Technical Service Report on the Mutagenicity Analyses of Cansolv Nitrosamine Samples

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SaskPower
Boundary Dam BD3 Carbon Capture System

Submitted to

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April 11, 2013

Executive Summary: Non-Proprietary

The generation of electricity and steam from fossil fuel-fired power plants accounted for 41% of global carbon dioxide (CO_2) emissions. Postcombustion carbon sequestration and storage will play a critical role in reducing global greenhouse gas emissions over the next several decades. Preeminent among CO_2 capture technologies are amine-based technologies. With this technology flue gases are passed through an "absorber" column with a countercurrent amine-based solvent. Following solvent partitioning, the CO_2 forms a carbamate complex with the amine. The amine- and carbamate-containing solution proceeds to a second "desorber" column, where high temperatures reverse this reaction, regenerating the amine sorbent and releasing the CO_2, which is compressed and routed to underground storage. There is significant public health and environmental concerns regarding the potential for nitrosamine contamination of air and drinking water supplies downwind of amine-based CO_2 capture plants. As a chemical class, nitrosamines are potent mutagens and some are strong carcinogens. The primary objective of this project was to conduct quantitative analyses of the mutagenicity for selected nitrosamine agents using a *Salmonella typhimurium* (strain YG7108) suspension assay. The nitrosamines analyzed were, N-Nitrosodimethylamine, (NDMA), Cansolv Nitrosamine-1 (Cansolv-N1), Cansolv Nitrosamine-2 (Cansolv-N2) and Cansolv Nitrosamine-1 (Cansolv-N3),. The results of this project are the following. NDMA was an exceedingly potent mutagen after mammalian microsomal metabolism with a mutagenic potency of 47,695 revertants per µmol. Cansolv-N3 was a clear mutagen and the agent required mammalian microsomal metabolic activation with a mutagenic potency of 915 revertants per µmol. Cansolv N-1 was an exceedingly weak mutagenic agent that required S9 metabolic activation with a mutagenic potency of 23 revertants per µmol. Cansolv N-2 was a direct, albeit very weak mutagen (54 revertants per µmol); S9 metabolic activation detoxified the nitrosamine and reduced its mutagenic activity. Cansolv N-1 was 4.8×10^{-4} times as mutagenic as NDMA; Cansolv N-2 was 1.1×10^{-3} times as mutagenic as NDMA. When compared to environmental nitrosamines such as nitrosamine drinking water disinfection by-products, both Cansolv N-1 and Cansolv N-2 were considered very weak mutagens.

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1. Introduction and Background

Recent data demonstrate that the generation of electricity and steam from fossil fuel-fired power plants accounted for 41% of global carbon dioxide (CO_2) emissions [1]. In the United States approximately 75% of power generation capacity is produced by fossil fuels. Postcombustion carbon sequestration and storage will play a critical role in reducing global greenhouse gas emissions over the next several decades. Preeminent among CO_2 capture technologies are amine-based technologies [2]. With this technology flue gases are passed through an "absorber" column with a countercurrent amine-based solvent. Following solvent partitioning, the CO_2 forms a carbamate complex with the amine. The amine- and carbamate-containing solution proceeds to a second "desorber" column, where high temperatures reverse this reaction, regenerating the amine sorbent and releasing the CO_2, which is compressed and routed to underground storage. There is significant public health and environmental concerns regarding the potential for nitrosamine and nitramine contamination of air and drinking water supplies downwind of amine-based CO_2 capture plants [2]. Environmental contamination could arise when nitrosamines and nitramines formed within the amine based solvent are stripped into the washwater and thence transferred back into the exhaust gas. Also nitrosamines and nitramines could form downwind if amines released in the exhaust gas react with ambient NOx [3]. As a chemical class, nitrosamines are potent mutagens and some are strong carcinogens [4-6]. Recently, we characterized the formation conditions for the generation of nitrosamines and nitramines associated with amine based carbon capture technologies [7]. Of interest is the finding that N-nitroso derivatives of piperazine form at elevated pH in carbon capture solutions that contain piperazine [8].

2. Scope of Work

2.1 *In vitro* Mutagenicity Analyses of Nitrosamine Compounds

Our primary objective was to conduct a quantitative analyses of the mutagenic potential for selected nitrosamine agents using a *Salmonella typhimurium* suspension assay according to the procedures published by Wagner et al., 2012 [9]. The agents that were analyzed in this project are listed in Table 1.

Table 1. Nitrosamine chemicals analyzed in this project.

Chemical Name	Abbreviation	CASN	Purity	Source
N-Nitrosodimethylamine	NDMA	62-75-9	99.9%	Sigma-Aldrich, USA
1-Nitrosopiperazine	NPZ (Cansolv-N3)	5632-47-3	94.3%	Harlan Laboratories, UK
1-Nitroso-(4-hydroxyethyl)-piperazine	Nitroso-HEP (Cansolv-N1)	48121-20-6	97.9%	Harlan Laboratories, UK

Casolv-N2	- Cansolv-N2	N/A	97.3%	MATRIC, USA

Cansolv-N1 (Nitroso-HEP) is generated in Cansolv's absorbent No.1 under regular operation and was supplied by Cansolv Technologies. The molecular weight for Nitroso-HEP as 159 g mol^{-1} was provided by Tan-Trung Nguyen, Project Manager, Cansolv Technologies Inc. Cansolv-N2 is generated in Cansolv's absorbent No.2 under research development and was supplied by Cansolv Technologies.

2.2 Study Plan

2.2.1 *Salmonella typhimurium* Cells

We employed *S. typhimurium* tester strain YG7108 (*hisG46*, *rfa*; △(*chl*, *uvrB, bio*) △ada_{ST}; ogt_{ST}; Cm^r; Amp^r) with and without mammalian microsomal activation (S9 mix). Reverse mutation is measured at *hisG46* (Figure 1) [10, 11]. *S. typhimurium* strain YG7108 is highly sensitive to nitrosamines and other alkylating agents and was provided by Dr. T. Nohmi, National Institute of Health Sciences, Tokyo, Japan [12].

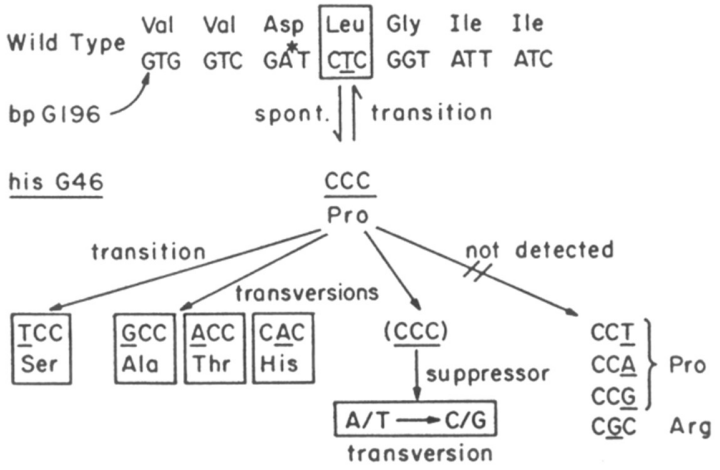

Figure 1. Reverse mutation at *hisG46* in *S. typhimurium* that leads to the growth of a visible colony on selective VB medium. The mutant colonies are referred to as revertants.

In our laboratory strain YG7108 was stored as a frozen culture in Luria-Bonner (LB) plus 10% dimethylsulfoxide (DMSO) at −80°C. To construct a master plate, frozen log-phase *S. typhimurium* YG7108 cells were thawed and grown in 5 mL LB with kanamycin (25 µg/mL) and

chloramphenicol (10 µg/mL) at 37°C overnight with shaking. The culture was streaked across an LB plate and incubated overnight at 37°C. The master plate was stored at 4°C [10]. We followed the published method for the mutagenicity and cytotoxicity (survivorship) experiments for the test agents [9]. The day before the experiment a single colony isolate of *S. typhimurium* YG7108 from the master plate was grown overnight in 100 mL LB medium with kanamycin (25 µg/mL) and chloramphenicol (10 µg/mL) at 37°C with shaking (200 rpm) [12]. The following day the culture was centrifuged for 5 min at 5000 rpm (4096 ×g) using the GSA rotor in a RC5B Superspeed Centrifuge (Sorvall). The supernatant was discarded, and the bacterial pellet suspended in 50 mL of 100 mM potassium-phosphate buffer, pH 7.4 (PPB). The suspension was centrifuged again for 5 min at 5000 rpm, and the supernatant fluid discarded. The pellet was suspended in 2.5-3 mL PPB. An aliquot (50 µL) of the bacterial suspension was added to 4.95 mL PPB and the optical density determined spectrophotometrically at 660 nm. The titer of the suspension was adjusted to 6×10^9 cells/mL; the cells were kept on ice until used in the microsuspension mutagenicity assay [9, 13, 14].

Most nitrosamines require metabolic activation to exhibit mutagenicity. Metabolic activation of nitrosamines is primarily dependent on cytochrome P-450 which converts nitrosamines into DNA alkylating agents [15]. Oxidative transformation is an important route of metabolism of xenobiotics. These oxidative reactions are largely catalyzed by the monooxygenase system based on cytochrome P-450. The monooxygenase system is formed by the enzyme system of cytochrome P-450 and NADPH cytochrome P-450 reductase. The haemoprotein cytochrome P-450 functions as the terminal oxidase involved in the hydroxylation of xenobiotics such as nitrosamines. A description of the cytochrome P-450-mediated monooxygenation of dimethylnitrosamine (NDMA) is presented in Figure 2 [15].

