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Mutagenicity and Chemistry of *N*-Nitroso-*N*-(*p*-substituted-benzyl)methylamines†

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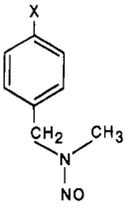
The relative mutagenicities of *N*-nitroso-*N*-(*p*-substituted-benzyl)methylamines in *Salmonella typhimurium* TA 1535 were tested in order to determine whether biological activity is affected by the electron density at a nitrosamine α carbon. The order of potency was as follows: X = Cl > CN > Br > NO_2 > H > CH_3O > CH_3 > F ≫ COOH. No direct correlation was apparent, nor was there any obvious correlation between biological activity and the extent of base-catalyzed hydrogen–deuterium exchange at the α carbons.

There have been numerous attempts to identify the structural features of nitrosamines that determine carcinogenic potency. The relative carcinogenicity of a series of related compounds has generally been measured by long-term feeding studies in experimental animals. A few studies have sought a relationship between this observed carcinogenicity and physical or chemical properties. Often this property has been a form of liposolubility that would be expected to affect in vivo transport. The liposolubility has been measured either in terms of the classical octanol–water partition coefficient[1] or ether–water partitioning,[2] or simply the number of carbon atoms in the molecule.[3] More sophisticated mathematical treatments have recently been developed that attempt to correlate the carcinogenicity of large groups of nitrosamines of differing organotropy with physical properties.[4–6] It has been generally hypothesized that a nitrosamine must undergo a chemical reaction, i.e., metabolic activation, at an α carbon before it can produce its carcinogenic effect. Only a few studies have examined relative carcinogenic potency in terms of chemical reactivity. A previous study examined the relative ease with which an α proton could be removed by base, i.e., the ease with which carbanions are formed, in a variety of nitrosamines.[7]

A related area, which has not received much attention, is the effect of systematically varying the electron density at the α position. A priori, this should affect the reactivity of the nitrosamine's reactive intermediate, whatever its nature (carbonium ion, carbanion, or free radical) and thus, presumably, the carcinogenicity.

To test this hypothesis, we prepared a series of *N*-nitroso-*N*-(*p*-substituted-benzyl)methylamines (**1–8**). We sought to relate chemical and biological reactivities to established physical parameters (in a semiquantitative manner) using a series of substituents whose electronic effects and abilities to transmit these effects have been firmly established.

The effect of the different substituents on mutagenicity was evaluated with the *Salmonella* microsome mutagenicity assay developed by Ames[8] as modified by Andrews et al.[9] The mutagenicity data were then compared with several aspects of the chemical behavior of these compounds.



1 X = H **5** X = Cl
2 X = CH_3 **6** X = CN
3 X = CH_3O **7** X = NO_2
4 X = F **8** X = COOH

Results

None of these *N*-nitroso-*N*-(*p*-substituted-benzyl)-methylamines was mutagenic without activation. With activation by the hamster S9 mix, a wide range of mutagenicities was observed. The data from a representative mutagenicity dose–response assay are given in Table II.

Based on the slopes of the dose–response curves (Figure 1), the order of mutagenic potency of the test compounds was as follows: *p*-chloro > *p*-cyano > *p*-nitro > *p*-hydrogen > *p*-methoxy > *p*-fluoro > *p*-methyl > *p*-carboxy. The *p*-chloro and *p*-cyano derivatives were the most mutagenic. The *p*-nitro and *p*-hydrogen compounds had about the same mutagenicity as did the compounds with *p*-methoxy and *p*-fluoro substituents. The *p*-methyl compound was mutagenic only at 500 and 1000 μg/plate doses. The *p*-carboxylic acid was not mutagenic under these test conditions.

