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Mutagenicity and Toxicity of Carcinogenic and Other Hydrazine Derivatives: Correlation Between Toxic Potency in Animals and Toxic Potency in *Salmonella typhimurium* TA1538

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Eleven hydrazine derivatives and an aromatic amine were examined for mutagenicity and toxicity to *Salmonella typhimurium*. Phenylhydrazine, 2-nitrophenylhydrazine, 4-nitrophenylhydrazine, 2,4-dinitrophenylhydrazine, *p*-tolylhydrazine, and 4-nitroaniline were found to be frameshift mutagens (strain TA1538). Benzylhydrazine, *m*-hydroxybenzylhydrazine, *p*-hydrazinobenzoic acid, L-tyrosine hydrazide, *p*-aminobenzoyl hydrazide, and isoniazid were not mutagenic. All chemicals were toxic to strain TA1538. A qualitative correlation was found between the pK of the compounds and their mutagenicity. Relative toxicities of hydrazines to bacteria were found to be closely correlated with the relative toxicities of the same compounds in animals. Described herein is a methodology for the rapid prescreening of chemicals which may be used as drugs for those with a high benefit/risk ratio.

Hydrazines and hydrazides are widely used in industry, agriculture, and medicine (8, 11). The alarming fact that all 38 different hydrazines tested so far in animals were found to be carcinogenic (4, 7, 20, 26) has prompted research concerning the detection of genetic and DNA-damaging activity of hydrazines in a number of short-term assays. Many of those compounds were found to be genetically active (8). Of particular interest are those hydrazine derivatives which are used in medicine: phenylhydrazine (PH), a hemolytic agent (23), is carcinogenic (19, 26), a bacterial mutagen, and a DNA-damaging agent (19); procarbazine [*N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide], an antitumor agent (16), is carcinogenic (13, 14), mutagenic (5, 18, 19), and DNA damaging in vivo; the antidepressant and antituberculotic drug isoniazid (isonicotinic acid hydrazide; INH) (11) is a carcinogen (19, 29), a mutagen, and DNA damaging (9, 19, 34); the antidepressants isocarboxazid, nialamide, and phenelzine (11) are mutagens (19) and DNA damaging (9, 19). Many other hydrazine derivatives are mutagens or DNA-attacking agents (19, 21, 25, 35).

A number of naturally occurring hydrazines, mainly secondary mushroom metabolites (e.g., mucotoxins produced by *Gyromytra*), are carcinogens and mutagens (27, 32, 33; B. Toth, Fed. Proc. 39:884, 1980). Recent experiments have demonstrated that β-*N*[γ-L-(4)-glutamyl]-4-hydroxymethylphenylhydrazine (agaritin), a secondary metabolite of several species of the edible mushroom *Agaricus*, may be converted to carcinogenic molecules (12, 26, 28, 30, 31).

The fact that many hydrazines were tested for carcinogenicity by one group of workers, using a single experimental protocol (26), has facilitated the research of others (19) who have clearly demonstrated the quantitative relationship between the carcinogenic and DNA-damaging potencies of 16 hydrazine derivatives. Although the degree of mutagenicity in bacteria is not quantitatively correlated with the carcinogenic potency, the Ames system is highly sensitive in detecting "positive" hydrazines (4, 19). There are, however, some inconsistencies between positive activity in the Ames system and carcinogenicity. The weak carcinogen INH is not mutagenic in the hands of most laboratories using the classical Ames method (plate incorporation technique), but was shown to be mutagenic when the modification of the host-mediated assay was employed (5).

All of the above has prompted us to use several aromatic hydrazines and related molecules as model compounds to test how relatively small changes in the protocol of a mutagenesis assay affect the mutagenicity. We were also interested in finding conditions at which the toxicity to the cells can be quantitated and meaningfully interpreted due to the following.

In a survey of various hydrazine derivatives as possible cytotoxic agents currently being car-

ried out at our laboratory, it was desirable to screen quickly for the most toxic and least mutagenic hydrazine derivative. The criteria for the desirable properties were: (i) toxicity should not be quantitatively related to mutagenicity, because if killing and mutagenesis are closely related, it may mean that cell death may occur due to the induction of lethal mutations; (ii) if there is some mutagenic activity, it should be weak and should not be increased by microsomal metabolism; (iii) toxicity should remain constant, or increase as a function of microsomal metabolism. The criteria for undesirable properties were the reverse of the above.