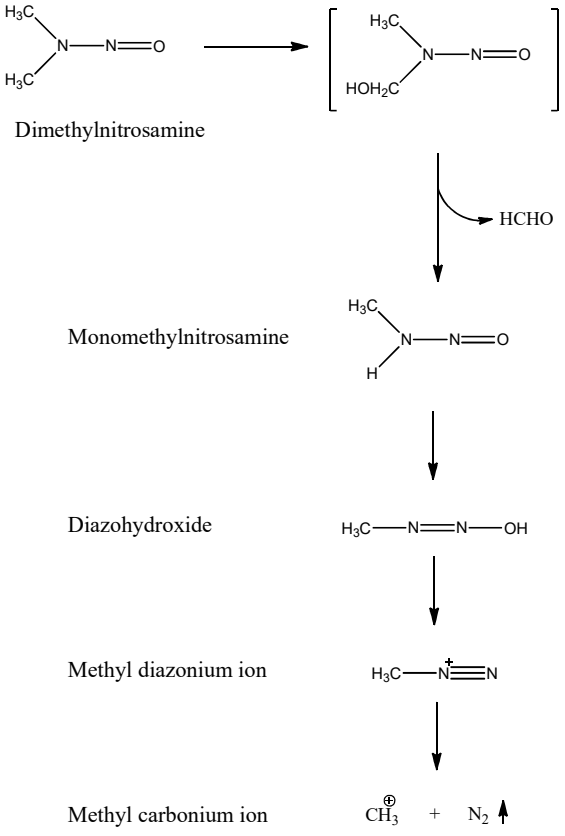

Figure 2. Metabolic activation of dimethylnitrosamine via cytochrome P-450 leading to the formation of a reactive methyl carbonium ion. This radical can alkylate DNA and induce mutation.

2.2.2 *S. typhimurium* Microsuspension Assay

The *S. typhimurium* microsuspension assay was conducted in sterile round bottom 96-well microplates. Each reaction mixture (well) was composed of a known test chemical concentration, 3×10^8 cells, and PPB in a total volume of 100 µL. When including a mammalian microsomal-mediated activation system, 35 µL of an S9 mix was added for a total volume of 100 µL. S9 (post

mitochondrial hepatic supernatant) from Aroclor 1254-induced male rats was purchased from Molecular Toxicology, Inc. (Moltox). The S9 mix consisted of 50 mM PPB pH 7.4, 10 mM $MgCl_2$, 5 mM glucose-6-phosphate, 30 mM KCl, and 4 mM NADP plus 200 µL/mL of Aroclor 1254-induced rat hepatic microsomal suspension (S9). The final S9 concentration in the treatment well was 7%. In general, for each experiment there were 3-6 microplate wells for each test agent concentration plus a concurrent negative control (buffer only) and concurrent positive controls (with S9 activation, NDMA, 50-1000 µM; without S9 activation, ethylmethanesulfonate (EMS), 188 µM). Each experiment was repeated. Each microwell is an independent clone and the number of repeated measurements was determined based on the power function of the ANOVA test statistic. We routinely require a power value ($1-\beta$) of ≥ 0.8 with $\alpha \leq 0.05$. This ensures that the statistical analyses have the sensitivity to reject the null hypothesis. The microplate wells were sealed with Alumnaseal to prevent any crossover volatilization or evaporation, and the microplate was placed in a plastic container and incubated at 37°C with shaking at 200 rpm for 60 min. After treatment, the entire contents of each well were added to 2 mL of molten histidine and biotin supplemented Vogel-Bonner (VB) top agar, mixed and poured onto selective VB plates. The VB selective plates were placed in a static 37°C incubator for 72 h. Histidine revertant colonies were counted by hand or with a New Brunswick Biotran III automatic colony counter. The data were saved as an Excel spreadsheet. The data were analyzed using the statistical and graphical functions of SigmaPlot 11.

Cell survivorship was measured by making serial dilutions from the treatment wells in PPB and plating on LB (complete media) plates. After incubation for 24 h the colonies were counted. The number of colonies from the negative control was set at 100% and the colonies from the treatment wells were compared and expressed as a percent of the negative control.

To confirm the genotype of the YG7108 cells, 100 µL of the titered cell suspension was added to an LB plate and spread with a flamed glass rod. Flamed tweezers were used to place a crystal violet disk onto the center of the plate, and the disk was tapped lightly in place. Due to the presence of the *rfa* mutation [10], the large molecules of crystal violet are able to enter and kill the YG7108 cells. This is indicated by a clear zone around the crystal violet disk (Figure 3).

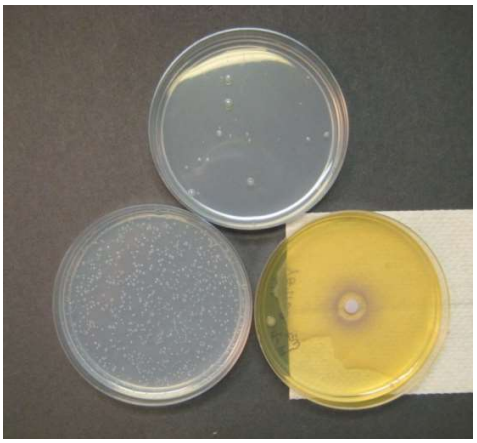

Figure 3. Diagnostic plates for the *S. typhimurium* microsuspension mutagenicity assay. A negative control is illustrated by the top plate, the left plate is the positive control (250 µM NDMA +S9 activation) and the right plate is the crystal violet LB plate showing a clear zone of inhibition identifying the presence of the *rfa*$^-$ mutation.

2.2.2.1 Comparison of the *S. typhimurium* Microsuspension Mutagenicity Assay and the Plate Incorporation Test

We employed a quantitative microsuspension methodology for the *S. typhimurium* mutagenicity assay instead of the more commonly used plate incorporation Ames test method. An overall comparison is outlined in Table 2. In the plate incorporation and preincubation methods, the cell titer is not determined; an aliquot (usually 100 µL) of an overnight culture in complete growth medium is used. For the plate incorporation assay the test agent is added to a 2-mL volume of supplemented molten top agar along with the cells and ± S9. The top agar is poured upon a VB minimal plate. Thus the actual exposure concentration to the cells is difficult to determine. It is impossible to calculate a molar concentration and usually a unit of grams per plate is used to express the concentration. The volume during the exposure time for the cells is impossible to quantify. Cells are exposed in approximately 2 mL of molten top agar which is then poured onto a VB selective plate that consists of approximately 20 mL. The cells are retained in the top agar, but the test chemical diffuses throughout the approximately 22 mL volume of agar in the petri plate.

Table 2. Comparison between the plate incorporation method and the microsuspension method for the *S. typhimurium* mutagenicity assay.

Metric	Plate Incorporation Method	Microsuspension Method
Single colony isolate grown overnight in complete medium	Yes	Yes
Centrifugation and washing bacteria before treatment	No	Yes
Determination of titer before treatment (same number of cells treated for each experiment)	No	Yes. By treating a consistent number of cells, the results can be presented in revertants per 10^8 cells plated. This provides a uniform level of mutagenicity response.
Treatment times can be adjusted	No	Yes
Precise control of concentration of test agents	No — With this method the test compound and bacteria are combined in 2 mL of molten agar and poured immediately onto a plate from which diffusion occurs throughout the plate. The concentration of agent can be expressed as the concentration in the treatment tube or as the concentration in the petri plate.	Yes – With this method a known number of bacteria are exposed to the agent while shaking for defined time periods. After treatment the mixture is added to molten agar and poured onto a petri plate. The concentration of agent is expressed as the concentration during treatment.
Determination of cytotoxicity	Not possible. A background lawn that is thin or sparse is only qualitative and not a quantitative measure of toxicity.	Yes, an aliquot of treated cells may be serially diluted and plated onto complete medium, allowing for determination of toxicity.

Another difficulty in the plate incorporation method is that the test agent is in contact with S9 monooxygenase enzymes and co-factors initially in the 2 mL overlay agar and then this volume changes when the contents are poured upon the VB plate. Finally, in the plate incorporation and preincubation methods no quantitative cytotoxicity measurement can be made. It is important that the *S. typhimurium* cells are not killed as the concentration of the test agent is increased throughout the concentration range. A qualitative visual inspection of the background lawn of cells is part of the plate incorporation and preincubation procedures, but this is not a true measurement of cytotoxicity.

In the microsuspension assay, *S. typhimurium* cells are washed, titered, and a specific number of cells are exposed to a test agent in a known volume of buffer with and without S9 for a specific time period. The concentrations of the test agent are expressed in molar units. An aliquot of the suspension is reserved after treatment, serial dilutions are prepared and known volumes are plated onto complete (LB) medium. All living bacterial cells will grow on LB medium and we can determine if we are conducting the assay at concentrations of test chemical that do not induce a cytotoxic response. Thus, the microsuspension procedure is a quantitative assay that provides control over cell titer, a precise measure of cytotoxicity and an exact determination of the concentration of test agent to which a specific number of cells are exposed for a specific time period. This level of precision provides high quality data on the relative mutagenic capacities and allows for a quantitative comparison amongst the test chemicals.

2.3 Quality Assurance Quality Control

Weekly project team meetings were held and the results were discussed. At all times good laboratory practices associated with a toxicology laboratory were followed. All data were kept in data books and transferred to computer files. Ultimately, with the consent of all parties involved in this project the results will be published in a peer-reviewed scientific journal. Chemicals were of the highest purity available and were kept in a freezer or at room temperature as specified by the vendors in a locked room. Manipulations of toxic and mutagenic chemicals were conducted in certified biological/chemical safety hoods. All materials were handled using disposable papers and gloves. Liquid mutagens were dispensed with disposable pipet tips. All personnel were instructed in the correct handling of dangerous chemicals and the procedures were reviewed periodically. All items that were in contact with mutagens were disposed of in accordance with regulations of the Division of Research Safety of the University of Illinois at Urbana-Champaign. The quality assurance objectives for data obtained in this project were defined in terms of accuracy and precision. Our chemical balances are periodically calibrated and our automated pipettes are checked and calibrated by Rainin Co. (or other certified vendor) on an annual basis.

3. Communication and Review

3.1 Project Director

For this project the Study Director is Dr. Michael Plewa, University of Illinois at Urbana-Champaign. Dr. Plewa will be the focal point for all discussions on the scope of work.

3.2 Project Manager

The Project Manager for this study is Tan-Trung Nguyen, Cansolv Technologies Inc., an affiliate of Shell Global Solutions International BV, 400 de Maisonneuve Ouest, Suite 200, Montréal, Québec, H3A 1L4 CANADA.