The pseudo-first-order rates for base-catalyzed hydrogen–deuterium exchange of the α protons of most of the

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Table I. Pseudo-First-Order Rate Constants of Hydrogen–Deuterium Exchange in *N*-Nitroso-*N*-(*p*-substituted-benzyl)methylamines

X	$k_{CH_2} \times 10^4$ $(r)^a$	rel k_{CH_2}	$k_{CH_3} \times 10^4$ $(r)^a$	rel k_{CH_3}	Hammett σ
CH_3O	0.78 (0.99)	0.61	0.78 (0.98)	0.89	−0.27
CH_3	1.00 (0.99)	0.78	1.25 (0.99)	1.42	−0.17
H	1.28 (0.99)	1	0.88 (0.99)	1	0.0
F	1.54 (0.99)	1.20	1.04 (0.99)	1.18	0.06
Cl	2.55 (0.96)	1.99	1.23 (0.99)	1.40	0.23

[a] sec^{-1}; r = correlation coefficient from least-squares calculation of rate constant data.

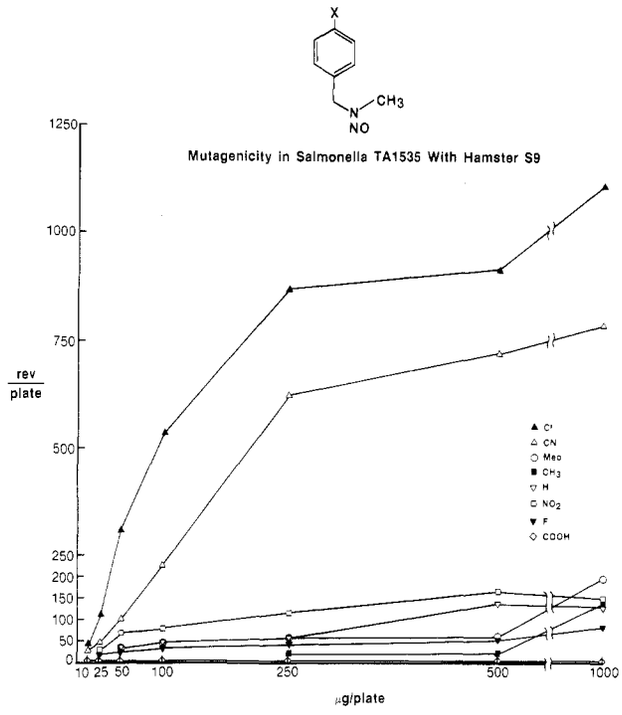

Figure 1. Dose–response of the mutagenicity of *N*-nitroso-*N*-(*p*-substituted-benzyl)methylamines toward *Salmonella* TA 1535 in the presence of hamster S9 mix.

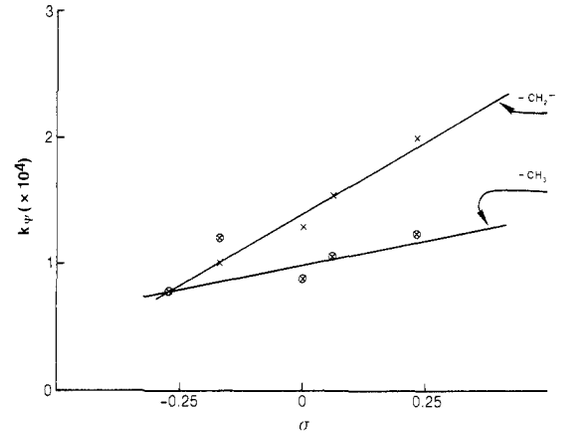

Figure 2. The variation in the rates of base-catalyzed hydrogen–deuterium exchange of *N*-nitroso-*N*-(*p*-substituted-benzyl)methylamines with σ.

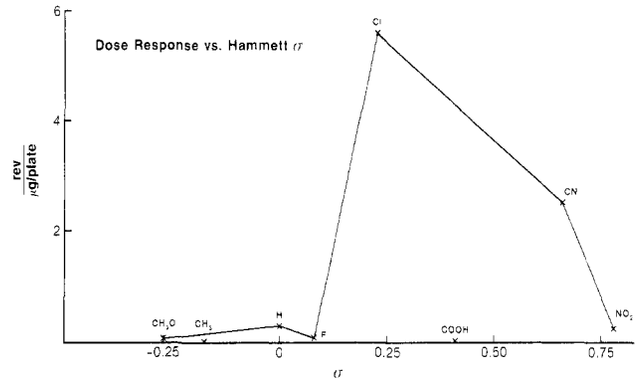

Figure 3. The variation of the slope of the dose–response curves of the mutagenicity of *N*-nitroso-*N*-(*p*-substituted-benzyl)-methylamines with σ.