In this work, we describe a rapid methodology which, as a first approach to screening compounds as potential drugs, yields results which may be helpful in deciding which compound has the best benefit/risk ratio and should be further tested in animals.

MATERIALS AND METHODS

Bacterial strains. *Salmonella typhimurium* strains TA1535, TA100, TA1537, TA1538, and TA98 were kindly provided by B. N. Ames, University of California, Berkeley.

Chemicals. PH, reagent grade, was from Merck & Co., Darmstadt. 2-Nitrophenyl hydrazine (2NPH) (>98% pure), 4-nitrophenylhydrazine (4NPH) (>98% pure), 2,4-dinitrophenylhydrazine (2,4DNPH) (99% pure), *p*-tolylhydrazine (TH) (99% pure), *p*-hydrazinobenzoic acid (HBA) (>98% pure), and 4-nitroaniline (4NA) (>99% pure) were from Fluka AG, Buchs, Switzerland. Benzylhydrazine dihydrochloride (BH) (99% pure), *m*-hydroxybenzylhydrazine dihydrochloride (MHBH) (99% pure), and *p*-aminobenzoyl hydrazide (ABHD) (95% pure) were from Aldrich Chemical Co., Milwaukee, Wis. L-Tyrosine hydrazide (THD) and INH were from Sigma Chemical Co., St. Louis, Mo.

All organic solvents and minerals were from Merck. L-Histidine, NADP, glucose 6-phosphate, and growth media components were from Sigma.

Handling of hydrazine derivatives. All hydrazines were weighed and dissolved in dimethyl sulfoxide immediately before use. Stock solution concentrations (M) were: PH, 0.4; 2NPH, 4.76; 4NPH, 4.34; 2,4DNPH, 1.36; TH, 1.13; BH, 2.13; HBA, 1.97; MHBH, 1.44; ABHD, 2.09; THD, 0.58; INH, 1.63; 4NA, 3.31.

Preparation of microsomes. S9 was prepared from liver homogenates of Aroclor 1254 (Monsanto Co., St. Louis, Mo.)-induced Sprague-Dawley male rats (1) as described (10). Protein concentration was 42 mg/ml (15). Optimal microsome concentration was determined as described (1).

S9 mixture. The S9 mixture used was a modified Ames reaction mixture and contained: 83 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma), pH 7.4 or 8.0; 27.5 mM KCl; 6.8 mM $MgCl_2$; 1.2 mM NADP; 4.1 mM glucose 6-phosphate; 100 mM glucose; 3.5 mg of microsomal protein per ml.

Preparation of bacterial cells. Cells were grown in Luria broth at 37°C for 18 h, diluted in the same medium, and grown until an absorbance reading at 550 nm was 4. Cells were kept at 0°C until used.

Spot tests. Spot tests were carried out by mixing 400 μl of cell culture with 600 μl of S9 mixture. Portions (500 μl) were then mixed with 3.0 ml of top agar and plated onto minimal plates (1). Paper disks (6.25-mm diameter), containing up to 1,000 μg of mutagen in 20 μl of dimethyl sulfoxide, were placed on the plates and incubated at 37°C for 48 h in the dark.

Plate incorporation assays. The mixtures as described for spot tests were used for plate incorporation assays. Hydrazine derivative solutions of various concentrations were added immediately before pouring onto minimal plates (50 μl/ml of reaction mixture). Incubation was as described above.

Mutagenesis and toxicity, liquid preincubation test. Reactions containing 400 μl of cell culture and 600 μl of S9 mixture were distributed in 1-ml portions. Appropriate volumes of mutagen solution in dimethyl sulfoxide were then added, and the reactions were incubated at 37°C with shaking (250 rpm) for 90 min. Samples (20 μl) for viable counts (see below) were withdrawn at zero time and at 90 min. At 90 min, 500-μl samples were mixed with 3.0 ml of top agar and plated as described above. Control reactions contained dimethyl sulfoxide without hydrazines. All experiments were run in duplicate and were repeated twice.

Viable counts. Samples (20 μl) were withdrawn and serially diluted in 0.85% saline. Samples (10 μl) from three dilutions were then plated onto rich (Luria) plates, 6 to 7 drops from each dilution, and the plates were incubated at 37°C for 18 h and then counted.

