationship between protein concentration and abolition of surfactant properties. Addition of sodium sulfide to normal surfactant causes no measurable alterations.

The influence of hydrogen sulfide on respiratory defense mechanisms has been considered in toxic and subclinical exposures in humans[61,62,112,185] and confinement animals.[55,63,64,66,118,171] Pulmonary edema predisposes to secondary bacterial infections by inhibiting mucociliary transport and macrophage activity.[151]

Cralley[116] produced ciliary stasis in rabbit tracheas exposed to sublethal levels of hydrogen sulfide (600 ppm for 5 minutes or 400 for 10 minutes). Sublethal treatment of male Fischer-344 rats at 0, 100, 200 or 400 ppm for 4 hours results in consistent nasal cavity lesions only in the highest dose group, which correlate with post-inhalation time.[150] Severe diffuse foci of degenerative and necrotic respiratory epithelium with minimal inflammatory response are reported after one hour. Necrotic cells, many with complete loss of cilia, defoliate to produce erosions and ulcerations. Twenty hours post-exposure, the erosions and

ulcerations are covered by thin basophilic cells having a high mitotic activity, and a severe diffuse neutrophilic response is present. Degeneration and inflammation have abated after 44 hours, and differentiation of respiratory epithelium cells is prominent.

Similarly, evaluation of male Fischer-344 rat lungs from sublethal exposures at 0, 83, or 440 ppm H_2S for 4 hours and sequential sampling at 1, 18 or 42 hours post-treatment reveals selective toxicity to terminal bronchiolar and proximal alveolar regions, especially to the ciliated cells.[148] Again, cell necrosis, edema and neutrophils are significant features at the highest dose, with only mild involvement at 83 ppm. As with the nasal epithelium,[150] degeneration and necrosis of the ciliated cells is immediate, with restoration and resolution of the accompanying pulmonary edema almost complete 42 hours after exposure.

Reduced capacity ($P < 0.01$) of the rat pulmonary defense mechanisms to inactivate *Staphylococcus epidermidis* after exposure to 45 ppm H_2S for 4 to 6 hours is attributed to impaired alveolar macrophages.[205] Robinson[203] cultured alveolar macrophages from male New Zealand White rabbits on gas-permeable membranes while treating with 54 ppm H_2S for up to 24 hours and noted a 95% reduction in phagocytosis of polyvinyl toluene beads. This impairment is more pronounced with continual exposure.

While investigating the effects of hydrogen sulfide on basal and particle-induced respiratory rates, and viability of alveolar macrophages from Fischer-344 rats exposed to 0, 50, 200, or 400 ppm for 4 hours, Khan *et al.*[126] reported basal oxygen consumption is not altered, but respiration associated with phagocytosis is significantly reduced at 200 (59%) and 400 (64%) ppm ($P < 0.05$). Macrophage viability is significantly decreased only at 400 ppm ($P < 0.05$). These results are confirmed *in vitro* with alveolar macrophages harvested from control rats and treated with sulfide, sulfite or sulfate solutions. Sulfide significantly ($P < 0.05$) inhibits basal (41%) and particle-induced (72%) respiration, while sulfite reduced the phagocytic respiration (41%). The basic mechanism for impairment of alveolar macrophage phagocytosis is suggested to be inhibition of oxidative metabolism,[203] specifically cytochrome oxidase.[125]

Although the primary function of the respiratory tract is gaseous exchange, no research has been directed at the effects of hydrogen sulfide on this process in other than clinical cases. Burnett *et al.*[36] evaluated 221 cases of industrial hydrogen sulfide toxicosis and noted "few" have pulmonary function or blood-gas determinations. Respiratory function tests from these cases demonstrate reduced diffusion capacity. A similar survey of 250 reports indicates only nine had pulmonary function tests performed (3.6%), with three diagnosing obstructive

patterns.[10] Histopathologically, Fischer-344 rats exposed at 83 or 440 ppm for 4 hours did not develop lesions in the air-blood barrier as indicated by lack of ultrastructural changes in alveolar endothelium, basement membrane or type-I pneumocytes.[148]

Biochemistry Using histochemical techniques to identify changes in lungs of rabbits inhaling 72 ppm for 30 min/d over 14 days, Jonek and Konecki[116] reported elevations in deoxyribonuclease II, adenosine triphosphatase and alkaline phosphatase that are attributed to an increase in alveolar macrophages. *In vitro* work with pulmonary microsomes exposed to 18 to 502 ppm for 1 hour notes reductions in acid phosphatase, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase and adenosine triphosphate. Arginase increases, however aldolase did not change.[110,111] Sensitivity of respiratory chain enzymes in pulmonary mitochondria from male Fischer-344 rats inhaling 0 to 400 ppm H_2S for 4 hours has been considered.[125] No significant ($P > 0.05$) effects are reported at 10 ppm for cytochrome c oxidase, succinate-cytochrome c reductase and NADH-cytochrome c reductase, or for succinate-cytochrome c reductase and NADH-cytochrome c reductase at 50, 200 and 400 ppm treatments. At sublethal (50 to 400 ppm) and lethal (500 to 700 ppm) exposures, cytochrome c oxidase activity is inhibited 15 to 68% and >90%, respectively. Succinate oxidase activity is reduced 40 to 63% at 200 to 400 ppm. Post-exposure (200 and 400 ppm) reactivation of cytochrome c oxidase at 24 and 48 hours is 88 to 90% and 74 to 71%, respectively. It is concluded that cytochrome c oxidase is the primary respiratory enzyme inhibited and this results in cytotoxic hypoxia in pulmonary tissue.

Analysis of respiratory tract lavage can detect biochemical and cellular alterations prior to development of histological lesions.[151] Evaluation of nasal lavages from rats treated at 0 to 400 ppm H_2S for 4 hours reveals significant ($P < 0.01$) increases in lactate dehydrogenase (320%) and protein (800%) at 400 ppm. Twenty hours post-inhalation these parameters are compatible with baseline. Alkaline phosphatase is not affected ($P > 0.05$) by 10, 200 or 400 ppm exposures. At the bronchoalveolar level, protein content significantly ($P < 0.01$) increases 3000, 400 and 450% at 1, 20 and 44 hours post 400 ppm exposure, but not at $\leq$ 200 ppm ($P > 0.05$). Lactate dehydrogenase and alkaline phosphatase activities are inhibited only at 200 and 400 ppm ($P < 0.05$) over 1 to 44 hours, while γ-glutamyl transpeptidase is increased 933% over the same periods. These alterations are attributed to severe cellular damage.[151] Green *et al.*[91] confirmed the elevations in lavage protein concentration and lactate dehydrogenase activity, in addition to no significant differences ($P > 0.05$) in phospholipid content from rats inhaling 0, 200 or 300 ppm for 4 hours. As with protein, phospholipid is considered a possible inhibitor of pulmonary surfactant.

Clinical signs and symptoms Prominent clinical signs and symptoms of acute and subacute hydrogen sulfide toxicosis are listed in Tables 5 and 7. Detailed discussions and epidemiologic information are available.[4,10,36,128,275]

Pathology Pathological results of hydrogen sulfide exposure to respiratory tissues are generally uniform throughout the tract and attributed to irritative or local action of the gas.[172] Pulmonary edema, often hemorrhagic, is the most common gross lesion;[36] although severity is dose dependent once the threshold is surpassed.[149] Edema was present in 6 to 15% of the cases industrial exposures.[10,36,128]

Light and electron microscopic descriptions of nasal and bronchoalveolar mucosal changes have been published.[91,148,150,198] In general, the nature of the respiratory lesions and distribution are similar to those of other mechanical, infectious or toxic agents.[149,150] Cytological alterations in nasal and bronchoalveolar lavages are published.[151] Robinson[203] described scanning and transmission electron microscopic changes in rabbit alveolar macrophages cultured under 54 ppm H_2S for 24 hours.

Hematopoietic system

Confusion and controversy historically exists concerning the effects of hydrogen sulfide on blood, especially its interaction with hemoglobin, due to rudimentary analytical techniques, vague definitions and misinterpretation.[21,172,222] Currently, results of *in vivo* hemoglobin-hydrogen sulfide interaction, such as sulfhemoglobin, are not pathophysiologically important since significant concentrations do not occur during toxicosis.[5,21,68,96,172,222] As with other organ systems, enzyme inhibitions and alterations in serum chemistry are reported.[21,26,124,172]

Blood proteins Curtis *et al.*[53] administered 0.8 mg $^{35}S^{-2}$/kg BW IV or PO to adult rats to determine the fate of sulfide in blood. Plasma versus cell distribution is 94.7 to 78.9% and 21.1 to 5.3%, respectively. Inorganic sulfate accounts for >95% for the radiolabled sulfur, with 5 to 17% binding to blood proteins. Electrophoresis and autoradiography of *in vitro* samples reveal sulfide binds to all major plasma proteins, with 50% of the protein bound activity in the albumin fraction.

Hemoglobin Effects of hydrogen sulfide on heme synthesis have been investigated. Injection of Wistar rats with 4.8 mg S^{-2}/kg BW IP significantly depresses δ-aminolevulinic acid synthetase and heme synthase activities.[210,237] The sulfide metabolite thiosulfate also inhibits these enzymes.[238] The reductions are attributed to the hypoxia resulting from inhibited oxidative metabolism. The activities of uroporphyrinogen I synthetase, uroporphyrinogen decarboxylase and RBC coporporphyrin are not altered.[238] Similar depression of heme

synthesis enzymes is reported after industrial exposures, although the degree of exposure cannot be concluded.[26,238] Reduced activities are reported at one month post-toxicosis, but associated peripheral anemia or abnormal bone smears are found.[238]

Interactions between hydrogen sulfide and hemoglobin have been reviewed (Figure 3).[172,226] Sulfhemoglobin (SHb) is a green pigmented compound with a sulfur atom incorporated into the porphyrin ring of oxyhemoglobin, which results in reduced oxygen affinity.[174,188] Its formation is associated with therapeutic drug use, occupational exposure to sulfur compounds, and air pollution.[188] The confusion about sulfhemoglobin stems from the vague original definition: bubbling H_2S through blood produces a pigment with an absorption maximum at 620 nm, which overlaps with methemoglobin, but is not eliminated by the addition of cyanide.[222] Today, sulfhemoglobinemia is considered a non-toxic syndrome in persons with normal hemoglobin,[188] and is not a significant factor in hydrogen sulfide toxicosis.[5,21,68,80,172,188,189,222]

The formation and structure of SHb are not completely elucidated because of the complexity of the reactions and unstable product.[115,172] Structurally, the porphyrin macrocycle is reduced to an iron chlorin with sulfur adding to the tetrapyrrole in a position away from the iron atom.[115,189,192] Partially and completely sulfurated hemoglobin molecules are formed.[189] Reversion to deoxyhemoglobin occurs readily, although the mechanism is not known.[68,115]

The green to slate grey discoloration occasionally reported during postmortem examination of hydrogen sulfide victims is likely sulfhemoglobin.[5,38,96] Although low concentrations potentially exist *in vivo,* [115,189] overt pigmentation is believed to be a postmortem change.[5,96,172]

Sulfmethemoglobin was discovered by Keilin.[123] It results from the reversible reaction of sulfide anions with methemoglobin in the sixth coordinate position of the ferric heme in a 1:1 stoichiometry (Figure 3).[174,224,225] Upon aeration, autoreduction of sulfmethemoglobin to oxyhemoglobin occurs rapidly at pH 7 to 5, and has a half-life ~2 hours.[50] Iatrogenically induced methemoglobinemia is an antidote for hydrogen sulfide toxicosis.[107,222,224,225,232,240,241]

Pseudo-sulfhemoglobin is an irreversible product of hemoglobin oxidation and denaturation that forms in the absence of sulfide anions, and persists in the RBC during circulation. It structurally may contain abnormal disulfide bridging with glutathione or other sulfhydryl compounds.[172,222]

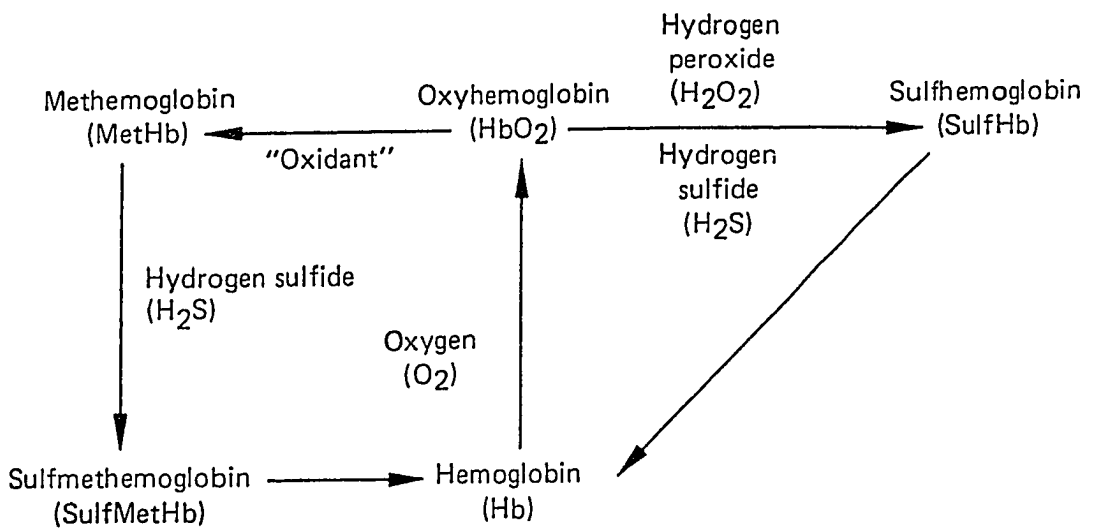


Figure 3. Proposed hydrogen sulfide interactions with hemoglobin. Reprinted with permission from the National Research Council. *Hydrogen Sulfide*. Committee on Medical and Biological Effects of Environmental Pollutants, subcommittee on Hydrogen Sulfide. Baltimore, Maryland:University Park Press, 1979.

Case Reports

Since problems with hydrogen sulfide have occurred for centuries, human and animal exposures at various concentrations are abundant. In all cases, toxicity, clinical signs, symptoms, progression and lesions are relatively similar, as are diagnostic and therapeutic principles.

Human exposure cases

Selected human exposures are summarized in Table 8. Although most cases are industrial accidents,[5,38,86,107,113,146,152,159,193,220,232,238,245,250,252,263,273] many agricultural[32,65,166,170,185,245] and domestic[58,80,81,239] reports exist. High-risk occupations are listed in Table 9.

Acute fatal and sub-lethal cases predominate (Table 8), while subacute[58,81,152] and chronic[80,238] toxicoses are less frequent. This may result from less dramatic exposures, therefore warranting less interest in publication, lack of confirmed cases, or the controversial nature. More recently, instances of non-fatal acute with chronic residual effects are being reported due to improved awareness, diagnostics and longer post-exposure studies.[246,263]

From the published literature, individual or small group exposures are typical, although instances of community wide accidents have been reported.[172] No cases of hydrogen sulfide suicides are known.[253]

Animal exposure cases

Cases of hydrogen sulfide toxicosis in domestic animals are presented in Table 10. Most result from human errors in livestock management such as confinement unit maintenance or operation, and inappropriate therapy or dietary formulation. Incidences of gas exposure tend to be peracute to acute in clinical signs and progression, while those resulting from oral ingestion of elemental sulfur are acute to subacute. One case of "chronic" toxicosis has been published.[24]

Confinement production units Intensified animal production has led to the development of confinement units to maximize use of mechanical feeding, watering and waste removal, and minimize animal handling and heating. The animals are typically housed on slotted floors over or near a manure holding pit which is periodically evacuated by agitation to resuspend solid materials prior to pumping out the waste. These facilities have been utilized in swine, poultry, beef, dairy and veal cattle, fur animals, and to some extent in catfish production.

Table 8. Selected case reports of human hydrogen sulfide toxicosis.

Nº	Reference	Situation (location)	Exposure source & factors
1	Breysse[32]	Poultry feather fertilizer plant (Washington)	Ruptured outfall line from steam cooker (2000 - 4000 ppm); Acute
2	Adelson & Sunshine[5]	State highway workers in sewer (Ohio)	Fish oil - crude petroleum plant discharging hydrogen sulfide into sewer system; Acute
3	Winek *et al.*[273]	Industrial park (Pennsylvania)	Working in a storage tank previously containing coal-tar resin (1900 - 6100 ppm); Acute
4	Miniats *et al.*[166]	Swine confinement unit (Ontario, Canada)	Agitation of manure pit without proper ventilation; Acute
5	Thoman[239]	Domestic accident (Iowa)	Homeowners poured one gallon HCl into basement well; Acute
6	Stine *et al.*[232]	Chemical tank-truck (Maryland)	Sodium sulfide waste dumped onto acidic waste; Acute
7	Matsuo *et al.*[157]	n/a	Accidental exposure to high pressure hydrogen sulfide; Acute
8	McAnalley *et al.*[159]	Petrochemical, sewer and leather workers (Texas & Utah) - 5 cases	Cleaning petrochemical tank, driver of tank-truck containing sludge waste, entered sewer pipe, miss-formulation of tanning vat, and exposure to tanning vat; Acute
9	Morse *et al.*[170]	Calf barn (Wisconsin)	Agitation of manure storage tank without adequate ventilation; Acute
10	Osbern & Crapo[185]	Underground liquid manure storage tank (Utah)	Entry into partially filled manure storage tank; Acute
11	Peters[193]	Hospital (Pennsylvania)	Cleaning out plaster of paris sludge (Ca sulfide) in a cast room drain with 90 % sulfuric acid; Acute

Nº	Clinical signs & symptoms	Basis for diagnosis	Treatment
1	(1/1/1)[a] Found dead	Hx[b]; PM[c]: hpe[d], ce[e], td[f]; + qual. blood analysis (distillation & vapor chromatography[g])	n/a
2	(5/5/3) Unconscious, death	Hx; odor; PM: odor, td, cyanosis, hpe; coins & keys black; + GC analysis (?) on blood	"Vigorous resuscitation"
3	(1/1/1) Unconscious, death	Hx; PM: pe, congestion, bronchitis; sulfide analysis:[g] 0.92 blood, 1.06 brain, 0.34 kidney, 0.38 ppm liver	n/a
4	(2/2/0) Odor, ocular & nasal irritation, dyspnea, collapse, headaches	Hx	Fresh air
5	(5/5/0) Unconscious, collapse, respiratory distress, nausea, vomiting, coma, tachycardia, spasms, hyperexcitable, irritable, pe[h]	Hx	Oxygen, amobarbital, aminophylline, levarterenol bitartrate, hydrocortisone
6	(1/1/0) Unconscious, seizure, cyanotic, agitated, disoriented, tachycardia, dyspnea, keratoconjunctivitis, neuropsychiatric sequelae	Hx	Oxygen, amyl nitrite, Na nitrate, Na thiosulfate
7	(?/1/1) Unconscious, respiratory distress, spasms, coma, chronic vegetative state, death	Hx; CT[i]: bilateral lucent lesions in cerebral hemispheres; PM: n/a	"Respiratory assistance", n/a
8	(5/5/5) Found dead, collapse	Hx; odor; blood sulfide analysis (ISE)[j]: 1.70 - 3.75 ppm (normal <0.05 ppm); copper pennies turned black	n/a
9	(3/3/1) Unconscious, collapse, cough, vomiting, respiratory arrest, death	Hx; PM: hpe, petechial brain hemorrhage, aspiration	Fresh air, n\a
10	(5/5/3) Unconscious, collapse, resp. irritation, vomiting, edema, irritable, death	Hx; PM: pe, aspiration; analysis blood (ISE)[j]: 5.0, 3.6 & 0.8 ppm sulfide	Fresh air, CPR, oxygen, IV fluids, antibiotics,
11	(4/4/0) Unconscious, collapse, apnea, cyanosis, tremors, headaches, chest pain	Hx; odor; analysis of reactants	Na bicarbonate, oxygen, amyl nitrite, Na nitrite, Na thiosulfate, diazepam, dexamethasone

Table 8. Continued.

12	Donham *et al.*[65]	Confinement manure facilities or septic tank (Iowa & Wisconsin) - 6 cases	Agitation without proper ventilation, entry into tank or associated facilities; Acute
13	Gann & Roseman[81]	Residential precious metal recovery (New Jersey)	Electroplating silver from a thiosulfate - silver solution; Subacute.
14	Tenhunen *et al.*[238]	Pulp mills (Finland)	Normal operation (0.05 - 5.2 ppm TWA[1] 8 hr); Acute & chronic
15	Smilkstein *et al.*[220]	Chemical tank-truck (Colorado)	Driver entered tank that had contained waste water from an oil-pumping operation (717 ppm); Acute
16	Hoidal *et al.*[107]	Roofing asphalt company (Washington)	Workers entering sealed asphalt cooling tank to repair pump (est. 650 - 700 ppm); Acute
17	Deng & Chang[58]	Natural hot-spring reservoir (Taiwan) - 2 cases	Improper aeration of reservoirs prior to entry for routine removal of organic debris; Acute & subacute
18	Gaitonde *et al.*[80]	Residential (England)	Family living near a coal mine, where a burning fire had been emitting hydrogen sulfide for ~1 year (0.6 ppm max. 4 mth); Chronic
19	Lindell *et al.*[146]	Pulp mills and oil refinery (Finland)	Twelve acute, non-fatal exposures
20	Vanthenen *et al.*[250]	Industrial (England)	Factory worker was bending over an open tank of sulfur, cystine HCl, Na bicarbonate, and methionine (50 - 200 ppm); Acute
21	Al-Mahasneh *et al.*[6]	n/a (Michigan)	n/a

12	(Total 14/9/8) Unconscious, collapse, dyspnea, resp. distress, nausea, neuropsychiatric, death	Hx; odor; PM: pe, n/a; n.b.[k] → manure gases ∴ not all cases diagnosed as hydrogen sulfide	Fresh air, n/a
13	(5/5/1) Cough, fatigue, sore throat, dyspnea, unconscious, nasal & ocular irritation, resp. distress, cardiopulmonary arrest, death	Hx; PM: hpe, black stained hands; hydrogen sulfide analysis[g]: 4.2 ppm lung, trace in blood	n/a
14	(18/1/0) Acute: unconscious; Chronic: appeared normal	Hx; depression of δ-aminolevulinic acid synthase (9/18) and RBC protoporphyrin (8/18); n.b.- other sulfur gases maybe involved	n/a
15	(1/1/0) Unconscious, apnea, pulseless, tremors, agitated, pulmonary edema, coma	Hx	Mouth-to-mouth, oxygen, Na bicarbonate, amyl nitrite, furosemide, Na nitrite, diazepam, hyperbaric oxygen
16	(2/2/0) Unconscious, apnea, cardiopulmonary arrest, coma, seizures, chronic vegetative state, conjunctivitis, agitation	Hx; n.b.- other gases (asphalt fumes) maybe involved	CPR, IV fluids, oxygen, epinephrine, Na bicarbonate, defibrillation, naloxone, dextrose, succinylcholine, pancuronium, Na nitrite
17	(8/8/3) Collapse, unconscious, dyspnea, agitated, vomiting, cyanotic, cold skin, aspiration, gray-green sputum, odor, pulmonary edema, keratoconjunctivitis, death	Hx; odor	Theophylline, oxygen corticosteriods, n.b.- nitrite treatment was not available
18	(?/1/0) 20 mth-old with paroxysmal tonic deviation of eyes, progressive involuntary body movements, ataxia, dystonia	Hx; ruled-out differentials; computer tomographic brain lesion	Spontaneous recovery
19	(?/12/0) Unconscious, "other symptoms compatible"	Hx; ISE[j] blood analysis: 0.04 - 0.60 ppm sulfide (n = 12)	n/a
20	(1/1/0) Dyspnea, collapse, unconscious, neuropsychiatric sequela	Hx	n/a
21	(?/1/1) Comatose, fixed dilated pupils, unresponsive,	Hx; blood sulfide analysis[g]: 0.04 ppm	Na nitrite, oxygen mask, hyperbaric oxygen

Table 8. Continued.

22	Campanyà *et al.*[38]	Chemical tank-truck (Spain)	Worker was cleaning a tank that had previously contained polysulfides with hydrochloric acid based solution; Acute
23	Goodwin *et al.*[86]	Petrochemical workers (Canada) - 2 cases	Pipefitter exposed by localized venting of gas in an allegedly flushed line (7500 ppm) & release of hydrogen sulfide contamination from water line; Acute
24	Luck & Kaye[152]	Sausage factory (England)	Putrefaction of intestines & contents in barrels prior to processing into sausage casing; Subacute
25	Wasch *et al.*[263]	Fishing boat & geothermal plant (California) - 3 cases	Fishing boat: rotting brine in holding tank. Geothermal plant: drying soil samples in ovens in a trailer & exposure to hydrogen sulfide vent; Acute
26	Vicas *et al.*[252]	Petroleum tanker truck (Canada)	Unloading hydrogen sulfide contaminated crude oil; Acute
27	Jäppinen & Tenhunen[113]	Pulp mills and oil refinery (Finland & Sweden)	Cases of acute toxicosis (n=21) associated with normal operation
28	Tvedt *et al.*[246]	Sewage treatment plant, swine confinement unit, fishing boat, herring oil industry, tannery, shipyard (Norway)	Opened lid of tank to test discharge water from sludge centrifuge, liquid manure agitation, spoiled fish in fishing boat hold, same, working in a waste tank, and overhauling an oil rig, respectively; Acute

[a](Initial number of people in exposure / number of people with some sign of clinical toxicosis / number of deaths due to exposure).
[b]Hx = history, circumstance, clinical signs or symptoms and progression.
[c]PM = postmortem examination.
[d]hpe = hemorrhagic pulmonary edema.
[e]be = brain edema.
[f]td = tissue discoloration, typically described as green, green-blue, slate grey, etc.
[g]Details of analysis are not published in the reference or literature.
[h]pe = pulmonary edema.

22	(2/2/2) Unconscious, apnea, death	Hx; PM: cyanosis, keratoconjunctivitis, hpe, td; ISE[j] blood sulfide analysis: >3.5 ppm (n=2); brass badge was blackened; analysis of contents in tank (reactants)	n/a
23	(5/5/3) Collapse, apnea, unconscious, cardiopulmonary failure, death	Hx; PM: pe, n/a; sulfide analysis (GD/IC)[m] of brainstem: 0.91 - 1.04 ppm (normal 0.69 ± 0.02 ppm; mean ± SE, n = 36)	n/a
24	(6/6/0) Blepharospasm, photophobia, lacrimation, keratoconjunctivitis, corneal erosions	Hx; + lead acetate paper	Chloramphenicol eye ointment
25	(4/4/0) collapse, unconscious, blurred vision, pe, weakness, neuropsychiatric sequela, dyspnea, nasal & ocular irritation	Hx; odor; EEG[n]: prolonged P-300 latency[o]	n/a
26	(1/1/0) Unconscious, tremors, amnesia, neuropsychiatric sequela	n/a	Oxygen mask, hyperbaric oxygen, other treatment not specified
27	(21/21/0) Unconscious, nausea, resp. & ocular irritation, etc.	Hx; ISE[j] blood sulfide analysis: 0.03 - 0.13 ppm, depressed δ-aminolevulinic acid synthase activity	n/a
28	(6/6/0) Unconscious, dyspnea, apnea, pe, blurred vision, amnesia, loss of smell, neuropsychiatric sequela	n/a	n/a

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[i]CT = computer tomography.

[j]ISE = ion-selective electrode sulfide determination generally based on the work of McAnalley *et al.*,[159] or a modification.[146]

[k]n.b. = *nota bene.*

[l]TWA = time-weighted average.

[m]GD/IC = sulfide analysis based on gas dialysis and ion chromatography.[86]

[n]EEG = electroencephalogram.

[o]P-300 latency = EEG potential that occurs 300 ms after a rare tone while listening to a specific paradigm.

Table 9. Occupations associated with hydrogen sulfide exposure.[a]

Animal fat & oil processors	Lithographers
Animal manure removers	Lithopone makers
Artificial-flavor makers	Livestock farmers
Asphalt storage workers	Manhole & trench workers
Barium carbonate makers	Metallurgists
Barium salt makers	Miners
Blast furnace workers	Natural gas production
Brewery workers	Painters using polysulfide caulking
Bromide-brine workers	compounds
Cable splicers	Paper manufacturing
Caisson workers	Petroleum production & refinery
Carbon disulfide makers	Phosphate purifiers
Cellophane makers	Photo-engravers
Chemical laboratory workers,	Pipeline maintenance workers
teachers & students	Pyrite burners
Cistern cleaners	Rayon makers
Citrus root fumigators	Refrigerant makers
Coal gasification workers	Rubber & plastics processors
Coke oven workers	Septic tank cleaners
Copper-ore sulfidizers	Sewage treatment plant workers
Depilatory makers	Sewer workers
Dye makers	Sheep dippers
Excavators	Silk makers
Felt makers	Slaughterhouse workers
Fermentation process workers	Smelting
Fertilizer makers	Soap makers
Fishermen & fish-processing	Sugar beet & cane processors
Fur dressers	Sulfur spa workers
Geothermal-power workers	Sulfur products processors
Glue manufacturing	Synthetic-fiber makers
Gold-ore workers	Tank gaugers
Heavy-metal precipitators	Tannery workers
Heavy-water manufacturers	Textile printers
Hydrochloric acid purifiers	Thiophene makers
Hydrogen sulfide production	Tunnel workers
Landfill workers	Well diggers & cleaners
Lead ore sulfidizers	Wool pullers
Lead removers	

[a]From NIOSH. *Occupational exposure to hydrogen sulfide*. No. 77-158. Cincinnati, Ohio:National Institute for Occupational Safety and Health, 1977.

Table 10. Selected case reports of hydrogen sulfide and sulfur toxicoses in animals.

Nº	Reference	Situation (location)	Exposure source & factors
Ruminant cases			
1	Coghlin[45]	Calves stabled in barn (Ontario, Canada) - acute to subacute.	Top-dressing 14 kg sulfur over silage as prophylactic treatment for ringworm and lice. Some animals selectively and "greedily" consumed. Water system was off 24 hr prior to feeding (?).
2	White[267]	Flock of postpartum ewes on pasture (England) - acute to subacute.	Sublimated sulfur formulated into barley-based pellets and fed to resolve chronic, low grade infections (est. 45 - 70 g/ewe/d).
3	Bengtsson[24]	Modern cattle barn with slatted floors & liquid manure system (Sweden) - chronic(?).	Malfunctioning sub-floor drainage system and poor building ventilation.
4	Haartsen[95]	Cattle barn with adjacent in-ground liquid manure tank (Germany) - acute.	Agitation of manure pit with ventilators closed (winter winds) but connecting manure channel open (est. 120 - 600 ppm).
5	Gunn *et al.*[94]	Replacement Holstein heifers housed in three separate barns according to age (Ontario, Canada) - acute to subacute.	Feeding concentrate ration supplemented with 33 kg "flowers of sulfur" (≥99.5% S) / 1000 kg grain, with additional flowers top-dressed (ave. est. 405 g S / head or 0.85 - 3.8 g S / kg BW).
6	Short & Edwards[216]	Case 1: Crossbred cows on range (Oklahoma) - acute to subacute.	Fed sulfur-based tick & Anaplasma control supplement with excessive sulfur level.

№	Clinical signs & symptoms	Basis for diagnosis	Treatment
1	(43/10/6)[a] Muscle spasms, uneasiness, ataxia, diarrhea, recumbency, depression, inappetence, blind, dyspnea, tachypnea, comatose, death, hydrogen sulfide on breath.	Hx[b]; hydrogen sulfide odor in barn & on breaths; PM[c]: pulmonary congestion & edema, pale livers, inflamed GI tract, some muscles were black.	Change diet, camphor in oil IM, calcium gluconate IV, fresh air, supportive care, oral solution: iron sulfate, liquid ammonia fortis, warm water
2	(480/160/32) Colic, depression, dyspnea, pyrexia, hydrogen sulfide on breath, black diarrhea, bloody feces	Hx; hydrogen sulfide on breath; PM: severe GI inflammation, ascites, inflamed & blackened kidneys, generalized petechial hemorrhage.	Glucose, saline, glycerine, bismuth carbonate PO, changed ration to bismuth carbonate in cubes.
3	(?/30/12) Inappetence, dull-rough coats, inelastic skin, loss of condition, reduced milk yield, tetany, SQ hematomas, lameness, tachycardia, tachypnea	n/a	n/a
4	(?/4/1) n/a	Hx; reenactment; n.b. - other manure gases may be involved.	n/a
5	(120/48-60/14) dehydration, rumen stasis, tachycardia, pale mucous membranes, pyrexia, dark diarrhea, colic, dyspnea, H_2S on breaths, metabolic acidosis, hypocalcemia & hypokalemia.	Hx; odor of H_2S in barn; PM: yellow granular S in GI, severe hemorrhagic rumenitis and enteritis, pulmonary edema, swollen pale renal cortices (acute tubular necrosis), secondary ruminal infections.	Changed ration, oral rumen antacid, Carmilax (Mg hydroxide + nux vomica + ginger + capsicum), supportive care.
6	(10/10/10) Ataxia, rumen stasis, bloat, death.	Hx; strong H_2S odor at PM.	n/a

Table 10. Continued.

		Case 2: Hereford-crossbred cows and calves on range Oklahoma) - acute to subacute.	Fed sulfur-based tick & Anaplasma control supplement with excessive sulfur level.
		Case 3: Crossbred cattle of various ages on range Oklahoma) - acute to subacute.	Fed sulfur-based tick & Anaplasma control supplement with excessive sulfur level.
Swine cases			
1	O'Donoghue[180]	Experimental exposure of pigs to hydrogen sulfide (Alberta, Canada) - acute to subacute.	Experimental inhalation studies (6) at variable intra-exposure concentrations
2	Molony[167]	Swine in confinement pen (Northern Ireland) - acute.	Associated with agitation of liquid manure pit behind pens.
3	Boothroyd[31]	Confinement unit with slatted floor over manure pit (England) - acute.	Forced-air agitation of liquid manure pit prior to emptying without pit fans functioning.
4	Miniats[166]	Modern swine confinement unit with slatted floors over manure pit (Ontario, Canada) - acute.	Mixing and evacuation of the eastern end manure pit without increasing that sections ventilation.
5	Vik[254]	Confinement unit with slatted floor over manure pit (n/a) - acute.	Extra vigorous agitation of liquid manure prior to evacuation of pit.
6	Donham *et al.*[65]	Case 4. Modern swine confinement unit with slatted floors over manure pit (n/a) - acute.	Vigorous agitation of liquid manure prior to evacuation of pit. Pit ventilation was off, although wall fans were running.
		Case 5. Modern swine confinement unit with slatted floors over manure pit (Iowa) - acute.	Vigorous agitation of liquid manure prior to evacuation of pit. Only one ventilation fan operating.

	(16/16/12) "Exhibited clinical signs," death.	Hx; PM: cyanotic mucus membranes, reddish-brown conjunctiva, SC hemorrhage, grey-green tracheal & bronchial mucosa with hemorrhage, pulmonary congestion, gastroenteritis, elemental S granules in contents, liver & kidney cyanotic, dark red-purple blood, strong H_2S odor in rumen & abomasum; positive lead acetate paper test on rumen and abomasal contents.	n/a
	(36/20/5) Recumbent, ataxia, listless, depressed, mania, death.	Hx; PM results not published; positive lead acetate paper test on rumen contents	n/a
1	(6/5/3) Discomfort, ocular irritation, salivation, semicomatose, spasms, shallow breaths, cyanosis, tetanic convulsions, ± dyspnea, death	Hx - "A confirmed diagnosis would have to be based on known exposure." PM: no significant lesions with acute deaths, cyanosis, marked hypostatic pulmonary congestion, minor pulmonary hemorrhage.	Fresh air
2	(23/19/19) Found dead.	Suspected carbon dioxide toxicosis; n.b. - hydrogen sulfide most likely (Lawson & McAllister[137]).	Fresh air
3	(7/14/14) Found dead.	Suspected carbon dioxide toxicosis; n.b. - hydrogen sulfide most likely (Lawson & McAllister[137]).	Fresh air
4	(670/ 21/16) Ataxia, dyspnea, listlessness, cyanosis, collapse, death.	Hx; PM: pulmonary edema and congestion, emphysema; n.b. - H_2S most likely, but other manure gases considered.	Fresh air
5	(177/ 65/65) Death, other clinical signs not published.	Hx	Fresh air
6	(400/200/60) Collapse, respiratory distress, death.	Hx; n.b. - H_2S most likely, but other manure gases considered.	n/a
	(400?/+224/224) Collapse, death.	Hx; odor; n.b. - H_2S most likely, but other manure gases considered.	n/a

Table 10. Continued.

Other species			
1	Eveleth *et al.*[75]	Chinchilla shed (North Dakota) - acute.	Burning high-sulfur coal for heat in enclosed building.
2	Blaxland *et al.*[29]	Laying hen house with sub-floor excreta pit (England) - acute.	Agitations of pit without ventilation fans running and improperly fitting manhole covers in the unit.
3	Corke[48]	Horse stable (England) - acute.	Accidental feeding of elemental sulfur to stabled horses (est. 0.2 - 0.4 kg/animal). It was initially being administered to one affected animal as an azoturia preventative (15 g/d).
4	Torrans[240] Torrans & Clemens[241]	Commercial catfish farm	Unconsumed fish food caused high sulfide levels in the feeding area and disturbance of sulfide rich sediments during harvesting in summer

[a](Number of animals initially exposed / animals developing some signs of clinical toxicosis / deaths due to hydrogen sulfide).

[b]Hx = history, circumstance, clinical signs and progression.

[c]PM = gross ± histologic postmortem examination.

1	(18/16/16) Found dead.	Hx; odor; PM: cyanotic mucous membranes, lungs grey & edematous, liver grey; silver coin placed in contact with tissues turned black in 20 minutes; production of lead sulfide when blood and liver distillates were reacted with a lead acetate solution	n/a
2	(4000/+130 regional/130) Comatose, found dead, recumbent, decreased feed consumption and egg production in affected sections.	Hx; PM: cyanotic muscles, straw colored pericardial fluid ± fibrin clot, friable fatty liver, congested kidneys, severe respiratory congestion & edema, serous fluid in body cavity; positive lead acetate paper test on uneaten ration.	n/a
3	(14/14/2) Dull, lethargy, colic, diarrhea, yellow nasal froth, respiratory distress & failure, cyanosis, terminal convulsions, pale yellow mucous membranes ± petechia, ± inappetence, ± hepatic failure.	Hx; PM: ventromedial pulmonary consolidation with emphysema, yellow froth in trachea & bronchi, unclotted blood in heart, pericardial transudate, subendocardial hemorrhage, GI edema & hemorrhage, yellow-green GI contents, liver congested & friable.	Mineral oil & aluminum hydroxide PO, methylene blue IV, colic: hyoscine N-butyl bromide & metamizole IV with pethidine IM, resp. fluid: furosemide, betamethasone, hydrochlothiazide, metabolic acidosis: Na bicarbonate IV & PO, supportive care & nutrition.
4	(n/a) Hyperpnea, bradypnea, tachycardia, apnea, spasms, death, abnormal feeding & poor growth post recovery.	Experimental.	Fresh water, change feeding practices, oxidize sulfide in water.