compounds were measured by NMR spectroscopy. It was not possible to measure rates of exchange for the cyano (**6**) or nitro (**7**) derivatives. The NMR spectra of the reaction mixture showed that the nitrile function was hydrolyzed during the course of the exchange. Addition of 40% $NaOD/D_2O$ to the solution of the nitro compound (**7**) in D_2O/CD_3OD immediately produced a bright red precipitate, characteristic of a Meisenheimer complex. Data for *p*-COOH are not given, since under the exchange conditions the species is *p*-COO^-, which has different electronic properties.

Data for the remaining compounds for which kinetic rates were obtained can be found in Table I. As one would anticipate, the rates of exchange for the methylene hydrogens reflect the different inductive effects of the para substituents more strongly than do the rates for exchange of the methyl hydrogens (Figure 2).

Discussion

The intent of this study of closely related nitrosamines was to seek a relationship between their physicochemical properties and the effect they have on biological systems. The Ames assay was used, since it was reasonable to expect that any systematic variation in biological response would be expressed through differences in mutagenicity.

The electronic properties of aromatic substituents have been extensively studied in a vast number of chemical reactions via Hammett linear free energy relationships. If the biological activity of the nitrosamine requires a chemical reaction at the methylene carbon or at the methyl carbon, then this activity should be affected by changing the substituent. The effect of a para substituent should be manifested most strongly at the benzyl carbon and in a predictable manner. In this instance, we did not find a direct linear relationship between the electronic properties of the substituents and the mutagenicity induced by the compounds in *Salmonella*.

There does appear to be a peak mutagenicity induced by the *p*-chloro compound (Figure 3). The electron-donating substituents (*p*-CH_3 and *p*-CH_3O) and the weakly electron-withdrawing substituant (*p*-F) were only weakly mutagenic, as was the compound with the strongest electron-withdrawing substituent (*p*-NO_2). The *p*-cyano derivative was intermediate in activity. The lack of reactivity of the *p*-COOH derivative may be due to the polar nature of the substituent preventing it from entering the cell. The lack of mutagenicity of the *p*-CH_3 compound (**2**) at low doses could be due to its conversion to the inactive *p*-COOH derivative, as has been shown in vivo.[10]

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Table II. Dose-Responses of *N*-Nitroso-*N*-(*p*-substituted-benzyl)methylamines in Strain TA1535 with and without Hamster S_9 (3 mg/plate)

				number of revertants per plate [a]																			
				dose																			
				cells only		50 μg		1 μg		2.5 μg		5 μg		10 μg		25 μg		50 μg		100 μg		250 μg		500 μg		1000 μg	
no.	X	σ [b]	S_9:	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
3	CH_3O	−0.27		11	8	11	11	10	13	21	19	15	10	24	14	18	17	19	*26*	15	*44*	20	*56*	14	*62*	12	*199*
2	CH_3	−0.17		22	9	32	10	20	12	21	11	22	16	15	9	19	14	23	15	13	19	18	20	20	23	17	*139*
1	H	0.0		14	20	10	16	14	21	18	24	15	24	12	12	18	24	24	32	17	*44*	23	*58*	15	*136*	15	*134*
4	F	0.06		14	6	10	7	15	8	16	7	12	10	13	8	13	17	10	23	18	33	17	39	21	*50*	16	77
5	Cl	0.23		13	19	12	10	13	9	18	15	20	17	12	*32*	7	*109*	17	*307*	10	*531*	13	*870*	10	*913*	6	*1109*
8	COOH	0.27		11	6	8	5	12	8	12	6	13	6	12	3	6	4	10	7	13	10	15	7	10	8	14	9
6	CN	0.66		21	13	16	8	25	14	26	19	29	*30*	23	*32*	29	*45*	27	*101*	11	*222*	16	*623*	9	*718*	14	*789*
7	NO_2	0.78		13	9	10	6	17	11	18	15	14	11	10	12	13	*27*	8	*50*	12	*72*	6	*115*	11	*149*	12	*145*

[a] Mean values based on duplicate plates. Italic numbers indicate that the number of revertants is significant (greater than twice the historical control mean or twice the current control mean, whichever was greater). The historical means ($N = 18$) were as follows. Cells only: without S9, 15 ± 7; with S9, 12 ± 5; Me_2SO: without S9, 12 ± 5; with S9, 9 ± 3. [b] Hammett constants.