3.3 Shell's Study Monitors

All study plans (protocols) from the laboratory were reviewed by one of the Shell's study monitors prior to test execution. The study followed the protocols as published by Wagner et al., 2012 [9].

3.3.1 Study Monitors

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4. Results and Discussion

4.1 N-Nitrosodimethylamine

4.1.1 NDMA Mutagenicity and Cytotoxicity Data

The mutagenicity of NDMA was analyzed with and without S9 mammalian microsomal activation using *S. typhimurium* strain YG7108 in a concentration range from 5 to 1000 µM. The raw data from the selective VB plates (mutagenicity) and the LB cytotoxicity plates for NDMA are presented in Tables 3 and 4. These data were used to generate the concentration-response curves (Figure 4) and for the statistical analyses (Tables 5 and 6).

Table 3. Plate counts of NDMA (VB plates) and percent survivorship (LB plates) +S9.

NDMA +S9 *S. typhimurium* YG7108 Experiments 020813 and 022813

	Revertants/Plate VB								
Conc µM	0	5	10	25	50	100	250	500	1000
	12	36	61	196	314	688	1026	1337	1375
	16	64	86	196	315	605	964	1324	1369
	15	51	90	164	292	547	981	1248	1453
	22	53	86	194	303	694	1119	1290	1506
	12	50	75	173	358	694	1027	1327	1579
	18	40	81	169	306	569	984	1056	1341
	23	57	71	163	248	540	794	1003	1439
	11	47	92	151	318	516	845	1189	1487
	35		69	152	288	628	811	1223	1434
	34				343	558	874		
	33					663	848		
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	17								

Table 3. Plate counts of NDMA (VB plates) and percent survivorship (LB plates) +S9.

NDMA +S9 *S. typhimurium* YG7108 Experiments 020813 and 022813

Revertants/Plate VB

Conc µM	0	5	10	25	50	100	250	500	1000	
	26									
	25									
	30									
Number	30	8	9	9	10	12	11	9	9	Number
Average	23.7	49.8	79.0	173.1	308.5	616.5	933.9	1221.9	1442.6	Average
SE	1.5	3.2	3.6	6.0	9.5	19.9	31.7	40.2	25.0	SE
SD	8.4	8.9	10.9	18.1	30.2	69.0	105.2	120.7	75.1	SD

NDMA +S9 *S. typhimurium* YG7108 Experiments 022813

LB Survivorship

Conc. µM	0	100	250	500	1000	
	314	346	315	355	309	
	331	355	336	303	312	
				331		
						% Negative
	97.4	107.3	97.7	110.1	95.8	Control
	102.6	110.1	104.2	94.0	96.7	
				102.6		
Number	2	2	2	3	2	Number
Average	100.0	108.7	100.9	102.2	96.3	Average
SE	2.6	1.4	3.3	4.7	0.5	SE
SD	3.7	2.0	4.6	8.1	0.7	SD

Table 4. Plate counts of NDMA (VB plates) and percent survivorship (LB plates) −S9.

NDMA −S9 *S. typhimurium* YG7108 Experiments 030713

Revertants/Plate VB

Conc µM	0	100	250	500	1000	
	21	25	26	26	36	EMS 554
	19	26	29	20	28	
	25	25	30	32	25	
	26	39	38	32	22	
	30	32	29	22	35	
	17	26	26	26	26	
Number	6	6	6	6	6	Number
Average	23.0	28.8	29.7	26.3	28.7	Average
SE	2.0	2.3	1.8	2.0	2.3	SE
SD	4.9	5.6	4.4	5.0	5.6	SD

NDMA −S9 *S. typhimurium* YG7108 Experiments 022813

LB Survivorship

Conc. µM	0	100	250	500	1000	
	384	349	339	315	358	
	360	344	327	378	389	
	355	394	357	371	362	
						% Negative Control
	104.8	95.2	92.5	85.9	97.7	
	98.2	93.9	89.2	103.1	106.1	
	96.9	107.5	97.4	101.2	98.8	
Number	3	3	3	3	3	Number
Average	100.0	98.9	93.1	96.8	100.9	Average
SE	2.4	4.3	2.4	5.4	2.7	SE
SD	4.2	7.5	4.1	9.4	4.6	SD

4.1.2 NDMA Concentration-Response Curves

The concentration-response curves for the mutagenicity and cytotoxicity of NDMA in the *S. typhimurium* strain YG7108 microplate suspension assay are presented in Figure 4. The top panel illustrates the cytotoxicity (LB plate experiments) for NDMA with S9 metabolic activation (open squares) and without S9 activation (filled squares). The bottom panel represents the mutagenicity of NDMA with S9 activation (open circles) and without S9 activation (filled circles).

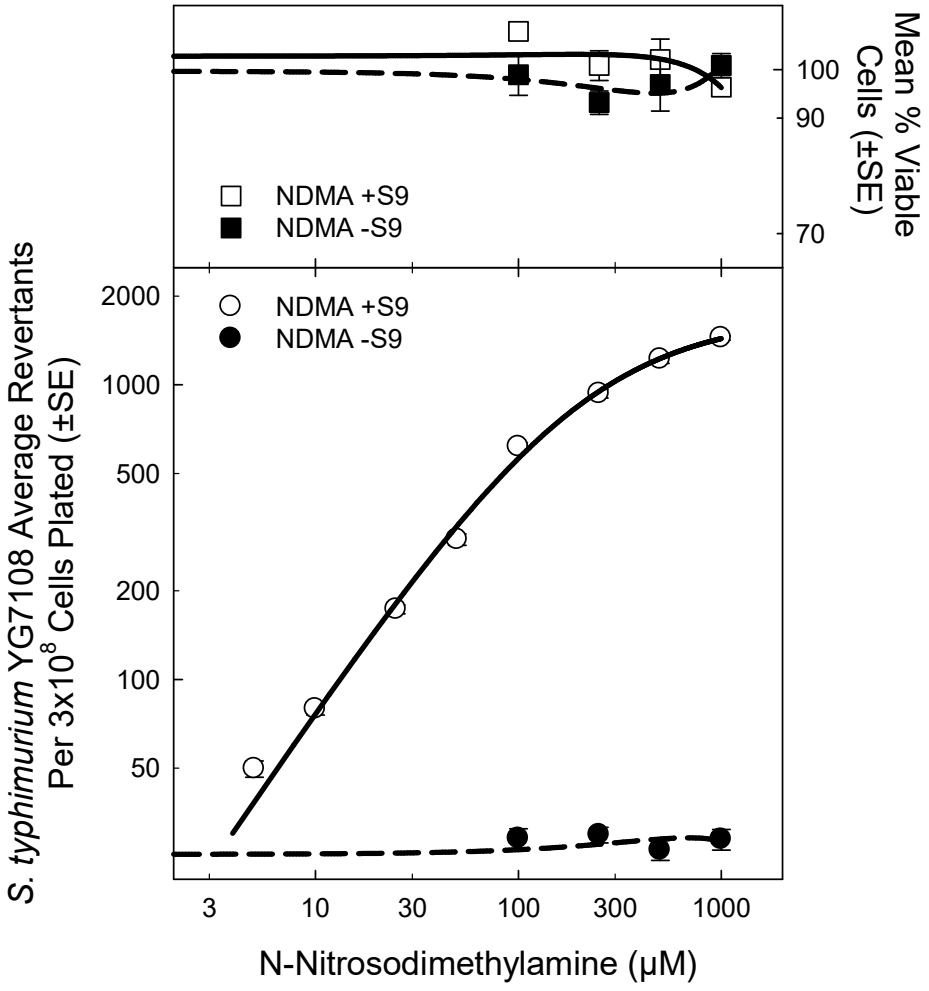

Figure 4. Log-log plot showing NDMA concentration-response curves for cytotoxicity (upper panel) and mutagenicity (lower panel) in *S. typhimurium* strain YG7108 with and without S9 metabolic activation.

4.1.3 Statistical Analyses of the Mutagenicity Data for NDMA

An ANOVA test statistic for the mutagenicity data of NDMA with S9 metabolic activation was conducted and the results are presented in Table 5.

Table 5. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses NDMA +S9

Data source: Data 6 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NDMA+S9 0	30	0	23.700	8.400	1.534
NDMA+S9 5	8	0	49.750	8.940	3.161
NDMA+S9 10	9	0	79.333	10.874	3.625
NDMA+S9 25	9	0	173.111	18.100	6.033
NDMA+S9 50	10	0	298.500	42.022	13.288
NDMA+S9 100	12	0	616.500	68.975	19.911
NDMA+S9 250	11	0	933.909	105.163	31.708
NDMA+S9 500	9	0	1221.889	120.707	40.236
NDMA+S9 1000	9	0	1442.556	75.083	25.028

Source of Variation	DF	SS	MS	F	P
Between Groups	8	26026785	3253348	919.7	<0.001
Residual	98	346652	3537		
Total	106	26373437			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
NDMA+S9 0 vs. NDMA+S9 1000	1418.856	62.770	<0.001	0.006	Yes
NDMA+S9 0 vs. NDMA+S9 500	1198.189	53.008	<0.001	0.007	Yes
NDMA+S9 0 vs. NDMA+S9 250	910.209	43.418	<0.001	0.009	Yes
NDMA+S9 0 vs. NDMA+S9 100	592.800	29.181	<0.001	0.010	Yes
NDMA+S9 0 vs. NDMA+S9 50	274.800	12.654	<0.001	0.013	Yes
NDMA+S9 0 vs. NDMA+S9 25	149.411	6.610	<0.001	0.017	Yes
NDMA+S9 0 vs. NDMA+S9 10	55.633	2.461	0.016	0.025	Yes
NDMA+S9 0 vs. NDMA+S9 5	26.050	1.101	0.274	0.050	No

An ANOVA test statistic for the mutagenicity data of NDMA without S9 metabolic activation was conducted and the results are presented in Table 6.

Table 6. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses NDMA -S9

Data source: Data 6 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NDMA-S9 0	6	0	23.000	4.858	1.983
NDMA-S9 100	6	0	28.833	5.636	2.301
NDMA-S9 250	6	0	29.667	4.412	1.801
NDMA-S9 500	6	0	26.333	4.967	2.028
NDMA-S9 1000	6	0	28.667	5.645	2.305

Source of Variation	DF	SS	MS	F	P
Between Groups	4	175.46	43.86	1.670	0.188
Residual	25	656.83	26.27		
Total	29	832.30			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.188).