Environmental quality, especially particulate and air contamination, is cited as the major limiting factor in performance of animals raised in confinement.[54,63,64,208] Reported particulates include feed, animal dander, feces, mold, pollen, mite, insect parts, mineral ash, microbes and proteins, and endotoxins.[54,61,62,195] Of the numerous gases and vapors produced by anaerobic digestion of liquid manure,[62,67,171] ammonia, carbon dioxide, hydrogen sulfide, and methane are considered the major confinement unit gases.[8,35,54,83,144,166,171,178,179,195]

Gases in confinement units do not reach harmful concentrations under normal operation.[54,171,178] However, with poorly designed buildings, improper ventilation rates (pit and house), and poor manure pit management, elevated manure gas levels and fatal exposures can result.[54,63,67,83,178,208] Vigorous mixing and evacuation of an established anaerobic manure pit, especially with insufficient ventilation, poses the greatest potential for gas associated fatalities.[35,54,83,144,171,208] Agitation results in massive release of gases due to increased diffusion, freeing gas trapped in viscous matter, and disrupting the organic surface layer.[65,195] Hydrogen sulfide is responsible for most animal and human deaths associated with manure gases.[54,65,137,166,178,208]

Ruminants Hydrogen sulfide toxicosis in ruminant livestock has been attributed to gas exposure and ingestion of elemental sulfur. The sources of gas exposure have been primarily anaerobic degradation of manure,[24,56,59,95,251] although industrial accidents in oil and natural gas fields are cited.[180,198] A majority of cases result from the therapeutic or prophylactic feeding of elemental sulfur, especially when overdosing occurs.[45,94,216,267]

Swine Improper agitation of the manure pit prior to unloading, especially in combination with ventilation failure, accounts for all the reported swine cases. Resulting clinical signs and deaths are related to the location of the animals in the confinement unit relative to site of agitation and ventilation.[31,65,166,167]

Other species Any animal raised in confinement in association with liquid manure management or in close proximity to hydrogen sulfide generating industry is at risk. Some of the cases reported include poultry,[29,215] horses,[48] chinchillas,[75] and catfish.[240,241]

Diagnosis of acute - subacute toxicosis

History and environment Diagnosing hydrogen sulfide toxicosis, as other disease processes without pathognomonic changes, relies on collecting various facts and establishing prioritized differentials for further consideration or confirmation. The basis for a positive diagnosis ranges from solely historical to tissue confirmation (Tables 8 and 10).

A major factor in establishing a diagnosis in all cases is an implicating history or circumstance under which an exposure could occur. Typical would be acute deaths in swine confinement units associated with manure pit agitation,[65,166,170,254] feeding elemental sulfur to ruminants,[45,94,216,267] or an industrial accident (Table 9).[38,250,273]

Some environmental evidence may still exist although the gas has often dissipated. If residual levels are not below the olfactory threshold, then rescuers or investigators may note the characteristic "rotten egg" odor.[5,45,58,65,94,159,193,252] This is typical in barns housing cattle or sheep poisoned by ingesting elemental sulfur.[45,94]

Discoloration of metallic objects in the area or on the victims is occasionally reported. Copper and brass buttons, badges, keys and coins from the victims may turn black due to the formation of copper sulfide.[5,38,159,268] Metal objects in the surrounding area may include copper in thermostats, wiring and piping (black), zinc in galvanized steel (white), or lead in paints (black).[172,208,268]

Measuring environmental gases is often attempted; although with time the agent has often dissipated completely or significant levels are not currently regenerated. In industrial accidents, the actual exposure or recreated atmosphere frequently reveals diagnostic levels of hydrogen sulfide[32,107,250,273] when compared with similar conditions in agricultural cases;[65,166,254] especially if a mechanized process, sealed tank or chemical vat is involved. However, exceptions do exist for manure pit associated cases.[170,185] Since manure derived hydrogen sulfide results from anaerobic microbial metabolism in the pit, a recently agitated pit will likely have released most of the trapped or dissolved gas and will not produce more until anaerobic conditions are reestablished.[29,65]

Clinical signs and symptoms Onset and progression of clinical signs or symptoms in acute-subacute exposures are strongly suggestive of a highly toxic, irritative, respiratory and CNS poison (Tables 5, 7, 8 and 10), but not diagnostic for hydrogen sulfide. One exception is that eructated gases and exhaled air from ruminants consuming sulfur will usually smell of hydrogen sulfide.[45,94,106] Human victims will occasionally smell of "rotten eggs,"[5,58] but no reports exist for livestock.[180]

Pathology Gross and histologic pathology may also implicate the nature and mechanism of the toxicant, but not a specific compound (Tables 8 and 10). One occasional variation seen in human gross pathology is the slate grey, greenish blue, or black pigmentation of internal organs that disappears with formalin fixation.[5,38,96] Various descriptions of tissue discoloration exist in the livestock literature, but they are not consistent in colors or organs.[29,45,75] The gastrointestinal contents of ruminants ingesting sulfur typically smell of

"rotten eggs" and yellow granules are common findings.[94,216] A histochemical technique in which "heavy metal sulfides formed in tissues are visualized with aid of physical development" has been applied experimentally to determine organ deposition in rats and guinea pigs.[256]

Several clinical pathology parameters are altered after acute to subacute hydrogen sulfide exposure. In human case reports, blood gases and acid-base balance are usually determined upon admission to a hospital,[58,107,185,193,232,239] although prior to arrival the patient is usually treated with 40 to 100% oxygen which may falsely elevate the oxygen values. Hoidal *et al.*[107] used blood gases to document impaired oxygen transport ($\%O_2$ saturation gap) and extraction (increased PvO_2 with normal PaO_2) attributed to altered hemoglobin and inhibited oxidative metabolism, respectively. Sulfhemoglobin is not a significant factor in hydrogen sulfide toxicosis,[5,21,68,80,172,188,189,222] but elevated levels have been reported in clinical cases.[107,193] Metabolic acidosis is a common derangement, but elevated serum lactate has only been reported twice.[185,241]

Long-term decreases in δ-aminolevulinic acid synthase and heme synthase activity occur with nonlethal hydrogen sulfide exposures and have been used to document cases.[113,238] In addition to the analyses being tedious, reduced enzyme activities do not correlate with degree of H_2S exposure, and other sulfur compounds common in the same environment produce similar results.[113]

Since hydrogen sulfide *in vivo* is predominately oxidized and excreted in the urine, urinary analysis of thiosulfate concentrations as a bromobimane complex by high pressure liquid chromatography has proposed application in industrial monitoring and sublethal or fatal forensic testing.[121] The method is sensitive to concentration and length of exposure, therefore it may have value in predicting environmental levels.

Ancillary procedures Ancillary diagnostic procedures are often used in human cases, although the alterations reported are not specific for hydrogen sulfide toxicosis. In nonfatal accidents, thoracic radiographs may reveal pulmonary edema or subsequent aspiration pneumonia.[36,58,185] Abnormalities have been reported on electrocardiography,[36,133,250] electroencephalography,[36,263] magnetic resonance imaging,[246] and computer tomography.[80,157,246] Wasch *et al.*[263] measured prolonged P-300 event-related cortical potentials (occurs 300 ms after onset of a rare tone) with an electroencephalograph in recovered victims of acute toxicoses with persistent cognitive impairments.

Qualitative analysis Several techniques based on reaction with metals have been utilized in case investigations to incriminate hydrogen sulfide as the toxicant. In a case of sudden deaths in chinchillas, Eveleth *et al.*[75] noted that a silver coin placed in contact with

liver or lung turned brown to black after 20 minutes (silver sulfide), and distillate from these tissues produced lead sulfide when reacted with lead acetate. Control samples are not mentioned. Similarly, sludge and water from a plaster of paris trap which a victim was cleaning with a 90% sulfuric acid solution when afflicted were acidified and resulting gases reacted with a silver nitrate solution to yield silver sulfide.[193] Blackened coins from dead sewer workers react with acid to generate the characteristic odor of "rotten eggs."[5] Lead acetate paper has confirmed the presence of hydrogen sulfide in feed samples collected from a poultry confinement unit experiencing sudden deaths,[29] and rumen contents of cattle ingesting elemental sulfur.[216]

Quantitative analysis A definitive diagnosis of hydrogen sulfide toxicosis can be strengthened by establishing significant sulfide concentrations in fresh tissues of the victim. Various methods have been developed to analyze different matrices for sulfide or hydrogen sulfide; many of these general techniques have been reviewed elsewhere.[21,90,134,172,191,268] Discussed below are published separations and determinations of sulfide with emphasis on diagnostic methods.

Gas chromatography (GC) Kage *et al.*[117] developed a GC method for biological samples based on extractive alkylation using pentafluorobenzyl bromide, tetradecyldimethylbenzylammonium chloride as phase-transfer catalyst, and potassium dihydrogenphosphate to buffer against sulfide formation. Separation is achieved on a glass column (2.1 m x 3 mm id) of 5% Apiezon grease L on Chromosorb W, at 200°C and a nitrogen flow of 0.3 kg/cm^2. A Ni^{63} electron capture detector connected to a computerized recorder provides quantitation. Sharp, symmetrical peaks, without interference, and linear calibration curves (0.0 to 0.8 ppm sulfide) are reported. The lowest detection limit is 0.01 ppm sulfide. Confirming the method with male Wistar rats fatally exposed to 500 to 600 ppm hydrogen sulfide, blood sulfide levels ranged from 0.19 to 0.61 ppm (n = 5), versus <0.01 ppm for controls. Statistical descriptions of performance and spike recovery are not provided.

High-performance liquid chromatography (HPLC) Savage and Gould[209] developed a determination for sulfide in bovine brain and rumen fluid while investigating the role of dietary sulfate in the pathogenesis of nutritionally induced polioencephalomalacia.[89] Pre-column derivatization of sulfide with *p*-N,N-dimethylphenylenediamine in 9.0 M sulfuric acid and ferric chloride as the oxidizing agent yields methylene blue. This is separated on an ion-interaction reversed-phase HPLC system and measured by spectrophotometric detection. The mobile phases (1.5 ml/min) are 35% acetonitrile, 0.5% acetic acid and 64.5% water, and 5.0 mM *p*-toluenesulfonic acid. Reproducibility is 1.8% and 1.6% coefficient of variation, 100 ±

1% and 86.3 ± 3% recovery, and 0.8% and 0.1% injection-to-injection for rumen fluid (n = 10) and cerebral cortex (n = 7 to 8), respectively. Normal sulfide concentrations for rumen fluid and brain are 1.8 ± 0.60 ppm (0.70 to 2.26 ppm) and 5.3 ± 0.99 ppm (3.3 to 6.1 ppm), respectively.

Recently, Ogasawara *et al.*[181] used pre-column derivatization with *p*-phenylenediamine and ferric iron to form the fluorescent compound thionine prior to ion-pair reversed-phase HPLC separation and fluorometric detection. After liberating the sulfide from washed human red blood cells by Conway microdiffusion,[46] the trapped sulfide is then derivatized and analyzed using a mobile phase of acetonitrile and sodium phosphate buffer (50/50) with sodium dodecyl sulphate added. Thionine elutes at 6.9 minutes as a sharp, well resolved peak. Calibration is linear from 0.003 to 1 ppm S^{-2} (r = 0.999), and relative standard deviations of peak areas for 0.006 and 0.3 ppm S^{-2} are 2.54 and 1.74%, respectively. Aqueous spike recoveries for 0.15 to 16 S^{-2} ppm range from 99.7 to 100.6%, and 54.1 to 60.5% in human red blood cell samples. The detection limit is 48 ppt sulfide. In application with red blood cells from healthy humans age 21 to 48 years, the mean (± SD; n = 5) sulfide contents are 0.053 ± 0.008 ppm for males and 0.048 ± 0.004 ppm sulfide for females. Relative standard deviations for five replications are 5.6% intra-assay and 8.89% inter-assay.

Gas dialysis / Ion chromatography Based on a method for measuring trace sulfides in turbid water,[87] Goodwin *et al.*[86] uses a continuous flow gas dialysis pretreatment to separate sulfide from brain homogenates, and subsequent injection into an ion chromatograph (anion column) with electrochemical detection by a silver working electrode, a silver/silver chloride reference electrode and stainless steel counter electrode for determination. Spike recovery from rat brain homogenate is 81.3 ± 1.35% (mean ± SE, n = 3), and a detection limit of 0.02 ppm sulfide. Similar analysis of bovine serum albumen, cysteine or methionine solutions for sulfide generated by alkaline hydrolysis yielded negative to negligible amounts.

Sulfide concentrations from brains of male Sprague-Dawley rats injected with the 5, 9 or 17 mg S^{-2}/kg BW IP produced a linear log dose-response curve with a highly significant dose effect ($P < 0.001$). Resulting brain sulfide levels are 2.3, 3.1 and 4.5 ppm, respectively; compared to 1.57 ppm for the controls. In two separate human cases of acute toxicosis in petrochemical workers, one exposure estimated at 7500 ppm H_2S, the brain stem sulfide concentrations are reported at 0.91 and 1.04 ppm S^{-2}. Human brain stems from non-hydrogen sulfide related deaths contain 0.69 ± 0.02 ppm S^{-2} (mean ± SE, n = 36) in males and 0.59 ± 0.074 (n = 9) in females. This analytical method has also been used by the same group to study brain concentrations resulting from sulfide injection versus gas inhalation,[202] selective

uptake of sulfide by the brain stem,[258] inhibition of monoamine oxidase by hydrogen sulfide,[261] and liberation of non-acid labile sulfide by dithiothreitol from brain tissues post toxicosis.[259]

Ion-selective electrodes (ISE) Potentiometric determination with ion-selective electrodes is a relatively recent addition to analytical chemistry as gauged by the number of theoretical and new design publications, versus those on application. Analysis may involve a solid-state electrode and double-junction reference electrode that together constitute an electrochemical cell, as illustrated by:[13,169]

[Ref Electrode | Ref Solution | J_1 | Salt Bridge] * J_2 * Sample Solution * ISE Membrane.

Where the double-junction reference electrode is encompassed within the brackets, and J_1 and J_2 are liquid-junction interfaces. The cell potential is the sum of local potentials at various solid-solid, solid-liquid or liquid-liquid interfaces, and depends on the physical designs of the electrochemical cell and component selection. Reference electrode solutions are ideally selected so resulting liquid-junction potential is negligible, and the net cell potential is defined by the membrane potential developing at the sample-ISE membrane interface.[169]

Response of an ideal and specific solid-state ISE to the ion activity of the analyte has been described as Nerstian:[13,34,132,169,184]

$$E = E^o + (2.303\ RT / n_xF)\ \log a_x, \qquad (1)$$

where E is the cell potential, E^o standard potential, R the gas constant, T temperature in Kelvins, n_x ionic charge of analyte, F the Faraday constant, and a_x the activity of the ion measured. If the ionic strength of the background solution is held constant by adding large concentrations of inert ions (buffer), then the activity coefficient is constant, and ion activity is directly related to concentration. Therefore, equation (1) can be rewritten in terms of molarity [X], or

$$E = E^o + (2.303\ RT / n_xF)\ \log [X]. \qquad (2)$$

With temperature constant and s, the Nernstian slope, substituted for (2.303 RT / n_xF), the equation simplifies to,

$$E = E^o + s\ \log [X]. \qquad (3)$$

At 25°C, the slope equals 59.16 mV/n_x, or 29.58 mV/decade for an ideal sulfide standard curve.[30,33,52,108,143,214] However, others believe the potential developing at sample-electrode membrane phenomena reflect stored electrical charge and therefore should be modeled as a capacitor.[40,84]

When a sensing electrode and appropriate reference are immersed in a solution containing a determinant at a concentration to which the pair is responsive, electrical potential develops at the electrode membrane due to a "selective spontaneous reaction."[183] Although much work has been directed at elucidating the mechanism by which the membrane potential forms, much is not understood.[130] Most theories center on ionic, elemental or electronic defects in the crystalline lattice, or ion exchange processes on the sensing electrode membrane, and are detailed elsewhere.[34,84,136,169] Ionic transport processes in silver sulfide electrodes are primarily the result of cation Frenkel defects,[136] which involve multifocal silver ions moving from normal crystalline positions to interstitial crystal positions. This "wandering" interstitial cation produces a separation of charge described by Ag^{+}_{Ag} + interstitial site ⇄ Ag^{+}_{i} + V^{-}_{Ag}.[34,136]

Potentiometric determination of sulfide with ion-selective electrodes offers many of the desired attributes for a diagnostic assay, namely being rapid, simple and inexpensive.[13,14,70,132,242] It is a rapid technique due to the fast, linear response of the electrode over six to eight decades of analyte concentration, and the minimum amount of sample preparation required prior to the determination because of insensitivity to color, viscosity and suspended solids. Simplicity is the main advantage of ion-selective electrode methods.[242] With few working parts, simple extractions, and no decisions to be made once the methodology is established, the whole analysis can quickly be performed by technicians with minimal training or experience. The low overhead costs of equipment and technical help lends to the test being inexpensive to establish and operate in a laboratory marginally equipped. Other advantages which may have application in a diagnostic setting include potential for automation, nondestructive and noncontaminating to the sample, use with dilute or microsamples, portability, and determination of ion activity.[13,14,70,132]

The primary disadvantage of potentiometric determinations with ion-selective electrodes is its relatively low precision.[13,14,169] Since reproducibility of the potential is typically 0.1 mV, the precision for measuring a monovalent ion is limited to >2 to 4%, and 5 to 10% with temperature variation.[14,70,169] The error would therefore be 4 to 8% for sulfide measurements. Under controlled experimental conditions or increased ion activity the precision is increased to 0.5%.[14]

Using the cross reactivity of a cyanide ISE, McAnalley *et al.*[159] developed an analysis for cyanide or sulfide ion in biological specimens. Sample acidification in a Conway microdiffusion cell separates the labile sulfide from the matrix and subsequently trapped in it in 1 M NaOH.[46] The latter basic solution is assayed for ion content. Calibration with

standardized sulfide solutions generated a linear standard curve from 0.01 to 10 ppm, with a detection limit "between 0.01 to 0.1 ppm sulfide." Five human blood samples from fatal, hydrogen sulfide cases in petrochemical, sewer and leather workers contain 1.70 to 3.75 ppm sulfide (2.75 ± 0.81; mean ± SE, n = 5). Random blood samples from non-hydrogen sulfide cases ranged from 0.05 to 0.08 ppm S^{-2} (n = 100). Other published human cases that report elevated blood sulfide concentrations determined by this technique[159] include an Italian truck driver poisoned while cleaning polysulfides from a tanker truck with an acid-based solvent (>3.5 ppm sulfide),[38] and three fatal liquid manure exposures (0.8 to 5.0 ppm sulfide).[185]

Attempts to measure blood sulfide in acute toxicosis cases where the victim recovers have failed due to the high detection limit of the method.[113,146] Based on the work of McAnalley *et al.*,[159] blood sulfide levels in non-fatal cases of hydrogen sulfide exposure are determined after preconcentrating the samples ten-fold in a wash-bottle reactor and trapping the acid-labile sulfide in sodium hydroxide.[146] The detection limit is lowered to 0.01 ppm sulfide using a 0.1 to 10 ppm S^{-2} standard curve. However, no estimates of repeatability, spike recovery, or normal values are reported. In twelve cases of non-fatal, acute hydrogen sulfide toxicosis, blood levels ranged from 0.04 to 0.6 ppm sulfide. Seventeen additional cases associated with pulp mills and oil refinery exposures are reported.[113] Blood samples drawn within two hours contain 0.039 to 0.130 ppm sulfide (0.075 ± 0.039 ppm; mean ± SD, n = 6) and those collected after two hours have <0.010 to 0.300 ppm.

Khan *et al.*[127] used a Johnson-Nishita sulfide reduction apparatus[114] to acid extract bovine blood before measuring the sulfide content with a silver/sulfide ISE and DJR. Repeatability and spike recoveries for blood are not reported.

Microdiffusion analysis Analysis of volatile compound can be performed using microdiffusion as the separation technique and colorimetric development for quantitation,[57,76,234] although other determination methods have been used.[127,159,181] The development and theory of the methodology is published.[46]

Feldstein and Klendshoj[76] used microdiffusion to liberate acid-labile sulfide from tissue and fluid and absorb it in 0.1N NaOH over three hours. Quantitation is by spectrophotometry after reaction with a bismuth reagent to produce brown colored bismuth sulfide. Detection limit is 1 ppm sulfide; recoveries from biological samples are 92 to 100%.

By redesigning the microdiffusion cell to increase the diffusion surface and using a shaker table, Debevere and Voets[57] reduced the diffusion time to 15 minutes. Measurement of the sulfide content of the basic absorption fluid is by spectrophotometry after development as methylene blue. A linear standard curve is reported over 3 to 16 ppm S^{-2}. Spike recoveries for various high protein, biological matrices are 92.0 to 104.0%.

Analysis of manure pit Dissolved sulfide levels have been used to predict potential for massive hydrogen sulfide release and post-exposure to strengthen a tentative diagnosis. In a clinical report involving the intoxication of 2 men and 21 hogs in a confinement unit during manure pit agitation, a pit sample collected the same day contained 25.7 ppm H_2S.[166] One month after another fatal, manure associated exposure, the dissolved hydrogen sulfide is reported to be 75 ppm.[61,65] While characterizing the chemical and physical nature of 23 liquid manure pits, Donham *et al.*[67] found total manure sulfides ranged from 32.7 to 274.6 ppm, and 0 to 37 ppm for soluble sulfides. Pomeroy[197] reported agitation of slurry containing 2 ppm sulfide generates 300 ppm hydrogen sulfide.

Differential diagnoses Ruling-out other clinical syndromes or diseases with similar presentations or laboratory results may be required. Causes of sudden death in livestock are listed in Appendix A. In confinement units without proper ventilation, other asphyxiating manure gases, or accumulated heat and moisture can produce high, acute mortality. If the unit uses a combustible energy source for heating, carbon monoxide toxicosis could result from defective heaters or ventilation. In cattle developing polioencephalomalacia due to hydrogen sulfide exposure, thiamine deficiency should be considered. Organic dust toxic syndrome (ODTS) occurs in humans after 4 to 6 hours exposure to dusty swine confinement units and may be associated with aerosolized endotoxins from Gram negative bacteria.[62]

MATERIALS AND METHODS

Reagents

All chemicals were of stated grade; and distilled, deionized water (Millipore) was used throughout the research. Manufacturers and sources are detailed in Appendix B. **Certified lead reference:** 1000 ppm Pb (Fisher). **Phosphate buffered saline (PBS):** 1.19 g anhydrous, reagent-grade dibasic sodium phosphate (Na_2HPO_4); 0.21 g reagent-grade monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$); 8.5 g sodium chloride (NaCl); and 1000 ml deionized water. Adjusted pH to 7.4 with dilute HCl or NaOH. **Poloxalene-mineral oil mixture:** 50:50 v/v. **0.85% Saline:** 8.5 g certified-grade sodium chloride (NaCl) in 1000 ml deionized water, adjusted pH to 7.4 with NaOH or HCl solutions; or commercial equivalent. **Sulfide antioxidant buffer (SAOB):** 40.0 g certified-grade sodium hydroxide (NaOH) and 20.0 g certified-grade L-ascorbic acid per liter of buffer. The above chemicals were dissolved in 1000 ml of deaerated water.[60,78] **100 ppm sulfide solution:** 50.0 g reagent-grade sodium sulfide ($Na_2S \cdot 9H_2O$) in 50 ml deaerated water (0.85% saline or phosphate buffered saline) to produce a stock 133,300 ppm sulfide solution. The above stock solution (~0.8 ml) was diluted to 500 ml with fresh SAOB to produce a 100 ppm S^{-2} standard after accounting for differences in hydration of the sodium sulfide and losses due to oxidation or interaction with other impurities in the reagents. The final sulfide concentration was confirmed by potentiometric titration.[184] **10,000 ppm sulfide solution:** 200.0 g reagent-grade sodium sulfide ($Na_2S \cdot 9H_2O$) dissolved in 200 ml deaerated water (0.85% saline or phosphate buffered saline) to produce a stock 133,300 ppm sulfide solution. Diluted 187.5 ml of the above to 1000 ml yield ≈10,000 ppm sulfide solution after accounting for oxidation, losses during formulation, and variation in hydration of sodium sulfide. Concentration was confirmed by potentiometric titration after dilution with SAOB.[184] **Deionized, deaerated water:** prepared by rapidly bubbling dry-grade nitrogen (N_2) for 30 minutes through a gas dispersion tube (Corning). **Sulfuric acid-ammonium persulfate bath:** 7.5 L reagent-grade sulfuric acid was added to a plastic tank (Nalge) with lid, to which 100 g reagent-grade ammonium peroxydisulfate was mixed. An additional 200 g ammonium peroxydisulfate was added every 3 to 4 weeks to recharge the bath.

Commercially Available Apparatus

The manufacturers or sources of the following commercial equipment are detailed in Appendix B. **Ion-selective electrodes (ISE):** Lazar cyanide micro-ISE, Lazar silver/sulfide micro-ISE, and Orion silver/sulfide ISE. **Double-junction references electrodes (DJR):** Lazar micro-reference and Orion reference. **pH/mV meters:** Orion model 701A and Corning 255 ion analyzer. **Circulating water bath:** high capacity water bath (Blue M), bilge pump (Atwood) and

regulated power supply (Tenna). **Data acquisition:** Personal computer (Televideo) running MS-DOS and GWBASIC, RS-232 cable, acquisition program (Appendix C), diskettes and printer. A second machine (Zeos) with a spreadsheet (Lotus) summarized the data (Appendix D). **Safety equipment:** hydrogen sulfide monitor (Industrial Scientific) and an emergency escape breathing apparatus (North Safety Equipment). **Other:** magnetic stirrer (Corning), micropipets (Rainin), gas detector system with 100 to 2000 ppm H_2S analyzer tubes (Sensidyne).

Procedures

Calibration of the standard sulfide solutions

The sulfide concentration of the standard 100 or 10,000 ppm S^{-2} solutions were confirmed by potentiometric titration[184] with a certified 1,000 ppm lead reference (Fisher), the Lazar silver/sulfide micro-ISE, and Orion DJR. For the 100 ppm standard, 10 ml of room temperature solution was transferred to a plastic beaker (Nalge) containing a stir bar (Fisher) and placed on an insulated (Dow) magnetic stirring plate. The electrode pair was immersed and initial potential noted after two minutes (0.00 ml 1,000 ppm Pb). Aliquots of the lead standard were added to the beaker with micropipets and the resulting millivoltage recorded after one minute. Titration continued so each addition produced a 5 to 10 mV change, and the end-point was surpassed to delineate a typical sigmoid curve. The electrodes were rinsed with copious amounts of water and dried with a soft tissue after each titration. The procedure was repeated at least three times for each sulfide standard solution.

Resulting negative millivoltages (abscissa) were graphed against total lead standard volume in milliliters (ordinate), and volume of titrant to the end-point determined at maximum inflection. The original sulfide concentration was for a 10 ml sample titrated with 1,000 ppm Pb (Appendix E):

(15.472973) x (titratant volume in ml) = ppm sulfide.

For the 10,000 ppm solution, 1 ml was diluted to 100 ml with SAOB, and titrated as described above. The final calculation thus reflected this dilution:

(1547.2973) x (titratant volume in ml) = ppm sulfide.

Confirming the slope of response of the ISE

With electrodes and meter set for sulfide determination, the slope of response of the sensing electrode was verified prior to each standard curve trial. First 1 ml of 100 ppm sulfide was added to 100 ml SAOB (~1 ppm) and the resulting millivoltage recorded at stabilization. Next, an additional 10 ml of sulfide standard was transferred to the beaker (~10 ppm) and the new potential recorded. The difference between these values, expressed as Δ mV/ten-fold change in concentration, is the electrode response slope or "check slope value."[184]

Calibration of the electrode pair

A modified "liter-beaker method" was used to calibrate the electrode pair.[13,52,168,182] The standard curve range of 0.02 to 10 ppm sulfide was selected based on published tissue sulfide levels.[146,159] Room temperature SAOB was transferred to a beaker containing a magnetic stirring bar (Fisher) using a 100 ml volumetric pipet (Corning). These were placed on an insulated (Dow) stirring plate and mixed at a moderate speed. The Orion silver/sulfide ISE and Orion DJR, connected to the Corning meter, were immersed 3 to 5 cm. Aliquots of the standardized 100 ppm sulfide solution were sequentially added (Table 11) without altering the measurement geometry, and the stabilized millivoltage recorded (1 to 2 minutes). Resulting potential values (-mV) were graphed against the formulated sulfide concentrations (log_{10}) to construct the standard curve.

Sulfide measurement in samples

After initial equipment and environment selection (experiment 1), all sulfide determinations were performed with the Orion silver/sulfide ISE with Orion DJR connected to the Corning meter equipped with an automatic temperature compensation probe. Samples >3 ml were transferred to a 30 ml plastic beaker (Nalge) with micro stir bar (Fisher) and mixed on a magnetic stirrer (no vortex) separated from the vessel by a piece of insulation (Dow). The electrodes were immersed 1 to 2 cm and the sample measured for 5.5 minutes; with equilibrium generally achieved within 1 to 3 minutes. However, dilute samples or volumes that required use of a microsample dish (1 to 3 ml) needed up to a 15 minute measurement period to reach steady state. Sulfide concentrations were extrapolated from the accompanying standard curve (see calibration of the electrode pair).

Data acquisition

During electrode calibration and sample analyses with the Corning meter and electrode pair, potentials (-mV) were recorded from data strings generated every 1.0 to 1.5 seconds by the meter. A dedicated personal computer running a data acquisition program (Appendix C) deciphered the signal and logged the values to magnetic disk and paper printout. These files were later processed with a spreadsheet (Appendix D) to calculate descriptive statistics for millivoltage over each minute of the analysis (mean, count, range, variance and standard error). In addition to establishing a standard curve and extrapolating sulfide concentrations in unknown samples, these statistics provided a basis for monitoring electrode performance and evaluating changes in analytical environment.

Table 11. A modified "liter-beaker" calibration method.[a] The nine point, 0.02 to 10 ppm sulfide standard curve was formulated by sequentially adding the stated volume of standard in column one to a beaker initially containing 100 ml sulfide antioxidant buffer to achieve the corresponding analyte concentration.

Volume of 100 ppm sulfide added (ml)	Accumulated Concentration (ppm S^{-2})	
	Approximate	Calculated
0.020	0.02	0.019996
0.030	0.05	0.049975
0.050	0.1	0.099900
0.200	0.3	0.299103
0.200	0.5	0.497512
0.50	1	0.990099
2.00	3	2.912621
2.00	5	4.761905
5	10	9.090909

[a]Based on Orion Research.[182]

Serum bottle storage system

Standardized sulfide solutions, extracted unknown samples, and SAOB were stored in 5, 10, 15, or 100 ml borosilicate bottles (20 mm O.D. mouth) after flushing with nitrogen (Air Products) and sealing. The capping system consisted of a 20 mm diameter x 0.5 mm thick Teflon® disk (Cadillac Plastics) under a 20 mm diameter x 3 mm thick silicon-rubber disk (Applied Science), both secured with an aluminum seal (Wheaton) applied with a hand crimper (Pierce) (Figure 4).[230] After sealing the bottles were refrigerated at 4°C.

Experimental design and statistical analysis

All experiments were completely randomized designs. Analysis of variance, Student's *t* test, and least significant difference (LSD) were used to establish the existence of significant differences (α = 0.05) between treatment means (SAS). Linear regressions for the standard curves were estimated by least-squares using a graphics program (Jandel Scientific) or data analysis software (SAS). Means with standard errors were reported.

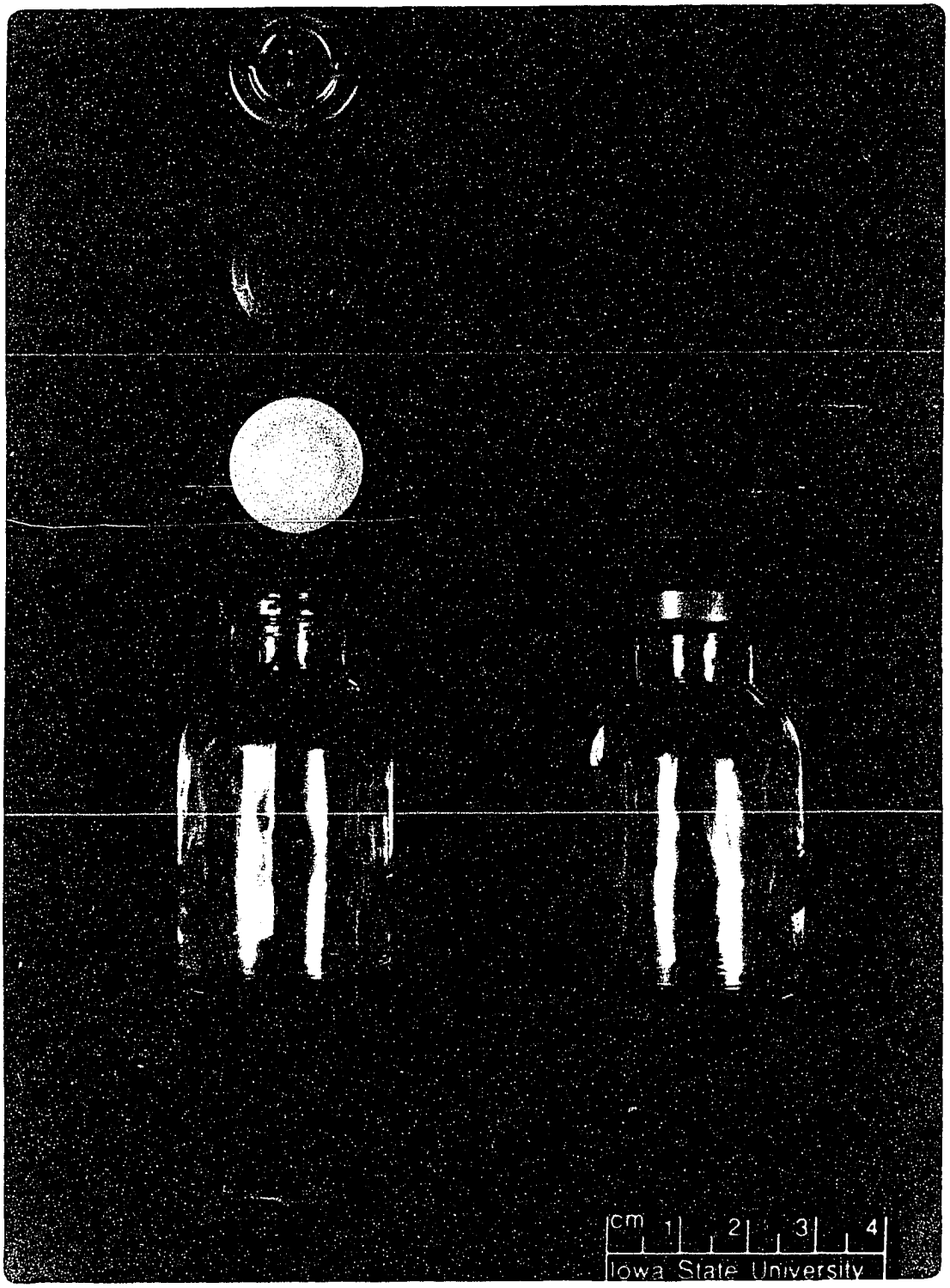
Experiments

Experimental work consisted of five phases: (1) establishment of a calibration and measurement technique for sulfide in an aqueous matrix with ion-selective electrodes; (2) development of an extraction method using known sulfide solutions; (3) application of the extraction and determination to normal tissues spiked with sulfide; (4) development of parenteral and inhalation exposure methods; (5) and animal exposures and an investigation of sample quality.

Phase 1: Measurement development

Selection of measurement equipment and environment (experiment 1) Accurate and precise ISE analyses require stable measurements under controlled apparatus configuration and environment. Selection of electrodes and pH/mV meter were based on published recommendations[159] and guidelines.[13,132,199] The initial electrodes were a Lazar silver/sulfide or cyanide micro-electrodes with matched double-junction micro-reference electrode coupled to a digital pH/mV meter (Orion). Unsatisfactory performances during calibration with standardized 0.01 to 10.0 ppm sulfide solutions required additional trials with other electrodes, meters and measurement environments. The final electrode configuration used for all the subsequent sulfide determinations in unknown samples was attached to a digital pH/mV meter (Corning) equipped with automatic temperature compensation probe (Corning). Filling solutions for the reference were saturated silver chloride for the inner chamber (Orion) and 10% potassium nitrate in the outer (Orion). All electrodes were maintained according to manufacturers recommendations.[138,139,184]

Figure 4. The capping system used to seal serum bottles of standardized sulfide solutions, extracted samples prior to analysis, or sulfide anti-oxidation buffer. After flushing the bottle with nitrogen, a Teflon® disk (TD) under a rubberized silicon disk (RSD) was immediately capped with an aluminum seal (AS) applied with a hand crimper.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Iowa State University

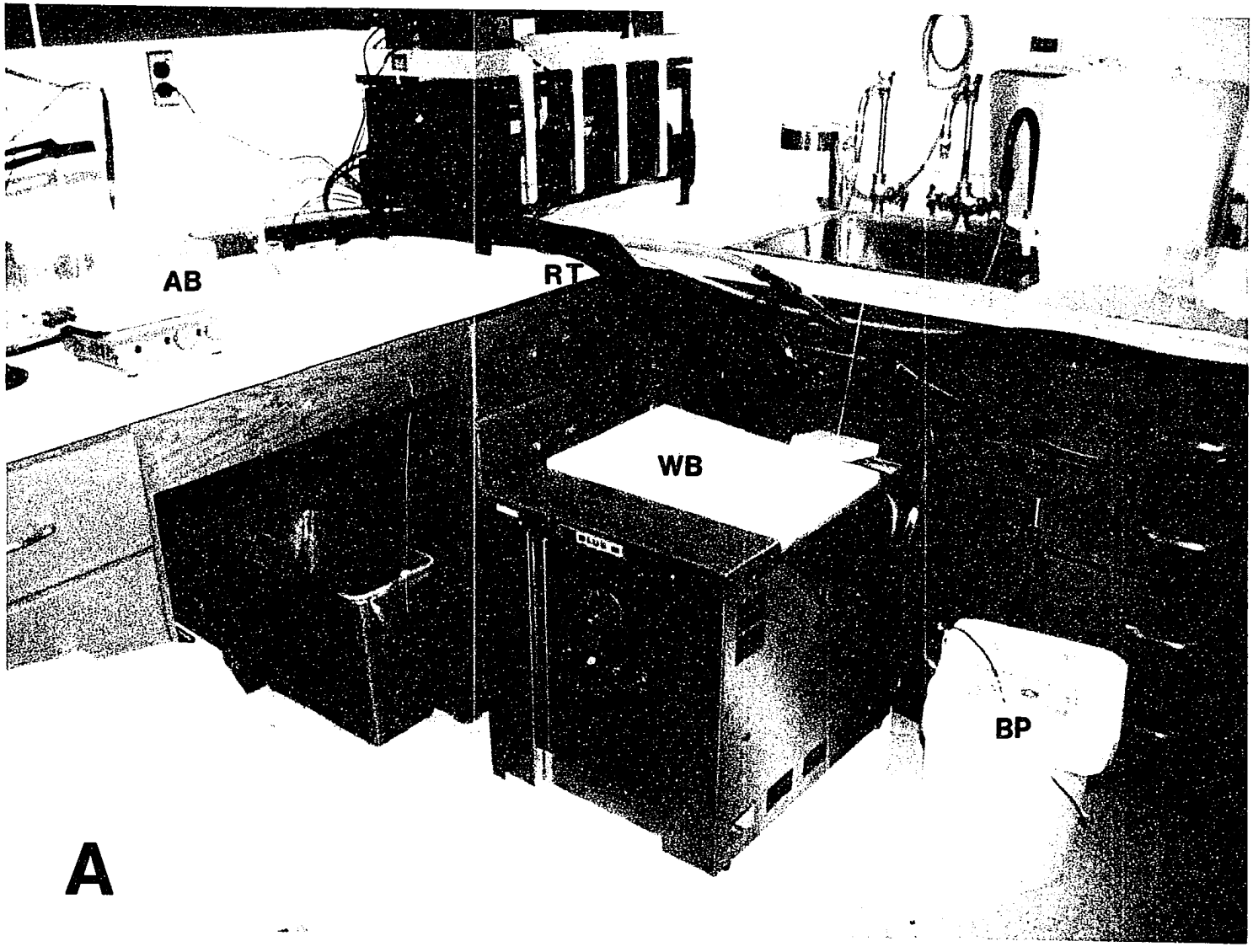
In addition to thermostatic room regulation, various techniques were devised to maintain the standards, samples and electrodes at a constant temperature in order to stabilize electrode potentials. Attempts ranged from insulation board (Dow) placed between the stirring plate and sample, an insulated jacket (Dow) constructed around a room temperature water reservoir (100 ml) with the sample immersed, and a circulation water bath system (Figure 5).