One possible activation reaction of a nitrosamine is the base-catalyzed abstraction of an α proton. The reactive intermediate thus produced could be hydroxylated or could react directly with a suitable macromolecule.[14] To test this hypothesis in conjunction with the mutagenicity assay, we measured the rates of base-catalyzed hydrogen–deuterium exchange for those *N*-nitroso-*N*-(*p*-substituted-benzyl)methylamines that were not subject to hydrolysis (**6**, X = CN) or nucleophilic substitution (**7**, X = NO_2). The rates were in accord with expectations based on Hammett σ constants (Figure 2). Exchange of the methylene protons of the *p*-chloro compound was fastest, and exchange of the methylene and methyl protons of the *p*-methoxy derivative was slowest. While the rate constants for the exchange of the methylene protons of all the compounds varied over a range of 3.3, the rate constants for the methyl protons varied only over a range of 1.6 times, a reflection of the decrease of the electronic effects through two additional bonds.

A recent study by Kroeger-Koepke et al.[15] of the mutagenicity of para-substituted nitrosomethylanilines did not find any obvious correlation between biological effect and electronic properties of the substituent. Their data and ours suggest that the mutagenicity of these nitroso compounds is not due solely to the electronic properties of para substituents. As the data in Figure 3 show, however, compounds with electron-withdrawing substituents may, in fact, be better mutagens, and there may, in fact, be an optimal amount of electron withdrawal for mutagenicity. On the other hand, there is no direct correlation between the exchange rates and mutagenicity. Clearly, other factors are also important. These may include transport, steric bulk, etc. of the parent compound or of activated metabolites.

Experimental Section

Analytical HPLC was performed on a μ-Bondapak C_{18} column from Waters Associates installed in a Waters Associates Model 440 HPLC. Preparative HPLC was done with two silica gel cartridges in a Waters Associates Prep 500 LC. IR spectra were recorded as films or KBr pellets on a Perkin-Elmer Model 297 IR spectrometer. Mass spectra were recorded on a Finnegan Model 3300 mass spectrometer. Kinetic experiments were run by following the disappearance of the methylene and methyl protons in a Varian Associates XL-100 NMR spectrometer equipped with a Nicolet 1080 computer. (The experiments with the *p*-methoxy and *p*-fluoro derivatives were performed with a Nicolet 300-MHz spectrometer equipped with a Nicolet 1280 computer.)

Chemicals. *N*-Nitroso-*N*-benzylmethylamine (**1**) was kindly provided by Dr. J. G. Farrelly of this facility. Substituted α-halotoluenes and the *p*-methoxy- and *p*-fluorobenzylamines were from Aldrich Chemical Co.

***N*-Nitroso-*N*-(*p*-cyanobenzyl)methylamine (6).** A solution of α-bromo-*p*-toluonitrile (10.0 g, 0.051 mol) in methanol (150 mL) was stirred at 0 °C while methylamine was bubbled through for 1.5 h. The reaction mixture was then boiled under reflux for 65 h. The chilled (0 °C) mixture was acidfied to pH 1.3 with 6 N HCl, concentrated in vacuo to 15 mL, and diluted with water (50 mL). This solution was extracted with ether (3 × 50 mL) and then was basified to pH 11 with 20% sodium hydroxide and

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reextracted with ether (3 × 50 mL), which was dried ($MgSO_4$) and evaporated in vacuo to an oil. This oil was nitrosated by stirring with sodium nitrite (24.8 g, 0.36 mol) in 30% acetic acid (100 mL) for 25 h at room temperature. The solution was basified with sodium hydroxide and extracted with chloroform (3 × 50 mL), which was dried (K_2CO_3) and evaporated to an oil (7.5 g). TLC (20% ethyl acetate/hexane) showed a principal spot at R_f 0.11 plus three impurities. The compound was purified by preparative LC (Waters Associates PREP 500) with two silica cartridges and 20% ethyl acetate/hexane as eluent to give the product, a pale yellow oil: 5.45 g (61%); UV (EtOH) λ_{max} 351 nm (ϵ 83); MS, m/z (relative intensity) 175 (M^+, 21.1), 116 (100), 89 (23.4). Anal. ($C_9H_9N_3O$) C, H, N.