Determination of toxic potency of hydrazines. Strain TA1538 was exposed in liquid to a series of hydrazine concentrations, and the level of cell survival was determined for each point. Survival curves were then plotted, with survival expressed as the number of lethal hits (−ln of the surviving fraction; see Results) as a function of hydrazine concentration. Since statistical fluctuation around the mean values obtained from the 12 to 14 samples (see above) for each point rarely exceeded 25% of the mean, statistical error bars are not presented in the figures. Linear regression analysis was carried out based on these survival curves, and the correlation coefficients of the regression lines fluctuated around 0.925 ± 0.067 (based on 52 regression lines); each was calculated on the basis of at least three concentration points of every hydrazine tested. The hydrazine concentration at which the survival level corresponding to one lethal hit was extrapolated or interpolated from the regression lines. For the sake of convenience (see below), toxicity was defined as the inverse of hydrazine concentration causing one lethal hit (90 min, 37°C).

Determination of the mutagenic potency of hydrazines. Calculation of mutagenicity was based on data from mutagenesis experiments in liquid culture as described above. Mutant yields (revertants per plate or per milliliter), determined for each point, were based on mean ± standard deviation of duplicate plates and repetition of experiments. Spontaneous mutant yields were subtracted, and the induced mutant yields were plotted versus hydrazine concentrations. A compound was defined as mutagenic when mutant yield in experimental plates was at least twice the yield obtained in control plates. Regression lines

were then calculated from the ascending parts of the dose-response curves. Correlation coefficients of those regression lines fluctuated around 0.926 ± 0.07 (based on 42 regression lines). Mutagenic potency was determined as the net (induced) mutant yield per milliliter at the survival level of one lethal hit, derived from the regression lines of the dose-response curves described above.

Determination of pK values of hydrazines. pK values were determined by (i) potentiometric titrations of aqueous solutions of hydrazines and (ii) spectrophotometric-potentiometric titrations, and were based on plots of pH as a function of log volume acid (base) added and log absorbance at appropriate wavelength as a function of pH, respectively. Calculation of the fraction of non-ionized species was carried out as described (22).

RESULTS

A rapid quantitative method for the determination of toxicity to bacteria is the measurement of the diameter of growth inhibition zones formed by diffusion (spot test). With the proper parameters known, minimal toxic doses at the edge of the growth inhibition zone can be calculated. This method requires the appropriate computer program (2, 3) and is based on the resolution of the human eye. In addition, the diameter is a function of the exponent of drug concentration, and therefore small changes in toxicity are difficult to measure. We therefore decided to determine toxicity in liquid cultures and to derive data for further calculations from survival curves.

In analogy to the determination of 50% lethal dose (LD_{50}) in animals, we have defined the toxicity of hydrazine derivatives in number of lethal hits, one lethal hit being the probability of survival (P_0) in a Poissonian population under conditions at which every organism receives the mean equivalent of one lethal dose: $P_0(1) = e^{-1}$ or $-\ln P_0(1) = 1$. Since the same bacterial strain (TA1538) was used, it may be assumed that the concentration of a certain hydrazine which induces one lethal hit reflects its toxicity. Additionally, relative toxicities can be expressed as the ratio between the respective concentrations of various chemicals at the survival point of one lethal hit at comparable conditions (e.g., type of medium, pH, initial cell concentration, exposure time). For convenience, we have expressed toxicity in "toxicity units," the reciprocal value of the concentration of the chemical (millimolar) which induces one lethal hit.

The toxicity value can now be utilized in the assessment of the mutagenic potential of the same compound. The mutagenic potential was defined as the number of induced mutants (per plate or per milliliter) at the survival level of one lethal hit, since at that point the effective exposure of the cells is standardized. Division of toxicity by mutagenicity yields a number reflecting the potential benefit/risk ratio of the compound. This methodology was applied in the examination of the hydrazines listed in Table 1. Mutagenicity spot tests (30) were carried out with *S. typhimurium* Ames strains TA1535, TA100, TA1537, TA1538, and TA98 with up to 1,000 μg of hydrazine per disk, with and without S9, and strain TA1538 was chosen for further work because it was the most sensitive. Tests with the base-pair substitution-detecting strains, TA1535 and TA100, were negative with or without S9 (data not shown). The dose-response curves of strain TA1538, describing cell killing and mutagenesis, are shown in Fig. 1. Plate incorporation assays yielded results similar to those shown in Fig. 1 (data not shown).