Fresh SAOB prepared with deionized, deaerated water was used in all determinations to control sulfide oxidation. In addition, a gas delivery system was installed to bubble nitrogen through or over the samples during the measurements. This consisted of ultra-pure carrier grade nitrogen (Air Products), two-stage pressure regulator with needle valve (Victor), in-line moisture trap, latex tubing, Pasteur pipets, and film wrap (American National Can) to cover the samples. Similarly, calibrated sulfide standards, extracted unknown samples, and SAOB were sealed in nitrogen flushed serum bottles and refrigerated at 4°C.

Noise generated from electromagnetic fields and capacitance effects are possible sources of error.[132] All extraneous electrical equipment was disconnected or removed from the laboratory. Essential pieces were positioned away from the measuring electrodes and grounded. In early trials, Faraday cages constructed from copper sheeting and copper screen over wooden frames were used around the support equipment (water bath with bilge pump, and regulated power supply) and measurement electrodes (Figure 6). Electrical cables were kept short, uncoiled or twisted, and shielded wire used if possible.

Stability and storage of SAOB based sulfide solutions (experiment 2) Standard sulfide solutions, calibration curves, and unknown samples were prepared or extracted into SAOB to slow oxidation. A seven point standard curve from 0.1 to 10 ppm was formulated by diluting a standardized 100 ppm sulfide solution with fresh SAOB to produce the required concentrations. Ten replications of this curve were individually sealed by concentration in serum vials. Two sets of the standard curve were warmed to room temperature and millivoltage of each concentration determined for five days ($n = 10$).

Bench-top versus controlled analysis conditions (experiment 3) The goal of the project was to develop a simple and inexpensive diagnostic test. Once the method for determining sulfide in aqueous solution was developed for highly controlled laboratory conditions, it was compared with the same procedure performed as a bench-top analysis under room conditions. Controlled measurement conditions consisted of the circulating water bath system (25.0°C), magnetic stirring, and nitrogen slowly bubbling through the sample in a film (American National Can) sealed beaker. Also, all controlled determinations were performed in a Faraday cage. Bench-top conditions were room temperature (23 to 25°C) and magnetic stir


AB
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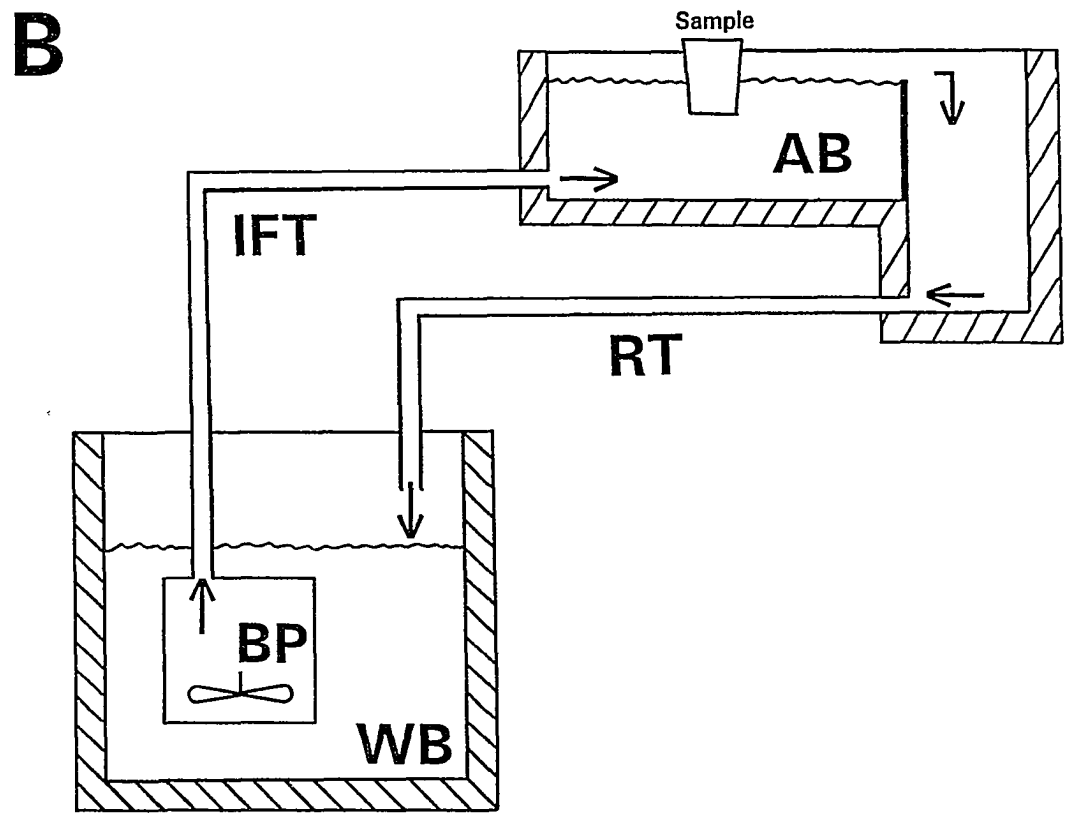


Figure 5. Circulating water bath system used to maintain uniform sample temperature during ion-selective electrode calibration and determination (both photograph A and illustration B). The flow path consisted of water bath (WB), bilge pump (BP) in flotation cradle, inflow tubing (IFT), analysis bath (AB) with magnetic stir plate, and return tubing (RT).

Figure 6. Faraday cages constructed from copper sheeting and screen over wooden frames and wired to the house ground system. Cages enclosed the bilge pump power supply (PS), water bath with pump (WB&P), and electrodes (ISE).

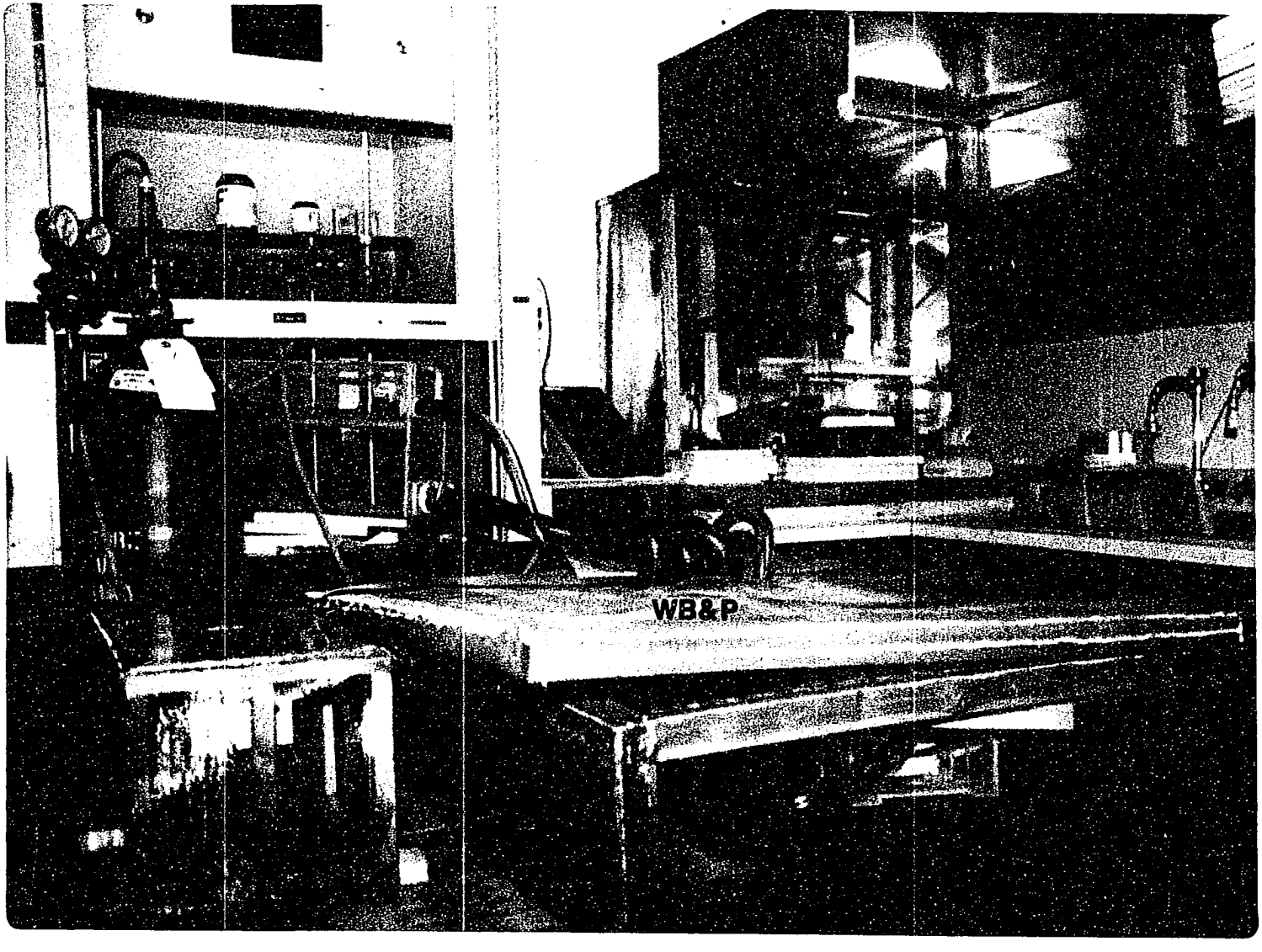

WB&P

plate with insulated pad (Dow). Standard curve sulfide solutions were formulated by diluting a 100 ppm S^{-2} standard to produce 200 ml of 0.1, 0.3, 0.5, 1, 3, 5, and 10 ppm solutions. Each concentration was divided into ten 10 ml portions which were individually sealed in serum bottles under nitrogen. Five sets of each concentration were measured as standard curves under both conditions (n = 5). The mean regression slopes and slope verification values, and point-by-point potentials were statistically compared.

Development of a modified liter-beaker calibration (experiment 4) The total laboratory time should be minimized in developing a rapid analysis for sulfide, including the calibration of electrodes. A calibration method based on the "liter-beaker technique" was devised (see above).[13,52,168,182,214,243] Two standard curves were formulated each day for five days to confirm the accuracy and reliability of the methods (n = 10). Resulting regression slopes were compared with the theoretical slope and slope verification values.

Phase 2: Extraction development with aqueous media

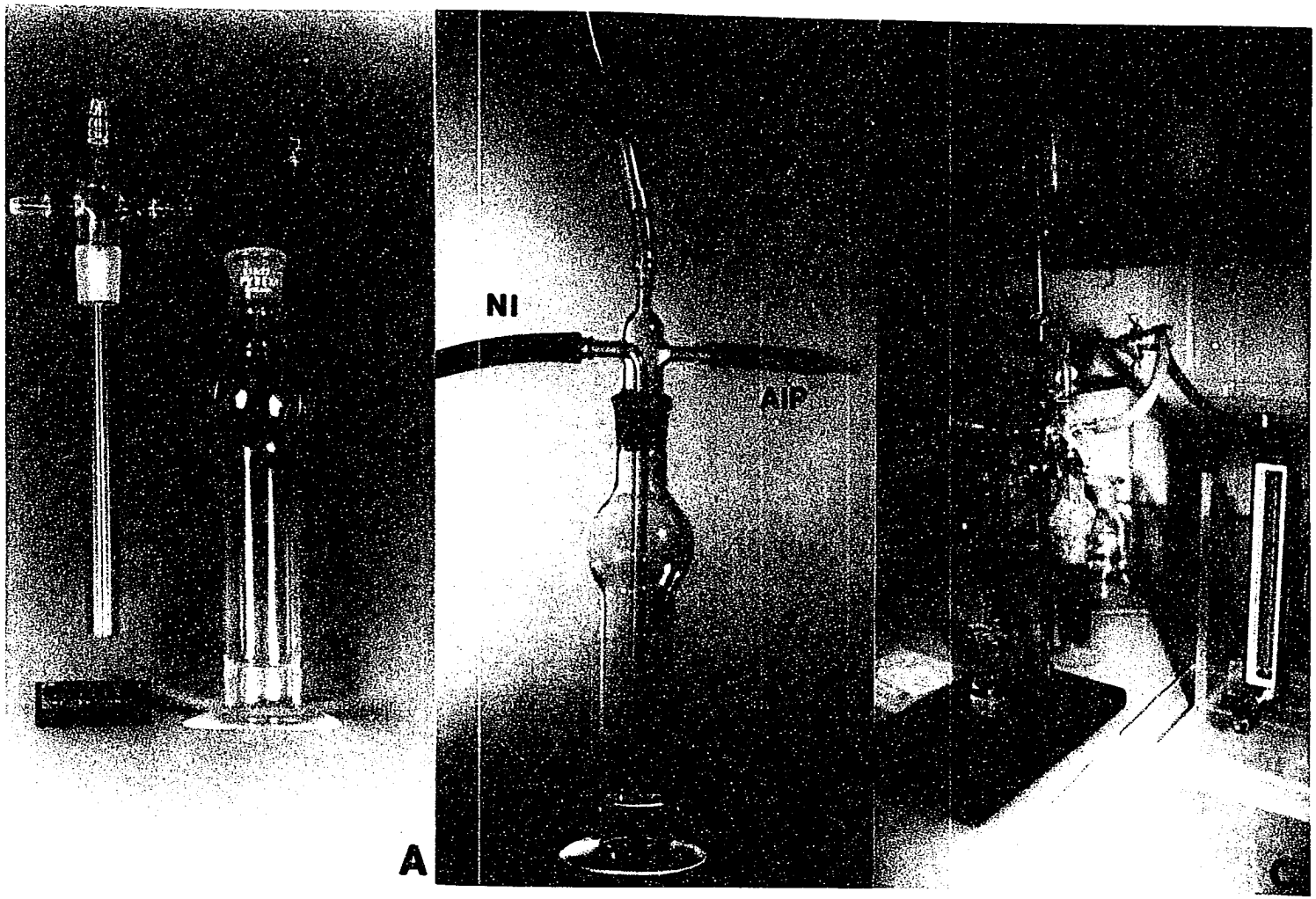
A wash-bottle chamber (Figure 7) with nitrogen gas as the carrier to transport acid-liberated sulfide as hydrogen sulfide to a basic trap solution was based on the work of Lindell *et al.*[146] The glassware consisted of 85 ml glass (Corning) wash-bottle base with a circular flare below the ~~S~~19/22 fitting. An elongated top held two lateral hose connections for injection and nitrogen influx, and one on the tapered apex for nitrogen egress. The nitrogen inflow port extended downward as a tube to within 2 mm from the bottom of the base when assembled.

Connectors for the chamber included an injection port fashioned from 5 cm of plastic tubing (6.4 mm I.D., 9.5 mm O.D., Norton) with a hypodermic needle guard firmly inserted distally into one end of the tube after a proximal portion of the guard was trimmed to receive a luer syringe tip (Becton-Dickenson). A tubing clamp (Fisher) regulated the patency. The outflow connector consisted of 30 cm of Teflon® tubing (0.5 mm I.D., Cadillac Plastics) heat sealed into the tapered end of a 1 ml disposable micropipet tip (Rainin), and 3 cm of plastic tubing (6.4 mm I.D., 9.5 mm O.D., Norton) firmly attached to the large end of the pipet tip. The nitrogen influx line was latex tubing (Fisher).

Vessels for the basic traps (SAOB) were heat sealed Pasteur pipettes for 1 ml volumes,[146] or either 5 or 10 ml volumetric flasks (Corning), depending on original sample size or desired preconcentration.

Comparison of measurement volumes (experiment 5) Use of wash-bottle extraction offers the option of concentrating the sample sulfide content by absorbing the released hydrogen sulfide into a smaller volume of base.[146] However, volumes <3 ml cannot be

Figure 7. Wash-bottle chamber for the acid extraction of sulfide from biological samples as hydrogen sulfide. (A) Dismantled chamber composed of top and bottom sections; note the wide diameter of the base with flared section to combat foaming. (B) Assembled wash-bottle with connectors attached. Connection points were for the acid injection port (AIP), nitrogen influx line (NI) and nitrogen out-flow (NE) adaptor. (C) An assembled unit during extraction of a blood sample. Foaming was a typical problem encountered with this matrix, note the lower section of the chamber.


NI
AIP
A

measured using the Orion ISE and DJR pairing in a 30 ml beaker (Nagle) with a micro stir bar (Fisher); therefore a micro-sample dish (Orion), which precludes agitation, was used. To determine if differences in measurement geometry and stirring influenced the resulting potentials over diagnostic ranges,[146,159] a set of standard curve solutions (0.1, 0.3, 0.5, 1, 3, 5 and 10 ppm S^{-2}) were formulated from a calibrated 100 ppm sulfide and ten sets of each concentration individually sealed in serum vials. The solutions as standard curves were sequentially measured as 1 or 10 ml samples at room conditions until five replications of each had been completed. The recording period was 5.5 minutes for all the 10 ml concentrations, 15 minutes for 0.3 to 10 ppm at 1 ml, and 30 minutes at 0.1 ppm for 1 ml samples to assure stabilization. The means of the resulting potentials were compared, as were the slopes of the standard curves.

Determination of optimal extraction variables (experiment 6) To evaluate the operational variables for acid-liberation of sulfide from aqueous medium and absorption in SAOB, a study of initial sulfide concentration (0.5 or 5 ppm), nitrogen carrier flow rate (30 or 60 ml/min), and absorption volume (1 or 10 ml) was performed in a factorial arrangement (2 x 2 x 2). SAOB based 0.5 and 5 ppm sulfide solutions were formulated from a 100 ppm standard and individually sealed in serum bottles to supply five replications per treatment combinations. After transferring 10 ml of the prescribed sample (0.5 or 5 ppm) to the wash-reaction chamber and establishing the SAOB trap (1 ml in a Pasteur pipet or 10 ml volumetric flask), the arrangement was flushed for one minute with nitrogen (60 ml/min) and then flow was set at 30 or 60 ml/min for the 30 minute extraction. Acidification was achieved by injecting 1 ml 6N HCl/min for the first 10 minutes. After a half-hour, the trap solution was sealed in a serum vial after flushing with nitrogen and refrigerated (4°C) until analysis the same day at room conditions. The electrodes were calibrated over 0.2 to 10 ppm sulfide (five points) by the modified liter-beaker method and samples measured for 5.5 minutes in a micro-sample dish (Orion) or beaker (Nalge). Five 10 ml samples of 0.5, 5 and 50 ppm S^{-2} were measured without undergoing the extraction process and served as controls. Percent recovery was calculated based on control samples extrapolated from the standard curves and expected values.

The effects of zinc preservation on extraction (experiment 7) Use of cationic elements to prevent oxidation of sulfide in analytical samples has been demonstrated.[127,196] To determine the effects of zinc precipitation on extraction recovery, 0.2 ml 2M zinc acetate was mixed into ten test tubes containing 0.5 ml 100 ppm S^{-2} in 10 ml SAOB. Ten similarly prepared tubes, but without the zinc solution, served as controls. A five point, standard curve

(0.2 to 10 ppm S^{-2}) formulated by the modified-liter beaker method was used for calibration. Potentiometric determinations were performed in plastic beakers (Nalge) with stir bars, under room conditions, and for 5.5 minute measurement periods. Percent recoveries were calculated based on the expected concentrations.

Phase 3: Method development with animal tissues

After establishing a technique for aqueous solutions, this knowledge was extrapolated to fresh tissue samples. Two schemes resulted, one for liquid samples (blood, serum and plasma) and another for solid tissues (brain, lung, heart, blood clot). The pH of the samples was measured with pH paper (Fisher) after extraction.

Anti-foaming agents (experiment 8) Preliminary work revealed that foaming of biological samples during extraction was a major obstacle, therefore extraction trials with prospective anti-foaming agents at various concentrations and volumes were conducted. Compounds considered were 10% silicone emulsion in water (Thomas Scientific), nonionic surface-active agent (Mallinckrodt), mineral oil U.S.P.,[145] and poloxalene (SmithKline Beecham).

Blood determinations - A (experiment 9) Fresh whole blood was collected daily in sodium citrate treated vacuum tubes (Becton-Dickenson) from healthy dairy cattle at the I.S.U. Veterinary Teaching Hospital. Each day, for five days, 10 ml whole blood was transferred to four test tubes treated with 0.2 ml 2M zinc acetate and mixed. To two of these tubes, 0.5 ml 100 ppm S^{-2} in SAOB was added and then all four slowly mixed for ten minutes (n = 10). These treatments were then transferred to poloxalene-mineral oil coated wash-bottles and an additional 3 to 5 ml of the anti-foaming mixture added prior to sealing the unit. The samples were acid extracted for 30 minutes by injecting 10 ml 6N HCl at 1 ml/min and the nitrogen flow at 60 ml/min. One and 10 ml SAOB absorption volumes were used for both spiked and untreated samples.

The electrodes were calibrated by using the modified liter-beaker method to formulate a nine point, 0.02 to 10 ppm S^{-2} curve. Trap volumes of 1 ml were analyzed in the micro-sample dish (Orion) and 10 ml samples in beakers (Nalge) with micro stir bars. All potentiometric determinations were performed under room conditions for 5.5 minute measurement periods for the standard curve and 15 minutes for the treatments. Percent recoveries were based on expected levels after correcting for endogenous sulfide levels estimated by assuming the linearity of the standard curve below 0.02 ppm.

Blood determinations - B (experiment 10) To improve extraction of acid-labile sulfide from blood, experiment 9 was repeated using SAOB or deoxygenated deionized water formulated sulfide standards, 2N HCl to acidify, and all absorbed into 10 ml SAOB traps.

Blood determinations - C (experiment 11) Experiment 9 was repeated using SAOB-based sulfide treatments, a milder acidification scheme, and 1:1 extraction. The dilute acid regime consisted of injecting 2 ml 0.25N HCl and then 8 ml 0.5N HCL, both at 1 ml/min.

Blood determinations - D (experiment 12) Based on experiment 9. Alterations consisted of 5 ml blood spiked with 0.25 ml 100 ppm S^{-2} in SAOB, and diluted with 10 ml deaerated 0.85% saline prior to extraction. Treatment and control sample were acid extracted with 2 ml 0.25N HCl and 8 ml 0.5N HCL (1 ml/min), and absorbed into 5 ml SAOB.

Serum and plasma analysis (experiment 13) Sulfide recovery from bovine serum and plasma was examined in conjunction with experiment 12. Fresh jugular bovine blood was collected daily into sodium citrate tubes (Becton-Dickenson) and centrifuged to yield plasma, or clotted in untreated blood tubes (Becton-Dickenson) for 45 minutes and centrifuged to separate the serum. To 10 ml serum or plasma, 0.5 ml 100 ppm S^{-2} and 0.2 ml 2M zinc acetate were added and mixed. Prior to extraction in a poloxalene-mineral oil coated wash-bottle, the above preparations were diluted with 5 ml deaerated saline. An additional 3 to 5 ml of the anti-foaming mixture was added to each unit before sealing and acidifying with 2 ml 0.25N HCl and then 8 ml 0.5N HCL (1 ml/min) for 30 minutes. The nitrogen flow rate was 60 ml/min and a 10 ml SAOB absorption volume. Calibration was by a nine point, 0.02 to 10 ppm sulfide standard curve prepared by the modified liter-beaker method. All potentiometric determinations were performed for 5.5 minutes under room conditions. Percent recoveries for ten replications were based on expected concentrations.

Sulfide determination in clotted blood (experiment 14) Fresh blood from dairy cows was collected daily and permitted to clot for one hour at room temperature. Sample preparation consisted of adding 5.0 g of clot to six tubes containing 0.2 ml 2M zinc acetate, 10 ml deoxygenated 0.85% saline, and 1 ml poloxalene-mineral oil mixture. Sulfide was added to three of these tubes (0.25 ml 100 ppm S^{-2}). All samples were homogenized (Tekmar) for 5 to 10 seconds at medium-high speed. The tubes were sealed with laboratory film (American National Can) and refrigerated at 4°C until extraction. After adding an additional 3 to 5 ml poloxalene-mineral oil, the samples were extracted for 30 minutes in wash bottle units by injecting 2 ml 0.25N HCl and then 8 ml 0.5N HCl (1 ml/min). Absorption vessels were 5 ml volumetric flasks (Corning) containing SAOB. The above steps were repeated over five consecutive days until there were fifteen replications per treatment.

A nine point, 0.02 to 10 ppm sulfide standard curve (modified-liter beaker method) with 5.5 minute measurement periods was used for calibration. Samples were assayed in 30 ml beakers (Nalge) with micro stir bars (Fisher) for 15 minutes. All work was performed under room conditions. Using the standard curve to extrapolate sulfide levels from the measured potentials, percent recoveries based on expected concentration were calculated after correcting for endogenous sulfide.

Analysis of acid-labile sulfide in solid tissues (experiment 15) Development of a sulfide determination for solid tissue was conducted using porcine brains from animals submitted live to the veterinary diagnostic laboratory for non-infectious, non-nervous system related problems. Immediately prior to acid extraction, 5.0 g of porcine cerebral cortex was added to a test tube containing 0.2 ml 2M zinc acetate, 1 ml poloxalene-mineral oil, and 20 ml fresh SAOB (n = 15). Sulfide spiked samples were prepared by adding 0.25 ml 100 ppm S^{-2} to the above tubes (n = 15). After homogenization for 5 to 10 seconds at medium-high (Tekmar), the resulting mixture was transferred to a wash bottle unit coated with anti-foaming agent. An additional 3 to 5 ml poloxalene-mineral oil was added to the chamber and acid extracted for 30 minutes with 10 ml 6N HCl (1 ml/min) and a nitrogen flow of 60 ml/min. Five milliliters of SAOB in a volumetric flask served as the absorption trap. Each treatment was replicated fifteen times.

After calibration by serial addition (nine point, 0.02 to 10 ppm S^{-2}), potentials of samples were measured for 5.5 minutes under room conditions. The percent recovery of the spiked samples was calculated after correcting for endogenous cerebral sulfide based on expected sulfide concentrations.

Phase 4: Development of exposure techniques

Experimental exposure of animals requires delivering prescribed doses at physiological conditions. For parenteral administration, pH, volume and stability are critical factors. In acute inhalation exposures, concentration and distribution are important.

Stability of standardized sulfide injection solution (experiment 16) After formulating and standardizing a liter of 10,000 ppm S^{-2} in PBS, the remaining solution was subdivided into 45 20 ml serum bottles which were flushed with nitrogen, sealed and stored at 4°C. The sulfide determination consisted of diluting 1 ml 10,000 ppm S^{-2} of a sample to 100 ml with SAOB and potentiometrically titrating 10 ml of the resulting solution with a 1000 ppm Pb reference (Fisher) under room conditions. Following the initial day (0), another set of five vials

were titrated on days 1, 2, 3, 5, 7, 10, 14, 21, and 28 (n = 5). Potentials (-mV) versus total titrant volumes (ml) were graphed, volume to end point estimated and sulfide concentrations calculated from the stoichiometry (Appendix E).

pH adjustment of the sulfide injection solution (experiment 17) A 10,000 ppm sulfide standard formulated in PBS was titrated with 2N HCl to determine the volume of acid required to produce physiologic pH (7.4). A dosage chart based on the LD_{50} of 14.6 mg $NaHS \bullet 2H_2O$/kg BW IP (5 mg S^{-2}/kg) for male 250 to 350 g Sprague-Dawley rats,[131,258] was calculated to determine the volume of 10,000 ppm sulfide required to deliver a lethal dose and for a given body weight (Figure 8). A plot of 2N HCl volume versus 10,000 ppm S^{-2} dose was generated using the pH titration data (Figure 9).

The final pH of doses formulated in test tubes with micropipets or tuberculin syringes (Becton-Dickenson) for hypothetical rats were compared to evaluate the above method. All pH determinations were performed in test tubes containing stir bars. The animals were a 250 g BW rat dosed at 2 x LD_{50} (10 mg S^{-2}/kg), and a 350 g BW injected with 4 x LD_{50} (20 mg S^{-2}/kg). The syringe formulation technique consisted of filling the needle and hub with PBS, and sequentially drawing the required volume of sulfide solution, corresponding 2N HCl volume, and finally an additional 0.2 ml PBS (n = 10). The initial and final volumes of PBS of the syringe method were also added to the micropipet prepared tubes (n = 10).

Confirmation of sulfide concentration in syringe formulated solutions (experiment 18) To confirm the sulfide dose in tuberculin syringe (Becton-Dickenson) formulated solutions (experiment 17), the 4 x LD_{50} (20 mg S^{-2}/kg) treatment for a 350 g BW, male rat was prepared, held for 5 seconds, and then injected into 10 ml SAOB for potentiometric analysis. To facilitate the sulfide determination, 1.57 ml of the latter was diluted to 10 ml with SAOB. After electrode calibration, sample measurement and extrapolation, the resulting sulfide concentrations for 30 replications were compared to the theoretical concentration.

Inhalation chamber design and validation (experiment 19) A hydrogen sulfide generation system, horizontal flow inhalation chamber, and scrubbing mechanism, all driven by in-house vacuum, were designed for acute, lethal exposure of individual adult rats (Figure 10) according to published recommendations.[3,103,160,161,217] All connections were 7 mm (O.D.) Pyrex® (Corning) and 6.35 mm (I.D.) Tygon® (Norton) tubing. The in-house vacuum valve was set to maintain a flow rate of 200 ml/min through the system, as measured at the gas generation flask.

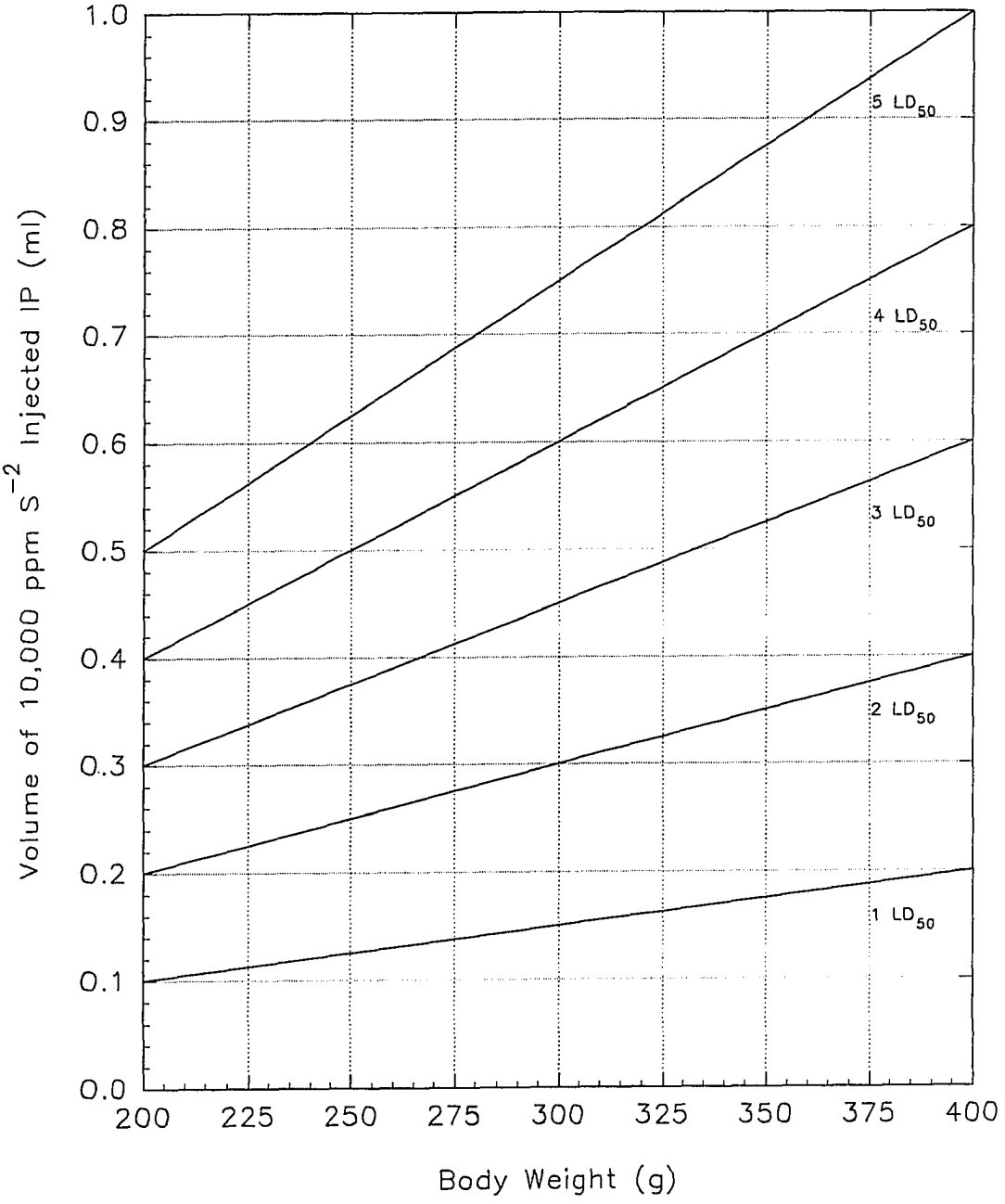


Figure 8. Lethal sulfide doses for adult rats at a given body weight. Lethal dose estimation of LD_{50} = 5.0 mg S^{-2}/Kg BW IP for adult male Sprague-Dawley rats.[131,258]

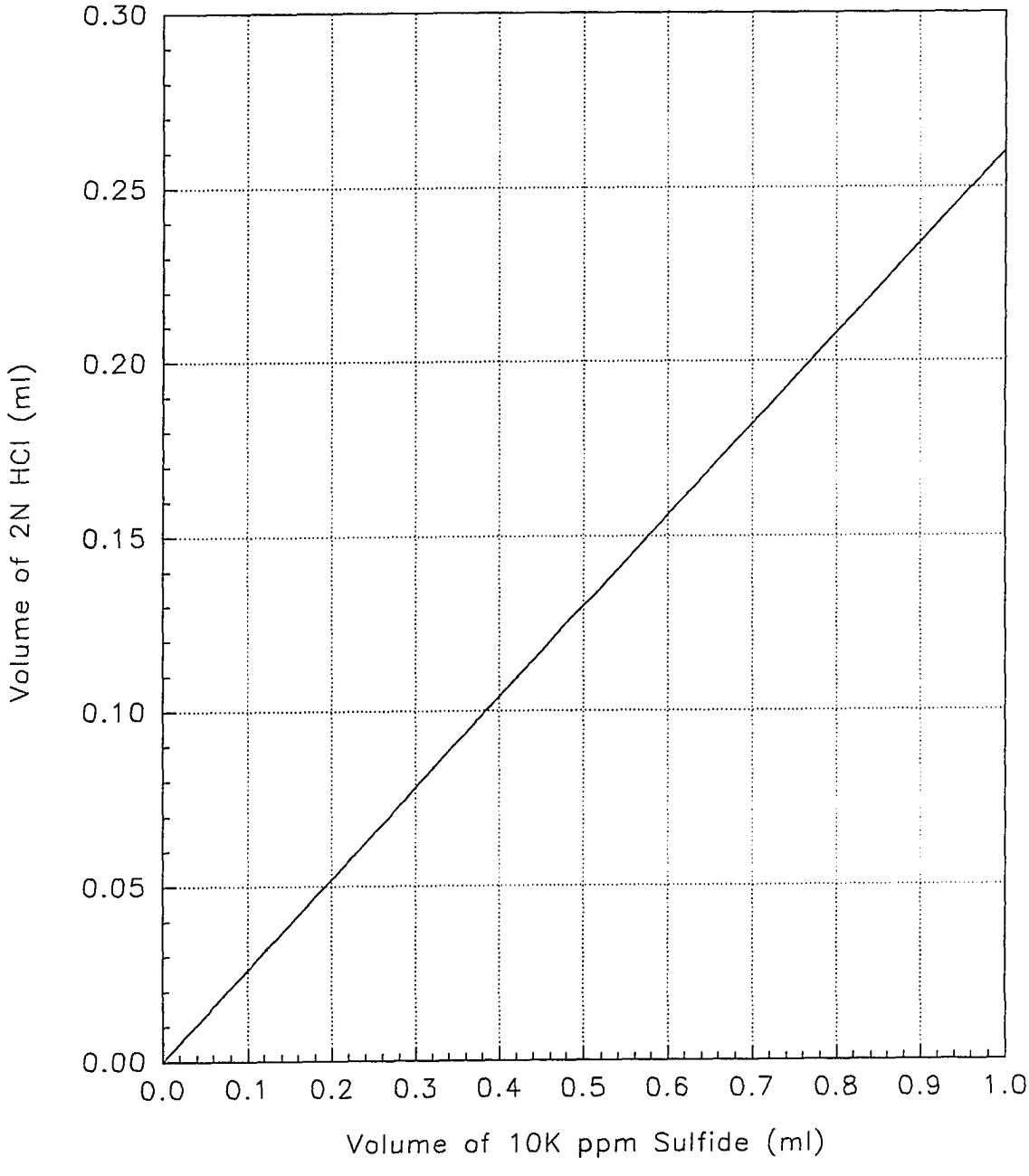
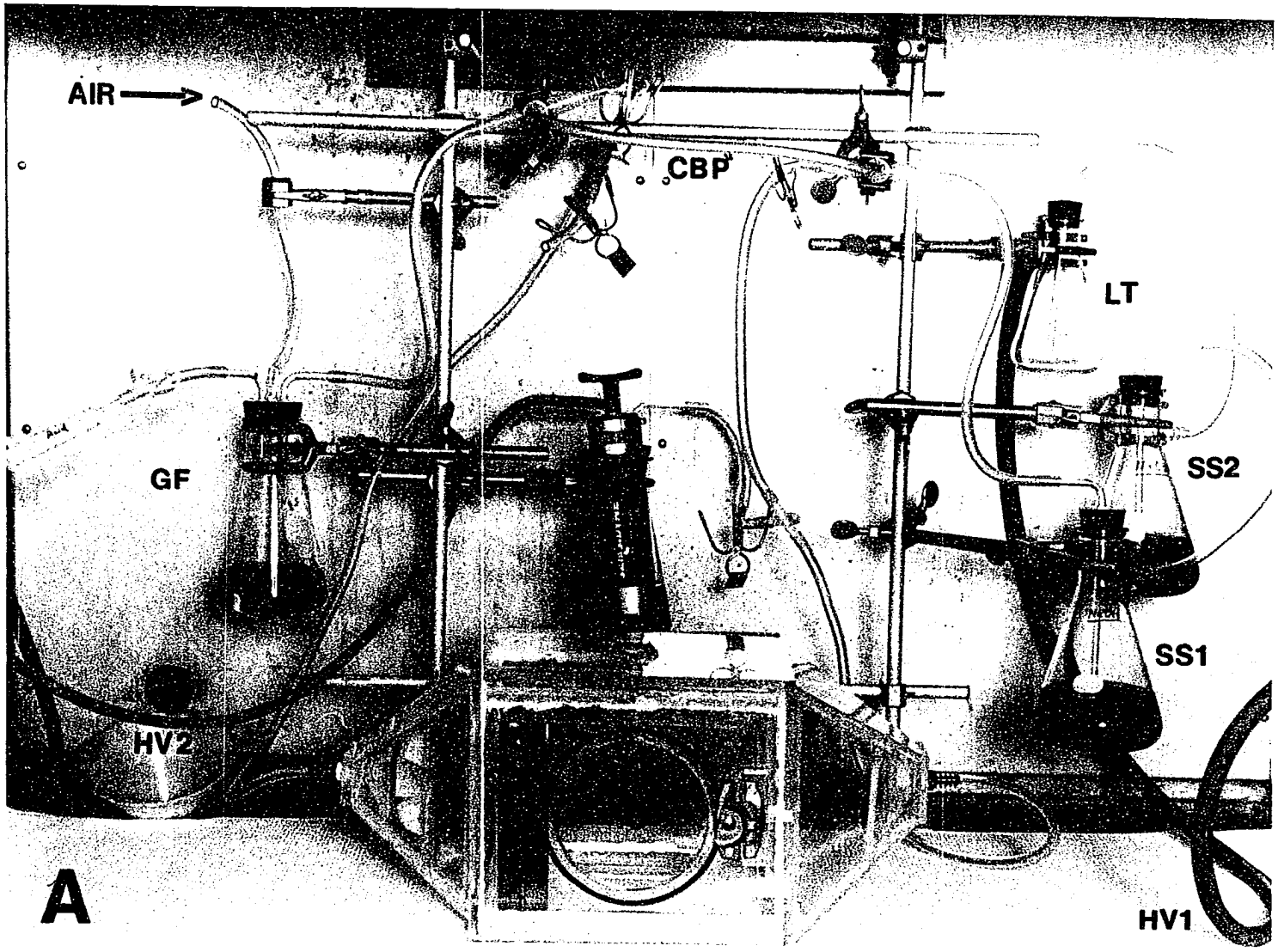


Figure 9. Volume of acid required to alter the pH of a given sulfide dose to physiological range.