***N*-Nitroso-*N*-(*p*-methylbenzyl)methylamine (2).** α-Chloro-*p*-xylene (8.44 g, 0.06 mol) was treated as described above to give the nitrosomethylamine derivative, 7.67 g (78%), which was indicated by GLC to contain no impurities: bp 95–98 °C (0.2 mm); UV (EtOH) λ_{max} 351 nm (ϵ 92). Anal. ($C_9H_{12}N_2O$) C, H, N.

***N*-Nitroso-*N*-(*p*-chlorobenzyl)methylamine (5).** *p*-Chlorobenzyl chloride was converted to the nitrosomethylamine as described above: bp 100–102 °C (0.05 mm); UV (EtOH) λ_{max} 351 nm (ϵ 76). Anal. ($C_8H_9ClN_2O$) C, H, N.

***N*-Nitroso-*N*-(*p*-nitrobenzyl)methylamine (7).** *p*-Nitrobenzyl bromide was converted to the crude nitrosamine as described above and purified by preparative HPLC on silica gel with 20% ethyl acetate/hexane as the eluent. Analytically pure nitrosamine was obtained in 59% yield after chromatography: mp 60–62 °C; MS, m/z (relative intensity) 195 (M^+, 31), 136 (37), 118 (28), 106 (41), 90 (32), 89 (43), 78 (82). Anal. ($C_8H_9N_3O_3$) C, H, N.

A minor byproduct (4.5%) was tentatively identified as *N,N*-bis(*p*-nitrobenzyl)methylamine on the basis of its mass spectrum: mp 102–103 °C; MS, m/z (relative intensity) 302 (8), 301 (M^+, 17), 179 (100), 136 (61), 106 (33).

α-(*N*-Nitroso-*N*-methylamino)-*p*-toluic Acid (8). α-Bromo-*p*-toluic acid (4.3 g, 0.02 mol) was kept with 25% aqueous methylamine (100 mL) in a tightly stoppered flask at room temperature for 5 days. The solution was concentrated at 60 °C in vacuo to a yellow oil. This was redissolved in water (50 mL) and 6 N HCl (10 mL), and the solution was washed with ether (3 × 25 mL). Sodium nitrite (7 g, 0.1 mol) was added to the aqueous solution, which was then stirred for 2 h at room temperature. The white crystalline solid was removed by filtration: yield 2.9 g (74%); mp 185–190 °C. Recrystallization from ethanol/water gave the product as colorless needles: yield 2.1 g; mp 195–197 °C (lit.[10] mp 196 °C); UV (EtOH) λ_{max} 351 nm (ϵ 100).

***N*-Nitroso-*N*-(*p*-methoxybenzyl)methylamine (3).** The *p*-methoxy and *p*-fluoro derivatives could not be prepared satisfactorily by the procedure described above.

p-Methoxybenzylamine (8.23 g, 0.06 mol) was boiled under reflux with ethyl formate (25 mL) for 48 h. The reaction mixture was stirred with 5% Na_2CO_3 (75 mL) for 1 h and extracted with ether (4 × 25 mL). The ether solution was washed with 2 N HCl (1 × 15 mL) and brine (1 × 10 mL) and dried (K_2CO_3). Crystallization from the ether solution gave the formamide as a white crystalline solid: yield 5.2 g (53%); mp 81–82.5 °C; MS, m/z (relative intensity) 165 (M^+, 52), 136 (67), 121 (100), 109 (33), 78 (35), 77 (58).