The following compounds were mutagenic to TA1538: PH, 2NPH, 4NPH, 2,4DNPH, TH, and the aromatic amine, 4NA. BH, MHBH, and HBA, as well as all of the hydrazides (ABHD, THD, and INH), were not mutagenic. In all positive cases, the compounds acted as direct mutagens, and microsomal metabolism decreased mutagenicity. These results are in agreement with those of others, who have demonstrated the mutagenicity of PH (19), 4NPH (12), 2,4DNPH (12, 19) and TH (21). To our best knowledge, the mutagenicity of 2NPH and 4NA has not been previously reported. Hydrazides were shown to act as base-pair substitution mutagens rather than frameshift mutagens (19, 35), which may explain the lack of activity with strain TA1538. The toxicity of all hydrazides increased in the presence of S9, whereas that of hydrazine derivatives containing no functional group in addition to the hydrazine moiety (phenyl-, tolyl-, benzyl-) remained essentially unchanged.

Since cells are more permeable to nonpolar or non-ionized molecules, we examined whether the changes in toxicity or mutagenicity observed at different pH values (Fig. 1) were mainly due to the degree of ionization of the hydrazines. pH 7.4 and 8.0 were chosen because of severe growth limitation of the bacterial cells at more extreme pH conditions (data not shown). No pattern of correlation existed between the actual concentration of the non-ionized compound at different pH conditions and its toxic or mutagenic potency. In general, toxicity increased at pH 8 as opposed to pH 7.4, regardless of the pK of the compound.

Since pH 7.4 and the presence of microsomes are the conditions which resemble the in vivo situation more than the other assay conditions, the toxic and mutagenic potency values obtained under these conditions were used for further calculations. Table 2 presents an elaboration of data pertaining to pH 7.4 in the presence of

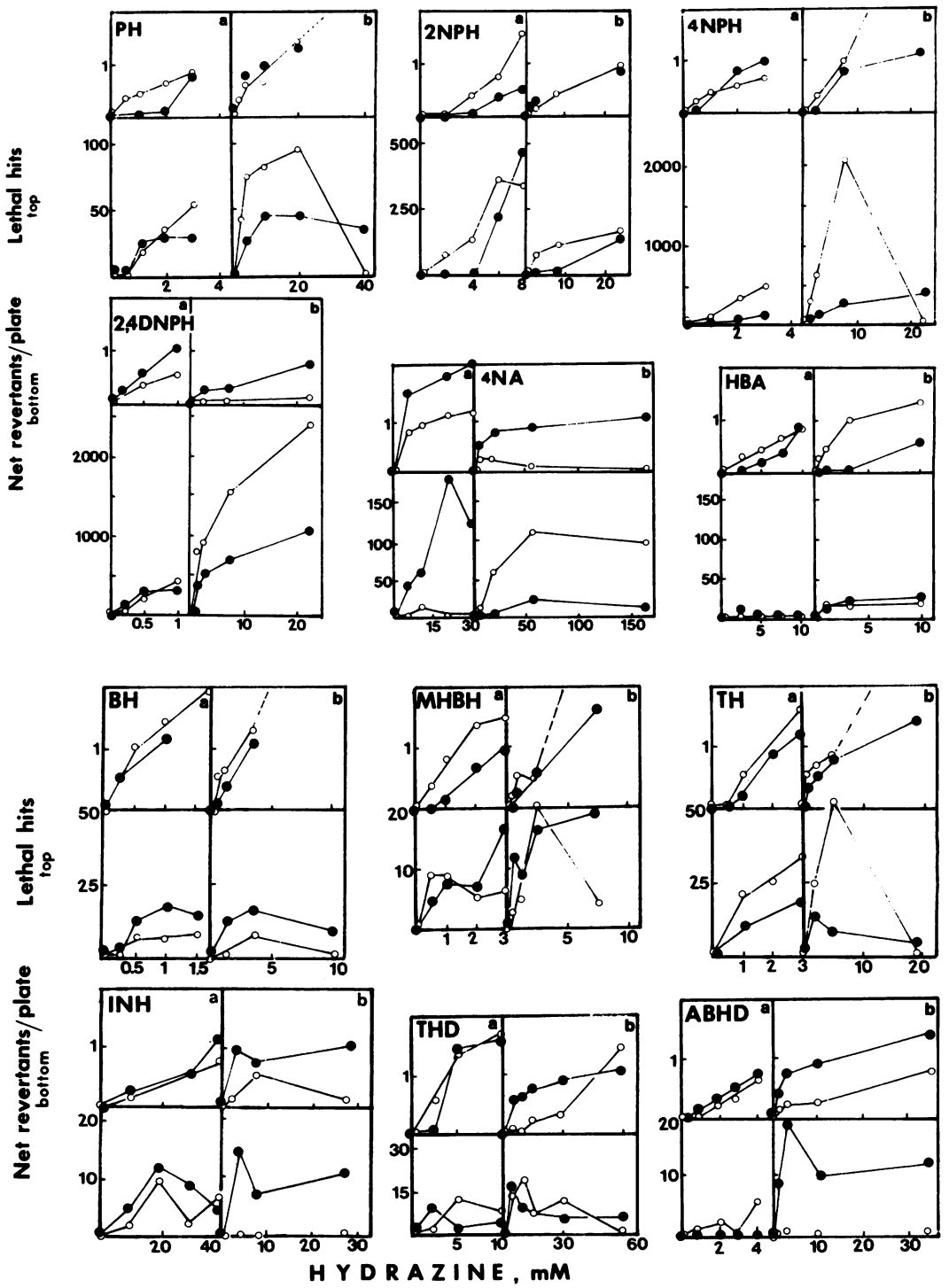
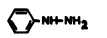
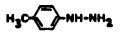
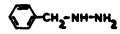
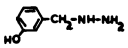
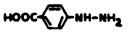
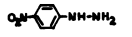
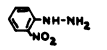
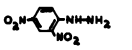
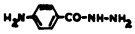
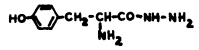
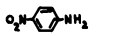