AIR
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HV2
HV1
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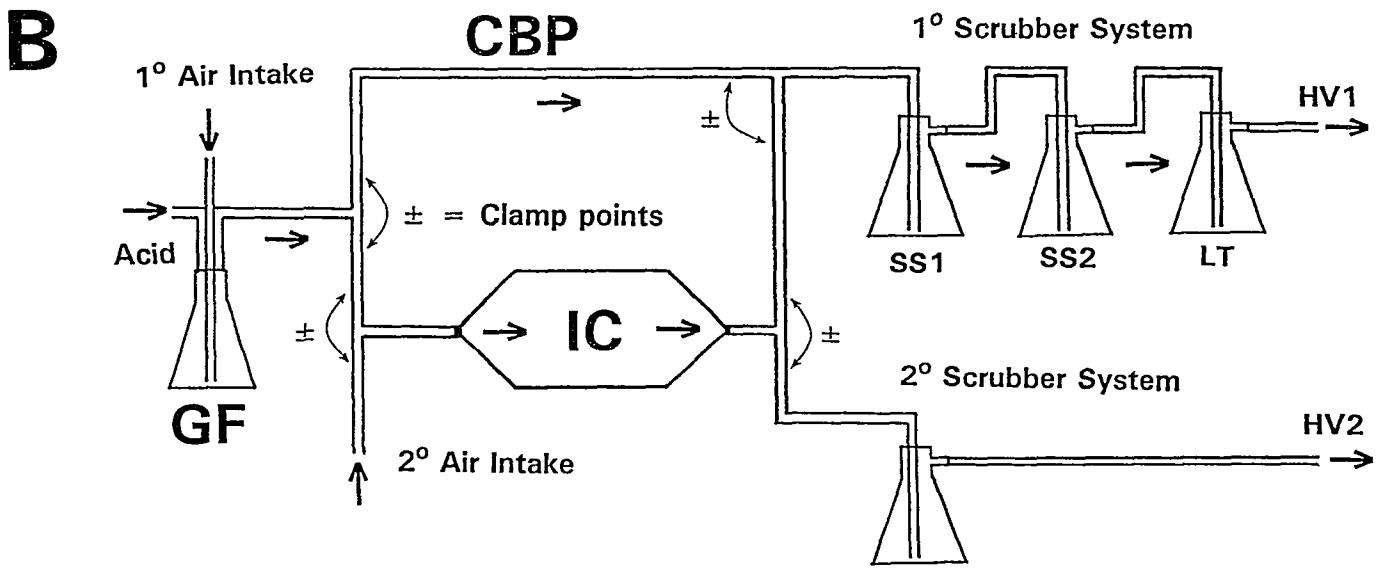


Figure 10. Vacuum driven gas exposure system for rodents (both photograph A and illustration B). Hydrogen sulfide produced by acidifying sulfide solution in the generation flask (GF) was drawn into the inhalation chamber (IC), and then through the two basic traps in the scrubber system (SS1) and liquid trap (LT) prior to entering the house vacuum (HV1) system. A second vacuum powered path (HV2) was used in conjunction with the chamber by-pass (CBP) and second basic trap (SS2) to vent the unit post-exposure.

The hydrogen sulfide generation vessel was a 500 ml wide-mouth Erlenmeyer flask (Corning) with a number 10 rubber stopper (Fisher) into which two short and one long stemmed glass tubes with 90° bends were inserted (Figure 11). With the stopper securely positioned, the longer tube extended to the base of the flask and agitated the 100 ml 13,300 ppm Na_2S solution with fresh air during operation. One of the short glass tubes was the injection port, which consisted of a 12 cm length of tubing with a hypodermic needle guard modified to receive a luer syringe (Becton-Dickenson) and a pinch clamp (Fisher) to control the patency. At time zero, 2 ml of 9N sulfuric acid was injected into the generation flask containing the sulfide solution to produce hydrogen sulfide. The other short stemmed tube was connected to the plastic outflow line, which led to the proximal gas port on the inhalation chamber (Figure 10).

The horizontal exposure chamber (12.5 L) was constructed from 6.35 mm thick acrylic plastic (Rohm and Hass) sealed with a solvent cement (Industrial Polychemical Service), except for a 9.5 mm thick piece used for the hatch. The chamber consisted of three functional sections (Figure 12). On the ends of the unit were the inflow and outflow diffuse cones, each consisting of three grates to facilitate mixing and distribution. The last grate of the inflow cone and the first of the outflow defined the animal holding area (11 L), which was accessed through the o-ring sealed hatch in the front wall. A sampling port centered in the ceiling held a rubber stopper from a blood tube (Becton-Dickenson) with a hole bored through it to receive a gas sampling tube (Sensidyne). When in place the sampling apparatus held the opening of the detection tube at the midpoint of the exposure chamber. From the outflow cone, the atmosphere was evacuated via the distal gas port and plastic tubing to the scrubbing system.

To remove the residual hydrogen sulfide prior to entering the in-house vacuum system, a series of basic solution traps were configured from three 500 ml filter flasks equipped with number 7 rubber stoppers (Fisher) traversed by glass inflow tubing extending to the bottom of the vessel (Figure 13). Except for the first trap which received gas from the inhalation chamber and last which vented to the vacuum line, the flasks were interconnected by plastic tubing from the side arm of the previous trap to the next inflow tube of the next. Flasks one and two contained 300 ml 2M NaOH through which the gas was bubbled to be absorbed as sulfide; the last was a liquid overflow trap.

Figure 11. Hydrogen sulfide generation flask for the inhalation exposure system. Acid entering through the injection port (IP) lowered the pH of the sulfide solution (Na_2S) and generated H_2S which exited via the out-flow port (OFP) was drawn by vacuum to the exposure chamber (EC).

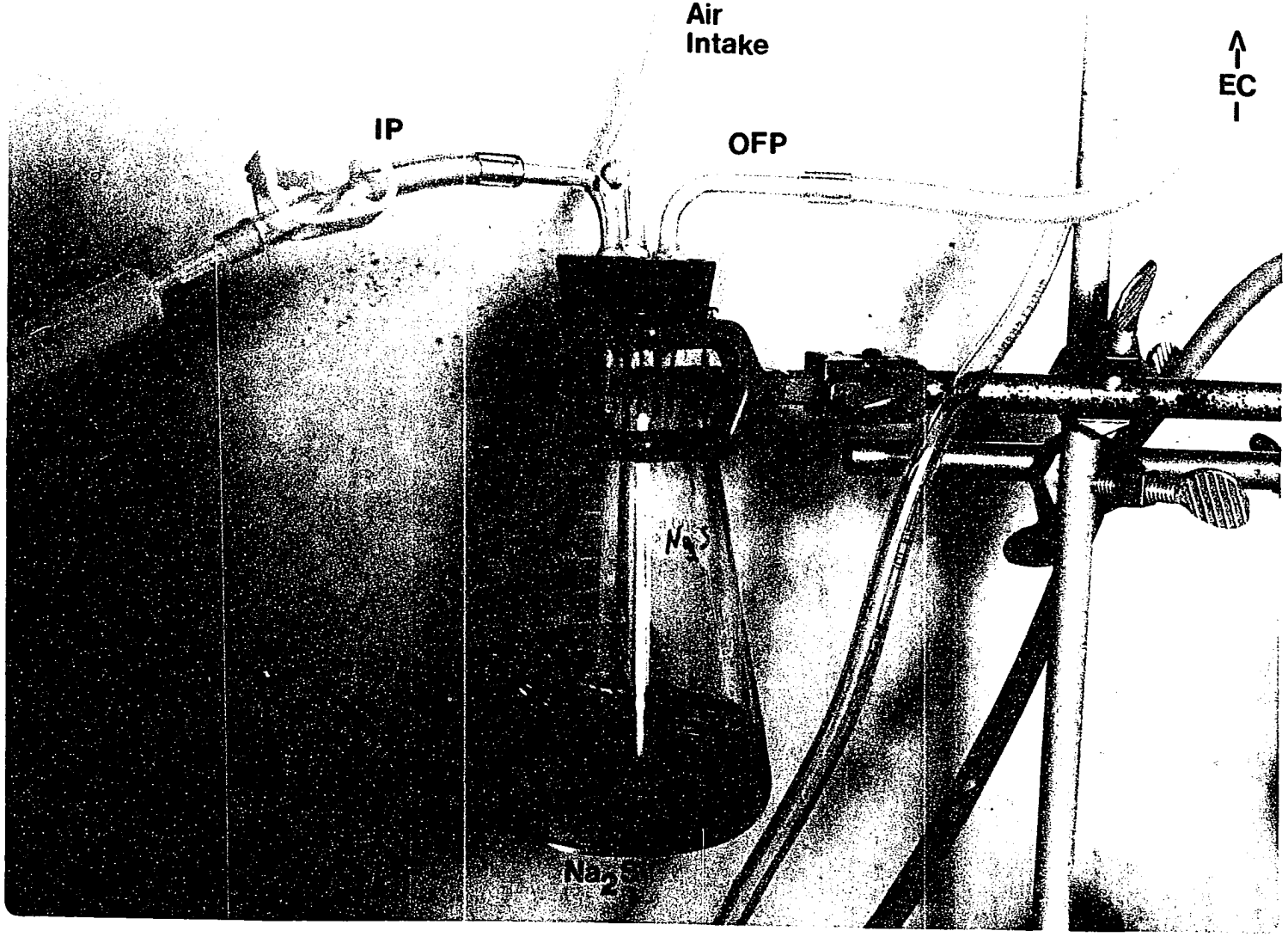

Air
Intake
EC
IP
OFP

Figure 12. The inhalation exposure chamber. Hydrogen sulfide gas from the generation flask entered the inflow diffusion cone (IDC) through the inflow port (IP), passed into the rodent exposure area (EA) before exiting via the collection cone (CC) and outflow port (OFP), respectively, to the scrubbers. For monitoring the gas concentration within the exposure area, a sampling syringe (SS) was attached to a detector tube (DT) via the sampling port (SP).

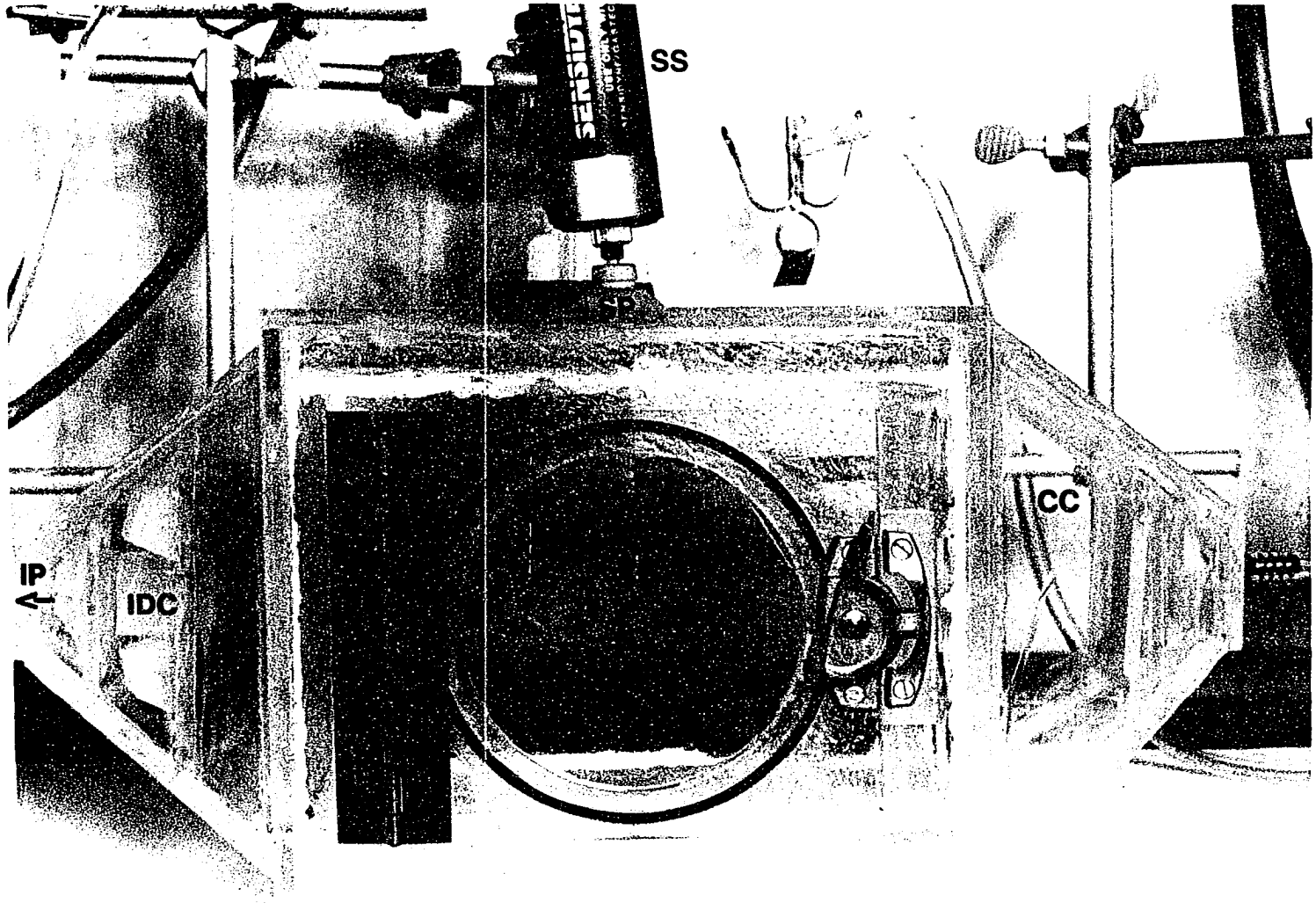
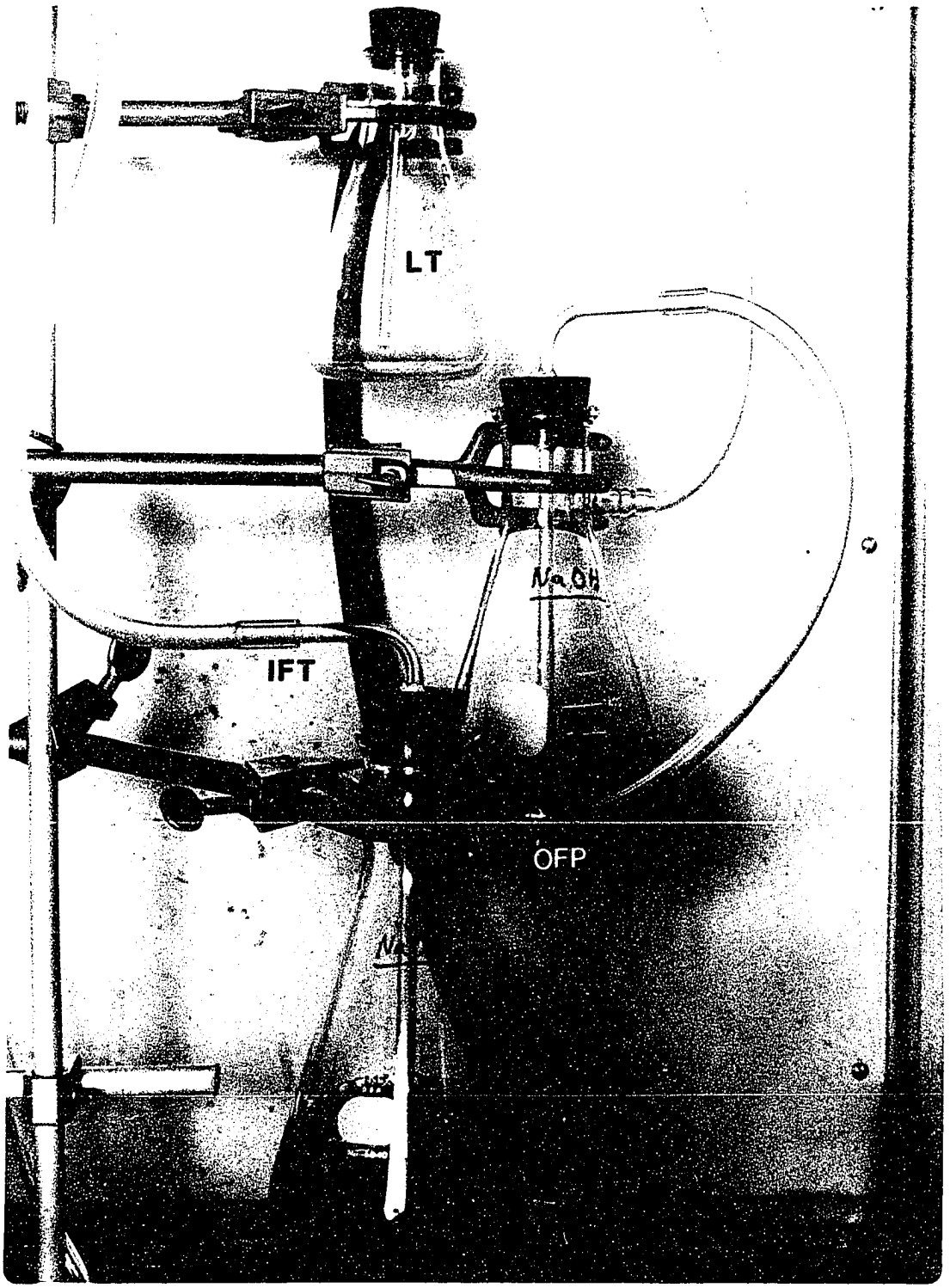

SS
IP
IDC
CC

Figure 13. Hydrogen sulfide scrubbing flasks for the inhalation exposure system. Gas exiting the exposure unit was bubbled through two basic traps (NaOH) and a liquid trap (LT) to remove H_2S from the air as S^{-2}. Each vessel in the series contained rubber stopper with inflow tube (IFT) with the sidearm of the vacuum flask serving as the outflow port (OFP).


LT
NaOH
IFT
OFP

The chamber was vented once an exposure had been completed by opening the generation system-to-scrubber bypass. After repositioning pinch clamps (Fisher) at the t-connectors (Nalge) in the generation system-to-chamber and chamber-to-scrubber lines, and turning on vacuum for the second scrubber system, the now isolated exposure unit was evacuated (Figure 10).

The inhalation chamber was checked for gas and liquid leaks after construction by placing dry-ice in hot water and sealing it in the unit; and by filling the chamber with water and letting it stand for 12 hours. Uniformity of gas flow was evaluated by setting up the system for exposure, injecting hot water on to dry-ice contained within the gas generation flask, and observing the flow pattern and vapor density within the chamber. It was also evaluated by positioning the hydrogen sulfide monitor (Industrial Scientific) within the chamber at various locations and noting the concentrations during trials. The hydrogen sulfide generation profile was established by plotting the mean gas concentration, determined with the monitor (Industrial Scientific) centered in the chamber, versus time after acid injection over 10 minutes for nine runs.

For safety, the whole exposure system was housed within a fume hood (Kewaunee Scientific Equipment). The laboratory was also monitored for hydrogen sulfide (Industrial Scientific) and equipped with an emergency escape breathing apparatus (North Safety Equipment).

Phase 5: Animal exposures

With techniques developed for acute, lethal exposure of animals to sulfide by injection or inhalation of hydrogen sulfide, exposure trials were conducted to confirm the previous tissue recovery work and evaluate the wash bottle extraction and silver/sulfide ISE analysis in diagnosing hydrogen sulfide toxicosis.

Dose-relationship study (experiment 20) Eighteen male Sprague-Dawley rats (Nasco) weighing 265 to 300 g were administered a 0 x LD_{50}, 2 x LD_{50} or 4 x LD_{50} sulfide dose (0, 10 or 20 mg S^{-2}/kg BW IP) formulated in a tuberculin syringe (experiment 17) and immediately injected intraperitoneally ($n = 6$).[141] Six controls (0 x LD_{50}) and three additional rats for spike recovery tissues were treated with an appropriate volume of PBS. The rats were observed for 3 minutes after injection, during which clinical signs and time-to-death were recorded. At the end of observation, or death, the animal was decapitated (Braintree Scientific) and tissues collected for sulfide analysis. One person collected blood by exsanguinating the rat into a sterile plastic bag (Nasco) treated with 0.2 ml 2M zinc acetate and 100 USP units sodium heparin (Elkins-Sinn). Each sample was mixed, sealed, and refrigerated at 4°C until extraction.

The amount of blood collected was determined by weighing the sample into the bottom half of a wash bottle unit at time of acid extraction. A second person immediately removed the brain (cerebrum to medulla), rinsed it with ice cold deaerated saline, obtained the weight, wrapped it with the weighing paper in aluminum foil, and flash froze the sample on dry-ice. The lungs were collected and preserved as described for the brain. Both organs were stored on dry-ice until acid extraction. Gross pathology was assessed as samples were collected.

Immediately after the sample collection period, the blood, brain and lung were acid extracted and resulting sulfide levels determined by the methods outlined in experiment 12 (blood) and experiment 15 (brain and lung). Whole specimens were extracted due to small sample sizes. Spiked treatments were prepared by adding 0.25 ml 100 ppm S^{-2}/5.0 g tissue. Sulfide determinations were performed under room conditions after calibrating the electrodes by formulating a nine point, 0.02 to 10 ppm sulfide standard curve (modified liter-beaker method). Acid-labile sulfide was extrapolated from the curve and corrected for variation in sample sizes. Linearity of the plot below the calibration range was assumed for statistical comparison of blood samples with <0.02 ppm sulfide. Percent recovery for spiked blood, brain and lung specimens were calculated after correcting for endogenous contributions.

Inhalation exposure (experiment 21) Eight 300 to 330 g male Sprague-Dawley rats (Nasco) were exposed to acutely toxic levels of hydrogen sulfide as outlined in experiment 19. The hydrogen sulfide concentration at mid chamber was sampled during each exposure (Sensidyne) at 4.5 minutes after injection of the acid into the generation flask. Seven rats exposed to room air served as controls, and three additional animals provided tissues for spiked samples. Clinical signs and progression were reported. Blood, brains and lungs were collected, preserved, and analyzed with sulfide levels calculated as described in experiment 20.

Sample quality study (experiment 22) Thirty-six 290 to 310 g male Sprague-Dawley rats (Nasco) were asphyxiated on day 0 with carbon dioxide (Air Products) and assigned to six groups (n = 6). The first set of rats was necropsied immediately, with brains collected and analyzed as in experiment 20. The others were stored under room conditions until sampled at 0.25, 0.5, 1, 1.5 and 2 days postmortem.

RESULTS

Phase 1: Measurement Development

Selection of measurement equipment and environment (experiment 1)

Unsatisfactory calibration results with the electrode pair prompted the investigation into a more stable sensing and reference set and measurement environment. Combinations of Lazar ISE / Lazar DJR, Lazar ISE / Orion DJR and Orion ISE / Orion DJR performed differently under similar sulfide concentrations and analysis environments. Potentials at different sulfide concentrations recorded over time from standard curve trials revealed the Lazar / Lazar pair to yield relatively stable readings after the first minute (Figure 14, plot A), however the values tended to drift at concentrations ≤ 0.5 ppm S^{-2}. The standard curve from this electrode pair ($Y = 635.3 + 161.9\ X$) was nonlinear and greatly deviated from the theoretical slope, even when only considering ≥ 0.5 ppm S^{-2} (Figure 15, plot A).

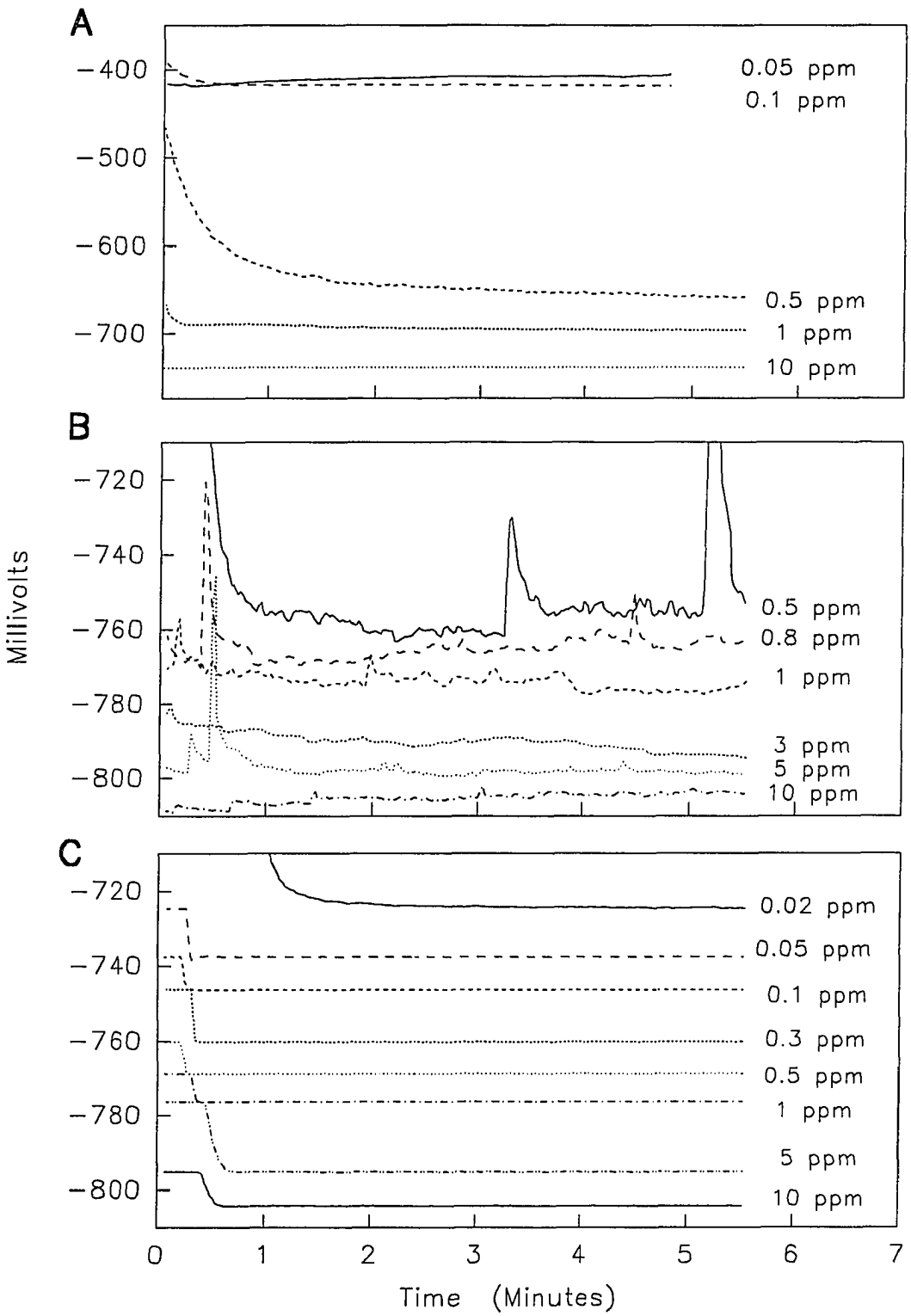
The Lazar ISE / Orion DJR pair produced erratic potential readings (Figure 14, plot B), even under highly controlled laboratory conditions (circulating water bath and Faraday cages). The resulting standard curve ($Y = 765.0 + 42.2\ X$) could only be produced after averaging the logged data over time (Figure 15, plot B).

The Orion ISE / Orion DJR combination provided rapid, stable potential readings without drift at sulfide concentration of ≥ 0.02 ppm S^{-2} (Figure 14, plot C). Standard curves formulated from prepared solutions or by serial addition ($Y = 775.2 + 29.3\ X$), and as controlled or bench-top analyses, were linear and the slope within acceptable limits (Figure 15, plot C). Performance of this ISE did not degrade until the third year of use, when the response time for 0.02 ppm S^{-2} gradually increased from 1.5 to 2 minutes (Figure 14, plot C) to 15 to 20 minutes. Polishing the crystal with fine wet-sand paper was palliative.

Thermally isolating the sample by shielding it from the stir plate or environment with extruded polystyrene (Styrofoam®, Dow) or a polystyrene water bath was effective unless combined with the nitrogen delivery system or wide changes in room temperature. Thus resulting in cooling of the sample. The circulating water bath system (Figure 5) maintained the samples at 25.0°C regardless of environmental changes, and could be combined with the nitrogen gas system to prevent oxidation.

Use of the Faraday cages (Figure 6) produced either no or minimal improvement in the poor performance of the Lazar / Lazar or Lazar / Orion combinations. No effect was noted with the Orion / Orion pair, although there was little room for improvement.

Figure 14. Potentials recorded during calibration trials with standard sulfide solutions for the ion-selective electrode / double-junction reference electrode pairs. Plot A, the Lazar / Lazar set under controlled laboratory conditions; plot B, Lazar / Orion pair as controlled analyses; and plot C, the Orion / Orion combination as a bench-top determination. Controlled conditions involved using a circulating water bath system to maintain the samples at 25.0°C, a nitrogen system to combat sulfide oxidation, and the Faraday cages. Bench-top determinations were performed at room temperature, and without the nitrogen delivery system and Faraday cages.


A
−400
−500
−600
−700
0.05 ppm
0.1 ppm
0.5 ppm
1 ppm
10 ppm
B
−720
−740
−760
−780
−800
0.5 ppm
0.8 ppm
1 ppm
3 ppm
5 ppm
10 ppm
C
−720
−740
−760
−780
−800
0.02 ppm
0.05 ppm
0.1 ppm
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1 ppm
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Millivolts
0
1
2
3
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7
Time (Minutes)

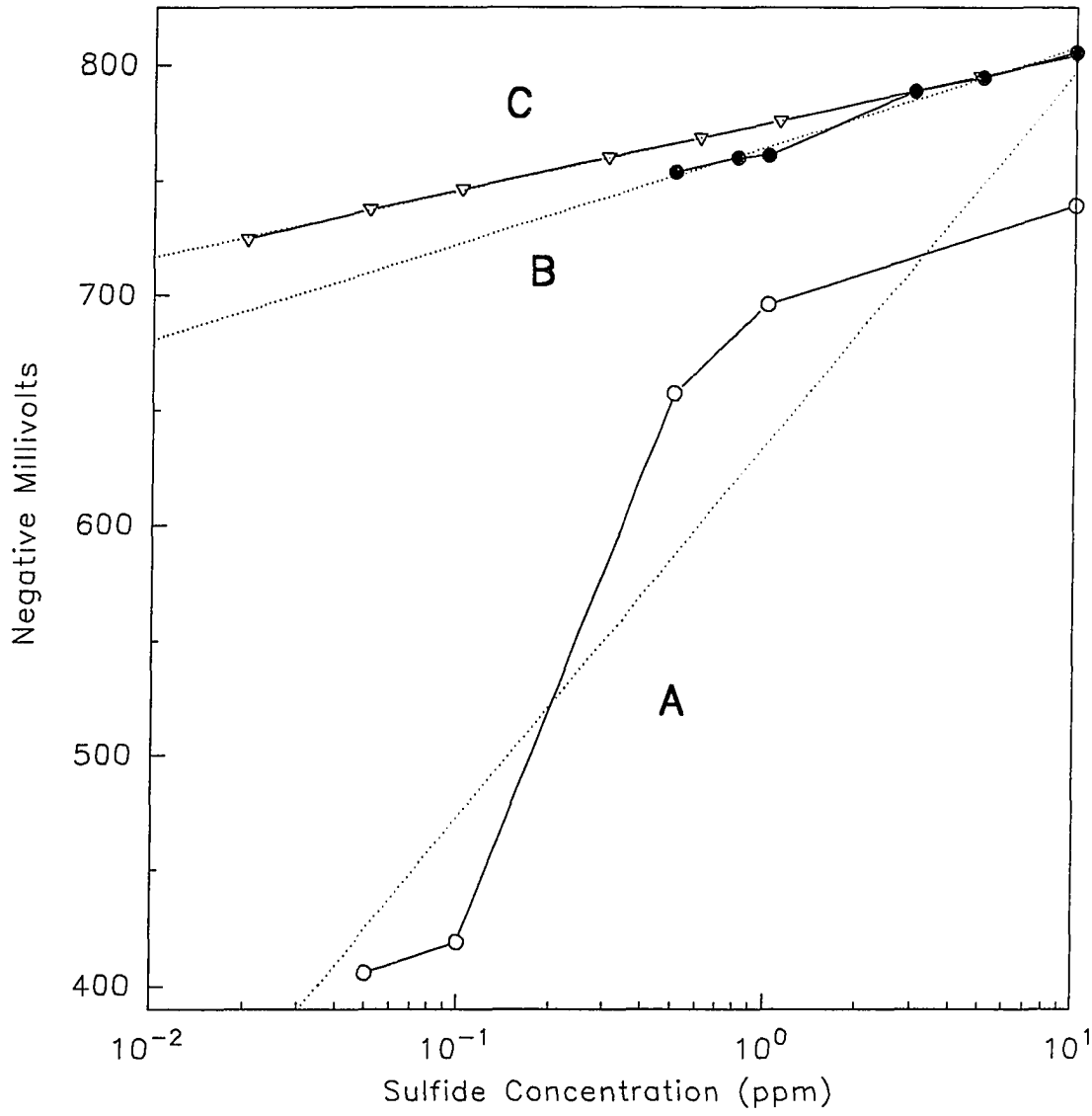

Figure 15. Resulting sulfide standard curves and regressions for the potential value tracings (Figure 14). Plot A (Lazar ISE / Lazar DJR) and B (Lazar ISE / Orion DJR) were performed under controlled conditions, and plot C (Orion ISE / Orion DJR) as bench-top analysis. Controlled conditions involved using a circulating water bath system to maintain the samples at 25.0°C, a nitrogen system to combat sulfide oxidation, and the Faraday cages. Bench-top determinations were performed at room temperature, and without the nitrogen delivery system and Faraday cages. Points graphed were the means of values recorded during 4.5 to 5.5 minutes of the measurement period (n = 50 to 60).

Stability and storage of SAOB based sulfide solutions (experiment 2)

Standardized sulfide solutions formulated in SAOB and stored under nitrogen in sealed serum bottles did not significantly ($P > 0.05$) degrade over five days (Figure 16). Means of the standard curve points were significantly different ($P < 0.0001$) with a LSD of 0.97 mV (Figure 17). Regression analysis indicates that the linear relationship between negative millivoltage and common logarithm of the sulfide concentration was highly significant ($P < 0.0001$). The relationship $Y = 763.5 + 31.2\ X$ was found to account for 99.97% of the variation in negative millivoltage (Figure 17).

Bench-top versus controlled analysis conditions (experiment 3)

Comparing the sulfide analysis performed under room conditions with measurements conducted in a regulated environment was done by calibrating the Orion / Orion electrode pair with the same standard curve solutions at each treatment. Resulting millivoltages from 0.1 to 10 ppm were nonsignificantly higher ($P > 0.05$) for the bench-top analysis (Figure 18). Highly significant ($P < 0.0001$) linear relationships were established by regression analysis of the mean negative millivoltages and common logarithms of the sulfide standards. The linear equations of the means, $Y = 764.3 + 30.8\ X$ and $Y = 763.7 + 31.6\ X$, accounted for 99.94% and 99.91% of the variation in potential, for bench-top and controlled treatments, respectively (Figure 18).

Slopes from the standard curves and slope verifications performed prior to each trial were highly significantly different ($P < 0.0001$) for both conditions (Table 12). Paired *t*-tests between the regression slope and slope-check values compared to the theoretical response of a silver/sulfide ISE revealed the bench-top calibrations were significantly different ($P < 0.002$) from the expected figure, but that the slope-checks were similar ($P > 0.196$). Contrarily, the slopes from the standard curves of the controlled trials were not significantly different ($P > 0.095$), while the check values differed significantly ($P < 0.0011$) from the theoretical slope.