4.1.4 Interpretation of NDMA Data

NDMA is a potent promutagen that requires mammalian microsomal activation (S9) to generate an ultimate alkylating mutagen that induced base-pair mutations in *S. typhimurium* strain YG7108. This result is clearly demonstrated in the comparison of the NDMA concentration-response curves of NDMA +S9 versus NDMA –S9 (Figure 4 lower panel and Table 5 versus Table 6). Mutagenic potency is a metric developed to compare different mutagens, for the purposes of this report to compare different nitrosamines. Note that throughout the concentration range of 5 to 1000 µM, NDMA with or without S9 was not cytotoxic to *S. typhimurium* (Figure 4 upper panel and Tables 3 and 4, LB plate data). Thus we did not have to correct for cytotoxicity in the calculation of the mutagenic potency of NDMA. The NDMA potency values were derived from all concentrations analyzed in the concentration-response curve. This metric provides a measure of mutagenic activity as induced mutants per micromole (µmol) of NDMA. The calculated mutagenic potency of NDMA with S9 metabolic activation was 47,694.7 revertants/µmol. The mutagenic potency of NDMA without S9 activation was 0.

4.2 1-Nitroso-(4-hydroxyethyl)-piperazine (Cansolv-N1)

4.2.1 Nitroso-HEP Mutagenicity and Cytotoxicity Data

Nitroso-HEP was analyzed with and without S9 mammalian microsomal activation in a concentration range from 1 to 20 mM. The raw data from the selective VB plates (mutagenicity) and the LB cytotoxicity plates for Nitroso-HEP are presented in Tables 7 and 8. These data were used to generate the concentration-response curves (Figure 5).

Table 7. Plate counts of Nitroso-HEP (VB plates) and percent survivorship (LB plates) +S9.

Nitroso-HEP (Cansolv-N1) +S9 *S. typhimurium* YG7108 Experiments 021313 and 021913

Revertants/Plate VB

Conc. mM	0	1	2.5	5	7.5	10	15	20	NDMA + control
	18	23	31	26	37	32	36	35	0.25 mM NDMA 984
	23	34	28	38	36	32	40	43	0.1mM NDMA 569
	11	24	32	34	33	26	45	35	
	35	44	35	34	46	46	70	48	
	34	33	31	46	57	49	54	45	
	33	40	29	48	33	40	53	52	
	36	37	31	36	55	35	52	46	
	22	35	40	42	36	59	52	64	
	34	35	31	38	46	42	40	55	
Number	9	9	9	9	9	9	9	9	Number
Average	27.3	33.9	32.0	38.0	42.1	40.1	49.1	47.0	Average
SE	3.0	2.3	1.2	2.2	3.1	3.4	3.4	3.1	SE
SD	9.1	6.8	3.6	6.7	9.3	10.2	10.3	9.3	SD

Nitroso-HEP (Cansolv-N1) +S9 *S. typhimurium* YG7108 Experiment 021313

LB Survivorship

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	349	390	333	310	300	347	255	278	
	317	335	311	280	328	287		306	
	334	336	324	279	265	251		288	
	104.7	117.0	99.9	93.0	90.0	104.1	76.5	83.4	% Negative Control
	95.1	100.5	93.3	84.0	98.4	86.1		91.8	
	100.2	100.8	97.2	83.7	79.5	75.3		86.4	
Number	3	3	3	3	3	3	1	3	Number
Average	100.0	106.1	96.8	86.9	89.3	88.5	76.5	87.2	Average
SE	2.8	5.5	1.9	3.1	5.5	8.4		2.5	SE
SD	4.8	9.4	3.3	5.3	9.5	14.5		4.3	SD

Table 8. Plate counts of Nitroso-HEP (VB plates) and percent survivorship (LB plates) −S9.

Nitroso-HEP (Cansolv-N1) −S9 *S. typhimurium* YG7108 Experiments 021313 and 021913

Revertants/Plate VB

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	19	21	18	23	24	27	20	23	EMS = 943
	26	18	20	30	34	15	22	34	EMS = 939
	26	28	22	31	15	21	30	22	
	43	40	36	38	50	36	43	36	
	39	39	36	46	42	33	37	49	
	23	41	40	28	41	48	44	46	
	26	36	45	45	41	50	41	37	
	48	34	39	29	37	36	47	49	
	38	44	31	42	49	40		39	
Number	9	9	9	9	9	9	8	9	Number
Average	32.0	33.4	31.9	34.7	37.0	34.0	35.5	37.2	Average
SE	3.4	3.1	3.2	2.8	3.8	3.9	3.6	3.3	SE
SD	10.1	9.2	9.7	8.3	11.4	11.6	10.3	10.0	SD

Nitroso-HEP (Cansolv-N1) −S9 *S. typhimurium* YG7108 Experiments 021313

LB Survivorship

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	291	287	323	339	277	236	265	319	
	334	283	287	313	289	239	316	310	
	178	313	296	324	300	229	266	257	
									% Negative Control
	108.7	107.2	120.6	126.6	103.4	88.1	99.0	119.1	
	124.7	105.7	107.2	116.9	107.9	89.3	118.0	115.8	
	66.5	116.9	110.5	121.0	112.0	85.5	99.3	96.0	
Number	3	3	3	3	3	3	3	3	Number
Average	100.0	109.9	112.8	121.5	107.8	87.6	105.4	110.3	Average
SE	17.4	3.5	4.0	2.8	2.5	1.1	6.3	7.2	SE
SD	30.1	6.1	7.0	4.9	4.3	1.9	10.9	12.5	SD

4.2.2 Nitroso-HEP Concentration-Response Curves

The concentration-response curves for the mutagenicity and cytotoxicity of Nitroso-HEP in the *S. typhimurium* strain YG7108 microplate suspension assay are presented in Figure 5. The top panel illustrates the cytotoxicity (LB plate experiments) for Nitroso-HEP with S9 metabolic activation (open squares) and without S9 activation (filled squares). The bottom panel represents the mutagenicity of Nitroso-HEP with S9 activation (open circles) and without S9 activation (filled circles).

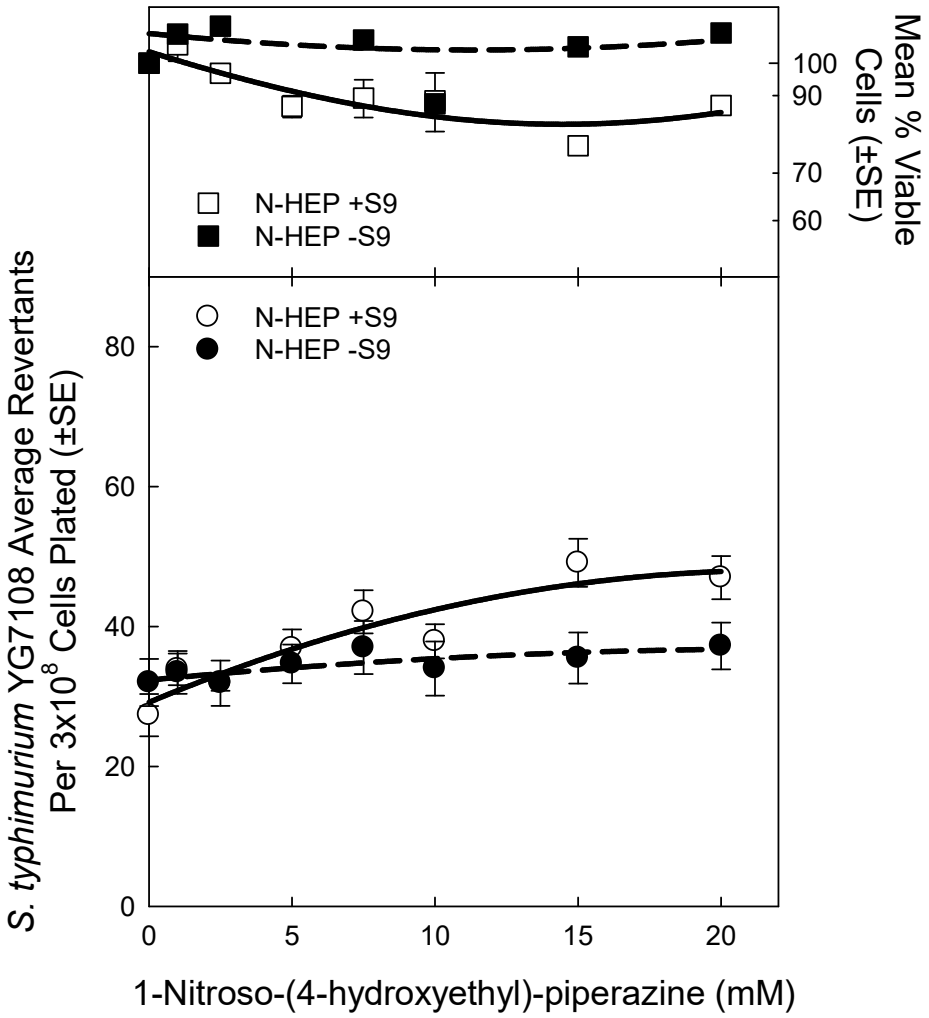

Figure 5. Nitroso-HEP concentration-response curves for cytotoxicity (upper panel) and mutagenicity (lower panel) in *S. typhimurium* strain YG7108 with and without S9 metabolic activation.

4.2.3 Statistical Analyses of the Mutagenicity Data for Nitroso-HEP

An ANOVA test statistic for the mutagenicity data of Nitroso-HEP with S9 metabolic activation was conducted and the results are presented in Table 9.