The formamide (5.0 g, 0.03 mol) was dissolved in dry tetrahydrofuran (25 mL) and reduced by $LiAlH_4$ (2.0 g, 0.05 mol) in the usual way to give the corresponding methylbenzylamine as a colorless liquid: yield 4.23 g (93%); MS, m/z (relative intensity) 151 (M^+, 28), 150 (52), 122 (20), 121 (100), 120 (27), 109 (12), 91 (12), 78 (18), 72 (22); IR (film) no carbonyl.

The amine was nitrosated in the usual way, and the *N*-nitroso-*N*-(*p*-methoxybenzyl)methylamine was obtained as a yellow oil: bp 122–124 °C (0.3 mm); MS, m/z (relative intensity) 180 (M^+, 20), 150 (15), 148 (16), 135 (44), 122 (63), 121 (100), 120 (42), 106 (19), 92 (18), 91 (32), 78 (60), 77 (63), 65 (28), 63 (26), 41 (39). Anal. ($C_9H_{12}N_2O_2$) C, H, N.

***N*-Nitroso-*N*-(*p*-fluorobenzyl)methylamine (4).** Using the procedure described above, we obtained the formamide of *p*-fluorobenzylamine as a colorless solid after crystallization from ether: yield 6.6 g (72%); mp 80–82 °C.

Reduction with $LiAlH_4$ gave *N*-(*p*-fluorobenzyl)methylamine in 78% yield: MS, m/z (relative intensity) 139 (M^+, 48), 138 (83), 119 (31), 118 (13), 110 (13), 109 (100), 83 (28); IR (film) no carbonyl.

Nitrosation gave a yellow liquid: bp 97–98 °C (0.4 mm); MS, m/z (relative intensity) 168 (M^+, 6), 136 (4), 123 (5), 122 (9), 110 (10), 109 (100), 83 (15), 75 (7), 63 (6), 58 (7), 41 (25). Anal. ($C_8H_9FN_2O$) C, H, N.

Hydrogen–Deuterium Exchange Studies. Approximately 30 mg of the nitroso compound was dissolved in CD_3OD (0.50 mL) and D_2O (0.25 mL). At initial time, 40% NaOD/D_2O (0.25 mL) was added, and the NMR sample tube was throughly shaken and then placed in the probe of the NMR spectrometer (50 °C). Kinetic data points were taken every 10 min under computer control by accumulating 8 FID pulses (16 pulses for the *p*-methoxy and *p*-fluoro derivatives). The spectra were Fourier transformed, integrated, and plotted. The areas of the exchanging hydrogens were normalized to the area of the nonexchanging aromatic protons, and the rates of exchange were obtained from pseudo-first-order plots of the data [ln (a/x) vs. t]. The slopes (rate constants) were calculated by a least-squares fit (Table I).

Mutagenicity Studies. The mutagenicity of the nitrosamines was assessed with *Salmonella* tester strain TA 1535, which detects base-pair substitution mutations, and hamster Aroclor 1254-induced S9 mix instead of rat S9, since nitroso compounds have been reported to be more sensitive to hamster S9 activation.[11-13] There were at least two independent dose–resonse studies with duplicate plates over a 10 point range of 1–1000 μg/plate. The plate incorporation test was used throughout. Plates were counted by using a hand-held tally. The number of revertants above the background considered significant was established at twice the value of the historical control mean or twice the value of the current control mean, whichever was greater.

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Registry No. **1**, 937-40-6; **2**, 62783-50-0; **3**, 84174-20-9; **4**, 84174-21-0; **5**, 84174-22-1; **6**, 84174-23-2; **7**, 84174-24-3; **8**, 72782-13-9; α-bromo-*p*-toluonitrile, 17201-43-3; α-chloro-*p*-xylene, 104-82-5; *p*-chlorobenzyl chloride, 104-83-6; α-bromo-*p*-toluic acid, 6232-88-8; *p*-methoxybenzylamine, 2393-23-9; ethyl formate, 109-94-4; *N*-(*p*-methoxybenzyl)formamide, 17061-63-1; *N*-(*p*-methoxybenzyl)methylamine, 702-24-9; methylamine, 74-89-5; *p*-nitrobenzyl bromide, 100-11-8; *N*-(*p*-fluorobenzyl)formamide, 84174-25-4; *N*-(*p*-fluorobenzyl)methylamine, 405-66-3.