Development of a modified liter-beaker calibration (experiment 4)

A facilitated method for calibrating the Orion / Orion electrode pair by serial addition of a standardized sulfide solution to a beaker of SAOB was devised and evaluated. Millivoltages recorded at each of the resulting concentrations were significantly different ($P < 0.0001$) as illustrated in Figure 19. Regression analysis revealed the linear relationship between the potentials (-mV) and sulfide concentration ($\log_{10}$), $Y = 764.2 + 29.5\ X$, was significant ($P < 0.0001$), and 99.994% of the variation in the potential was accounted for by the said equation.

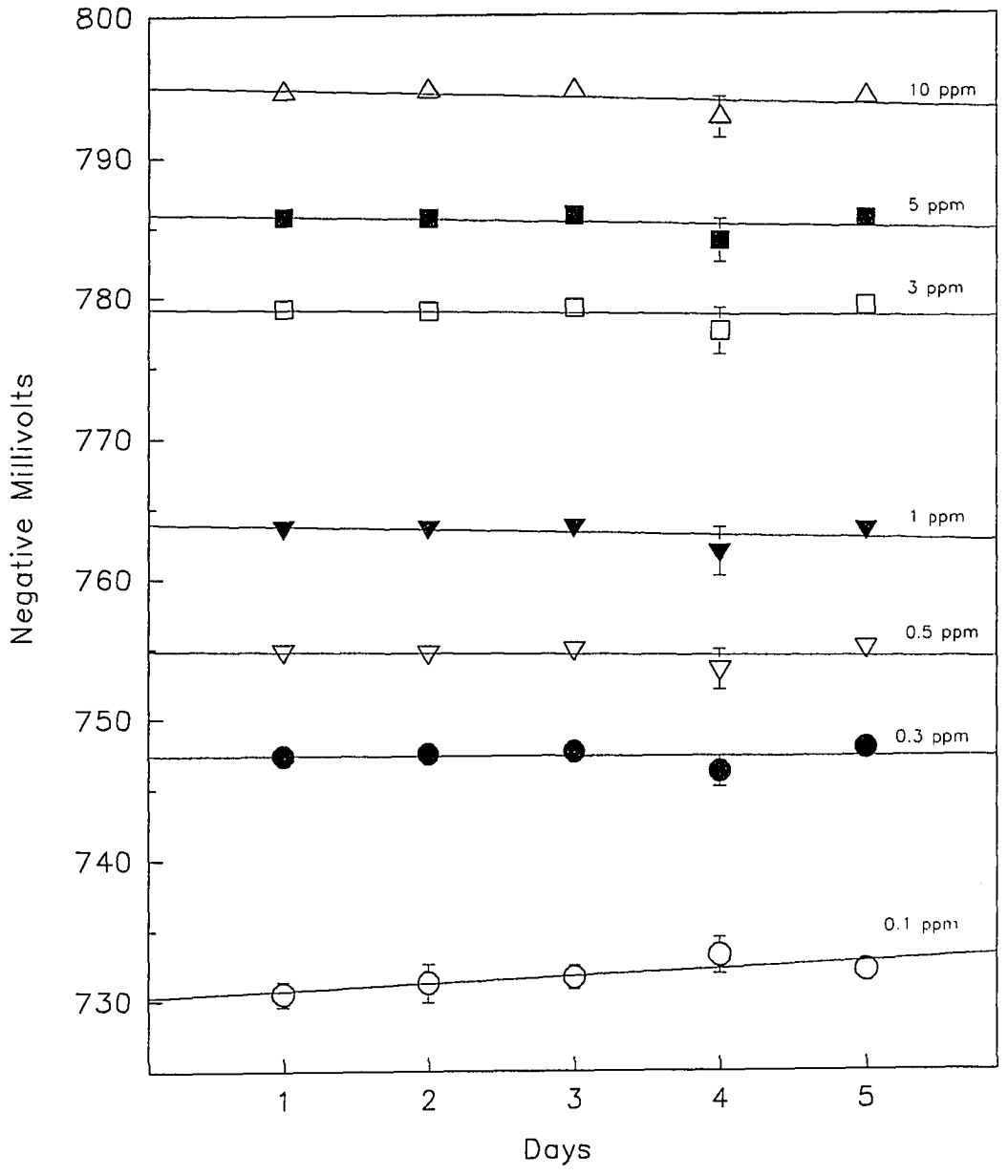

Figure 16. Stability of sulfide standards formulated in sulfide antioxidant buffer, sealed under nitrogen in serum bottles, and stored in darkness at 4°C.

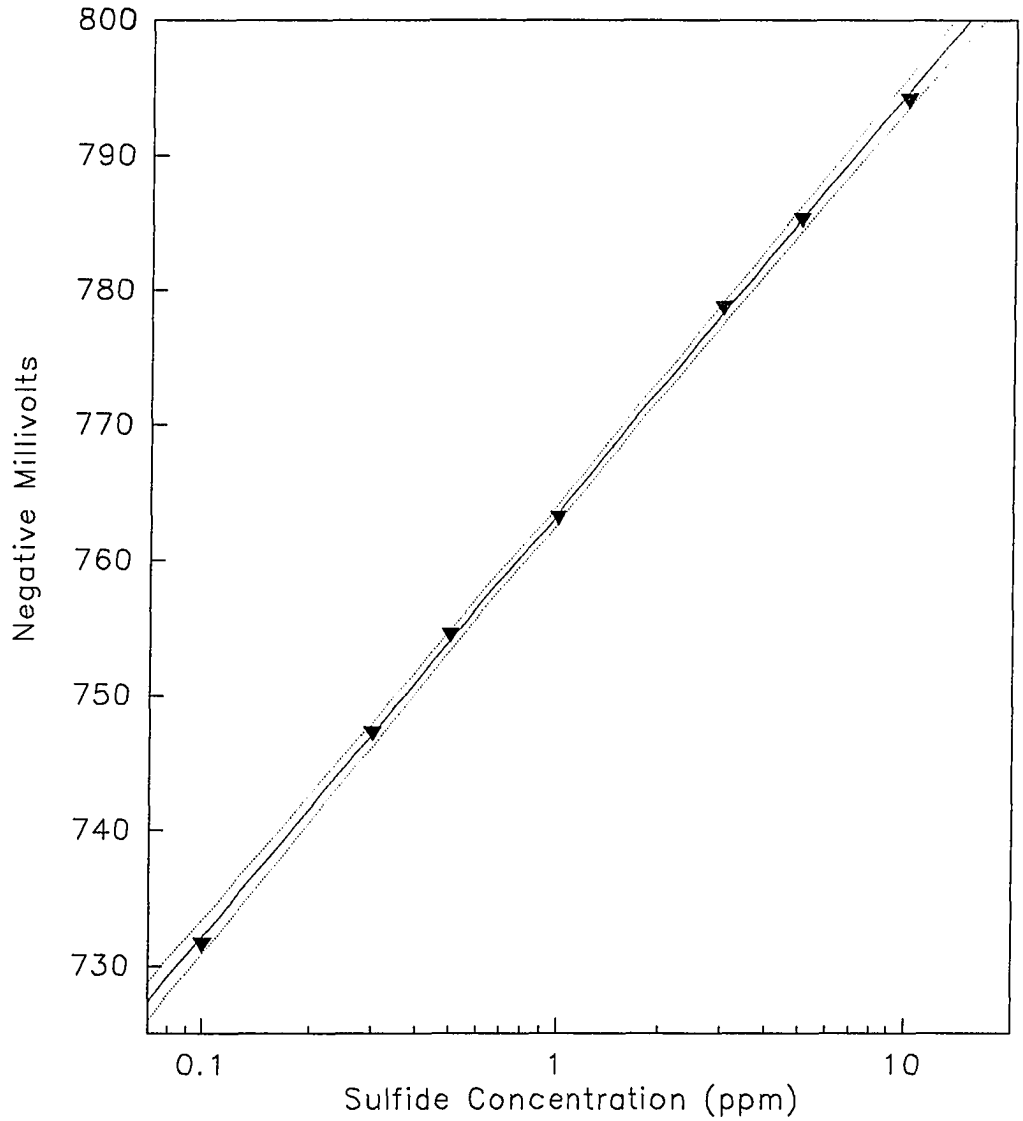

Figure 17. Standard curve and 99% confidence intervals for the Orion silver/sulfide ion-selective electrode with Orion double-junction reference. Mean values were graphed (n = 10).

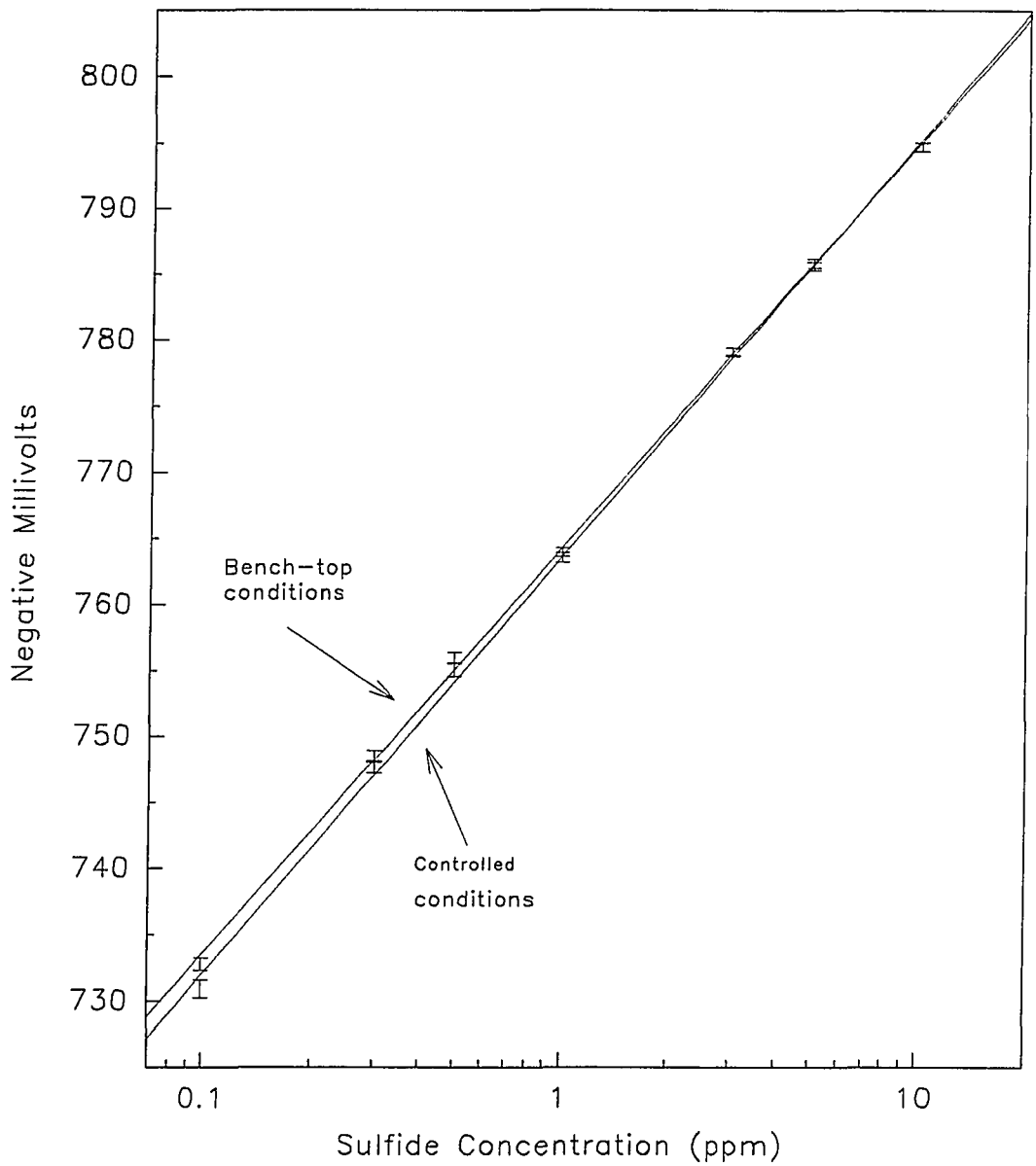

Figure 18. Standard sulfide curves measured under controlled laboratory conditions and as a bench-top analysis. Means ± SE were plotted for 5 replications.

Table 12. Comparison of slope estimates from regression of the standard curve points and electrode slope-check performed under bench-top or controlled experimental conditions.

Analysis Condition	Source of Slope	N	Slope (-mV/decade ppm S^{-2})	
			Mean	Standard Error
Bench-top	Regression of standard curve	5	30.83[a]	0.101
Bench-top	Slope verification check	5	29.70[b]	0.077
Controlled	Regression of standard curve	5	31.64[c]	0.188
Controlled	Slope verification check	5	29.78[b]	0.092

[a,b,c]Means with identical superscripts are not significantly different ($P < 0.05$), least significant difference = 0.368.

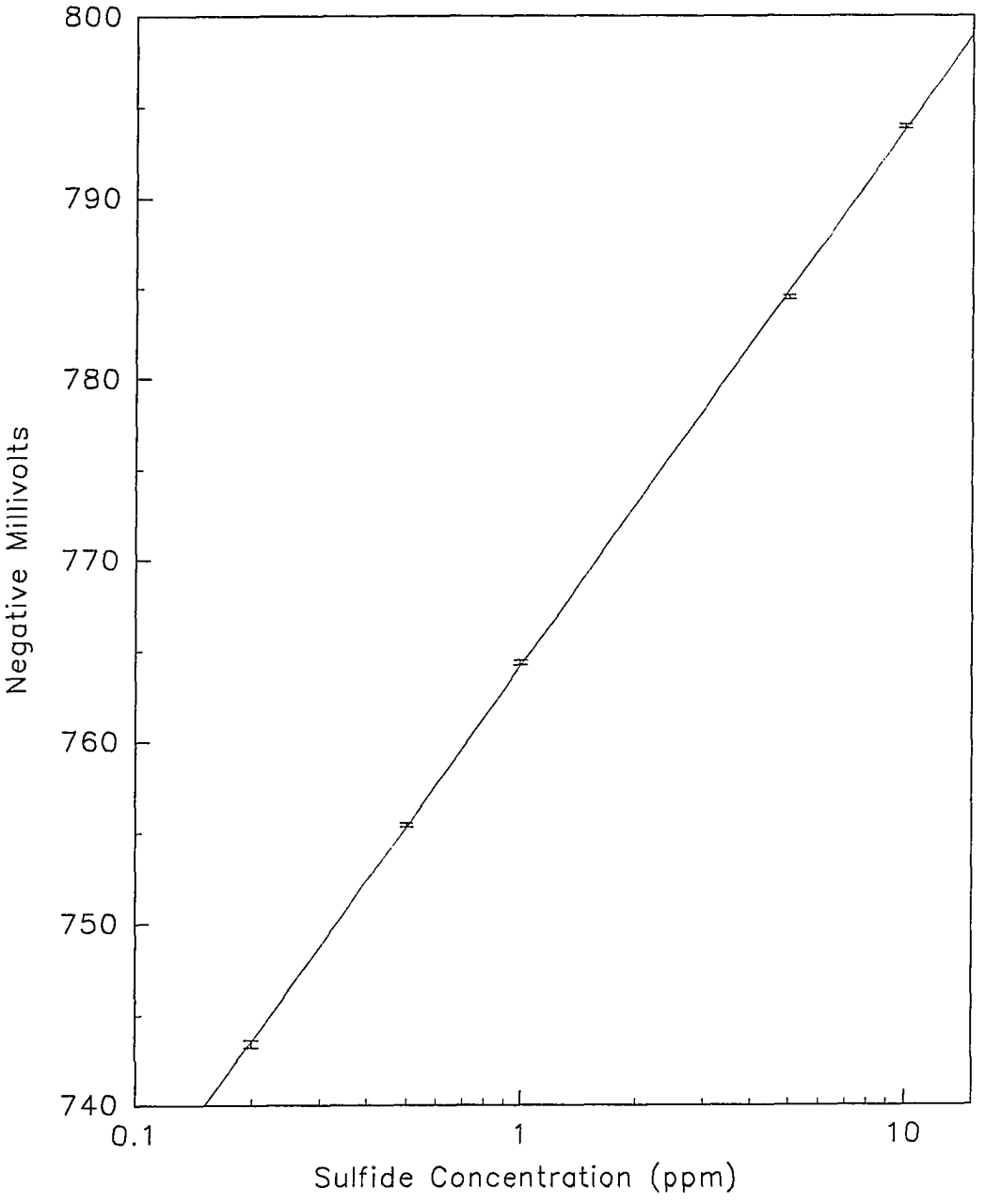

Figure 19. Standard sulfide curve formulated by the modified liter-beaker method. Means ± SE for 10 replications were plotted.

Slope estimates from the regression of the standard curve data (29.67 ± 0.094, n = 10) and the slope verification procedure performed prior to each replication (29.55 ± 0.037, n = 10) were not significantly different ($P > 0.05$) from each other or theoretical (29.58 -mV/decade ppm S^{-2}).

Phase 2: Extraction Development with Aqueous Media

Comparison of measurement volumes (experiment 5)

Graphs of the resulting 0.1 to 10 ppm standard curves (Figure 20) measured in 1 ml (micro-dish) or 10 ml (beakers) were similar ($P > 0.05$), except at 0.1 ppm S^2 ($P < 0.0094$). Both sets of points were at stabilization, although the unstirred 1 ml samples at lower concentrations required 5 to 10 minutes to stabilize. Regression analysis for each plot was highly significant ($P < 0.0001$), with $Y = 756.2 + 38.1\ X$ ($r = 0.957$) and $Y = 759.2 + 31.6\ X$ ($r = 0.998$) describing the 1 and 10 ml data, respectively. Without the 0.1 ppm point in the 1 ml regression, the linear equation was $Y = 759.4 + 31.3\ X$ ($r = 0.998$).

The regression, verification check and theoretical slopes, even without the 0.1 ppm data, were significantly different ($P < 0.05$), except for the slope-check (29.69 ± 0.046, n = 10) and theoretical values (29.58 -mV/decade ppm S^{-2}) ($P > 0.05$).

Determination of optimal extraction variables (experiment 6)

The effects of various initial sulfide concentrations placed in the wash-bottle prior to extraction, the flow rate of nitrogen gas through the chamber during acidification, and volume of the SAOB absorption trap were evaluated (Table 13). Volume of SAOB in the trap was a significant ($P < 0.05$) factor in the extraction process. Recoveries for the 1 ml samples were 10% lower than traps containing 10 ml SAOB, 87 ± 3.3% versus 96.4 ± 1.28% (n = 20), respectively. For the 30 or 60 ml N_2/min flow rates, spike recoveries were 91.5 ± 2.24 and 92 ± 3.1% (n = 20), and 92.5 ± 2.91 versus 91.3 ± 2.44% (n = 20) for initial sulfide concentrations of 0.5 and 5 ppm, respectively ($P > 0.05$). Unextracted controls were 99.2, 99.4 and 99.2% (n = 10) of the theoretical 0.5, 5 and 50 ppm S^{-2}, respectively. Loss due to the transfer process was 3 to 12%.

The effects of zinc preservation on extraction (experiment 7)

Zinc acetate added to a SAOB based sulfide solution resulted in nonsignificantly ($P > 0.05$) lower percent recoveries. The ten control samples yielded 93.1 ± 1.28% of the spiked sulfide after acid extraction, while the zinc treatments produced 89 ± 4.8% (n = 10).

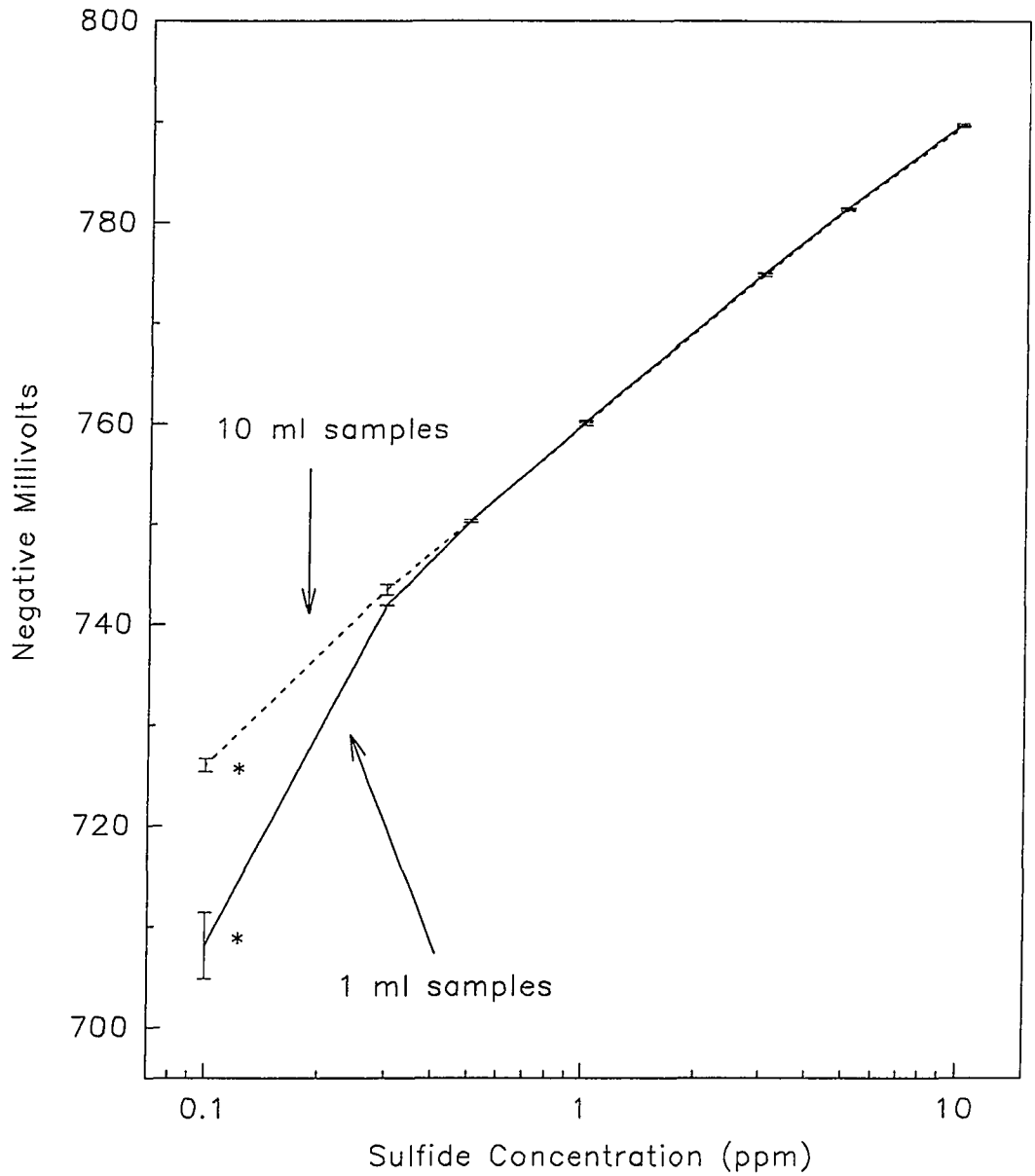

Figure 20. The same standard sulfide curve solutions measured in 1 ml micro-sample dishes or 10 ml volumes in plastic beakers with micro-stir bars. Means ± SE for 10 replications were plotted.
*Significantly different ($P < 0.05$) by the Students t-test.

Table 13. Analysis of variance for operational factors influencing acid extraction in the wash-bottle reaction chamber.

Source	Degrees of Freedom	Mean Square	F Value	Pr > F
SAOB Trap Volume (1 or 10 ml)	1	797	5.96	0.020 *
Nitrogen Flow Rate (30 or 60 ml/min)	1	6	0.05	0.826
Initial Sulfide Concentration (0.5 or 5 ppm)	1	14	0.10	0.751
Volume * Flow Rate	1	18	0.13	0.719
Volume * Sulfide Concentration	1	1	0.01	0.920
Flow Rate * Sulfide Concentration	1	124	0.93	0.341
Volume * Flow Rate * Sulfide Concentration	1	295	2.21	0.147
Error	32	134		

* Significant ($P < 0.05$).

Phase 3: Methods Development with Animal Tissues

Anti-foaming agents (experiment 8)

To prevent contamination of the SAOB trap by sample foaming during acid extraction, several compounds with potential as anti-foaming agents for biological samples were tested. Silicone emulsion in water (Thomas Scientific) and a nonionic surface-active agent (Mallinckrodt) had no effect on the foaming of cattle blood during acid extraction while being agitated with nitrogen gas. Individually, either mineral oil or poloxalene (SmithKline Beecham) initially alleviated the problem, but after acidification had been completed the extent of foaming increased with time. A 50:50 (v/v) mixture of mineral oil and poloxalene prevented foaming during the 30 minute extraction period in most samples. A thin coating of this blend was applied to the inside wall and extended portion of the nitrogen inflow stem prior to adding the sample. After transferring the specimen an additional 3 to 5 ml was added. In some species or tissues with severe foaming problems, an additional 1 to 2 ml was injected during the extraction period via the injection port.

Blood determinations A to D (experiments 9 - 12)

Sample coagulation, foaming, and poor recoveries plagued the analysis during the series of trials on the recovery of sulfide spikes from cattle blood (Figure 21). In blood determination A, 10 ml 6N HCl was used to acidify the sample to liberate the sulfide as hydrogen sulfide. After injection of 2 ml 6N HCl, the blood would coagulate into a solid mass. This prevented proper agitation of the sample by the nitrogen during extraction as the flow of the gas was limited to small channels around or within the coagulum and thus could not access trapped bubbles. Foaming was a minor problem with use of the mineral oil-poloxalene mixture, and what occurred was alleviated by the curvature of the wash-bottle wall or reducing the nitrogen flow rate. Although increasing nitrogen flow did little to combat coagulation, decreasing it exacerbated the problem. As a factorial arrangement (2 x 2), no significant differences ($P > 0.05$) were present between the level of the sulfide spike (0 or 5 ppm) or volume of the SAOB trap (1 or 10 ml). A ten-fold concentration should occur by transferring the acid-labile sulfide from 10 ml blood to 1 ml SAOB. The mean level of sulfide in the spiked blood treatments absorbed into 1 ml (5.5 ± 2.95 ppm, $n = 5$) was sixteen times more than the 10 ml sample (0.34 ± 0.150 ppm, $n = 5$), but the difference was statistically and realistically nonsignificant ($P > 0.05$, LSD 6.821 ppm). Similar results ($P > 0.05$, LSD 8.10 ppb) were found in comparing the unspiked treatments recovered into 1 (9.7 ± 3.29 ppb) or 10 ml (4.2 ± 1.28 ppb), however, only a 2.3 fold concentration occurred. Spiked and

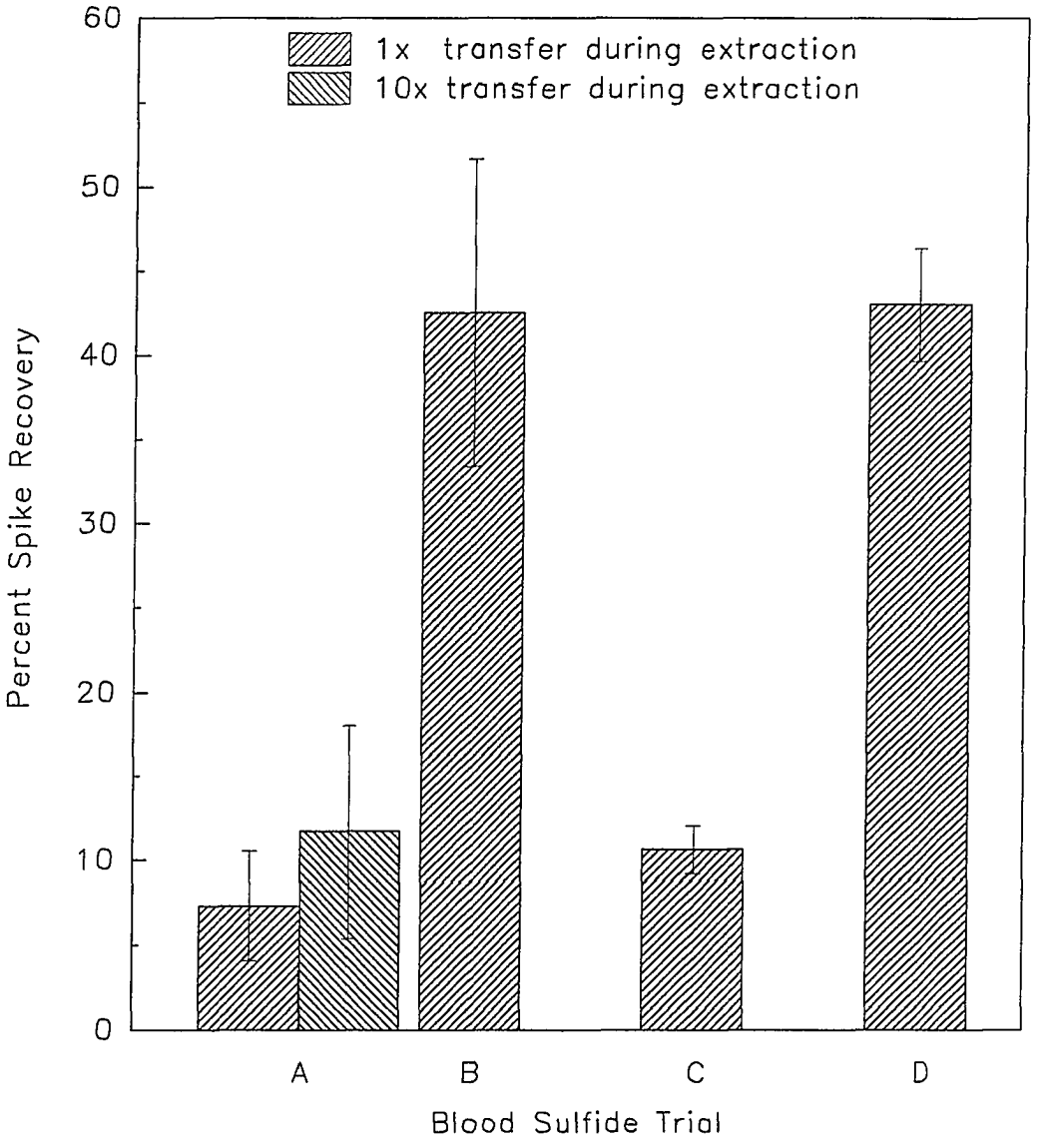

Figure 21. Recovery of sulfide from treated bovine blood specimens extracted by various acidification schemes. Means ± SE were plotted for 10 replications, except n = 5 for trial A.

unspiked treatments compared with the respective SAOB trap volumes were also nonsignificantly different ($P > 0.05$), for the 1 ml LSD was 6.81 ppm and 0.347 ppm with 10 ml.

In blood determination B, 10 ml 2N HCl was used to acidify the samples and 10 ml SAOB was the absorption solution. The milder acid produced moderate coagulation and excessive foaming occasionally resulted. Increasing the nitrogen flow in non-foaming samples to as high as 100 ml/min prevented or alleviated coagulation. Problematic foaming was resolved by injecting additional acid or anti-foaming agent. Sulfide transfer from spiked samples calculated as resulting sulfide concentration or percent recovery (Figure 21) were both significantly higher than the controls ($P < 0.003$). For sulfide levels, the 5 ppm spiked samples were 2.0 ± 0.43 ppm ($n = 20$) versus 7.00 ± 2.54 ppb ($n = 10$) in the controls (LSD 1.25 ppm).

The blood determination was repeated using 2 ml 0.25N HCl and then 8 ml 0.5N HCl with 1:1 transfer (trial C). Little to no foaming or coagulation developed. Resulting sulfide concentrations in control specimens were significantly lower ($P < 0.0001$) than the spiked treatments, although the percent recoveries were poor (Figure 21). Control samples contained 0.9 ± 0.58 ppb ($n = 10$) compared to 0.50 ± 0.066 ppm ($n = 10$) for the treatments (LSD 0.19 ppm).

In the final blood determination (trial D), 5 ml blood was diluted with deaerated saline prior to acidifying with 2 ml 0.25N HCl and then 8 ml 0.5N HCl. Extent of foaming was minimal and coagulation nonexistent. Percent recovery for the 5 ppm spike was the highest and least variable (Figure 21). Recorded sulfide concentration was 2.05 ± 0.160 ppm ($n = 10$) after 1:1 extraction.

The final pH of the wash-bottle mixture was checked after each extraction with indicator paper (Fisher Scientific). Regardless of the acidification scheme the pH was ≤ 2. Endogenous blood sulfide level in cattle, as estimated from the extrapolated control treatments, was 5.7 ± 1.33 ppb ($n = 30$).

Serum and plasma analysis (experiment 13)

To compare recoveries from blood derived matrices, spiked samples of cattle serum and plasma were extracted in conjunction with whole blood determination D (experiment 12). The foaming and coagulation problems encountered with blood were nonexistent with either serum or plasma. Final pH of the chamber solutions was ≤ 2.

Percent recoveries from sulfide spiked sera and plasma (5 ppm) were significantly different ($P < 0.05$), and both were higher ($P < 0.05$) than blood (Figure 22). The sulfide levels were 3.81 ± 0.112 and 2.75 ± 0.215 ppm for the ten treated plasma and serum samples, respectively. Endogenous sulfide was not estimated for plasma or serum.

Sulfide determination in clotted blood (experiment 14)

Freshly clotted cattle blood was analyzed as sulfide treated or control specimens to estimate the endogenous level and extraction recovery. Foaming and coagulation plagued the determinations, and additional mineral oil-poloxalene mixture and reduced nitrogen flow rate were required to combat foaming. Homogenization in ≥2:1 (v/w) saline to sample ratio reduced coagulation and foaming. The solution in the wash-bottle after extraction was ≤pH 2.

Spike recovery from blood clot was poor (Figure 22). Although sulfide concentrations extracted from the treated samples (0.61 ± 0.091 ppm; n = 15) were significantly ($P < 0.05$) higher than the estimated background samples (1.5 ± 0.40 ppb; n = 15).

Analysis of acid-labile sulfide in solid tissues (experiment 15)

Analysis of swine cerebrum as a model for sulfide extraction and determinations in solid tissues did not present problems with foaming or coagulation. Only one sample foamed during extraction, and coagulation was limited to 1 to 2 mm particles that freely circulated around the wash-bottle chamber as agitated by the nitrogen bubbles. Final pH of the extracted solutions was ≤2.

Sulfide recoveries from the brain specimens treated at 5 ppm were higher than any matrix previously tested (Figure 22). The endogenous sulfide level of 1.89 ± 0.193 ppm (n = 15) was significantly lower ($P < 0.05$, LSD 0.540 ppm) than 4.00 ± 0.179 ppm extracted from the treated specimens.

The same technique was applied to other solid tissues, such as heart and lung, and produced similar extraction characteristics and recoveries (experiments 20 and 21). Coagulation was a problem if homogenization was not complete or the saline:sample ratio was <2:1 (v/w).

Phase 4: Development of Exposure Techniques

Stability of standardized sulfide injection solution (experiment 16)

Saline injection media stored in nitrogen flushed serum bottles at 4°C did not significantly ($P < 0.05$) degrade until after day 10 post formulation (Figure 23).

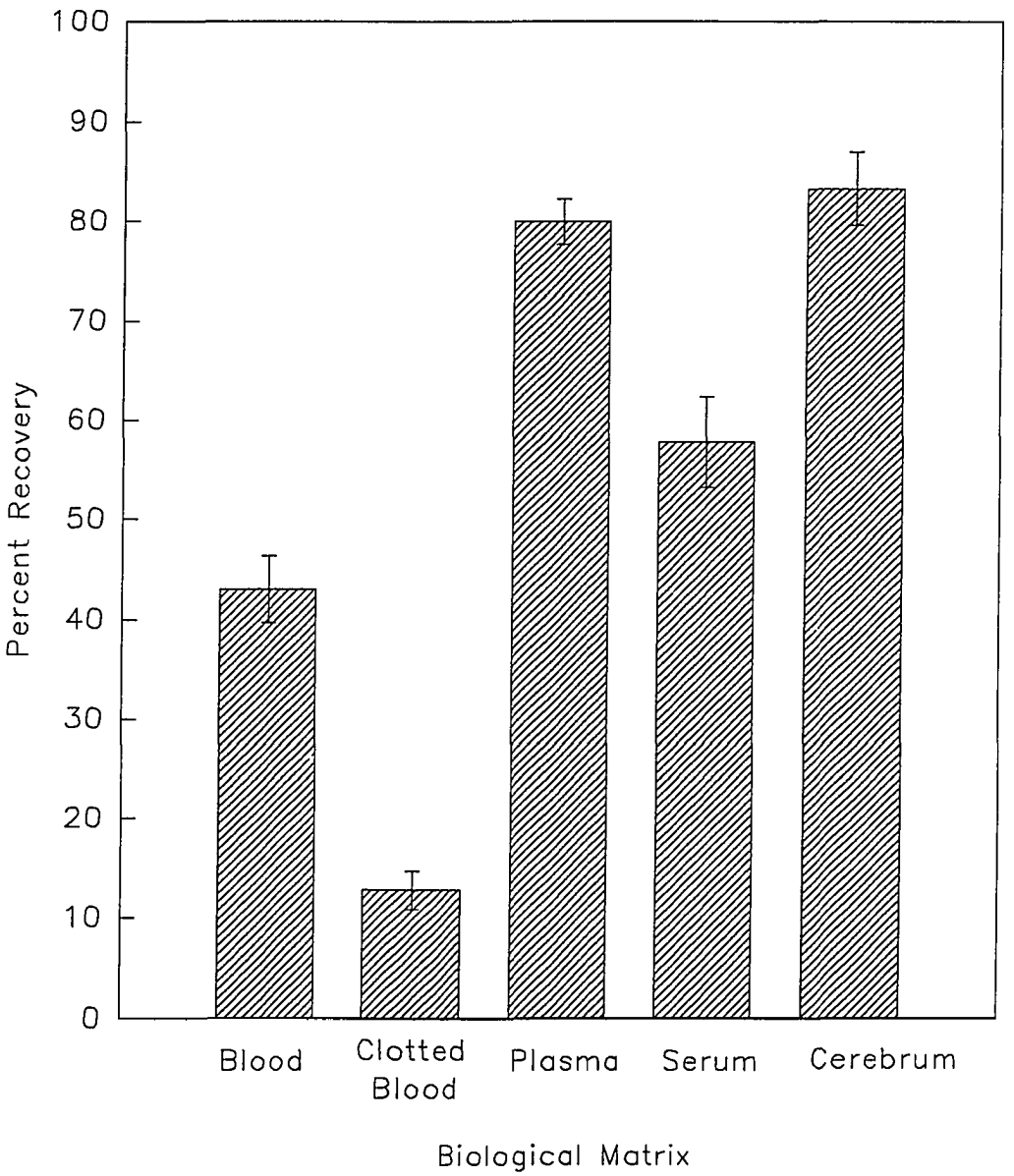

Figure 22. Sulfide recoveries from treated tissues. Blood, clotted blood, serum and plasma samples were collected from dairy cattle, and cerebrum specimens from swine. Means ± SE were plotted for 10 replications, except for blood clot and brain where n = 15.