Table 9. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Nitroso-HEP +S9

Data source: Data 2 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NHEP+S9 0.00	9	0	27.333	9.055	3.018
1.00 +S9	9	0	33.889	6.791	2.264
2.50 +S9	9	0	32.000	3.571	1.190
5.00 +S9	9	0	36.889	8.131	2.710
7.50 +S9	9	0	42.111	9.253	3.084
10.00 +S9	9	0	37.889	7.339	2.446
15.00	9	0	49.111	10.289	3.430
20.00 +S9	9	0	47.000	9.274	3.091

Source of Variation	DF	SS	MS	F	P
Between Groups	7	3498.000	499.714	7.426	<0.001
Residual	64	4306.444	67.288		
Total	71	7804.444			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
NHEP+S9 0.00 vs. 15.00 +S9	21.778	5.632	<0.001	0.007	Yes
NHEP+S9 0.00 vs. 20.00 +S9	19.667	5.086	<0.001	0.009	Yes
NHEP+S9 0.00 vs. 7.50 +S9	14.778	3.822	<0.001	0.010	Yes
NHEP+S9 0.00 vs. 10.00 +S9	10.556	2.730	0.008	0.013	Yes
NHEP+S9 0.00 vs. 5.00 +S9	9.556	2.471	0.016	0.017	Yes
NHEP+S9 0.00 vs. 1.00 +S9	6.556	1.695	0.095	0.025	No
NHEP+S9 0.00 vs. 2.50 +S9	4.667	1.207	0.232	0.050	No

An ANOVA test statistic for the mutagenicity data of Nitroso-HEP without S9 metabolic activation was conducted and the results are presented in Table 10.

Table 10. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Nitroso-HEP -S9

Data source: Data 2 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NHEP-S9 0	9	0	32.000	10.124	3.375
1.00 -S9	9	0	33.444	9.167	3.056
2.50 -S9	9	0	31.889	9.714	3.238
5.00 -S9	9	0	34.667	8.276	2.759
7.50 -S9	9	0	37.000	11.358	3.786
10.00 -S9	9	0	34.000	11.597	3.866
15.00 -S9	8	0	35.500	10.323	3.650
20.00 -S9	9	0	37.222	10.022	3.341

Source of Variation	DF	SS	MS	F	P
Between Groups	7	260.911	37.273	0.364	0.920
Residual	63	6452.667	102.423		
Total	70	6713.577			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.920).

4.2.4 Interpretation of Nitroso-HEP Data

Nitroso-HEP is a weak promutagen that requires mammalian microsomal activation (S9) to generate an ultimate mutagen that induced base-pair mutations in *S. typhimurium* strain YG7108. This result is demonstrated in the comparison of the Nitroso-HEP concentration-response curves of Nitroso-HEP +S9 versus Nitroso-HEP –S9 (Figure 5 lower panel and Table 9 versus Table 10). With S9 activation the lowest concentration of Nitroso-HEP that expressed a mutagenic response was 5 mM (Table 9). Throughout the concentration range of 1 – 20 mM, Nitroso-HEP without S9 was not cytotoxic. However, Nitroso-HEP +S9 was weakly cytotoxic to *S. typhimurium* (Figure 5 upper panel and Tables 7 and 8, LB plate data). The Nitroso-HEP mutagenicity concentration-response curves based on revertants per 1×10^8 surviving cells is presented in Figure 6. We adjusted for cytotoxicity in the calculation of the mutagenic potency of Nitroso-HEP +S9.

The Nitroso-HEP mutagenic potency values were derived from all concentrations analyzed in the concentration-response curve. The calculated mutagenic potency of Nitroso-HEP with S9 metabolic activation was 22.7 revertants/μmol. The mutagenic potency of Nitroso-HEP without S9 activation was 0.

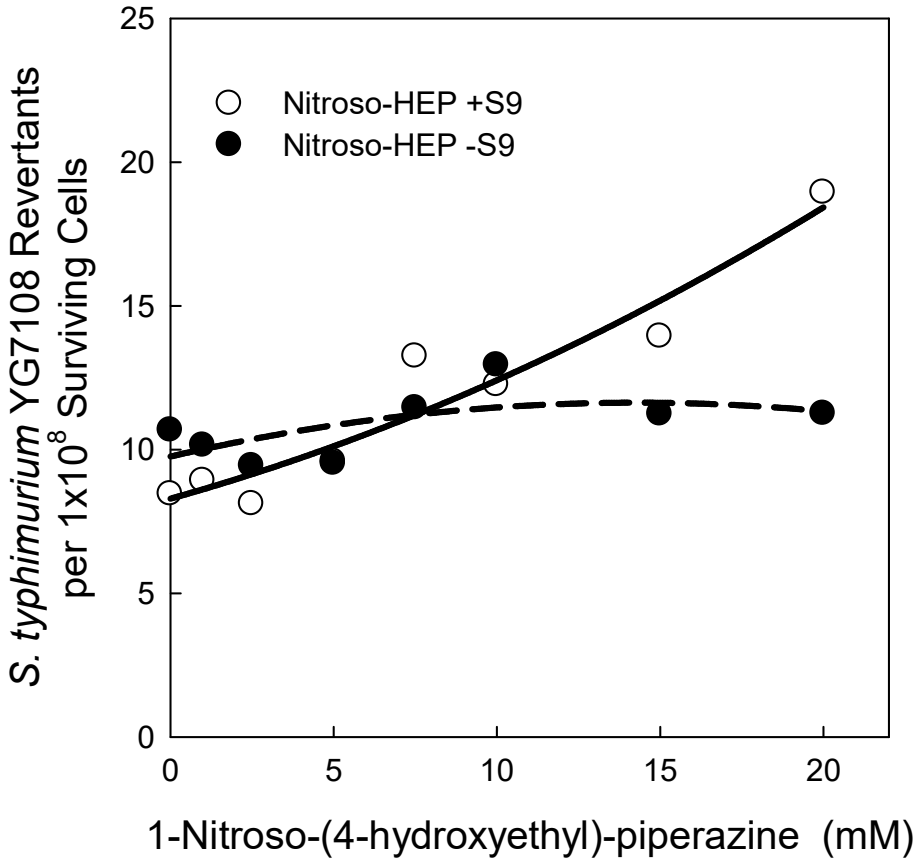

Figure 6. Nitroso-HEP mutagenicity concentration-response curves in *S. typhimurium* strain YG7108 with and without S9 metabolic activation. Data adjusted for cytotoxicity and expressed as revertants/1×10^8 surviving cells.

4.3 Cansolv-N2

4.3.1 Cansolv-N2 Mutagenicity and Cytotoxicity Data

Cansolv-N2 was analyzed with and without S9 mammalian microsomal activation in a concentration range from 1 to 20 mM. The raw data from the selective VB plates (mutagenicity) and the LB cytotoxicity plates for Cansolv-N2 are presented in Tables 11 and 12. These data were used to generate the Cansolv-N2 concentration-response curves (Figure 7).

Table 11. Plate counts of Cansolv-N2 (VB plates) and percent survivorship (LB plates) +S9.

Cansolv-N2 +S9 *S. typhimurium* YG7108 Experiments 021513 and 022113

Revertants/Plate VB

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	26	20	21	26	30	35	34	43	250 µM NDMA = 794
	21	19	23	24	34	29	40	35	100 µM NDMA = 540
	20	13	15	30	29	19	32	44	
	24	46	24	33	43	49	42	50	
	19	44	21	32	35	37	31	75	
	24	14	15	29	40	47	45	50	
	22	24	22	32	36	29	32	56	
	24	31	23	23	41	29	39	40	
	48	19	20	25	40	26	53	50	
Number	9	9	9	9	9	9	9	9	Number
Average	25.3	25.6	20.4	28.2	36.4	33.3	38.7	49.2	Average
SE	2.9	4.1	1.1	1.3	1.6	3.3	2.4	3.8	SE
SD	8.8	12.2	3.3	3.8	4.9	9.8	7.3	11.5	SD

(Cansolv-N2 +S9 *S. typhimurium* YG7108 Experiment 021513

LB Survivorship

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	337	367	360	356	336	335	296	370	
	364	310	289	330	311	306	320	259	
		328	235	346	318	313	357	282	
	96.1	104.7	102.7	101.5	95.8	95.5	84.4	105.5	% Negative Control
	103.8	88.4	82.4	94.1	88.7	87.3	91.3	73.8	
		93.5	67.0	98.7	90.7	89.3	101.8	80.4	
Number	2	3	3	3	3	3	3	3	Number
Average	100.0	95.5	84.0	98.1	91.7	90.7	92.5	86.6	Average
SE	3.8	4.8	10.3	2.1	2.1	2.5	5.0	9.6	SE
SD	5.4	8.3	17.8	3.7	3.7	4.3	8.8	16.7	SD

Table 12. Plate counts of Cansolv-N2 (VB plates) and percent survivorship (LB plates) −S9.

Cansolv-N2 −S9 *S. typhimurium* YG7108 Experiments 021513 and 022113

Revertants/Plate VB

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	17	37	52	51	48	83	65	91	EMS = 693
	22	38	42	47	50	56	95	87	EMS = 592
	20	27	43	51	39	76	66	52	
	20	32	33	54	34	48	60	64	
	53	23	39	46	43	61	56	64	
	23	21	29	42	30	56	50	60	
	21	18	37	34	35	59	71	51	
	29	36	20	45	38	48	55	49	
	13	28	53	37	36	48	52	65	
Number	9	9	9	9	9	9	9	9	Number
Average	24.2	28.9	38.7	45.2	39.2	59.4	63.3	64.8	Average
SE	3.9	2.4	3.5	2.2	2.2	4.2	4.6	5.0	SE
SD	11.6	7.3	10.5	6.6	6.6	12.5	13.7	15.0	SD

Cansolv-N2 −S9 *S. typhimurium* YG7108 Expts. 021513

LB Survivorship

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	314	325	306	328	309	292	233	28	
	275	277	300	321	273	305	133	66	
	287	312	316	343	375	284	153	57	
	107.53	111.3	104.8	112.3	105.8	100.0	79.8	9.6	% Negative Control
	94.2	94.9	102.7	109.9	93.5	104.5	45.6	22.6	
	98.3	106.8	108.2	117.5	128.4	97.3	52.4	19.5	
Number	3	3	3	3	3	3	3	3	Number
Average	100.0	104.3	105.2	113.2	109.2	100.6	59.2	17.2	Average
SE	3.9	4.9	1.6	2.2	10.2	2.1	10.5	3.9	SE
SD	6.8	8.5	2.8	3.8	17.2	3.6	18.1	6.8	SD

4.3.2 Cansolv-N2 Concentration-Response Curves

The concentration-response curves for the mutagenicity and cytotoxicity of Cansolv-N2 in the *S. typhimurium* strain YG7108 microplate suspension assay are presented in Figure 7. The top panel illustrates the cytotoxicity (LB plate experiments) for Cansolv-N2 with S9 metabolic activation (open squares) and without S9 activation (filled squares). It is clear that Cansolv-N2 is cytotoxic to *S. typhimurium* cells. The bottom panel represents the mutagenicity of Cansolv-N2 with S9 activation (open circles) and without S9 activation (filled circles).