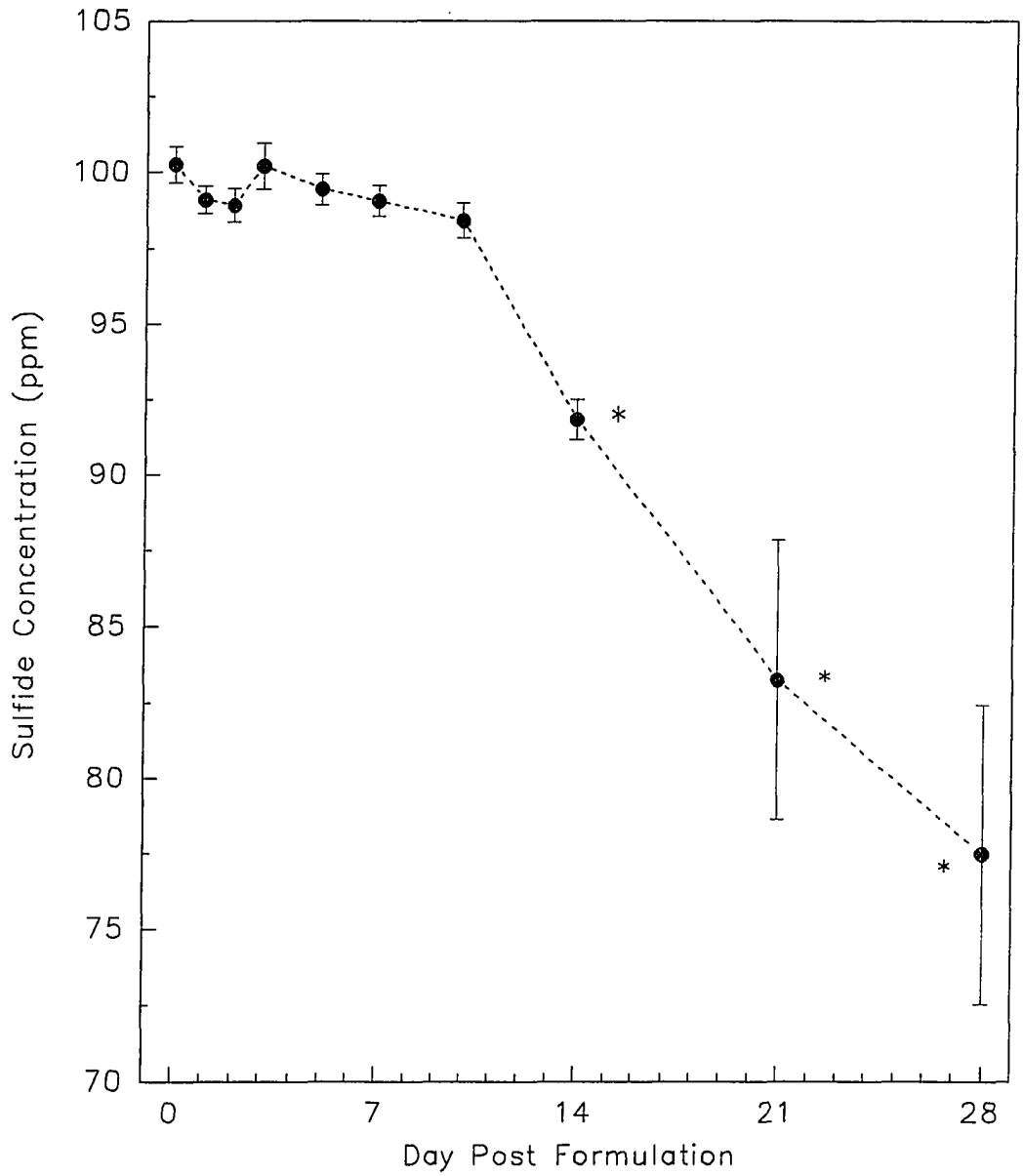

Figure 23. Stability of 10,000 ppm sulfide injection solution stored at 4°C in serum bottles sealed under nitrogen. Means ± SE for 5 replications were plotted.
*Significantly different from the day 0 ($P < 0.05$).

pH adjustment of the sulfide injection solution (experiment 17)

Titration of 15 ml 10,000 ppm sulfide formulated in PBS required 3.9 ml 2N HCl to produce the physiologic pH 7.4 (Figure 24). The volume of acid required to adjust the pH of a given sulfide dose (Figure 8) was extrapolated from this relationship (Figure 9).

The final pH of tuberculin-syringe formulated solutions for two hypothetical rats were compared to the same formulations prepared with micro pipets (Table 14). The resulting solutions for both doses were lower in pH for the pipet prepared solutions than the syringe solutions and the pH 7.4 target value ($P < 0.05$). Deviation from ideal pH and variability within the pipet solutions were 13 to 17% lower than the syringe formulations. The 2 x LD_{50} (250 g BW) sample prepared in the hypodermic had the highest final pH and largest deviation from pH 7.4 (+0.46), but the high variance in the treatment (± 0.296) prevented it from being statistically different ($P < 0.05$) from physiological conditions.

Confirmation of sulfide concentration in syringe formulated solutions (experiment 18)

Tuberculin syringe prepared doses for a hypothetical rat (350 g BW) injected at 4 x LD_{50} were diluted and analyzed for sulfide content. The formulations prior to dilution contained 7490 ± 64 ppm S^{-2} (n = 30), which was 5% higher than the estimated 7128 ppm ($P < 0.05$).

Inhalation chamber design and validation (experiment 19)

The exposure chamber did not leak under positive pressure tests conducted with dry-ice vapor or water. Vapors formed in the generation flask by injecting hot water onto dry-ice were drawn uniformly into the exposure chamber as demonstrated by the flow pattern within the inflow diffusion cone, exposure chamber and outflow collecting cone.

After injecting 2 ml 9N sulfuric acid into 100 ml 13,300 ppm sulfide contained in the generation flask, the hydrogen sulfide levels in the center of the chamber rose rapidly during the first 4.5 minutes to a peak concentration of 1330 ± 112 ppm, n = 9 (Figure 25). During the following six minutes levels gradually declined to 880 ± 160 ppm H_2S. The equation for the generation curve was $Y = 60.56 - 152.26 X + 367.03 X^2 - 95.62 X^3 + 9.25 X^4 - 0.31 X^5$ ($r = 0.9947$). Therefore, 4.5 minutes post-injection was selected as the time for collecting the single sample during subsequent animal exposures.

Acidification of the scrubbing solutions and monitoring for subsequent hydrogen sulfide release with the hand-held detector, and the addition of zinc acetate solution with observation for zinc sulfide precipitation, revealed that all the hydrogen sulfide was removed by the first flask.

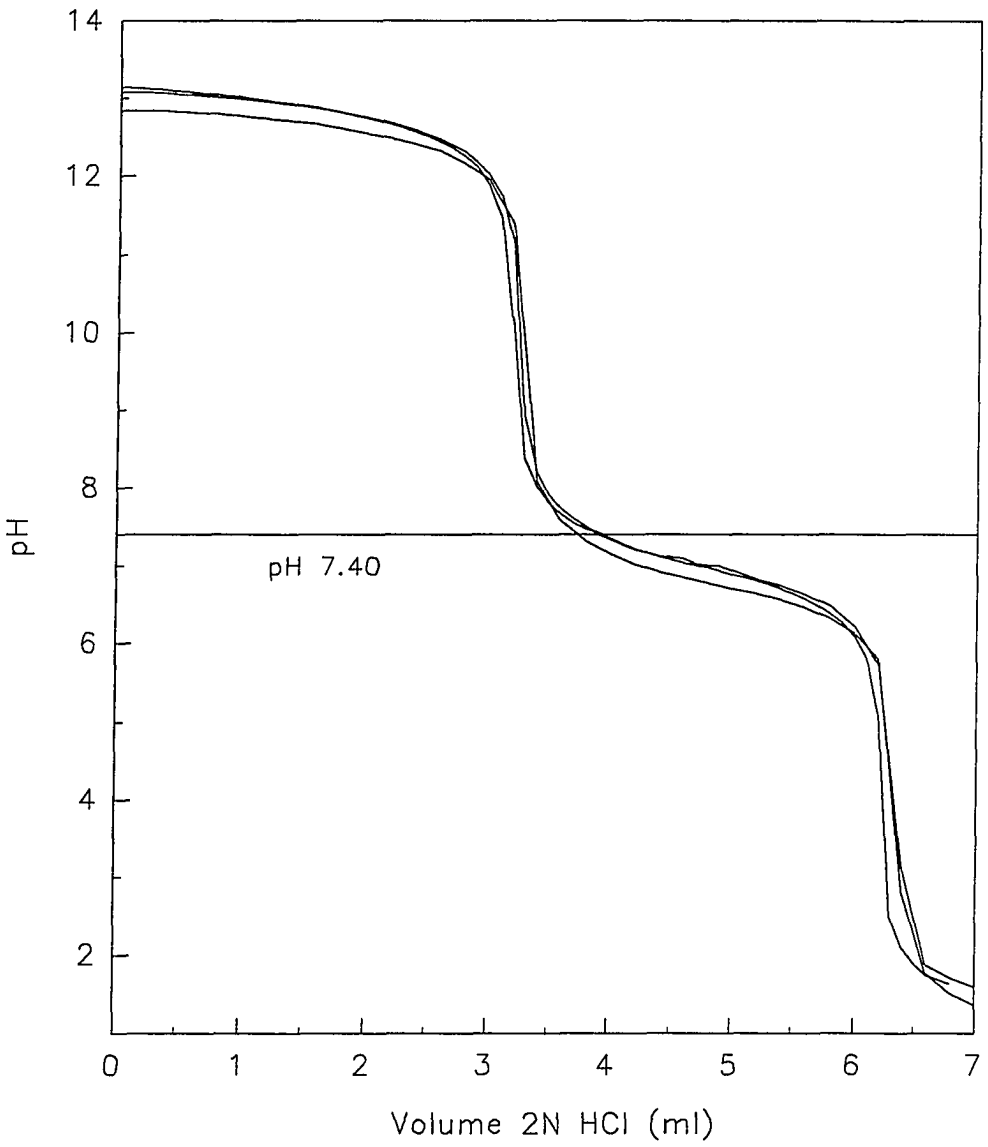

Figure 24. Titration of the sulfide injection solution with acid to determine the volume required to adjust the pH to physiological range (pH 7.4).

Table 14. Confirmation of pH adjustment to physiological levels in micro-pipet versus tuberculin syringe formulated lethal sulfide doses.

Method of Formulation	Hypothetical Rat for Dosage[a]	
	2 x LD_{50} (250 g BW)	4 x LD_{50} (350 g BW)
Micro-pipet & Test Tubes -		
Final pH (mean ± SE)[b]	7.26 ± 0.051	7.247 ± 0.0113
Deviation[c] (mean ± SE)	-0.13 ± 0.051*	-0.153 ± 0.0113*
Tuberculin Syringes[d] -		
Final pH (mean ± SE)	7.85 ± 0.296	7.48 ± 0.086
Deviation (mean ± SE)	0.46 ± 0.296	0.08 ± 0.086

[a]Based on 14.6 mg NaHS/kg BW IP (5 mg S^{-2}/kg) = LD_{50} for male 250 to 350 g Sprague-Dawley rats.[131,258]

[b]Sample size, n = 10.

[c]Deviation from normal pH 7.4.

[d]Disposable tuberculin syringe with 3/8" 26 G needle.

* Significantly different by the paired Student's t-test with pH 7.4 ($P < 0.05$).

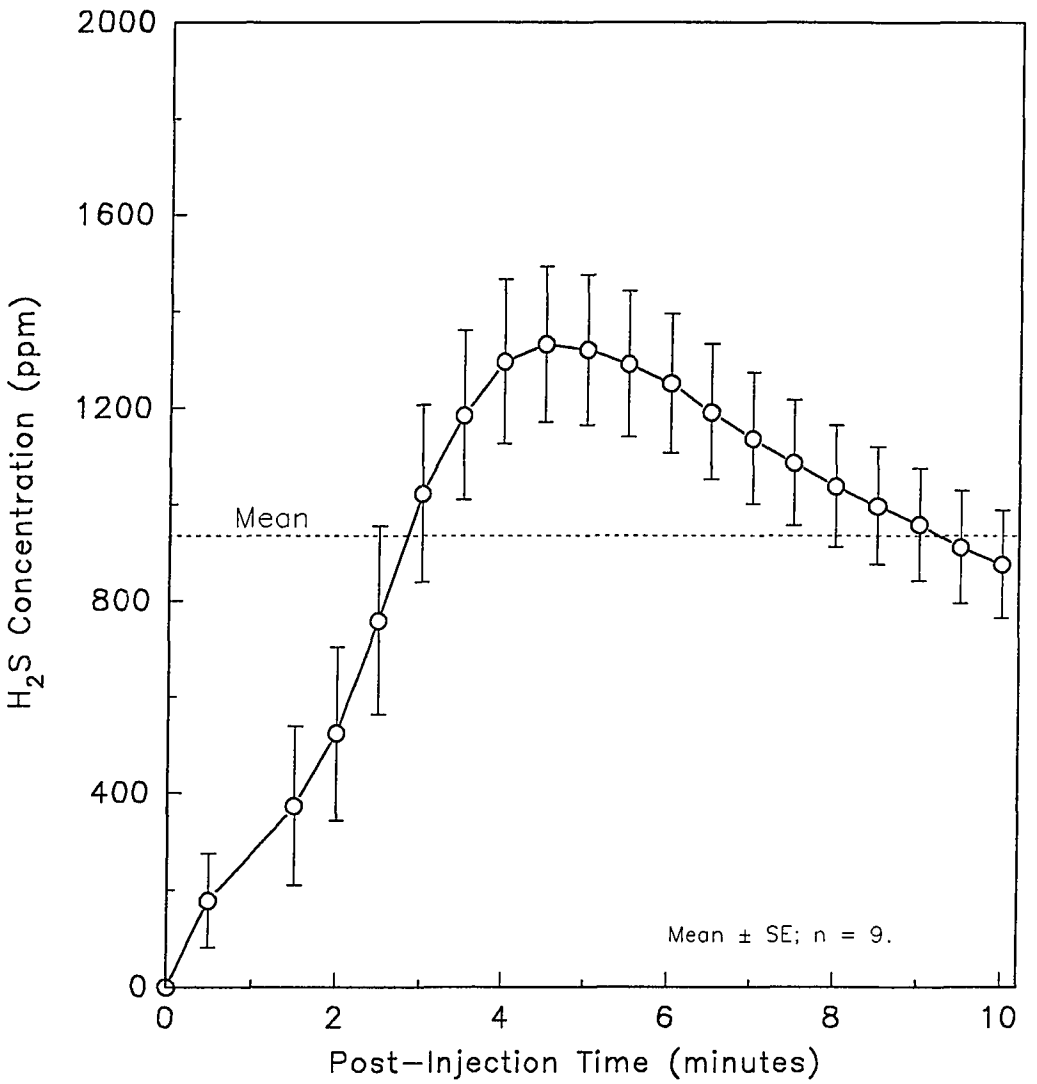

Figure 25. Hydrogen sulfide generation pattern for the inhalation exposure system as measured at the center of the animal chamber.

Phase 5: Animal Exposures

Dose-relationship study (experiment 20)

Adult male rats were injected at one of two lethal dosages of sodium sulfide and the resulting brain, lung and blood sulfide concentrations compared with the control group. Onset and progression of clinical signs were faster in the 4 x LD_{50} (20 mg S^{-2}/kg) group than the 2 x LD_{50} (10 mg S^{-2}/kg), as were the intensity of signs. Immediately after completion of the intraperitoneal injection all rats appeared and acted normal. Signs developed after 30 to 45 seconds in the high dose group, versus 45 to 60 seconds in the low. Initially the rats would cease their exploring and appear apprehensive; followed by sternal recumbency, tachypnea and tremors which rapidly progressed to seizures, apnea and death. The mean time to death was 2.71 ± 0.245 minutes (n = 6) for the 2 x LD_{50} group and 2.26 ± 0.238 minutes (n = 6) at the high dosage ($P < 0.05$). Controls remained normal during the three minute observation period. No lesions or signs of aberrant injections were present at postmortem examination.

Acid-labile sulfide levels in the brain, blood and lungs increased in a dose-response relationship (Figure 26). The low and high treatments resulted in 140 and 170% increases in brain sulfide content, respectively, although brains from the 4 x LD_{50} group were the only treatment significantly different from the controls ($P < 0.05$). Even though the sulfide dosages caused 150 to 175 fold increases in blood sulfide (Figure 26), both groups were nonsignificantly different ($P > 0.05$) from each other and the 0 x LD_{50} treatment. The statistical relationship among the three dosages were similar for the pulmonary tissue ($P > 0.05$), although the 2 x LD_{50} group was 30% lower than the controls. Only 14 ppt sulfide (extrapolated) was recovered from the lungs of one of the 2 x LD_{50} rats, which was 99,000 fold lower than the endogenous level reported (0.99 ± 0.032 ppm, n = 6). However, the high dose group was 170% higher than the controls. Spike recoveries (n = 3) for the tissues were 88.5 ± 2.69% for brain, 8.9 ± 2.93% with blood, and 102 ± 4.4% from lung.

Inhalation exposure (experiment 21)

During the inhalation trial, rats were exposed to acutely fatal concentrations of hydrogen sulfide gas in the exposure chamber and brain, blood, and lung collected for sulfide analysis and comparison with levels from control animals. After 20 to 60 seconds exposure, the animals would vigorously sniff the air and then display avoidance activities, including facing away from the gas flow and positioning themselves against the outflow grate. Next they would paw at their eyes and noses for 5 to 10 seconds before developing tachypnea and collapsing. This very rapidly progressed to generalized seizures, apnea, and death. Although all exposures were set for the same concentration, as the experiment progressed the capillary

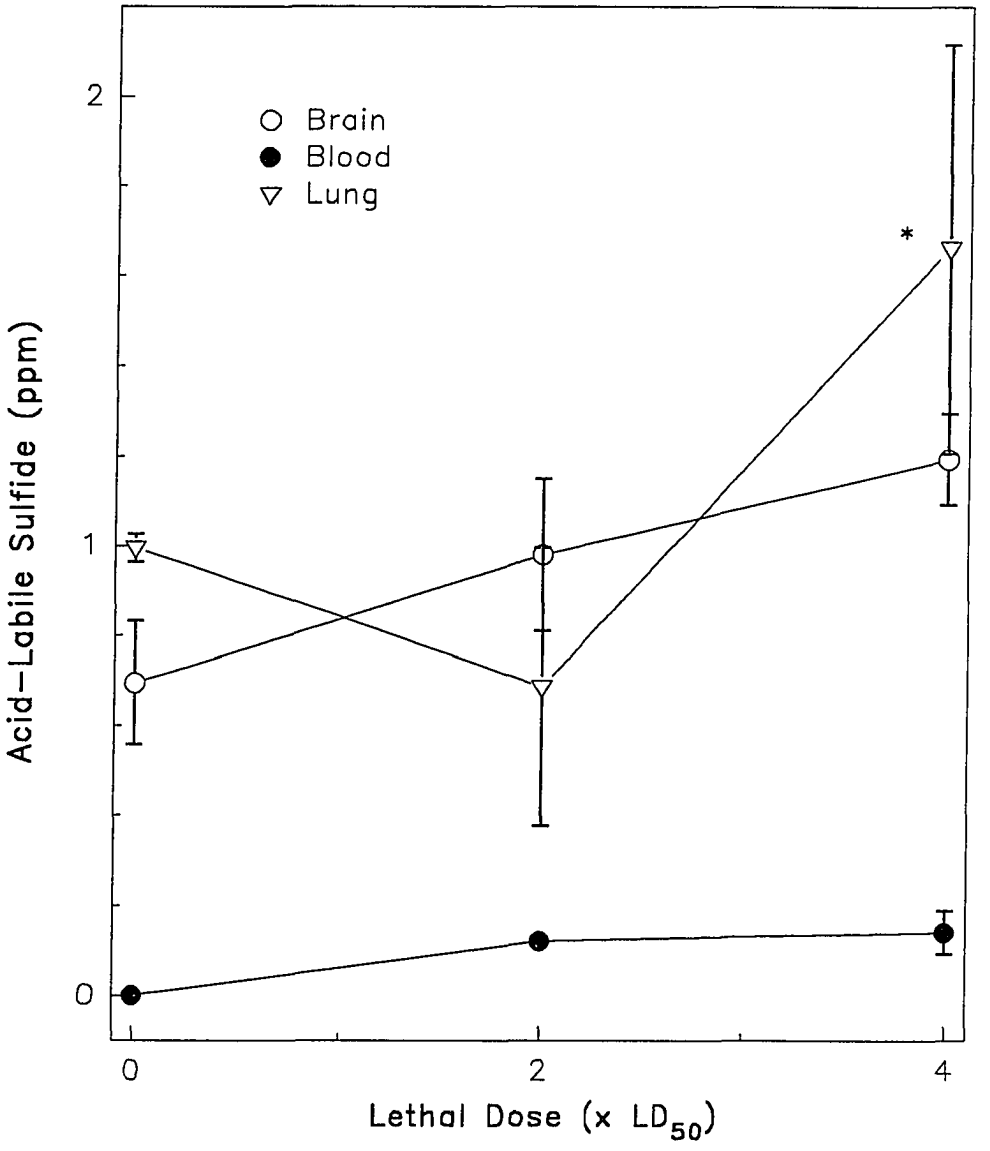

Figure 26. Concentrations of acid-labile sulfide in tissues for Sprague-Dawley rats injected intraperitoneally with 0, 2 or 4 x LD_{50} sulfide doses. The LD_{50} estimation of 5 mg S^{-2}/kg BW IP was based on Kombian *et al.*[131] and Warenycia *et al.*[258] Means ± SE for 6 replications were plotted (Appendix F).
*Significantly different from other treatments of the same tissue ($P < 0.05$).

action in the acid injection tube decreased so additional acid dripped into the sulfide solution after initiation. This resulted in generation of higher hydrogen sulfide levels, shorter onset periods, quicker progression, and peracute deaths. The mean peak exposure level collected at 4.5 minutes was 2090 ± 260 ppm H_2S (n = 8).

Postmortem lesions were limited to the respiratory tract, with severity related to the duration of clinical signs. Normal to mildly congested lungs and trachea were present after peracute exposure, but with longer clinical courses the severity of congestion and fluid increased. Extreme cases included severe pulmonary congestion with multifocal hemorrhage, and tracheal irritation and froth. No gross lesions were present in the other major organ systems.

Acid-labile sulfide levels in the exposed animals were higher than for the control group (Figure 27). Although the brain, blood and lung were increased 227, 210 and 230%, respectively, brain was the only tissue significantly ($P < 0.05$) elevated (Figure 27). Spike recoveries (n = 3) for the tissues were 101.1 ± 2.54% for brain, 23 ± 10.8% with blood, and 76 ± 11.0% from lung.

Sample quality study (experiment 22)

Asphyxiated rats stored under room conditions for up to 2 days postmortem were analyzed for acid-labile sulfide. At day 0.25 the bodies were in rigor mortis and the brains in good condition; by 0.5 days the brains were beginning to soften and rigor mortis was still present. After the first day, rigor had resolved and the brains were mildly autolytic. At the last two sampling periods the malacia progressed to moderate by day 1.5 and marked on day 2. During the experiment the room was 21.09 ± 0.048°C with 54.57 ± 0.239% humidity (n = 37).

The sulfide content of the initial group was 0.66 ± 0.215 ppm (n = 6). Results from subsequent sampling periods did not significantly differ ($P > 0.05$) from the controls until after the 1.5 day (Figure 28). By day 2, sulfide increased eight fold over the previous sampling period, or twelve times the day 0 concentration to 7.7 ± 1.74 ppm (n = 6) ($P < 0.05$).

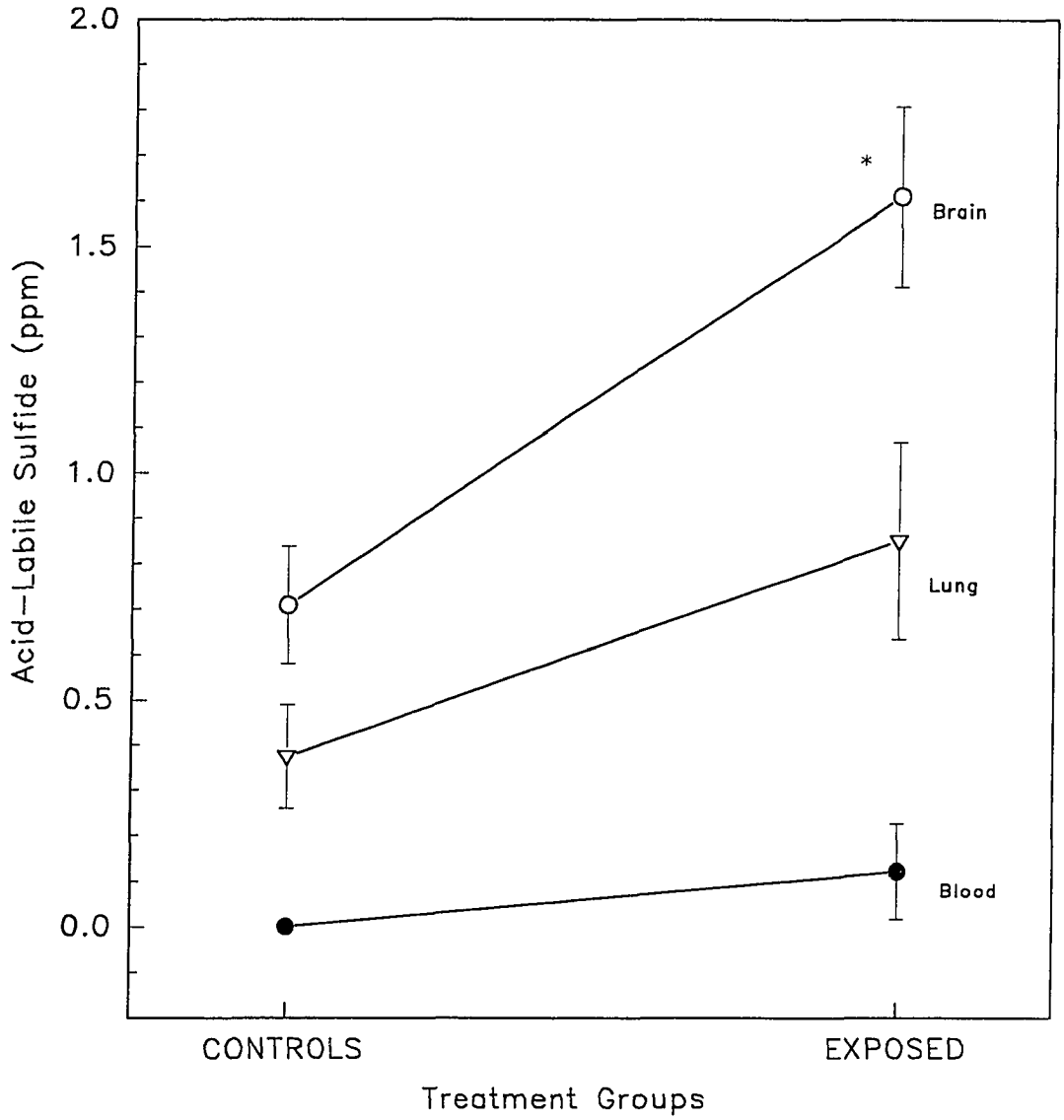

Figure 27. Acid-labile sulfide in tissues from Sprague-Dawley rats exposed to 2090 ± 260 ppm (n = 9) hydrogen sulfide. Means ± SE were plotted for 7 control and 8 exposed animals (Appendix G).
*Significantly different from corresponding control ($P < 0.05$).

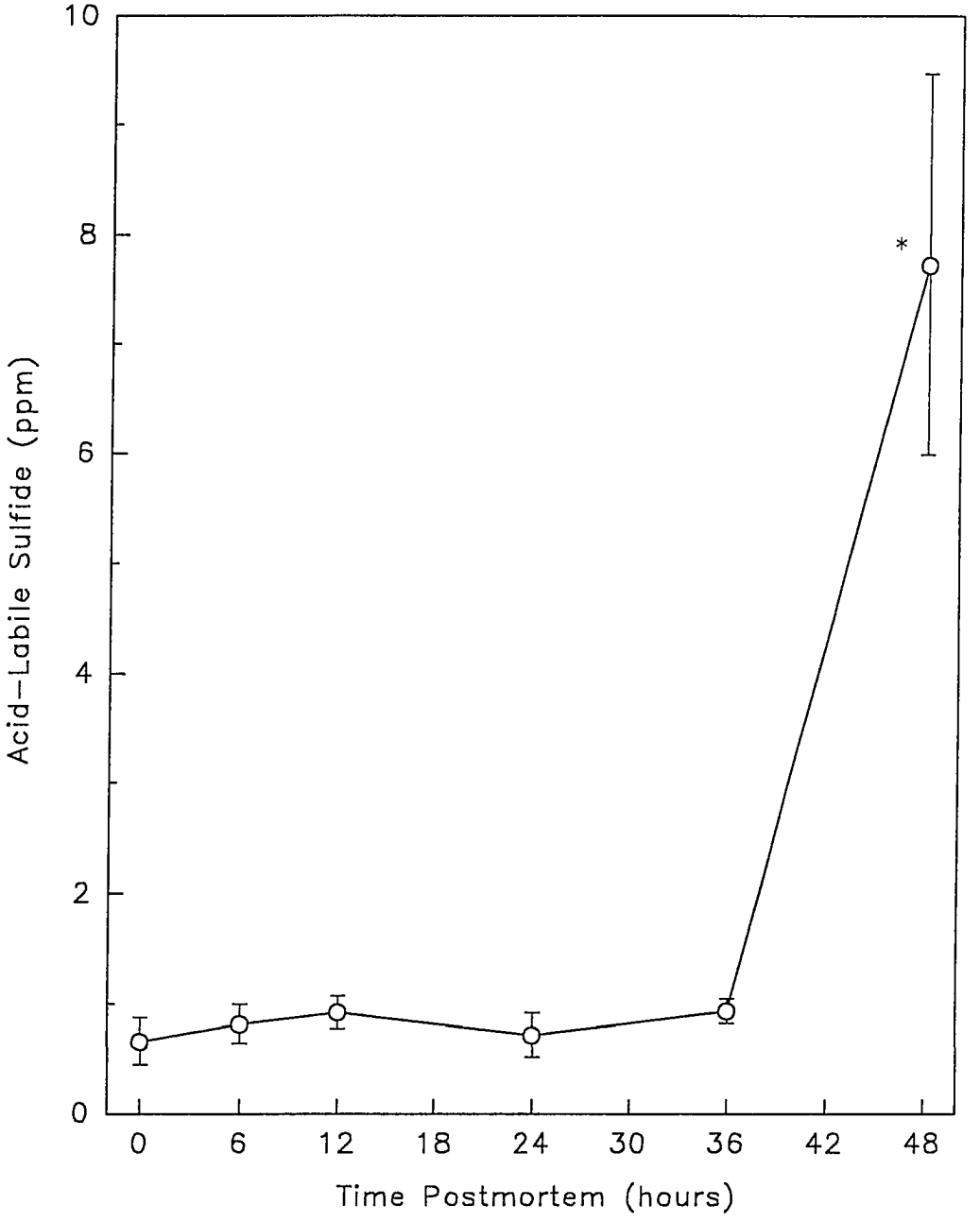

Figure 28. Postmortem changes in endogenous sulfide concentrations in the brains of Sprague-Dawley rats stored at 21°C and 54% humidity. Means ± SE of 6 replication were plotted.
*Significantly different from the 0 hour mean ($P < 0.05$).

DISCUSSION

The goal of this project was to develop a rapid, simple and inexpensive quantitative diagnostic analysis to confirm hydrogen sulfide toxicosis in livestock. The potentiometric method of McAnalley *et al.*[159] based on Conway micro-diffusion extraction and sulfide determination with an ion-selective electrode was the principle method detailed in the literature for postmortem confirmation with human blood samples. Since the beginning of this research project, forensic techniques based on gas chromatography with electron capture detection,[117] high-performance liquid chromatography and spectrophotometric determinations,[89,181,209] and gas dialysis-ion chromatography extraction with electrochemical detection[86] have been published.

Initial selection of electrodes, laboratory equipment and methodologies was based on published recommendations.[13,37,49,69,70,132,183,184,199,218] McAnalley *et al.*[159] found both silver/sulfide and cyanide ion-selective electrodes from Lazar and Orion suitable for sulfide determinations. Based on price and availability of 3 mm diameter sensing and reference electrodes for measurements in small volumes, the Lazar silver/sulfide and cyanide solid-state electrodes with matched double-junction reference were selected for use with an available digital pH/mV meter (Orion) and magnetic stirring plate (Fisher Scientific). However, unstable, noisy electrode potentials or slow stabilization, electromotive force drift, poor response slopes compared to theoretical, and short linear ranges on the resulting calibration curves plagued the establishment of the basic potentiometric measuring system (Figures 14 and 15). Durst states:[70]

> Often the users approach the application of ion-selective electrodes with great anticipation, assuming that since they have used the pH electrode without difficulties, the analogous ion-selective electrode will be equally well behaved. This naive approach often leads to considerable disappointment because the potential sources of trouble are almost as varied as the sensors and the samples.

Common sources of error include interfering ions, fouling or poisoned electrodes, sample-matrix effects, instability at the reference liquid junction, improper calibration, temperature variation, and user inexperience (Table 15).[14,70,132,164]

A useful diagnostic test requires that minimal effort is spent trouble shooting an established technique. During the present work, the first years were spent determining which combination of indicating and reference electrodes, pH/mV meter and environment would fulfill

Table 15. Sources of error in making potentiometric determinations.[a]

Sources	Nature of the Problems	
pH/mV Meter -	Electronic malfunction	Calibration
	Input impedance	
Indicator Electrode -	Activity vs concentration	Fouling and poisoning
	Species	Water activity
	Ionic distribution	Disease states
	Electrode interferences	Static electricity
	Solution interferences	Storage
Reference Electrode -	Residual liquid junction	Streaming potential
	Salt-bridge concentration	Tip potential
	Salt-bridge composition	Cells without transference
	Liquid-junction structure	Increment techniques
	Suspension effect	
Potential Drift -	Parallel direction	Random direction
	Concentration dependent	
Standards -	Preparation and calibration	Operational scale
	Dilution	Range
	Matrix	

[a]Reprinted with permission from Durst RA. Sources of error in ion-selective electrode potentiometry. In: Freiser H, ed. *Ion-Selective Electrodes in Analytical Chemistry*. v. 1. New York, New York:Plenum Press, 1978;311-338.

the basic requirements (experiment 1). The initial micro-electrode pair and meter produced standard curves with poor response slope, slow stabilization, drift and a short linear range (Figure 14 and 15). However, using five minute measurement periods, a suitable working range and slope was achieved for >0.5 ppm sulfide, which encompasses normal and diagnostic brain levels,[86] and confirmatory blood values[159] previously published.

The final configuration of Orion silver/sulfide sensing and double-junction reference electrodes with Corning meter did provide stable, rapid, Nernstian calibration to ≥0.02 ppm S^{-2} (Figures 14,15 and 17 thru 20). The difference between sensing electrode designs and construction could account for some of the problems since both can result in unstable and noisy potential readings.[70] Equally different were the reference electrodes. The Lazar micro-reference contained a fiber wick junction and relatively longer distances between the components, compared to the Orion porous sleeve interface and compact design. Since the most common problem with an electrode configuration arises from the reference electrode at the liquid junction, which is poorly understood,[70] the reference was changed before a new indicator electrode was purchased.

Problems with meters designed to the minimum specifications for potentiometric readings are rare,[13,37,70,132] and others have reported satisfactory results with Orion 701A based systems.[33,146,159,183,243] However, changing to the Corning Ionanalyzer® produced stabler measurements and facilitated method development. Other than an undetected electronic malfunction, the improvement with the Corning meter may have resulted from newer solid-state electronic technology and design, and the automatic temperature compensation probe. Since ion-selective electrodes respond (E) to ion activity ($a_{x'}$) according to the Nernst equation [$E = E^{o} + (2.303RT/n_xF) \log a_{x'}$; T = degrees Kelvin], all measurements should be made at the same temperature for high precision and accuracy.[13,70,132,218] Also, the RS232 interface on the Corning meter permitted recording data strings every 1.0 to 1.5 seconds (Appendix B), therefore standard curve data could be analyzed to monitor the performance (Appendix C) for subtle degradation in response or document improvements in methodology (Figure 14). The latter was not required for routine diagnostic work, and actually increased analysis time.

Once the potentiometric method was established for highly controlled laboratory conditions, the same analysis was confirmed as a bench-top test without the water bath system, nitrogen delivery system, and Faraday cages to fulfill the requirement of simplicity (experiment 3). Point-by-point comparison of the resulting standard curves (Figure 18) were not significantly different ($P > 0.05$), although the estimated slope response varied ($P < 0.05$)

with the method of calculation (Table 12). As predicted by the Nernst equation for measurements at 25°C, the theoretical response for a divalent ion sensing electrode is 29.58 mV/decade ppm.[33,108,127,143] Orion Research[184] states that silver/sulfide electrodes yielding around 25 to 30 mV/decade ppm S^{-2} are considered properly functioning for sulfide determinations. The resulting slopes from this experiment (Table 12), and the whole project, were compatible with this recommendation (experiments 4 and 5). Other published experimental results range from 28.0 to 30.2 mV/decade ppm S^{-2}.[30,33,52,108,127,143,214] Sample temperatures between the two analysis conditions (25.0 versus 23 to 25°C) may have accounted for some variation, although Bock and Puff[30] only reported a 1mV/decade ppm S^{-2} difference for controlled measurements at 20 versus 30°C. Significant differences ($P < 0.05$) among slope estimates, while remaining within the acceptable response range, resulted from low variance in the treatment means, thus attesting to the high precision of calibration for the electrode pair.

To reduce the analysis time a rapid and accurate method for calibrating the electrodes was developed based on the "liter-beaker" technique (experiment 4).[13,52,168,182,214,243] Improved speed results from not having to clean the electrodes between each standard point, and because each concentration is generated as required and not by serial dilution prior to calibration. The process is further facilitated by eliminating measurement geometry (electrode positions, depth) and environmental (stirring rate, temperature, nitrogen flow, liquid junctions) variables since both are held constant. Considering the rapid response rate of the silver/sulfide electrode within the 0.02 to 10 ppm sulfide range (Figure 14, plot C), an eight to nine point standard curve compatible with theoretical response could be established in about 10 minutes (Figure 15, plot A; and Figure 19). The two to three decade ranges were selected to encompass the expected diagnostic levels (>1 ppm S^{-2})[86,159] and provide lower concentrations with which to monitor for electrode degradation.

Two stated disadvantages of the liter-beaker method[182] are that it involves only one calibration, and that some uncertainly may exist if calibrated within a dilute concentration range after storage. Tóth *et al.*[242] recommended calibrating three times with a series of standards, in ascending, descending and finally ascending concentrations, and then averaging the results. The present work found that intra- and inter-daily variation in the calibration trials were negligible, with the first plot equivalent to the last. Also, Orion Research does not advise pretreating the indicator electrode with moderate concentrations of sulfide prior to calibration,[184] but Lazar Research does recommend pretreatment.[139]

A sulfide anti-oxidation buffer of some formulation is used in sulfide analysis with ion-selective electrodes[17,60,78,127,214] to maintain the analyte in the detectable sulfide form, prevent oxidation of sulfide during storage and measurement, fix the ionic strength of the solutions so concentration can be determined, and remove possible interference.[14,70,168,214,242] The original SAOB contained 80 g sodium hydroxide, 320 g sodium salicylate and 72 g ascorbic acid, per liter of water.[78] Baumann[17] recommended replacing the sodium salicylate with disodium EDTA to chelate heavy metal impurities. During the present work, SAOB was used in all standard curve and sample determinations, but because zinc chelation was used to preserve the sulfide from tissues, the EDTA was deleted.