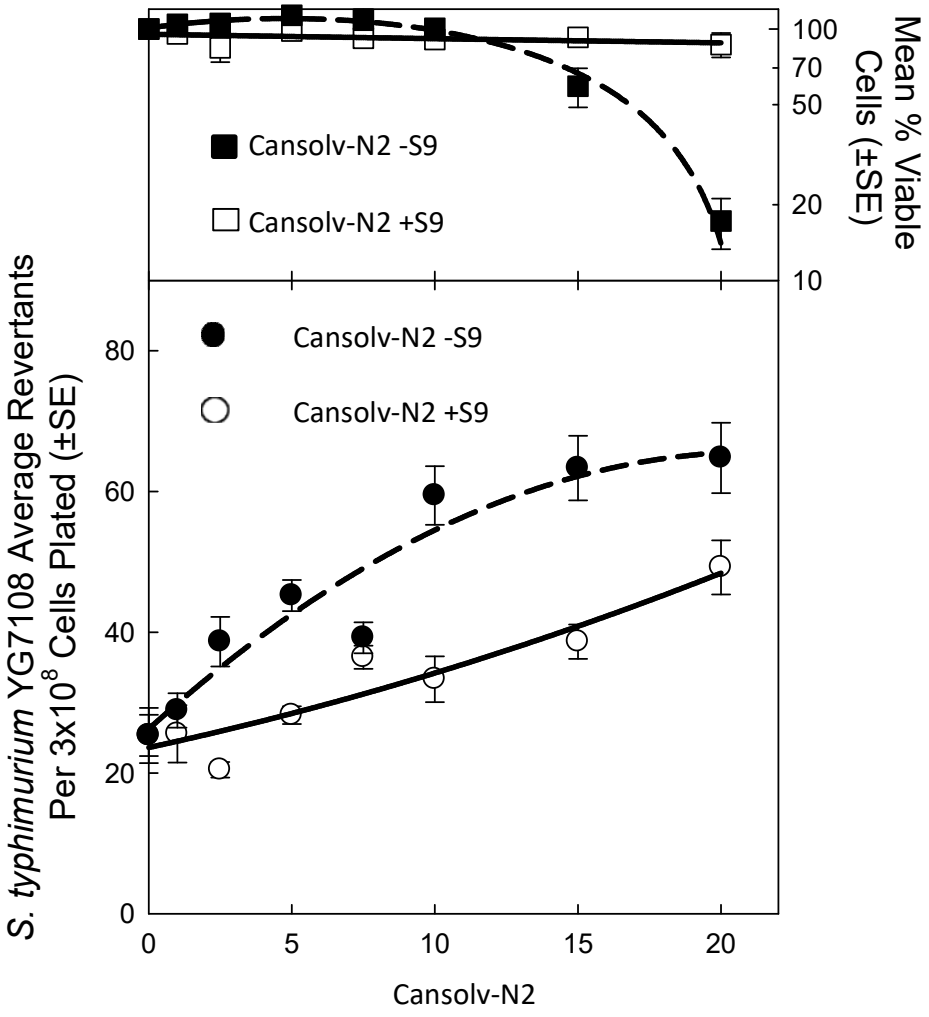

Figure 7. Cansolv-N2 concentration-response curves for cytotoxicity (upper panel) and mutagenicity (lower panel) in *S. typhimurium* strain YG7108 with and without S9 metabolic activation.

4.3.3 Statistical Analyses of the Mutagenicity Data for Cansolv-N2

An ANOVA test statistic for the mutagenicity data of Cansolv-N2 with S9 metabolic activation was conducted and the results are presented in Table 13.

Table 13. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Cansolv-N2 +S9

Data source: Data 4 in Shell Cansolv Project

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
0.00 Canv-N2 +S9	9	0	25.333	8.789	2.930
1.00 Can-N2+S9	9	0	25.556	12.238	4.079
2.50 Can-N2 +S9	9	0	20.444	3.321	1.107
5.00 Can-N2 +S9	9	0	28.222	3.801	1.267
7.50 Can-N2 +S9	9	0	36.444	4.927	1.642
10.00 Can-N2 +S9	9	0	33.333	9.772	3.257
15.00 Can-N2 +S9	9	0	38.667	7.314	2.438
20.00 Can-N2 +S9	9	0	49.222	11.541	3.847

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	7	5365.542	766.506	10.970	<0.001
Residual	64	4471.778	69.872		
Total	71	9837.319			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).
Power of performed test with alpha = 0.050: 1.000
Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**
0.00 Can-N2 +S vs. 20.00 Can-N2 +S9	23.889	6.063	<0.001	0.007
0.00 Can-N2 +S vs. 15.00 Can-N2 +S9	13.333	3.384	0.001	0.009
0.00 Can-N2 +S vs. 7.50 Can-N2 +S9	11.111	2.820	0.006	0.010
0.00 Can-N2 +S vs. 10.00 Can-N2 +S9	8.000	2.030	0.046	0.013
0.00 Can-N2 +S vs. 2.50 Can-N2 +S9	4.889	1.241	0.219	0.017
0.00 Can-N2 +S vs. 5.00 Can-N2 +S9	2.889	0.733	0.466	0.025
0.00 Can-N2 +S vs. 1.00 Can-N2 +S9	0.222	0.0564	0.955	0.050

Comparison	**Significant?**
0.00 Can-N2 +S vs. 20.00 Can-N2+S9	Yes
0.00 Can-N2 +S vs. 15.00 Can-N2+S9	Yes
0.00 Can-N2 +S vs. 7.50 Can-N2+S9	Yes
0.00 Can-N2 +S vs. 10.00 Can-N2+S9	No
0.00 Can-N2 +S vs. 2.50 Can-N2+S9	No
0.00 Can-N2 +S vs. 5.00 Can-N2+S9	No
0.00 Can-N2 +S vs. 1.00 Can-N2+S9	No

An ANOVA test statistic for the mutagenicity data of Cansolv-N2 without S9 metabolic activation was conducted and the results are presented in Table 14.

Table 14. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Cansolv-N2--S9

Data source: Data 4 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
0.00 Can-N2 -S9	9	0	24.233	11.658	3.919
1.00 Can-N2 -S9	9	0	28.889	7.322	2.441
2.50 Can-N2 -S9	9	0	38.667	10.548	3.516
5.00 Can-N2 -S9	9	0	45.222	6.629	2.210
7.50 Can-N2 -S9	9	0	39.222	6.610	2.203
10.00 Can-N2 -S9	9	0	59.444	12.491	4.164
15.00 Can-N2 -S9	9	0	63.333	13.748	4.583
20.00 Can-N2 -S9	9	0	64.778	15.031	5.010

Source of Variation	DF	SS	MS	F	P
Between Groups	7	14875.333	2125.048	17.677	<0.001
Residual	64	7693.778	120.215		
Total	71	22569.111			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0.00 Can-N2-S vs. 20.00 Can-N2–S9	39.444	7.632	<0.001	0.007	Yes
0.00 Can-N2-S vs. 15.00 Can-N2–S9	38.000	7.352	<0.001	0.009	Yes
0.00 Can-N2-S vs. 10.00 Can-N2–S9	34.111	6.600	<0.001	0.010	Yes
0.00 Can-N2-S vs. 5.00 Can-N2–S9	19.889	3.848	<0.001	0.013	Yes
0.00 Can-N2-S vs. 7.50 Can-N2–S9	13.889	2.687	0.009	0.017	Yes
0.00 Can-N2-S vs. 2.50 Can-N2–S9	13.333	2.580	0.012	0.025	Yes
0.00 Can-N2-S vs. 1.00 Can-N2–S9	3.556	0.688	0.494	0.050	No

4.3.4 Interpretation of Cansolv-N2 Data

Cansolv-N2 is a weak direct-acting mutagen that induced base-pair substitution mutations in *S. typhimurium* strain YG7108. The lowest Cansolv-N2 (-S9) concentration that induced a statistically significant increase in mutagenicity as compared to the negative control was 2.5 mM (Table 14). At concentrations above 10 mM Cansolv-N2 induced substantial cytotoxicity (Table 12, LB survivorship plates; Figure 7 upper panel). Thus this sample of Cansolv-N2 expresses direct mutagenic and cytotoxic activity. When Cansolv-N2 was analyzed with S9 activation we observed a reduction in mutagenicity (Figure 7 lower panel); the lowest concentration that induced a significant increase in mutagenic activity was 7.5 mM (Table 13). Throughout the concentration range from 1 – 20 mM Cansolv-N2 +S9 did not induce

cytotoxicity (Figure 7 upper panel). It appears that the mammalian microsomal monooxygenases detoxified the direct-acting activity of Cansolv-N2.

In order to calculate the mutagenic potency of Cansolv-N2 with and without S9 activation we normalized the data on a survivorship basis. The mutagenicity concentration-response curves of Cansolv-N2 +S9 and –S9 based on revertants per 1×10^8 surviving cells is presented in Figure 8. The Cansolv-N2 mutagenic potency value was derived from all the concentrations analyzed in the concentration-response curve. The calculated mutagenic potency of Cansolv-N2 with S9 metabolic activation was 13.1 revertants/µmol. The mutagenic potency of Cansolv-N2 without S9 activation was 53.8 revertants/µmol.

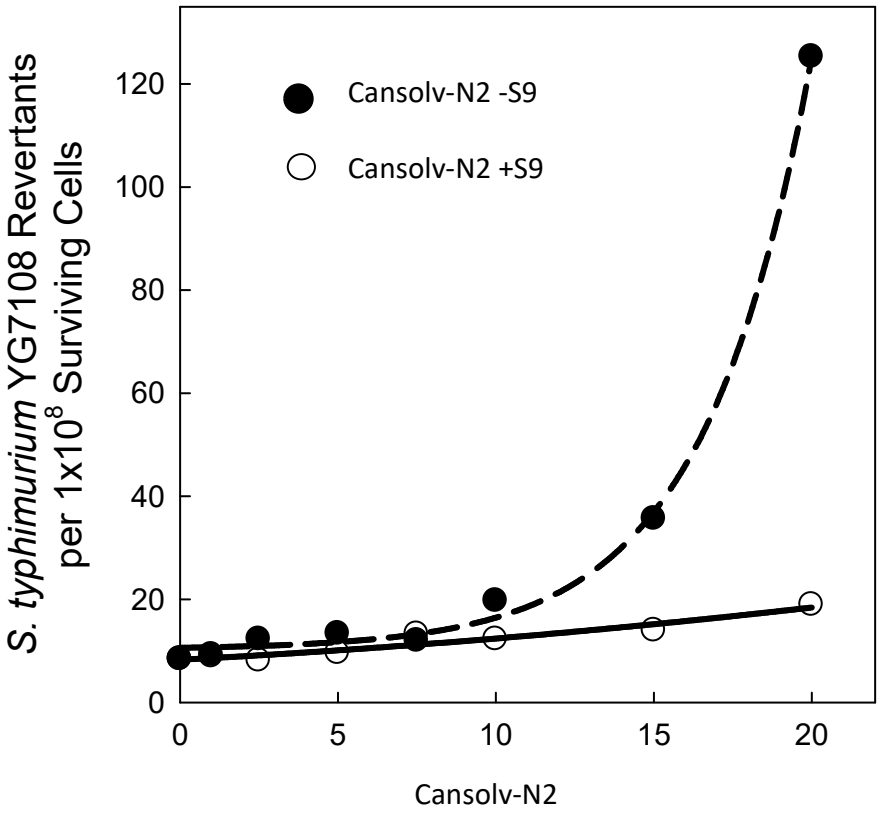

Figure 8. Cansolv-N2 mutagenicity concentration-response curves in *S. typhimurium* strain YG7108 with and without S9 metabolic activation. Data adjusted for cytotoxicity and expressed as revertants/1×10^8 surviving cells.

4.4. Nitrosopiperazine Cansolv-N3

4.4.1 Nitrosopiperazine Mutagenicity and Cytotoxicity Data

Nitrosopiperazine was analyzed with and without S9 mammalian microsomal activation in a concentration range from 1 to 20 mM. The raw data from the selective VB plates (mutagenicity) and the LB cytotoxicity plates for Nitrosopiperazine are presented in Tables 15 and 16. These data were used to generate the Nitrosopiperazine concentration-response curves (Figure 9).

Table 15. Plate counts of Nitrosopiperazine (VB plates) and percent survivorship (LB plates) +S9.

Cansolv Nitrosopiperazine +S9 *S. typhimurium* YG7108 Experiments 022613, 030513, 030713

Revertants/Plate VB

Conc. mM	0	1	2.5	5	7.5	10	15	20	NDMA
	28	185	259	404	465	649	624	803	50 µM = 306
	14	168	289	467	480	549	626	821	1 mM = 1376
	20	205	256	428	549	567	816	859	0.1 mM=696
	18	261	326	406	716	641	689	749	
	21	306	367	477	618	692	696	836	
	12	236	357	449	700	711	787	794	
	26	267	361	444	512	666	764	796	
	20	275	419	470	594	664	685	683	
	20	252	393	432	473	591	786	841	
	30				538	502	663	822	
	21				545	720	747	715	
	29								
Number	12	9	9	9	11	11	11	11	Number
Average	21.6	239.4	336.3	441.9	562.7	632.0	716.6	792.6	Average
SE	1.6	15.1	19.3	8.9	26.0	21.3	20.2	16.6	SE
SD	5.7	45.2	57.9	26.7	86.3	70.6	66.8	55.1	SD

Cansolv Nitrosopiperazine +S9 *S. typhimurium* YG7108 Experiments 022613

LB Survivorship

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	289	335	330	317	332	295	327	309	
	304	300	296	292	313	287	303	300	
	304	300	296	292	313	287	303	300	
									% Negative
	96.7	112.0	110.4	106.0	111.0	98.7	109.4	103.3	Control
	101.7	97.3	104.0	111.0	99.3	111.0	111.0	98.0	
	101.7	100.3	99.0	97.7	104.7	96.0	101.3	100.3	
Number	3	3	3	3	3	3	3	3	Number
Average	100.0	103.2	104.5	104.9	105.0	101.9	107.2	100.6	Average
SE	2.0	4.5	3.3	3.9	3.4	4.6	3.0	1.5	SE
SD	2.9	7.8	5.7	6.8	5.9	8.0	5.2	2.7	SD

The revertant plate count and the percent survivorship data for Nitrosopiperazine without S9 metabolic activation are presented in Table 16.

Table 16. Plate counts of Nitrosopiperazine (VB plates) and percent survivorship (LB plates) −S9.

Cansolv Nitrosopiperazine −S9 *S. typhimurium* YG7108 Experiments 022613 and 030513

Revertants/Plate VB

Conc. mM	0	1	2.5	5	7.5	10	15	20	EMS = 870
	27	29	25	32	33	33	22	28	EMS = 615
	25	30	22	38	27	32	24	24	
	31	25	25	32	32	25	27	31	
	29	18	23	25	26	24	25	30	
	23	24	24	27	29	20	22	34	
	24	26	23	26	30	28	19	25	
	23	28	20	24	19	25	18	24	
	26	29	19	22	28	23	26	28	
	27	23	26	24	28	30	31	23	
Number	9	9	9	9	9	9	9	9	Number
Average	26.1	25.8	23.0	27.8	28.0	26.7	23.8	27.4	Average
SE	0.9	1.3	0.8	1.7	1.4	1.5	1.4	1.2	SE
SD	2.7	3.8	2.3	5.2	4.1	4.4	4.1	3.7	SD

Cansolv Nitrosopiperazine −S9 *S. typhimurium* YG7108 Experiments 022613

LB Survivorship

Conc. mM	0	1	2.5	5	7.5	10	15	20	
	286	300	324	317	308	299	232	295	
	320	349	292	362	304	314	298	328	
	345	335	364	325	337	296	273	343	
									% Negative Control
	90.2	94.6	102.2	100.0	97.2	94.3	73.2	93.1	
	100.9	110.1	92.1	114.2	95.9	99.1	94.0	103.5	
	108.8	105.7	114.8	102.5	106.3	93.4	86.1	108.2	
Number	3	3	3	3	3	3	3	3	Number
Average	100.0	103.5	103.0	105.6	99.8	95.6	84.4	101.6	Average
SE	6.6	4.6	6.6	4.4	3.3	1.8	6.1	4.5	SE
SD	9.3	8.0	11.4	7.6	5.7	3.0	10.5	7.7	SD

4.4.2 Nitrosopiperazine Concentration-Response Curves

The concentration-response curves for the mutagenicity and cytotoxicity of Nitrosopiperazine in the *S. typhimurium* strain YG7108 microplate suspension assay are presented in Figure 9. The top panel illustrates the cytotoxicity (LB plate experiments) for Nitrosopiperazine with S9 metabolic activation (open squares) and without S9 activation (filled squares). The bottom panel represents the mutagenicity of Nitrosopiperazine with S9 activation (open circles) and without S9 activation (filled circles).

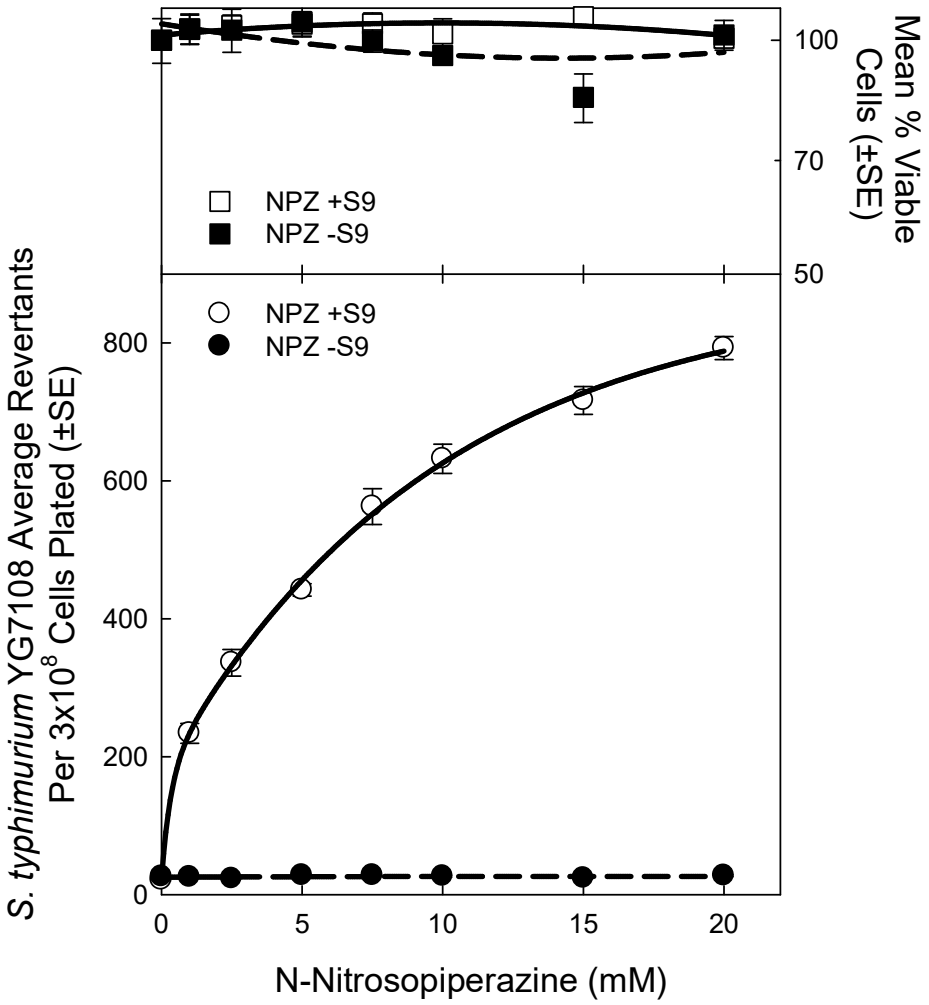

Figure 9. Nitrosopiperazine concentration-response curves for cytotoxicity (upper panel) and mutagenicity (lower panel) in *S. typhimurium* strain YG7108 with and without S9 metabolic activation.