After hours of formulating and calibrating SAOB-based sulfide solutions, these laboriously produced solutions would oxidize, even when stored in volumetric flasks with minimum air space, flushed with nitrogen, and sealed with a ground glass stopper and laboratory film. Freshly formulated SAOB has a golden color which browns as the ascorbic acid is oxidized[60,78] to dehydroascorbic acid.[85] SAOB and the serum bottle storage system (Figure 4) were used to preserve the standardized sulfide solution and extracted sample prior to analysis, facilitate the determination by permitting long term storage of preformulated standards, and reduced intra-experimental variation. No significant ($P > 0.05$) degradation occurred in 0.1 to 10 ppm sulfide standards stored in sealed serum bottles after flushing with nitrogen and refrigeration at 4°C for five days (Figure 16). This time period was selected because it included the average analysis period for an experiment in this project. However, extra standard solution bottles from previous experiments have retained the initial concentrations for up to six months, the longest time examined post formulation.

One of the longest steps in the analysis was the formulation and calibration of sulfide standards. The analysis time was greatly reduced by having calibrated standards available for an extended period of time. Stability of sulfide solutions under these storage conditions would facilitate longer term experiments by permitting multiple day studies in which the samples for each day are extracted and sealed in serum bottles until the final day, at which time all sulfide determinations are made during one measurement period. Published reports confirm that SAOB-based sulfide solutions are generally stable for 24 hours,[60,177] and flushing with nitrogen can extend the storage life to several months.[60]

Analytical procedures are generally composed of a sample preparation step to isolate, purify or concentrate the analyte, followed by qualitative measurement or quantification. Acid extraction of sulfide as hydrogen sulfide, use of nitrogen as the carrier gas, and subsequent trapping of the gas in an alkaline solution as sulfide is common.[46,57,76,86,87,114,127,146,159,234]

The wash-bottle reaction chamber was based on the work of Lindell *et al.*,[146] except that the diameter of the base was widened and a circular flare added (Figure 7A) to combat sample foaming (Phase 3). The head of Lindell's chamber contained two ports, with the top exit accommodating two fused-silica capillary tubes (0.32 mm ID) glued into place which functioned as the in- and outflow. The present design (Figure 7B) consisted of three standard laboratory hose adapters (12.7 mm ID) to which interchangeable connectors could be attached. This facilitated cleaning of glassware in experiments or case work where reaction chambers had to be used frequently.

Since acid extraction in the wash-bottles can preconcentrate the samples by trapping the hydrogen sulfide in smaller volumes of base,[146] and determinations in volumes of 1 to 3 ml require use of micro-sample dishes (Orion), calibration of the electrode pair in a beaker with a stirring bar would not be valid for extrapolations of sulfide concentration <0.5 ppm (Figure 20, experiment 5). Differences in response rates and potentials at stabilization are related to sample concentration, the beaker method accommodating stirring, and variation in measurement configuration. Also, with the smaller volumes which have larger air-to-exposed surface area versus total sample volume ratios, there is a greater potential for sample oxidation after depleting the antioxidative capacity of the SAOB during longer measurement periods.

This limited calibration curve (Figure 20) would suffice for diagnostic purposes in fatal exposures since previously reported tissue sulfide levels are above 0.5 ppm.[86,159] However, samples from non-fatal cases could not be estimated from the beaker calibrated standard curve since ranges <0.5 are expected.[146] In this instance, one could calibrate with standards measured in a micro-sample dish, or increase the sample size or absorber volume.

Of the variables associated with acid extraction of samples over 30 minutes (experiment 6), the volume of SAOB in the absorber trap influenced ($P < 0.05$) the efficiency of transfer from an aqueous matrix, and the nitrogen flow rate and initial sulfide content had no effect ($P > 0.05$). The absorbent vessels for 1 ml traps were heat sealed Pasteur pipets, versus 10 ml volumetric flasks. Since solution temperature and initial path lengths were similar, the difference between 4 and 13% loss for 10 and 1 ml, respectively, likely resulted from differences in surface contact between the nitrogen bubble carrying the liberated hydrogen sulfide and the alkaline buffer, and concentration gradients. Nitrogen bubbles in the Pasteur pipet would collide and coalesce shortly after exiting the outflow connector as contact with the glass slowed ascent. These larger bubbles would contain smaller surface to volume ratios, therefore providing less opportunity for hydrogen sulfide to absorb into the SAOB. Although a thin film of buffer appeared to flow between the bubble and the glass, there may

have been instances in which the lateral portion of the bubble only contacted the glass, further reducing the absorption area. However, the volumetric flasks have a relatively large reservoir at the base in which the ascending bubbles remained small and separated. Only at the neck of the flask did the bubbles coalesce as they contacted the glass and ascend as within the pipet. The concentration gradient in the larger volume container was also greater, therefore more conducive to diffusion of the hydrogen sulfide into the SAOB.

The extraction time per sample was set at 30 minutes based on previous works,[127,146,159] and accounted for most of the analysis time. Although extraction could be optimized, the longer time assured transfer in samples where the nitrogen flow rate was reduced due to severe foaming. In addition to carrying liberated hydrogen sulfide to the absorber vessel, the gas agitated the acidified sample and could break up mildly coagulated samples. Increasing the number of samples extracted at once would also shorten this step.

Sulfide precipitation with cadmium or zinc is used to prevent oxidative loss from samples.[82,89,127,196] Zinc precipitation of SAOB-based sulfide samples did not significantly ($P > 0.05$) alter the acid extraction as applied in the present project (experiment 7). This agrees with the work of Pomeroy[196] with sulfurous spring samples. He reported substantial losses of sulfide in oxygenated water samples treated with cadmium when comparing cadmium with sulfide precipitation, concluding that the cadmium sulfide is too susceptible to oxidation for use as a preservative. Based on current and published results, zinc acetate was added to the freshly collected or prepared samples to reduce potential losses that would yield falsely reduced concentrations. However, this preservation may not prevent false positive results (experiment 22).

In applying the established methodology to tissue samples (Phase 3), the foaming characteristics of the various organs impeded further development of the diagnostic assay until methods for controlling it were devised. Foaming was the primary factor in determining the final dimensions of the wash-bottle reactor (Figure 7A). The diameter of the base was expanded to prevent bubble formation, and in combination with the circular flare, increased the surface tension on ascending bubbles to promote rupture. A larger chamber volume permitted sample dilution to reduce the protein concentration, and thus the tendency to foam.

In the original paper by Lindell *et al.*,[146] human blood was extracted in a similar wash bottle chamber, but they do not mention foaming problems. However, in a personal communication they acknowledged the problem and suggested using mineral oil as an

antifoaming agent.[145] Plain mineral oil did not prevent bubbling in cattle blood, but a mineral oil-poloxalene mixture (50:50 v/v) added to all samples prior to acid extraction was successful (experiment 8).

Excessive foaming occasionally developed in individual samples and was combated by reducing the nitrogen flow rate, or injecting additional acid or antifoaming agent. With excessive bubbling, a portion of the sample being acidified could be transported from the acidic solution and deposited on the upper portions of the wash-bottle, thereby not being extracted and reducing the recovery. In addition, if the low pH solution bubbles over to acidify the trap, the sulfide content would be falsely lowered. Excessive foaming required close monitoring of the extraction process, therefore one could not perform other tasks that would shorten the analysis time. Every whole and clotted blood sample foamed to some extent, while serum and plasma were less of a problem. Solid tissues, such as brain and lung, rarely had complications associated with foaming.

Coagulation of the sample during acidification was a problem encountered during the tissue extraction trials. It was resolved by prediluting the sample and using lower concentrations of acids to slowly decrease the pH while further diluting the protein content. Higher nitrogen flow rates would also reduce or break up the coagulum. Whole blood was the worst matrix for this problem, especially if acidified with moderately concentrated acids without prior sample dilution. Lindell *et al.*[146] acidified 10 ml human blood with 10 ml 14M sulfuric acid but does not report coagulation problems.

Sulfide recovery from spiked samples ranged from poor for blood and clotted blood, to good in serum and plasma, and excellent with brain and lung (Figures 21 and 22). This pattern was evident in both the preliminary tissue trials (experiments 9 thru 15) and the acute lethal exposures (experiments 20 and 21). With the high-performance liquid chromatography-based method of Ogasawara *et al.*,[181] spike recoveries from aqueous medium were 99.7 to 100.6%, but 54.1 to 60.5% for human red blood cell samples. Published spike recoveries for brain tissues include 86.3 ± 3% from bovine cortex,[209] and 81.3 ± 1.35% for Sprague-Dawley rat brains.[86] Previous reports measuring blood sulfide with ion-selective electrodes unfortunately do not state recoveries.[127,146,159] Recoveries from spiked aqueous samples in the present project were 87 ± 3.3 to 96.4 ± 1.28% (experiment 6) and 83 to 101% for brain (experiment 15, 20 and 21), which were similar to the above results. Although showing the same trend in recoveries from aqueous to blood samples as Ogasawara *et al.*,[181] the blood values at best were only 43 ± 3.4% (experiment 12).

Low recoveries could result from the extraction technique, foaming, and coagulation of the sample, in addition to irreversible interaction of sulfide with sample subcomponents. Hydrogen sulfide can react with metallo-proteins and disulfide groups within the secondary and tertiary structure of proteins.[21,255] Warenycia *et al.*[259] suggested a portion of exogenous sulfide is bound to the brain tissue and not liberated by acidification. Curtis *et al.*[53] demonstrated rapid sulfide oxidation in blood samples, and that radiolabled sulfide bound to all major plasma protein fractions. Other blood components known to bind sulfide include free heme,[18,20,228,229] hemoglobin[74,228] or methemoglobin,[224-226,232] and ferritin.[18] In addition, cations of calcium, cobalt, copper, iron, magnesium, manganese, and nickel catalyze the oxidation of ions or metabolites of hydrogen sulfide in the presence of oxygen.[41]

Sulfide reacts with cystine and cystinyl peptides to produce persulfide groups under alkaline conditions (pH>8),[39,147,274] which would reduce recoveries. Elevating either sulfide concentration or pH will increase the rate and extent of the reaction.[39] However, alkaline hydrolysis or protein desulfuration[92,190,201,213] is also reported to generate artifactual acid-labile sulfide when samples are prepared at an elevated pH.[127,258] Thus pH may have been a factor in the sulfide recoveries from brain tissue being higher than that of the aqueous samples. The net sulfide recovery from biological samples appears to be a combination of spike concentration, losses due to sample metabolism or binding, and artifact.

The poor concentrating ability of the wash-bottle chamber with spiked blood samples (experiment 9) was likely due to the same factors that lead to low spike recoveries. Lindell *et al.*[146] utilized the ten-fold concentrating ability of a similar chamber to confirm sublethal hydrogen sulfide toxicosis in humans. They show two standard curves illustrating concentration after 10:1 extraction with a sodium hydroxide matrix, but do not report recoveries for spiked blood. In the present work, the sample concentration was attempted to define the endogenous blood sulfide levels for comparison with values resulting for experimental toxicoses. Due to the poor results with blood, the background sulfide levels were estimated by assuming linearity of the standard curve to the region required. This was incorrect since the standard curve only extends to 20 ppb sulfide and the plot would likely yield a non-Nernstian response in the regions extrapolated. Estimated values were used for statistical purposes since it was advised to be more meaningful than substituting 0.02 ppm for all the endogenous concentrations.[51]

Methods for delivering fatal, physiologically buffered sulfide doses by intraperitoneal injection or inhalation were developed to obtain tissues for sulfide analysis from controlled exposures (Phase 4). Sulfide salts are commonly dissolved and injected to simulate lethal

hydrogen sulfide exposure,[28,53,86,93,131,149,202,221,224,227,235,237,258-261] however, these solutions are highly alkaline.[74,172,224,272] Rarely does the methodology indicate if the final injection solution is buffered or pH corrected to a physiological range to minimize acid-base imbalances,[53,74,125,200,210] or if the potential loss of sulfide as hydrogen sulfide is considered. Smith and Abbanat[223] stated that alkaline solutions may delay absorption of sulfide from the peritoneal cavity, while acidic increases uptake.

When designing parenteral formulations, the pH, tonicity, and volume administered should be within physiological limits, or not to exceed the local and systemic capacity to accommodate any derivations.[9,155,244,264] A 10,000 ppm S^{-2} solution was selected to deliver a lethal dose under the recommended 5 to 10 ml injection volume range for adult rats[77] to assure absorption prior to death. After estimating the prescribed dose (Figure 8) and acid volume required to adjust this aliquot solution to physiological (Figure 9), the total volume including buffer to fill the dead space of the syringe was under one milliliter. The final pH and calculated dose from the syringe formulation trials were remarkably close to the expected, considering the accuracy of a disposable tuberculin hypodermic (experiment 17 and 18). Lack of excessive fluid in the peritoneal cavity suggested that the high tonicity of this small volume did not delay absorption or shift distribution of body fluids (experiment 20). A similar approach where by hydrogen sulfide gas is generated in a syringe prior to intraruminal injection is used by Bird[27] to study the effects of elevated sulfide concentrations in sheep.

Whole-body inhalation chambers have been developed for controlled hydrogen sulfide exposures with rats,[22,79,100,140,151,205,236] mice,[73,211,224] guinea pigs,[98] rabbits and pigs.[180] In developing an inhalation system, the generation, distribution, sampling and characterization, and cleanup of the test atmosphere must be considered.[103,160,161,217]

Generating hydrogen sulfide by acidifying a sodium sulfide solution (Figure 11) was a rudimentary, but effective method for producing the acutely toxic atmosphere for the small volume chamber (experiment 19). The generation profile suggests a massive initial release of hydrogen sulfide, followed by a lower rate of production or dilution with fresh room air (Figure 25). If concentrations >800 to 900 ppm are considered acutely toxic (Table 5), then this configuration provided 7 to 7.5 minutes of atmosphere prescribed for the experiment. The high variability associated with this generation system (experiment 21) resulted from loss of capillary action in the injection port, which permitted additional acid to drip into the sulfide solution after the initial injection and displacement of the acid used to prime the glass tubing. A redesigned injection port would combine the air intake and injection ports so that all acid volume would be aspirated directly into the sodium sulfide solution. This approach could yield

the control required to conduct multi-concentration inhalation exposures. Other methods would be to purchase an air-hydrogen sulfide blends at the required concentrations; or use bottled hydrogen sulfide and air with metered flows to formulate the atmosphere.

The flow and resulting atmospheric distribution within the animal exposure compartment was uniform, based on the carbon dioxide vapor pattern and density. Since hydrogen sulfide has a relative density of 1.19 (air = 1.00, S.T.P.), and carbon dioxide is 1.527 272, the CO_2/H_2S would be about 1.28. Atmospheric sampling during calibration and exposures was from the center point of the animal chamber and consisted of a single sample during animal trials (Figures 10 and 12). Although multiple samples from different locations during the trial would be better, based on the vapor pattern and the small chamber volume, the selected approach was economical and likely sufficient. Since no hydrogen sulfide was detected in the second scrubber trap (Figure 13), determining the sulfide concentration in the first flask, and knowing the NaOH volume, absorption time and flow rate, the average hydrogen sulfide level could have been estimated.

Although generating high concentrations of an acutely toxic chemical, all components of the exposure system fit within a fume hood that provided a high margin of safety (Figure 10). Since injection of a volume of acid into a sulfide solution produces a finite amount of hydrogen sulfide (Figures 11 and 25), the hood ventilation rate would accommodate a worst case accident during an exposure without requiring much more than completely lowering the sash. Therefore, secondary exposure to operators was negligible during normal chamber generation and removal of animals.

During the exposure trials using the injection and inhalation exposure methods (Phase 5), the clinical signs, progression, and time course were compatible with those reported in other experimental exposures[22,98,125,149,180] and cases involving humans (Table 8) and livestock (Table 10). Gross pathology was limited to the hydrogen sulfide exposed rats in the inhalation trial (experiment 21); no lesions were present in the sodium sulfide injected rats (experiment 20). This agrees with the gross and histopathological findings of Lopez *et al.*[149] in male Sprague-Dawley rats injected with 10 mg S^{-2}/kg BW IP or exposed to 1655.4 ± 390.9 ppm H_2S; which concludes that sulfide salt injections mimic gas exposures for the nervous system, but not the respiratory tract. Tissue sections were not collected for histological evaluation during the studies due to the sample sizes used in the analysis.

As determined by the ion-selective electrode method, there was good agreement among estimations of endogenous sulfide between experiments (20 to 22) for blood and brain, but less so for lung. Sulfide in the brains of Sprague-Dawley rats were 0.69 ± 0.14 (n = 6),

0.71 ± 0.129 (n=7) and 0.66 ± 0.215 ppm (n=6), for experiments 20 to 22, respectively. These brain levels are all lower, but with less inter-analysis variation, than published values of 1.06 to 3.49 ppm for Sprague-Dawley rats, as determined by a group using gas dialysis and ion chromatography with electrochemical detection.[86,202,258,259] Extrapolated estimates (<20 ppb) for blood sulfide in rats were 0.8 ± 0.67 ppb (n=6) for experiment 20, and 0.57 ± 0.172 ppb (n=7) in experiment 21. Previously published estimates of endogenous blood sulfide concentrations are similarly hindered by relatively high analytical detection limits.[117,159] Interestingly, the pulmonary endogenous sulfide estimations were higher than blood, and similar to the brain levels. However, the two sets of estimates from experiments 20 and 21, 0.99 ± 0.032 (n=6) and 0.37 ± 0.115 ppm (n=7), respectively, were in poor agreement. No lung sulfide data are available for comparison.

The physiological role and sources of endogenous sulfide are not clear.[181,258] Enzymatic metabolism involving sulfide has been reported,[158,233,249] and recent works demonstrate that the brain sulfide level measured is not artifactual.[86,209,258] Its role in neuronal excitability is suggested.[258]

Comparisons between published and present post-exposure tissue sulfide levels were limited by differences in treatment times, concentrations and method of delivery, tissues analyzed, and the possibility of species variations; however, trends can be examined. A dose-level relationship between exposure level and resulting sulfide concentration occurred in blood, lung and brain (Figures 26 and 27) in the injection and inhalation experiments (20 and 21); except for the lungs from the 2 x LD_{50} group in the parenteral treatments which was erroneously low due to one value estimated at 14 ppt sulfide. Removal of the latter point would bring the remaining data into the predicted range (Figure 26). Good dose-tissue concentration relationships are reported for sulfide levels in rat brains measured by the gas dialysis/ion chromatography method.[86,258]

The resulting blood sulfide concentrations from the injection and inhalation studies (0.12 to 0.14 ppm) were compatible with the low end of the range reported in blood from male Wister rats exposed to 500 to 600 ppm hydrogen sulfide.[117] As determined by a gas chromatography based method, the resulting values are 0.19 to 0.61 ppm sulfide, with controls <0.01 ppm (n=5). However, compared to fatal human cases these values were lower (0.92 to 5 ppm),[38,159,160,185] except for one with 0.04 ppm.[6] Nonfatal incidences also report higher values (0.03 to 0.6 ppm).[113,146]

Brain sulfide concentrations from rats receiving sulfide or hydrogen sulfide doses were lower (0.69 to 1.61 ppm S^{-2}) than any previous reports for whole rat brain by the group using the gas dialysis/ion chromatograph analysis. Published and estimated values from figures include 1.60 and 2.3 ppm sulfide after 5 mg S^{-2}/kg IP,[86,259] 3.1 ppm S^{-2} for 9 mg/kg,[86] 1.65 and 5.35 ppm S^{-2} after 10 mg/kg,[202,259] and 4.4 ppm S^{-2} for 17 mg/kg.[86] Inhalation exposure of 1400 ppm hydrogen sulfide yielded 5.00 ppm S^{-2}.[202] Similar intra-experimental variations in tissue levels post exposure were noted in the results from the current work, especially lung.

Estimates of the endogenous or post exposure sulfide content of pulmonary tissue have not been made under controlled conditions. In a letter describing a human case of subacute toxicosis, the 4.2 ppm H_2S measured in the lung tissue by an unspecified method is considered "above lethal threshold."[81] Only a "small amount" of hydrogen sulfide is reported in the blood.

Addition of dithiothreitol[44] to brain homogenates from control and sulfide injected rats results in higher sulfide recoveries in all treatments and wider differences between treatment groups.[259] Incorporation of the Cleland reagent (dithiothreitol), which reduces disulfide bonds, into the extraction process of the present work may improve the recoveries and statistical significance.

High variances in the resulting tissue concentrations prevented some of the treatment from being judged statistically different from the control or other dosed groups (Figures 26 and 27). This was especially disappointing with the blood samples since the endogenous levels (<20 ppb) were several decades lower than expected post-exposure values based on fatal and nonfatal human cases; compared to the reported endogenous and post-treatment brain sulfide concentrations which are considerably closer in range.[86,202,258,259] The variance within treatments likely resulted from a combination of problems. Especially dosage errors with the inhalation exposure since the injection port dripped additional acid into the generation flask as the capillary action of the glass tubing diminished. Higher hydrogen sulfide levels were generated which caused peracute deaths, shorted exposures periods, and therefore lower total sulfide inhaled. A three minute post injection observation period was used in experiment 20. Although all sulfide treated rats were clinically affected within this time frame, those surviving longer at a given dose would have more time to metabolize the sulfide than one dying more acutely. Losses during storage, the extraction procedure, and errors in calibration and analysis could also contribute to the variance.

As determined by the gas dialysis/ion chromatography method, the dose-level relationship for brain appears to have higher intra-experimental precision, thus resulting in a better ability to determine statistical differences over similar ranges.[86,258] Other than

difference in analysis technique, this group used two minute post-injection observation periods based on median time-to-death from a LD_{50} trial based on unbuffered sulfide solutions. This shorter period and potential for iatrogenically induced metabolic alkalosis due to the injection solution formulation could have resulted in a narrow range for time-to-death, less time for metabolism of the dose, and therefore more uniform tissue concentrations. One point against this is that basic solutions delay absorption of sulfide from the peritoneal cavity,[223] which could result in lower body concentration and prolong survival times if the potential alkalosis is not fatal. Brain sulfide levels from the present inhalation and injection experiments were all lower than those published for the parenteral based studies.[86,258]

The validity of a sulfide measurement involving diagnostic materials is more likely to be determined by the physical quality of the samples rather than the analytical method since sulfide is rapidly metabolized and artifactual sulfide is formed from postmortem protein degradation.[74,117,127,159] Most workers conclude samples should be collected as soon as possible, steps taken to preserve the sulfide content (refrigeration, flash freezing, or cationic precipitation), rapid delivery to the laboratory under preservation, and immediate analysis.[74,89,113,117,127,131,145,159,202,258] For field based submissions, it is unlikely that practitioners would have cadmium or zinc solutions readily available for use as a preservative.

In addition to the general recommendations above for submission, blood samples should be refrigerated at 4°C until extraction[113,145,159] with minimum air volume in the blood-tube[145] or establish a vacuum. The value of precipitating the sulfide with cadmium acetate has been demonstrated,[127] however Pomeroy[196] advises using zinc acetate. Stored blood samples older than two to three days can produce artifactual sulfide.[117,127] Although, human autopsy specimens, including some badly decomposed, did not yield >0.4 ppm S^{-2} ($n = 25$).[159] Antioxidant buffers should not be used as blood preservatives since falsely elevated sulfide concentrations can be generated.[127]

The regions of the brain selectively accumulate sulfide at different rates (Table 6).[258] If available, the brain stem should be considered the diagnostic region of choice since it has the lowest endogenous and highest net uptake of exogenous sulfide. Specifics for submitting brain for sulfide analysis include flash freezing with dry-ice or liquid nitrogen,[131,258] storage at similar temperature, and prompt analysis. Validity of brain as an analytical sample post exposure was investigated in rat cadavers stored at a moderate ambient temperature (experiment 22). Since most cases of hydrogen sulfide toxicosis in swine are associated with agitation of sub-floor manure pits[31,65,137,166,167,171,208,254] which are emptied in fall or spring when the contents can be spread on the open fields,[67] the 21°C temperature was selected

because it would fall in the range expected during these seasons and could easily be maintained in the laboratory. Results show that samples collected prior to 36 hours did not significantly differ ($P > 0.05$) from time zero in unexposed rats (Figure 28). Earlier work shows that sulfide concentration in control and treated rats are discernable at 15 hours postmortem when stored at -20 to 22°C, but not between 22 to 37°C.[86] This latter range could be a factor in swine cases since agitation mishaps are usually accompanied by ventilation deficits, which could elevate the temperature and humidity in the confinement unit.

Brain sulfide content in rats did not increase until after 72 hours when refrigerated at 4°C.[86] However, rats killed by a lethal sulfide injection (17 mg S^{-2}/kg BW IP) and stored at 4°C show a significant ($P < 0.01$) decrease within 12 hours after death which was attributed to mitochondrial metabolism, but did not drastically vary thereafter until after 96 hours postmortem. Sulfide levels in post toxicosis rats stored at 21°C were not examined in the present project.

CONCLUSIONS

The final equipment configuration consisted of an Orion silver/sulfide ion-selective electrode and Orion double-junction reference electrode attached to a digital pH/mV meter with an automatic temperature compensation probe. This arrangement provided rapid and stable measurement of 0.02 to 10 ppm S^{-2} when conducted under highly controlled or bench-top laboratory conditions. The basic analysis was further facilitated by using a serum bottle storage system with nitrogen gas and a sulfide antioxidant buffer to prevent oxidation of standardized solutions and extracted unknown samples. Adaption of a serial addition method, "liter-beaker technique," to the expected diagnostic analyte range permitted rapid calibration of the electrode pair over three decades of sulfide concentrations. Slopes from the resulting standard curves were in agreement with the theoretical response for a silver/sulfide electrode to sulfide. This calibration method was adequate for extrapolation of any unknown sulfide concentration when the potentiometric determination was conducted in plastic beakers with stirring bars, but for volumes ≤ 3 ml the samples required measurement in micro-sample dishes without mixing. In the former, sample concentrations <0.3 ppm S^{-2} could not be extrapolated from a liter-beaker calibrated standard curve; although fatal exposures to hydrogen sulfide should result in tissue sulfide levels higher than this point.

The wash-bottle extraction unit developed during the research provided excellent recoveries from sulfide spiked aqueous samples. Initial sulfide concentration and nitrogen flow rate in the chamber did not influence the recoveries, although a lower volume of sulfide antioxidant buffer as the absorbent solution will increase losses. This was reflected in the adequate but lower sulfide recoveries during extractions that resulted in concentrating the analyte ten fold. Zinc preservation of aqueous sulfide samples did not markedly reduce the percent recoveries.

Sulfide extraction from animal tissues was plagued by sample coagulation, foaming and poor percent recoveries. Diluting the samples prior to extraction, and acidification with low molarity acids prevented coagulation. Increasing the nitrogen flow rate could also break up moderate clots. Whole blood and clotted blood were the worst samples for coagulation. Foaming was combated by using a mineral oil-poloxalene mixture as the antifoaming agent in the extraction unit, redesigning the wash-bottle chamber, and reducing the flow rate of the nitrogen carrier gas. Again, the whole blood and clotted blood were the worst matrices for foaming compared to brain, lung, serum or plasma. Recovery of sulfide from spiked blood or blood clot was poor (Figures 21 and 22), while results were good to excellent for serum, plasma, brain and lung (Figure 22). Dismal recoveries from blood prevented the concentrating

ability of the extraction unit from reliably defining the background sulfide levels. Endogenous acid-labile sulfide concentrations estimated during the research project are summarized in Table 16. Future research could further define background sulfide levels in various tissues (blood, heart) and other species (poultry, fish).

Injection of alkaline sulfide solution is commonly used to reproduce acute hydrogen sulfide toxicosis in laboratory animals, although these solutions are possibly incompatible with physiologic acid-base balance. Using tuberculin syringes to formulate lethal sulfide doses and adjusting the pH to a physiological range was a relatively accurate method for conducting acute, multiple dosage exposures. When combined with the serum bottle storage system to prolong the sulfide content of non-buffered solutions, a larger experimental design could be conducted over several days or as a subacute, multiple dose study with the syringe formulation method. Although the injection port on the hydrogen sulfide generation flask should be redesigned to afford more control, the inhalation exposure system provided a safe and effective method for generating, delivering, sampling and cleaning up an acutely fatal atmosphere.

Since initiating the research, other methods for quantifying tissue sulfide have been published. In general, the present technique yields sulfide estimates that were lower than the other analyses for the same matrix. Variance in the sulfide concentrations established by the current determination were higher than reported by the other methods. This prevented the test from determining some of the elevated tissue sulfide levels that resulted from sulfide and hydrogen sulfide treatments as being statistically different, although several fold higher than the endogenous concentrations. Incorporation of dithiothreitol into the extraction procedure may increase the statistical sensitivity of the test. Where a research group has used one of the other techniques several times to determine tissue sulfide, the inter-experimental error for the ion-selective electrode method appeared to be lower. Comparative and collaborative studies involving the gas chromatography, high performance chromatography, gas dialysis-ion chromatography, and ion-selective electrode analyses would clarify the differences and similarities between each.

The validity of a sulfide measurement involving diagnostic materials is more likely to be determined by the physical quality of the samples rather than analytical method since sulfide is rapidly metabolized and artifactual sulfide is formed from postmortem protein degradation. Blood, brain and lung were demonstrated to be suitable samples for diagnostic confirmation of

Table 16. Endogenous acid-labile sulfide concentrations established during the research project.

Tissue	Source	Sulfide Concentration	
		n	Mean ± SE
Blood			
Whole blood	Rat	12	0.67 ± 0.281 ppb[a]
Whole blood	Cattle	30	5.7 ± 1.33[a]
Blood clot	Cattle	15	1.5 ± 0.40[a]
Brain			
Cerebrum-Medulla	Rat	12	0.69 ± 0.090 ppm
Cerebrum	Swine	18	1.89 ± 0.193
Lung	Rat	12	0.63 ± 0.113 ppm

[a]Blood sulfide concentrations estimated by assuming the linearity of the standard curve beyond the 0.02 ppm point.

hydrogen sulfide exposure. Specimens should be collected as soon as possible, steps taken to preserve the sulfide content (refrigeration, flash freezing, or zinc precipitation), rapid delivery to the laboratory under preservation, and prompt analysis.

In addition to evaluating future hydrogen sulfide cases with the ion-selective electrode determination, instances of suspected polioencephalomalacia in ruminants where the animal does not have well defined gross and histological lesions could be analyzed for elevated sulfide concentrations. Also, the technique could be used to evaluate the sulfide content of other biological samples, such as waters, soils, sediments, and plants.

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APPENDIX A: CAUSES OF SUDDEN DEATH IN LIVESTOCK[a]

Disease	Species				
	Bovine	Ovine	Caprine	Equine	Porcine
Infectious					
ABSCESS DUE TO CLOSTRIDIUM PERFRINGENS				X	
ACTINOBACILLOSIS					X
ACTINOBACILLUS PNEUMONIA, PLEUROPNEUMONIA					X
ACUTE ANTHRAX	X	X	X	X	X
ACUTE BOVINE VIRUS DIARRHEA, BVD	X				
AFRICAN HORSE SICKNESS - EXOTIC				X	
AFRICAN MALIGNANT CATARRHAL FEVER, ALCELAPHINE HERPESVIRUS-1	X				
AFRICAN SWINE FEVER - EXOTIC					X
ANAPLASMOSIS	X	X	X		
BACILLARY HEMOGLOBINURIA, CLOSTRIDIUM HEMOLYTICUM, (C. NOVYI TYPE D)	X	X			X
BACILLUS PILIFORMIS, TYZZER'S DISEASE, IN FOALS				X	
BACTERIAL PNEUMONIA		X			
BLACK DISEASE, INFECTIOUS NECROTIC HEPATITIS, CLOSTRIDIUM NOVYI	X	X	X	X	X
BLACKLEG, CLOSTRIDIUM CHAUVOEI	X	X	X		X
BLUETONGUE	X	X	X		
BOTULISM, CLOSTRIDIUM BOTULINUM	X	X	X	X	
BORDER DISEASE, HAIRY SHAKER DISEASE		X	X		
BOVINE MALIGNANT CATARRHAL FEVER	X				
BOVINE SPONGIFORM ENCEPHALOPATHY, BSE	X				
CAPRINE MYCOPLASMA MYCOIDES INFECTION			X		
CLOSTRIDIAL ENTERITIS, CLOSTRIDIUM PERFRINGENS TYPE C, IN SUCKLING PIGS					X
CLOSTRIDIUM PERFRINGENS TYPE B INFECTION	X				
CLOSTRIDIUM SEPTICUM ABOMASITIS, BRAXY	X	X			
CLOSTRIDUM SORDELII IN FEEDLOT CATTLE	X				
COLITIS IN WEANED LAMBS DUE TO CAMPYLOBACTER		X			
COLITIS X, ACUTE EQUINE COLITIS SYNDROME				X	

CONTAGIOUS CAPRINE PLEUROPNEUMONIA - EXOTIC			X		
CYSTITIS AND PYELONEPHRITIS					X
EDEMA DISEASE					X
ENCEPHALOMYOCARDITIS					X
ENDOCARDITIS	X	X	X	X	X
ENTERIC COLIBACILLOSIS	X				X
ENTEROTOXEMIA, CLOSTRIDIUM PERFRINGENS TYPE D	X	X	X		
ENZOOTIC ADULT LYMPHOSARCOMA, BOVINE LEUKOSIS	X				
EQUINE INFECTIOUS ANEMIA, EIA				X	
ERYSIPELAS					X
FOOT AND MOUTH DISEASE - EXOTIC	X	X	X		X
HEARTWATER, COWDRIOSIS - EXOTIC	X	X	X		
HEMOPHILIS PARASUIS, GLASSER'S DISEASE					X
HEMOPHILUS INFECTION		X			
HEMOPHILUS SOMNUS DISEASE COMPLEX, HEMOPHILOSIS	X				
HEMORRHAGIC ENTEROTOXEMIA, CLOSTRIDIUM PERFRINGENS TYPE C	X	X			
HEMORRHAGIC NECROTIZING CLOSTRIDIAL ENTEROCOLITIS IN FOALS				X	
HOG CHOLERA, SWINE FEVER, PESTIVIRUS INFECTION - EXOTIC					X
INCLUSION BODY RHINITIS, PORCINE CYTOMEGALOVIRUS					X
LAMB DYSENTERY, CLOSTRIDIUM PERFRINGENS TYPE B		X			
LEPTOSPIROSIS	X	X	X	X	
LISTERIOSIS	X	X	X		X
LIVER, HEPATIC, ABSCESS	X	X	X		
MALIGNANT EDEMA, CLOSTRIDIUM SEPTICUM	X	X	X	X	X
MELIOIDOSIS, PSEUDOMONAS PSEUDOMALLEI - EXOTIC	X	X	X	X	X
MENINGOENCEPHALITIS, MENINGITIS, MENINGOVENTRICULITIS	X	X	X	X	X
MYCOSIS OF THE GUTTURAL POUCH				X	
NEONATAL RHINOPNEUMONITIS				X	
PASTEURELLA HAEMOLYTICA-LIKE ORGANISM ASSOCIATED DIARRHEA					X
PASTEURELLA MASTITIS		X			
PASTEURELLA PNEUMONIA	X	X	X		
PERICARDITIS	X				X

PNEUMOCYSTIS CARINII PNEUMONIA			X	X	X
PROLIFERATIVE ENTERITIS, ILEITIS, OF POST-WEANED SWINE					X
PSEUDORABIES, AUJESKY'S DISEASE	X	X	X	X	X
PULMONARY LISTERIOSIS IN CATTLE	X				
RABIES	X	X	X	X	X
RIFT VALLEY FEVER IN RUMINANTS - EXOTIC	X	X	X		
ROTAVIRAL DIARRHEA IN CALVES AND LAMBS	X	X			
SALMONELLOSIS	X	X	X	X	X
SEPTICEMIA OF LAMBS OR KIDS		X	X		
SEPTICEMIC PASTEURELLOSIS OF SWINE					X
STREPTOCOCCAL SEPTICEMIA AND ARTHRITIS IN YOUNG PIGS					X
STAPHYLOCOCCUS AUREUS MASTITIS IN EWES		X			
STREPTOCOCCUS SUIS INFECTION IN SWINE					X
STRUCK, CLOSTRIDIUM PERFRINGENS TYPE C		X			
SWINE DYSENTERY, SERPULINA HYODYSENTERIAE					X
SWINE EPIDEMIC INFERTILITY, ABORTION AND RESPIRATORY SYNDROME					X
TERMINAL ILEITIS, REGIONAL ENTERITIS, CHRONIC ENTEROCOLITIS IN LAMBS		X			
TETANUS, CLOSTRIDIUM TETANI	X	X	X	X	X
TICKBORNE FEVER - EXOTIC	X	X	X		
TRYPANOSOMIASIS, NAGANA - EXOTIC					X
VISCERAL CASEOUS LYMPADENITIS		X	X		
YELLOW LAMB, CLOSTRIDIUM PERFRINGENS TYPE A		X			
YERSINIA ASSOCIATED DIARRHEA	X	X	X		

Nutritional

ABOMASAL BLOAT IN CALVES AND LAMBS	X	X			
ACUTE FLUORIDE TOXICITY					X
ACUTE ORAL COPPER TOXICITY	X	X	X		
ACUTE SELENIUM TOXICITY	X	X	X	X	X
BLOAT	X	X	X		
COBALT TOXICITY	X	X			
CHRONIC COPPER TOXICITY	X	X	X		X
COPPER DEFICIENCY, MOLYBDENUM TOXICITY	X				

DIETETIC MICROANGIOPATHY, MULBERRY HEART DISEASE					X
EARLY WEANING-ASSOCIATED RUMEN IMPACTION IN LAMBS		X			
ETHANOL TOXICOSIS	X				
FOCAL SYMMETRICAL POLIOMYELOMALACIA, SELENIUM TOXICITY					X
GENERALIZED GLYCOGEN STORAGE DISEASE IN CORRIEDALE LAMBS		X			
GOSSYPOL TOXICITY	X	X	X		X
HYPOCALCEMIA IN THE EWE		X			
HYPOMAGNESEMIA IN RUMINANTS, GRASS TETANY, WHOLE MILK TETANY	X	X	X		
IONOPHORES TOXICITY	X	X	X	X	
IRON TOXICITY	X		X	X	X
MAGNESIUM DEFICIENCY TETANY IN CALVES	X				
MOLDY SWEET CLOVER, DICUMAROL POISONING	X	X	X	X	X
PARENTERAL COPPER SUPPLEMENT TOXICITY	X	X	X		
PHOSPHORUS TOXICITY	X	X	X	X	X
PHYTOGENIC SELENIUM POISONING		X			
POLIOENCEPHALOMALACIA, CEREBROCORTICAL NECROSIS	X	X	X		
POTASSIUM TOXICITY	X			X	
PREGNANCY TOXEMIA, PREPARTURIENT KETOSIS		X	X		
PROTEIN-ENERGY MALNUTRITION, STARVATION	X	X	X	X	X
RUMINAL LACTIC ACIDOSIS, GRAIN OVERLOAD	X	X	X		
SOYBEAN OVERLOAD	X				
SULPHUR, SULFUR, TOXICITY	X	X	X	X	
UREA, NON-PROTEIN NITROGEN TOXICITY	X	X	X		
VITAMIN A DEFICIENCY	X	X	X	X	
VITAMIN E / SELENIUM DEFICIENCY					X
WATER DEPRIVATION, SALT TOXICITY	X	X	X		X
WATER TOXICITY, INTOXICATION, OF CALVES	X				
ZINC TOXICITY	X	X			
WHITE MUSCLE DISEASE, NUTRITIONAL MYODEGENERATION	X	X	X	X	