4.4.3 Statistical Analyses of the Mutagenicity Data for Nitrosopiperazine

An ANOVA test statistic for the mutagenicity data of Nitrosopiperazine with S9 metabolic activation was conducted and the results are presented in Table 17.

Table 17. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Nitrosopiperazine +S9

Data source: Data 7 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
NPZ +S9 0	12	0	21.583	5.696	1.644
+S9 1	9	0	239.48	45.214	15.138
+S9 2.5	9	0	336.33	57.881	19.294
+S9 5	9	0	441.88	26.746	8.915
+S9 7.5	11	0	562.72	86.315	26.025
+S9 10	11	0	632.00	70.590	21.284
+S9 15	11	0	716.63	66.832	20.150
+S9 20	11	0	792.63	55.102	16.614

Source of Variation	DF	SS	MS	F	P
Between Groups	7	5279880.6	754268.6	228.7	<0.001
Residual	75	247249.96	3296.6		
Total	82	5527130.6			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
NPZ +S9 0 vs. +S9 20	771.053	32.171	<0.001	0.007	Yes
NPZ +S9 0 vs. +S9 15	695.053	29.000	<0.001	0.009	Yes
NPZ +S9 0 vs. +S9 10	610.417	25.469	<0.001	0.010	Yes
NPZ +S9 0 vs. +S9 7.5	541.144	22.579	<0.001	0.013	Yes
NPZ +S9 0 vs. +S9 5	420.306	16.601	<0.001	0.017	Yes
NPZ +S9 0 vs. +S9 2.5	314.750	12.432	<0.001	0.025	Yes
NPZ +S9 0 vs. +S9 1	212.306	8.385	<0.001	0.050	Yes

An ANOVA test statistic for the mutagenicity data of Nitrosopiperazine without S9 metabolic activation was conducted and the results are presented in Table 18.

Table 18. One Way Analysis of Variance *Salmonella typhimurium* YG7108 Mutagenicity Analyses Nitrosopiperazine -S9

Data source: Data 7 in Shell Cansolv Project

Group Name	N	Missing	Mean	Std Dev	SEM
0 -S9	9	0	26.111	2.713	0.904
1 -S9	9	0	25.822	3.801	1.267
2.5 -S9	9	0	23.000	2.345	0.782
5 -S9	9	0	27.778	5.167	1.722
7.5 -S9	9	0	28.000	4.062	1.354
10 -S9	9	0	26.667	4.359	1.453
15 -S9	9	0	23.778	4.055	1.352
20 -S9	9	0	27.444	3.745	1.248

Source of Variation	DF	SS	MS	F	P
Between Groups	7	218.2	31.1	2.079	0.059
Residual	64	959.7	14.9		
Total	71	1178.0			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.059).

4.4.4 Interpretation of Nitrosopiperazine Data

Nitrosopiperazine is a promutagen that requires mammalian microsomal activation (S9) to generate an ultimate alkylating mutagen that induced base-pair substitution mutations in *S. typhimurium* strain YG7108. This result is clearly demonstrated in the comparison of the Nitrosopiperazine concentration-response curves of Nitrosopiperazine +S9 versus Nitrosopiperazine –S9 (Figure 9 lower panel and Table 17 versus Table 18). Note that throughout the concentration range of 1 to 20 mM, Nitrosopiperazine with or without S9 was not cytotoxic to *S. typhimurium* (Figure 9 upper panel and Tables 15 and 16, LB plate data). Thus we did not have to adjust for cytotoxicity in the calculation of the mutagenic potency of Nitrosopiperazine. The Nitrosopiperazine mutagenic potency values were derived from all concentrations analyzed in the concentration-response curve. This metric provides a measure of mutagenic activity as induced mutants per micromole (µmol) of Nitrosopiperazine. The calculated mutagenic potency of Nitrosopiperazine with S9 metabolic activation was 914.7 revertants/µmol. The mutagenic potency of Nitrosopiperazine without S9 activation was 0.

5. Summary and Conclusions

In this project we employed a highly sensitive *S. typhimurium* strain, YG7108, that was developed to evaluate the mutagenicity of alkylating agents such as nitrosamines to study the mutagenicity of two carcinogenic nitrosamines, Nitrosodimethylamine and Nitrosopiperazine (Cansolv-N3). The U.S. EPA risk level for increased cancer of 1/1000000 via oral consumption of NDMA is 7.0×10^{-4} µg/L for drinking water [16]. The Carcinogenic Potency Database (CPDB) was developed at the University of California, Berkeley, and Lawrence Berkeley Laboratory and is a standardized analysis of animal cancer tests. The primary CPDB metric is the TD_{50} value which is the "dose-rate in mg/kg body wt/day which, if administered chronically for the standard lifespan of the species, will halve the probability of remaining tumorless throughout that period" [17]. Both NDMA and Nitrosopiperazine (Cansolv-N3) are listed in the Carcinogenic Potency Database with TD_{50} values as 0.0959 and 8.78, respectively. Based on the TD_{50} values, NDMA is approximately 91.6 times more carcinogenic than Nitrosopiperazine (Cansolv-N3). NDMA is a potent promutagenic nitrosamine (Figure 4) while Nitrosopiperazine is also a promutagen that requires metabolic activation; it expressed less mutagenic activity than NDMA (Figure 9). Comparing the mutagenic potencies for NDMA and Nitrosopiperazine (Cansolv-N3) derived from this study, the former nitrosamine is approximately 52× more mutagenic (Table 19).

Table 19. Comparative mutagenicity of Cansolv nitrosamines in *S. typhimurium* strain YG7108 with and without S9 metabolic activation

		Salmonella typhimurium YG7108 Reversion Assay			
Compound	CASN[a]	Concentration Range	S9[b]	Lowest Mutagenic Conc. [c]	Mutagenic Potency (rev/µmol)[d]
NDMA	62-75-9	100-1000 µM	−S9	Negative	Negative
NDMA	62-75-9	5-1000 µM	+S9	10 µM	47,694.7
Nitrosopiperazine	5632-47-3	1-20 mM	−S9	Negative	Negative
Nitrosopiperazine	5632-47-3	1-20 mM	+S9	1 mM	914.7
Nitroso-HEP	48121-20-6	1-20 mM	−S9	Negative	Negative
Nitroso-HEP	48121-20-6	1-20 mM	+S9	5 mM	22.7
Cansolv-N2	-	1-20 mM	−S9	2.5 mM	53.8
Cansolv-N2	-	1-20 mM	+S9	7.5 mM	13.1

[a] American Chemical Society chemical abstract service number. [b] Aroclor-induced hepatic microsomal mixture (MolTox Inc.). [c] The lowest nitrosamine concentration that induced a statistical increase over the concurrent negative control as defined by an ANOVA test statistic. [d] The mutagenic potency was the average induced revertants per µmol calculated from the concentration-response curve. Higher mutagenic potency values denote greater mutagenicity.

For the Cansolv nitrosamines, Nitroso-HEP (Cansolv-N1) was 4.8×10^{-4} times as mutagenic as NDMA while Cansolv-N2 with and without S9 activation were 2.7×10^{-4} and 1.1×10^{-3} times as mutagenic as NDMA, respectively (Table 19). With respect to the mutagenicity of

Nitrosopiperazine (Cansolv-N3), Nitroso-HEP and Cansolv-N2 were 0.02 and 0.06 times as mutagenic.

In comparison to other environmental nitrosamines, such as those found in drinking water, we found that Nitroso-HEP was 0.003×, 0.002×, or 0.02× as mutagenic as Nitrosomorpholine, Nitrosopiperidine or Nitrosopyrrolidine, respectively. Similarly, for Cansolv-N2 its mutagenicity was 0.008×, 0.006× or 0.05× that of Nitrosomorpholine, Nitrosopiperidine or Nitrosopyrrolidine, respectively (Table 20). We conclude that Nitroso-HEP and Cansolv-N2 are very weak mutagens.

Table 20. Comparison of the mutagenic potency of project nitrosamines with selected nitrosamines found in drinking water

Nitrosamine	CASN	Lowest Mutagenic Concentration (mM)	Mutagenic Potency (rev/µmol)	Reference
N-Nitrosodimethylamine	62-75-9	0.010	47,694.7	this study
1-Nitrosopiperazine	5632-47-3	1.0	914.7	this study
1-Nitroso-(4-hydroxyethyl)-piperazine	48121-20-6	5.0	22.7	this study
Cansol-N2	-	2.5	53.8	this study
N-Nitrosomorpholine	59-89-2	0.5	6724	[9]
N-Nitrosopiperidine	100-75-4	0.1	9588	[9]
N-Nitrosopyrrolidine	930-55-2	0.75	997	[9]

The results from this study are the following.

1. NDMA is an exceedingly potent mutagen after mammalian microsomal metabolism.
2. The Nitrosopiperazine (Cansolv-N3) sample provided was mutagenic and the agent required mammalian microsomal metabolic activation. Without S9 activation Nitrosopiperazine was not mutagenic.
3. Nitroso-HEP (Cansolv N-1) was an exceedingly weak mutagenic agent that required S9 metabolic activation. Nitroso-HEP was not directly mutagenic.
4. Cansolv-N3 was a direct, albeit very weak mutagen and S9 metabolic activation detoxified the nitrosamine and reduced its mutagenic activity.
5. When compared to environmental nitrosamines such as nitrosamine drinking water disinfection by-products, both Nitroso-HEP and Cansolv-N2 were considered very weak mutagens.

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