Parasitism

ABOMASAL PARASITISM, HAEMONCHUS, OSTERTAGIA, TRICHOSTRONGYLUS	X	X	X		

BLUE WILDEBEEST EYE DISEASE, GEDOELSTIA LARVAE - EXOTIC	X	X	X	X	
COCCIDIOSIS	X	X	X		
COENURIASIS FROM TAENIA MULTICEPS	X	X	X		
ELAEOPHORIASIS		X			
EXSANGUINATION BY MOSQUITOES	X				
LICE	X				
LIVER FLUKE DISEASE	X	X	X		
PARAELAPHOSTRONGYLOSIS, ELAPHOSTRONGYLOSIS		X	X		
SURRA, TRYPANOSOMA EVANSI - EXOTIC	X				

Physical

AIR EMBOLISM				X	
ASPIRATION, FOREIGN BODY, PNEUMONIA	X	X	X	X	X
BRISKET, "HIGH-MOUNTAIN" DISEASE (LOCOWEED POISONING?)	X				
CEREBRAL TRAUMA	X	X	X	X	X
COMPLICATIONS OF INTRAVENOUS FLUID OR DRUG THERAPY	X	X	X	X	X
ELECTRICAL INJURY, ELECTROCUTION	X	X	X	X	X
ENCEPHALOMALACIA FOLLOWING DEHORNING	X	X	X		
ESOPHAGEAL OBSTRUCTION, FOREIGN BODY, CHOKE	X	X	X		
EXERCISE INDUCED EQUINE PULMONARY HEMORRHAGE, EPISTAXIS				X	
FRACTURED RIBS OR STERNUM, THORACIC TRAUMA	X	X	X	X	X
GASTRIC, GASTROSPLENIC TORSION					X
GASTRIC, INTESTINAL, RECTAL, ANAL FOREIGN BODIES					X
GUNSHOT INJURY	X	X	X	X	X
HEAT STRESS, HEATSTROKE	X	X	X	X	X
HYPOTHERMIA, COLD STRESS	X	X	X	X	X
INTRACAROTID INJECTION	X	X	X	X	X
LIGHTNING STRIKE, ELECTROCUTION	X	X	X	X	X
PONTO-MEDULLARY, BRAIN STEM TRAUMA OR THROMBOSIS	X	X	X	X	X
POST PARTUM HEMORRHAGE, UTERINE ARTERY RUPTURE IN MARES AND COWS	X			X	
PNEUMOTHORAX	X	X	X	X	X
PREDATION, ATTACKS, BITES	X	X	X	X	X

PRENATAL OR PERINATAL ASPHYXIA	X	X	X	X	X
RENAL AND URETERAL TRAUMA	X	X	X	X	X
RUPTURE OF THE LIVER IN NEONATAL LAMBS		X			
RUPTURE OF THE PREPUBIC TENDON IN COWS AND MARES	X			X	
SKULL, HEAD, FACIAL FRACTURE, TRAUMA	X	X	X	X	X
SUDDEN DEATH IN BUCKET-REARED CALVES AT FEEDING TIME	X				
TORSION OF THE LIVER					X
TORSION OF THE SPLEEN, SPLENIC TORSION					X
TRAUMATIC RETICULOPERITONITIS, HARDWARE	X	X	X		
VERTEBRAL FRACTURE, DISLOCATION, SPINAL CORD TRAUMA	X	X	X	X	X
WATER INHALATION, DROWNING, NEAR DROWNING	X	X	X	X	X

Toxic

ACACIA POISONING - EXOTIC	X	X			
ACONITUM SPP., MONKSHOOD POISONING	X				
ACORN, OAK POISONING	X	X	X	X	
ADONIS MICROCARPA (PHEASANT'S EYE) POISONING					X
AFLATOXIN TOXICITY, AFLATOXICOSIS	X			X	X
ALBIZIA POISONING - EXOTIC	X	X			
ALGAE POISONING	X	X	X	X	X
ALPHA-NAPHTHYL THIOUREA (ANTU) TOXICITY				X	
4-AMINOPYRIDINE TOXICITY	X		X	X	
ANTICOAGULANT TOXICITY	X	X	X	X	X
ANTIMONY TOXICITY					X
ARSENIC TOXICITY	X	X	X	X	X
ATROPA, DATURA, JIMSON WEED, HYOSCYAMUS, HENBANE POISONING	X				
AUTUMN CROCUS, COLCHICUM AUTOMNALE, POISONING	X	X	X		X
AVOCADO (PERSEA AMERICANA) POISONING			X		
BAGPOD, SESBANIA POISONING	X				
BITTERWEED (HYMENOXYS) POISONING	X	X			
BLINDGRASS POISONING - EXOTIC		X	X		
BLISTER BEETLE, CANTHARIDIN TOXICITY				X	
BORAX, BORON TOXICITY	X				

BOX, BUXUS POISONING	X	X	X	X	
BRASSICA SPECIES POISONING	X	X	X		
BROMIDE TOXICITY	X		X	X	
BRONOPOL TOXICITY IN CALVES - EXOTIC	X				
BRYOPHYLLUM TUBIFLORUM POISONING IN CATTLE - EXOTIC	X				
CARBON TETRACHLORIDE TOXICITY		X			
CHOCOLATE, THEOBROMINE, COCOA TOXICITY	X			X	
CESTRUM POISONING - EXOTIC	X	X	X		
CLOSANTEL TOXICITY - EXOTIC		X	X		
COAL TAR TOXICITY					X
COCKLEBUR POISONING	X	X			X
CRASSULACEAE POISONING, KRIMPSIEKTE - EXOTIC		X	X		
CROFTON WEED, EUPATORIUM ADENOPHORUM POISONING				X	
CRUCIFEROUS PLANT SEED POISONING	X				
CUCUMIS MYRIOCARPUS POISONING - EXOTIC	X	X			
CYANOGENIC PLANT POISONING, CYANIDE	X	X	X	X	X
DATISCA GLOMERATA, DURANGO ROOT POISONING	X				
DEADLY NIGHTSHADE POISONING					X
DEATH CAMUS, ZIGANDENUS SP. POISONING	X	X	X	X	
DICHAPETALUM POISONING - EXOTIC	X	X	X		
DIOXIN TOXICITY					X
EUPHORBIA AND SARCOSTEMMA POISONING - EXOTIC	X	X			
FENVALERATE TOXICITY			X		
FERN, NOTHOLAENA POISONING		X			
FIREWEED, SUMMER CYPRESS, KOCHIA SCOPARIA, POISONING	X				
FUSARIUM MONILIFORME, FUMONISIN B1 TOXICITY				X	X
GOAT'S RUE, GALEGA OFFICINALIS, TOXICITY IN SHEEP		X			
HEMLOCK (CONIUM) POISONING	X				
HYDROGEN SULFIDE GAS TOXICITY	X	X	X	X	X
INKWEED, DRYMARIA PACHYPHYLLA POISONING	X	X	X		
IRONWOOD POISONING - EXOTIC	X	X	X	X	
IVERMECTIN, AVERMECTIN TOXICITY IN CALVES	X				
JUTE SEED (CORCHORUS OLITORIUS) - ASSOCIATED POISONING - EXOTIC	X				

LABURNUM ANAGYROIDES, LABURNUM, GOLDEN CHAIN POISONING	X				
LARKSPUR, DELPHINIUM POISONING	X	X	X	X	
LEAD TOXICITY	X	X	X	X	X
LEVAMISOLE TOXICITY		X	X		
LIVER, HEPATIC, DISEASE DUE TO PLANT OR FUNGAL POISONING	X	X	X	X	X
MERCURY TOXICITY	X				
METALDEHYDE TOXICITY	X	X	X		
METHYL ALCOHOL, METHANOL TOXICITY	X				
MILKWEED, ASCLEPIAS, POISONING	X	X	X	X	
MOLDY SWEET POTATO POISONING	X				
MULGA, ROCK FERN, POISONING - EXOTIC	X	X			
MUSTARD SEED, SINAPIS POISONING	X	X			
NARTHECIUM ASIATICUM MAXIM POISONING - EXOTIC	X				
NICOTINE, BLACK LEAF 40 TOXICITY	X	X	X	X	X
NITRATE, NITRITE POISONING	X	X	X		
NITROGEN DIOXIDE TOXICITY					X
OLEANDER POISONING	X	X	X	X	
ORGANOCHLORINE, CHLORINATED HYDROCARBON TOXICITY	X	X	X	X	X
ORGANOPHOSPHATE OR CARBAMATE TOXICITY	X	X	X	X	X
OVINE HEART LEAF POISON BUSH, GASTROLOBIUM GRANDIFLORUM, POISONING - EXOTIC		X			
OXALATE POISONING OR ETHYLENE GLYCOL TOXICITY	X	X	X		
PALO SANTO TREE SEED (BULNESIA SARMIENTII) POISONING - EXOTIC	X				
PETROLEUM HYDROCARBON TOXICITY	X	X	X		
PHALARIS STAGGERS, TRYPTAMINE ALKALOID POISONING	X	X			
PIERIS POISONING			X		
PIGWEED, AMARANTHUS POISONING	X				X
POISON PEACH POISONING - EXOTIC				X	
PORCINE HEMORRHAGIC SYNDROME					X
PRIVET, LIGUSTRUM POISONING	X	X	X	X	
PURPLE MINT, PERILLA FRUTESCENS POISONING	X				
RED MAPLE LEAF, ACER RUBRUM, POISONING				X	
RHODODENDRON POISONING	X	X	X		

RUBBER VINE POISONING IN RUMINANTS - EXOTIC	X	X	X		
RUBIACEAE POISONING, GOUSIEKTE - EXOTIC	X	X	X		
SAWFLY LARVAL TOXICITY - EXOTIC	X	X	X		
SNAKE BITE	X	X	X	X	X
SODIUM FLUOROACETATE, 1080 TOXICITY	X	X	X	X	X
SODIUM MONOCHLOROACETATE TOXICITY	X	X			
SOLANUM POISONING	X				
STRYCHNINE TOXICITY	X	X	X	X	X
STYPANDRA GLAUCA POISONING - EXOTIC		X	X		
SWEET VERNAL GRASS POISONING - EXOTIC	X				
THERMOPSIS MONTANA POISONING CAUSING MYOPATHY	X				
TOBACCO, NICOTINIA POISONING	X	X			X
WATER DROPWORT, OENANTHE CROCATA, POISONING - EXOTIC	X		X	X	X
WATER HEMLOCK, CICUTA POISONING	X	X	X	X	X
WHITEHEADS, SPHENOSCIADIUM CAPITELLATUM POISONING	X				
YELLOW DAISY, WEDELIA ASPERRIMA POISONING - EXOTIC		X			
YEW (TAXUS) POISONING	X	X	X	X	

Other

ACUTE RESPIRATORY DISTRESS, BOVINE PULMONARY EDEMA AND EMPHYSEMA	X				
ACUTE RESPIRATORY DISTRESS SYNDROME IN LAMBS		X			
ALLERGIC SKIN DISEASE	X				
ANAPHYLAXIS OR ACUTE DRUG REACTION	X	X	X	X	X
AORTIC AND PULMONARY TRUNK RUPTURE				X	
AORTIC ANEURYSM	X				
ANHIDROSIS				X	
ANEURYSM OF THE AORTIC ARCH				X	
ATRIAL RUPTURE				X	
BACK MUSCLE NECROSIS					X
BLEEDING ABOMASAL ULCER	X	X	X		
BLOOD TRANSFUSION REACTION	X			X	
CARDIAC FAILURE					X
CARDIAC NEOPLASIA, HEART TUMORS	X			X	

CARDIOMYOPATHY AND WOOLY HAIRCOAT OF POLL AND HORNED HEREFORD CATTLE	X				
CARDIOMYOPATHY IN JAPANESE BLACK CALVES - EXOTIC	X				
CARDIOVASCULAR SUDDEN DEATH				X	
CECAL RUPTURE IN HORSES, PERFORATION OF THE CECUM				X	
CHOKING, ANOXIA, POSTANESTHETIC HYPOXIA	X	X	X	X	X
COMPLICATIONS OF ENUCLEATION OF THE CORPUS LUTEUM	X				
CONGENITAL HYPOMYELINOGENESIS OF HEREFORD CALVES	X				
CONGENITAL RENAL AGENESIS					X
DIAPHRAGMATIC HERNIA, DIVERTICULUM, RUPTURE				X	X
ENDOCARDIAL FIBROELASTOSIS				X	
EXERTIONAL RHABDOMYOLYSIS		X			
FACTOR VIII DEFICIENCY, HEMOPHILIA A	X			X	
FATTY LIVER ASSOCIATED SUDDEN DEATHS IN WETHERS		X			
GASTRIC ULCERS					X
GASTRIC, STOMACH RUPTURE				X	
GASTRODUODENAL, GASTRIC, DUODENAL, ULCERS				X	
GRASS SICKNESS, EQUINE DYSAUTONOMIA - EXOTIC				X	
GUTTURAL POUCH NEOPLASIA				X	
IDIOPATHIC BLOAT IN CALVES, RUMINAL DRINKING	X				
IDIOPATHIC PULMONARY HYPERTENSION IN CALVES	X				
IDIOPATHIC SUDDEN DEATH				X	
ILEAL DIVERTICULUM				X	
INHERITED, CONGENITAL, CYSTIC RENAL DYSPLASIA OF LAMBS		X			
INTESTINAL HEMORRHAGE SYNDROME	X	X			
INTESTINAL TORSION, VOLVULUS	X	X	X	X	X
ISOIMMUNE HEMOLYTIC ANEMIA	X				X
LYMPHOSARCOMA, LYMPHOMA, LEUKEMIA, IN SWINE					X
MARFAN SYNDROME IN LIMOUSIN CATTLE	X				
MAXILLARY ARTERY BLEEDING INTO THE GUTTURAL POUCH				X	
MESANGIOCAPILLARY GLOMERULONEPHRITIS IN LAMBS		X			
MULTIPLE HEART ANOMALIES IN FOALS				X	
NAVEL, UNBILICAL, BLEEDING IN PIGLETS					X

NEONATAL ISOERYTHROLYSIS				X	
NEONATAL ISOIMMUNE THROMBOCYTOPENIA IN PIGLETS					X
PERFORATED ABOMASAL ULCER	X	X	X		
PORCINE STRESS SYNDROME, PSS					X
PROCAINE PENICILLIN REACTION	X	X	X	X	
PULMONARY LOBAR HYPERTROPHY IN FOALS				X	
RENAL HYPOPLASIA					X
RUPTURE OF THE MITRAL CHORDAE TENDINAE				X	
SPLENIC RUPTURE OR HEMATOMAS				X	
SPORADIC CARDIAC ANOMALIES	X				X
THROMBOSIS OF THE CAUDAL VENA CAVA	X				
THYMOMA	X	X	X	X	X
VENTRICULAR, INTERVENTRICULAR SEPTAL DEFECT IN LARGE ANIMALS	X	X	X	X	X
VULVAR, VESTIBULAR, VAGINAL LACERATIONS, TRAUMA, RUPTURE, NECROSIS	X	X	X		X
VOLVULUS, TORSION, OF THE SMALL INTESTINE				X	
VOLVULUS, TORSION, ROOT OF THE MESENTERY	X	X	X	X	X

[a]Compiled from CONSULTANT (computer-assisted differential diagnosis program), Veterinary Computing Services, New York State College of Veterinary Medicine, Cornell University. Inclusion of this table should not convey that the author is in total agreement with its content.

APPENDIX B: MANUFACTURERS AND SOURCES

Air Products and Chemicals Incorporated
Box 538
Allentown, Pennsylvania

Research-grade carbon dioxide (99.9995%)
Ultra-pure carrier grade nitrogen gas (99.999%)

American Natioanl Can
Greenwich, Connecticut 06836

Parafilm M® laboratory wrap

Applied Science Laboratory
Post Office Box 440
State College, Pennsylvania 16081

Silicon rubber sheets for disks

Atwood Corportion
Lowell, Massachusetts

Mini-king 500 bilge pump

Becton Dickenson & Company
Rutherford, New Jersy 07070

10 & 20 ml syringes
Tuberculin syringe with 3/8" 26 G needle
B-D Vacutainer® tubes

Blue M Electric Company
Blue Island, Illinois

Water bath (MW-1116C-1)

Braintree Scientific
P.O. Box 361
Braintree, Massachusetts 02184

DecapiCones™restrainer (DC-200)
Rodent guillotine (RG-100)

Cadillac Plastics
North Bradley Industrial park
Davenport, Iowa 52802

Teflon® sheets for disks
Teflon® tubing (0.5 mm I.D.)

Corning Scientific Products
Corning Glass Works
Corning, New York 14831

Automatic temperature compensation probe (476277)
Ion analyzer® (255)
Magnetic stirrer (PC-320)
Pyrex® laboratory glassware:
- 250 ml beaker
- Gas dispersion tube
- 5 ml volumetric pipet
- 10 ml volumetric pipet
- 100 ml volumetric pipet
- 200 ml volumetric flask
- 500 ml volumetric flask
- 1000 ml volumetric flask

Dow Chemical
Midland, Michigan

Styrofoam® R value 3.8

E.I. duPont de Nemours & Company
Wilmington, Delaware

Teflon®

Elkins - Sinn, Incorporated
Cherry Hill, New Jersey 08034

Heparin (1000 USP units sodium heparin/ml)

Epson America
2780 Lomita Blvd
Torrance, California 90505

Computer printer (LX-800)

Fisher Scientific
711 Forbes Avenue
Pittsburg, Pennsylvania 15219

Laboratory timer
Latex laboratory tubing
Pasteur pipets
pH electrode
Reagents:
- certified 1000 ppm lead reference solution
- certified-grade L-ascorbic acid
- certified-grade disodium ethylenediamine tetraacetate
- certified-grade sodium chloride
- certified-grade sodium hydroxide
- certified-grade zinc acetate
- reagent-grade ammonium peroxydisulfate
- reagent-grade hydrochloric acid
- reagent-grade dibasic sodium phosphate
- reagent-grade monobasic sodium phosphate
- reagent-grade sodium sulfide
- reagent-grade sulfuric acid

Rubber stoppers
Teflon® magnetic stir bars
Tubing clamps

Industrial Polychemical Service
P.O. Box 471
Gardena, California 90247

Weld-On 16® acrylic cement

Industrial Scientific Corporation
355 Steubenville Pike
Oakdale, Pennsylvania 15071-1093

Hydrogen sulfide monitor (HS267)

ISU Glassblowing Shop
Iowa State University
Gilman Hall
Ames, Iowa 50011

Custom wash-bottle chamber

Jandel Scientific
65 Koch Road
Corte Madera, California 94925

SigmaPlot™(version 4.1)

Kewannee Scientific Equipment Corporation
Adrian, Michigan 49221

Fume hood

Lazar Research Laboratory
920 North Formosa Avenue
Los Angeles, California 90046

Micro double-junction reference electrode (DJM-146)
Micro silver/sulfide ion-selective electrode (ISM-146S)
Micro cyanide ion-selective electrode (ISM-146CN)

Lotus Developement Corporation
55 Cambridge Parkway
Cambridge, Massachusetts

Lotus 1 • 2 • 3® spreadsheet (version 2.2)

Mallinckrodt Incorporated
675 McDonnell Boulevard
St. Louis, Missouri 63134

Triton X-100 Scintillar® (3555)

Matheson Gas Products
30 Seaview Drive
Seacaucus, New Jersy 07096

Rotameter® (FM-10528-HA)
Flow tube (610A)

Microsoft Corporation
One Microsoft Way
Redmond, Washington 98052

MS-DOS® versions 3.3 and 5.0
GWBASIC® version 3.3

Millipore
Bedford, Massachusetts 01730

Milli-Q water purefication system

Nalge Company
A subsidiary of Sybron Corporation
Post Office 20365
Rochester, New York 14602

30 ^{cc} Nalgene® beakers
20 l Nalgene® tank with lid
T-shaped tubing connections

Nasco
901 Janesville Avenue
Fort Atkinson, Wisconsin 53538-0901

Sprague-Dawley rats
Whirl-pak sterile bags (6 oz)

North Safety Equipment
2000 Plainfield Pike
Cranston, Rhode Island 02920

Emergency escape breathing appartus (850)

Norton-Performance Plastics
Post Office Box 3660
Akron, Ohio 44309

Tygon® tubing

Orion Research Incorporated
The Schrafft Center
529 Main Street
Boston, Massachusetts 02129

Double-junction reference electrode (900200):
Saturated silver chloride inner solution (900002)
10 % potassium nitrate outer solution (900003)
Silver/sulfide ion-selective electrode (941600)
Microsample dishes (920014)

Pierce Chemical Company
Post Office Box 117
Rockford, Illinois 61105

Hand crimper (No. 13212)

Rainin Instrument Company
Mack Road
Woburn, Massachusetts

Disposible microliter pipette tips (RC200)
Gilman Pipetman P-200 micro pipet
Gilman Pipetman P-1000 micro pipet

Rohm & Hass
Philadelphia, Pennsylvania

Plexiglas®

SAS Institute Incorporated
SAS Circle, Box 8000
Cary, North Carolina 27512-8000

SAS System (version 6.06)

Sensidyne Incorparated
12345 Starkey Road, Suite E
Largo, Florida 33543

Gastec gas sampling pump (800)
Gastec hydrogen sulfide detector tubes (4H)

SmithKline Beecham Animal Health
Whiteland Business Park
812 Springdale Drive
Exton, Pennsylvania 19341

Polaxalene (Therabloat®)

Swan (Cumberland-Swan) Incorporated
Smyrna, Tennessee 37167

Swan X-heavy mineral oil U.S.P.

Tenna
Korea

Regulated power supply (Pyramid Phase III, PS-3)

Tekmar Company
Post Office Box 37202
Cincinnati, Ohio 45222

Tissumizer® (SDT-1810)
Probe (SDT-182EN)
Power unit (TR-10)

TeleVideo Systems
1170 Morse Avenue
Sunnyvale, California 94086

Tele-PC computer

Thomas Scientific
99 High Hill Road
Swedesboro, New Jersey 08085

Thomas antifoam spray (1130D15)
(10 % silicone emulsion in water)

Transcoject
2350 Neumünster
West Germany

10 ml polypropylene syringes

Wheaton Scientific
1501 North 10th Street
Millville, New Jersy 08332

Aluminum seals
Borosilicate glass serum bottles
5, 10, 15, 100 ml

Victor Equipment Company
San Franisco, California

Two-stage pressure regulator (VTS-250C)
Needle valve (1030)

Zeos International Ltd.
530 5th Avenue, N.W.
St. Paul, Minnesota 55112

80386 / 33 MHz computer

APPENDIX C: DATA ACQUISITION SOFTWARE FOR THE CORNING 255 ION ANALYZER[a]

```
10    CLS : WIDTH 40
20    ON ERROR GOTO 3000
30    ON KEY(10) GOSUB 1030 : KEY(10) ON
60    LOCATE 5,5 : PRINT "    Data Acquistion Software"
70    LOCATE 7,5 : PRINT " For The Corning 255 Ion Analyzer"
80    LOCATE 12,3: PRINT "Press The Read Button On The Analyzer"
90    LOCATE 13,1: PRINT "Only After Completing The Input Requests"
100   LOCATE 18,2: PRINT "S.T.Witte  5/27/89  Iowa Vet. Diagn. Lab"
120   LOCATE 22,5 : PRINT "Press F10 To Exit This Program"
130   GOSUB 2000
200   LINE INPUT "  File: "; F$
210   LINE INPUT "  Date: "; D$
220   LINE INPUT "   ISE: "; I$
230   LINE INPUT " Sample: "; S$
240   LINE INPUT "  Units: "; U$
250   OPEN F$ FOR OUTPUT AS #2
260   OPEN "LPT1:" FOR OUTPUT AS #3
320   PRINT F$ : PRINT D$ : PRINT I$ : PRINT S$ : PRINT U$
325   PRINT " "
330   PRINT#2,F$ : PRINT#2,D$ : PRINT#2,I$ : PRINT#2,S$ : PRINT#2,U$
335   PRINT#2," "
336   PRINT#3,F$ : PRINT#3,D$ : PRINT#3,I$ : PRINT#3,S$ : PRINT#3,U$
338   PRINT#3," "
340   PRINT " No.  Time   Temp  Reading"
366   PRINT "                    "
368   PRINT#2," No.  Time   Temp  Reading"
375   PRINT#2,"                  "
376   PRINT#3," No.  Time   Temp  Reading"
379   PRINT#3," "
380   Y% = 100
400   TIME$ = "00:00:00"
410   OPEN "COM1:9600,E,7,2,RS,CS,DS,CD"  AS #1
420   WHILE LOC(1) <25 : WEND
430   A$ = INPUT$(LOC(1),#1)
440   READING$=MID$(A$,2,7)
450   TEMP$=MID$(A$,10,5)
500   PRINT#2,Y%;TIME$;TEMP$;READING$
510   PRINT Y%;TIME$;TEMP$;READING$
515   PRINT#3,Y%;TIME$;TEMP$;READING$
600   Y%=Y%+1
700   IF TIME$ > "00:05:30" , THEN GOTO 1000 , ELSE GOTO 420
1000  X$=STRING$(9,42) : PRINT X$ "Measurement Completed" X$
1010  BEEP : BEEP
1030  END
2000  LOCATE 24,5 : PRINT "Press The Space Bar To Continue"
2010  WHILE INKEY$="" : WEND : CLS : RETURN 200
3000  CONT
```

---

[a]Written for an IBM-compatible personal computer running GW-BASIC (Version 3.3) under DOS.

APPENDIX D: CODE AND FORMULAS, BY CELL ADDRESSES, FOR THE LOTUS 1-2-3 (VERSION 2.2) SPREADSHEET MONITORING PERFORMANCE OF THE ION-SELECTIVE ELECTRODES[a]

Data Import and Parse

A7: U |*L>>***L>>>********L>>>>>>
A8: ' No. Time Reading
A10: U |*V>>>*T>>>>>>>>*****V>>>>>
D10: U "No.
E10: (D8) U ^Time
F10: U "Reading

Calculation Formulas

I2: ^1 MIN
J2: ^2 MIN
K2: ^3 MIN
L2: ^4 MIN
M2: ^5 MIN
H3: "Mean:
I3: @DAVG($STATS,2,I$12..I$13)
J3: @DAVG($STATS,2,J$12..J$13)
K3: @DAVG($STATS,2,K$12..K$13)
L3: @DAVG($STATS,2,L$12..L$13)
M3: @DAVG($STATS,2,M$12..M$13)
H4: "Std:
I4:
@SQRT(@DCOUNT($STATS,2,I$12..I$13)/(@DCOUNT($STATS,2,I$12..I$13)-1))*@DSTD($STATS,2,I$12..I$13)
J4:
@SQRT(@DCOUNT($STATS,2,J$12..J$13)/(@DCOUNT($STATS,2,J$12..J$13)-1))*@DSTD($STATS,2,J$12..J$13)
K4:
@SQRT(@DCOUNT($STATS,2,K$12..K$13)/(@DCOUNT($STATS,2,K$12..K$13)-1))*@DSTD($STATS,2,K$12..K$13)
L4:
@SQRT(@DCOUNT($STATS,2,L$12..L$13)/(@DCOUNT($STATS,2,L$12..L$13)-1))*@DSTD($STATS,2,L$12..L$13)
M4:
@SQRT(@DCOUNT($STATS,2,M$12..M$13)/(@DCOUNT($STATS,2,M$12..M$13)-1))*@DSTD($STATS,2,M$12..M$13)
H5: "Var:
I5:
@DCOUNT($STATS,2,I$12..I$13)/(@DCOUNT($STATS,2,I$12..I$13)-1)*@DVAR($STATS,2,I$12..I$13)
J5:
@DCOUNT($STATS,2,J$12..J$13)/(@DCOUNT($STATS,2,J$12..J$13)-1)*@DVAR($STATS,2,J$12..J$13)
K5:
@DCOUNT($STATS,2,K$12..K$13)/(@DCOUNT($STATS,2,K$12..K$13)-1)*@DVAR($STATS,2,K$12..K$13)
L5:
@DCOUNT($STATS,2,L$12..L$13)/(@DCOUNT($STATS,2,L$12..L$13)-1)*@DVAR($STATS,2,L$12..L$13)
M5:
@DCOUNT($STATS,2,M$12..M$13)/(@DCOUNT($STATS,2,M$12..M$13)-1)*@DVAR($STATS,2,M$12..M$13)
H6: "Max:
I6: @DMAX($STATS,2,I$12..I$13)
J6: @DMAX($STATS,2,J$12..J$13)
K6: @DMAX($STATS,2,K$12..K$13)
L6: @DMAX($STATS,2,L$12..L$13)
M6: @DMAX($STATS,2,M$12..M$13)
H7: "Min:
I7: @DMIN($STATS,2,I$12..I$13)
J7: @DMIN($STATS,2,J$12..J$13)
K7: @DMIN($STATS,2,K$12..K$13)
L7: @DMIN($STATS,2,L$12..L$13)
M7: @DMIN($STATS,2,M$12..M$13)
H8: "Count:
I8: @DCOUNT($STATS,2,I$12..I$13)
J8: @DCOUNT($STATS,2,J$12..J$13)
K8: @DCOUNT($STATS,2,K$12..K$13)
L8: @DCOUNT($STATS,2,L$12..L$13)
M8: @DCOUNT($STATS,2,M$12..M$13)
H11: ^CRITERION:
I12: ^Time
J12: ^Time
K12: ^Time
L12: ^Time
M12: ^Time
I13:
($E11>=@TIME(0,0,30))#AND#($E11<@TIME(0,1,30))
J13:
($E11>=@TIME(0,1,30))#AND#($E11<@TIME(0,2,30))
K13:
($E11>=@TIME(0,2,30))#AND#($E11<@TIME(0,3,30))
L13:
($E11>=@TIME(0,3,30))#AND#($E11<@TIME(0,4,30))
M13:
($E11>=@TIME(0,4,30))#AND#($E11<@TIME(0,5,30))

Report Generation

P8: "File No.:
P9: "Date:
P10: "ISE:
P11: "Sample:
P12: "Units:
Q15: ^1 MIN
R15: ^2 MIN
S15: ^3 MIN
T15: ^4 MIN
U15: ^5 MIN
P16: "Mean:

```
Q16: +I3
R16: +J3
S16: +K3
T16: +L3
U16: +M3
P17: "Std:
Q17: +I4
R17: +J4
S17: +K4
T17: +L4
U17: +M4
P18: "Var:
Q18: +I5
R18: +J5
S18: +K5
T18: +L5
U18: +M5
P19: "Max:
Q19: +I6
R19: +J6
S19: +K6
T19: +L6
U19: +M6
P20: "Min:
Q20: +I7
R20: +J7
S20: +K7
T20: +L7
U20: +M7
P21: "Count:
Q21: +I8
R21: +J8
S21: +K8
T21: +L8
U21: +M8
```

Macro Commands

```
Y1: [W30] 'Macro Functions For The SpreadSheet
X3: ^Macro Name
Y3: [W30] ^Macro Content
Z3: [W30] ^Description of Macro

X5: 'Alt-X
Z5: [W30] '***Repositions Data, Calc. & Stores***
X6: '\x
Y6: [W30] '/ma11.c15~a1~
Z6: [W30] 'Moves file no., date, ISE, sample & units
information.
Y7: [W30] '/ca1.a5~q8~
Z7: [W30] ^Copies file information into summary.
Y8: [W30] '{GOTO}a17~
Z8: [W30] 'Relocates cursor to address A17.
Y9: [W30] '/rea17.c19~
Z9: [W30] 'Range-erases the extra title & blank spaces.
Y10: [W30] '/rea8.c8~
Z10: [W30] 'Range-erases extra title.
Y11: [W30] '{GOTO}a21~
Z11: [W30] 'Relocates cursor to address A21.
Y12: [W30] '/ma21.c8100~a11~
Z12: [W30] 'Moves the raw data up.
Y13: [W30] '{GOTO}a1~
Z13: [W30] 'Position cursor at A1.
Y14: [W30] '/dpg
Z14: [W30] '/Data/Parse/Go. Input & Output range
preset.
Y15: [W30] '{CALC}
Z15: [W30] 'Calculates the worksheet.
Y16: [W30] '{GOTO}p1~
Z16: [W30] 'Relocate cursor to P1.
Y17: [W30] '/ppagq
Z17: [W30] '/Print/Printer/Align/Go/Quit.
Y18: [W30] '{BEEP 2}
Z18: [W30] 'Beep

X21: 'Alt-D
Z21: [W30] '***Retreives Raw Data - Hard Drive***
X22: '\d
Y22: [W30] '/fit
Z22: [W30] '/File/Import/Text.
Y23: [W30] '{ESCAPE}
Z23: [W30] 'Clears data transfer path.
Y24: [W30] 'C:
Z24: [W30] 'Types part of path.
Y25: [W30] '{BEEP 2}{?}~
Z25: [W30] 'Pauses for file no.

X29: 'Alt-A
Z29: [W30] '***Retreives Raw Data - Drive A***
X30: '\a
Y30: [W30] '/fit
Z30: [W30] '/File/Import/Text.
Y31: [W30] '{ESCAPE}
Z31: [W30] 'Clears data transfer path.
Y32: [W30] 'A:
Z32: [W30] 'Types part of path.
Y33: [W30] '{BEEP 2}{?}~
Z33: [W30] 'Pauses for file no.

X37: 'Alt-C
Z37: [W30] '*** Reconditions Worksheet ***
X38: '\c
Y38: [W30] '/rea1.b6~
Z38: [W30] 'Erases old file information.
Y39: [W30] '/rea11.b8100~
Z39: [W30] 'Clears the previous data set.
Y40: [W30] '/red11.f8100~
Z40: [W30] 'Removes last parsed data.
Y41: [W30] '/req8.q12~
Z41: [W30] 'Erases old file summary information.
Y42: [W30] '/cz42.z42~a8~
Z42: [W30] ' No.  Time      Reading
Y43: [W30] '{HOME}{GOTO}a11~
Z43: [W30] 'Repositions cursor.

X47: 'Alt-P
Z47: [W30] "**** Prints the Worksheet Results ****
X48: '\p
Y48: [W30] '/ppagq
Z48: [W30] '/Print/Printer/Align/Go/Quit.
```

Defined Ranges in Spreadsheet

```
AD3: '****** Table of Ranges ******
AD5: 'NO.
AE5: 'D1
AD6: 'READING
AE6: 'F1
AD7: 'STATS
AE7: 'D10..F300
AD8: 'TIME
AE8: 'E1
AD9: '\A
AE9: 'Y30
AD10: '\C
AE10: 'Y38
AD11: '\D
AE11: 'Y22
AD12: '\P
AE12: 'Y48
AD13: '\X
AE13: 'Y6
```

---

[a]This program calculates descriptive statistics from the data collected by the data acquistion software (Appendix C).

APPENDIX E: DERIVATION OF THE CALIBRATION EQUATION

This equation is used to determined the sulfide concentration of a standard solution after potentiometric titration of a 10 ml sample with 1000 ppm Pb reference standard.[a]

Moles of lead (Pb) added:

$$(X \text{ ml}) \cdot (1000 \text{ mg Pb} / 1000 \text{ ml}) = X \text{ mg Pb}$$
$$(X \text{ mg Pb}) \cdot (0.001 \text{ g/mg}) = 0.001 \cdot X \text{ g Pb}$$
$$(0.001 \cdot X \text{ g Pb} / 207.2 \text{ MW Pb}) = \text{Moles Pb}$$

Moles Pb = Moles sulfide, therefore:

$$wt_g / MW = \text{Moles}$$
$$wt_g = (\text{Moles}) \cdot (MW)$$
$$= [0.001 \cdot X \text{ g Pb} / 207.2 \text{ MW Pb}] / (32.06 \text{ MW S})$$

$$\text{mg } S^{-2} = [(0.001 \cdot X \text{ g Pb} / 207.2 \text{ MW Pb}) \cdot (32.06 \text{ MW S})] \cdot [1000 \text{ mg/g}]$$
$$\text{mg } S^{-2} = (0.1547297) \cdot (X \text{ mg})$$

$$\text{ppm } S^{-2} = (0.1547297 \cdot X \text{ mg}) / (0.010 \text{ l})$$
$$= (15.472973) \cdot (X)$$

Summary:

$$\textit{ppm } S^{-2} = (15.472973) \cdot (X),$$

where X is the volume of 1000 ppm Pb (titrant) in millilters to reach the end-point of the potentiometric titration of a 10 ml sample.

---

[a]Based on Orion Research.[184]

APPENDIX F: CONCENTRATIONS OF ACID-LIABLE SULFIDE RECOVERED FROM RATS INJECTED WITH LETHAL LEVELS OF SULFIDE.[a]

Tissue	Sulfide Dose		
	0 x LD_{50}	2 x LD_{50}	4 x LD_{50}
Brain	0.69 ± 0.14 ppm	0.98 ± 0.170 ppm[b]	1.19 ± 0.101 ppm*
Blood	0.0008 ± 0.00067[c]	0.12 ± 0.086	0.14 ± 0.048
Lung	0.99 ± 0.032	0.69 ± 0.310	1.7 ± 0.46

[a]LD_{50} equals 14.6 mg NaHS/kg BW IP (5 mg S^{-2}/kg) for male 250 to 350 g BW Sprague-Dawley rats.[131,258]

[b]Means ± SE, n = 6.

[c]Estimated by assuming linearity of the standard curve beyond 0.02 ppm S^{-2}.

*Significantly different from other treatments of the same tissue as determined by the Student's t-test ($P < 0.05$).

APPENDIX G: TISSUE ACID-LABILE SULFIDE FROM RATS EXPOSED TO ROOM AIR OR ACUTELY TOXIC HYDROGEN SULFIDE CONCENTRATIONS

Tissue	Exposure Treatment		Least Significant Difference ($P < 0.05$)
	Room Air (n = 7)	Hydrogen Sulfide[a] (n = 8)	
Brain	0.71 ± 0.129 ppm	1.61 ± 0.198 ppm	0.527
Blood	0.00057 ± 0.000172[b]	0.12 ± 0.105[b]	0.244
Lung	0.37 ± 0.115	0.85 ± 0.218	0.556

[a]Mean peak exposure concentration of 2090 ± 260 ppm H_2S.
[b]Estimated from the standard curve by assuming linearity beyond 0.02 ppm S^{-2}.