FIG. 1. Mutagenicity of hydrazine derivatives in *S. typhimurium* TA1538. Mutagenesis assays in liquid were carried out as described in text. The figure is based on data presented in Table 2. Dots are mean values. For the sake of visual clarity, standard deviations shown in Table 2 are not illustrated here. For each compound: left sections (a), results obtained at pH 8.0; right sections (b), results obtained at pH 7.4; upper panels, survival curves, expressed in number of lethal hits as a function of dose; lower panels, net (induced) revertants per plate; open circles, absence of microsomes; closed circles, presence of microsomes.

TABLE 1. Hydrazine derivatives used in this study.

Structure	Compound	LD_{50}		pK[a]
		Rat, oral	Mouse, i.p.[b]	
	Phenylhydrazine (PH)	188	170[c]	5.21
	*p*-Tolylhydrazine (TH)	NA[d]	NA	5.4
	Benzylhydrazine (BH)	50[e]	NA	7.12
	*m*-Hydroxybenzylhydrazine (MHBH)	NA	NA	8–8.2
	*p*-Hydrazinobenzoic acid (HBA)	NA	NA	4.48
	4-Nitrophenylhydrazine (4NPH)	NA	250[e]	3.95
	2-Nitrophenylhydrazine (2NPH)	NA	NA	NA
	2,4-Dinitrophenylhydrazine (2,4DNPH)	NA	450[c]	~3.0
	*p*-Aminobenzoyl hydrazide (ABHD)	NA	NA	NA
	L-Tyrosine hydrazide (THD)	NA	NA	9.3
	Isonicotinic acid hydrazide (isoniazid; INH)	142[e]	132[c]	12.2
	4-Nitroaniline (4NA)	750[e]	NA	1.1

[a] Determined as described in the text.
[b] i.p., Intraperitoneal.
[c] Reference 19.
[d] NA, Not available.
[e] Reference 6.

microsomes. It is shown that potent mutagens having low toxicity/mutagenicity ratios can be easily excluded as potential drugs. Thus, BH turned out to be the compound of choice in this series of hydrazines because of its high toxic potency, high toxicity/mutagenicity ratio, and low mutagenicity and because its toxicity was not decreased by metabolism.

Toxic potency in bacterial cultures does not reflect the potency of the same chemical in animals: bacterial systems cannot provide information concerning pharmacokinetics, metabolism by systems other than cytochrome P_{450}, target organs, receptor molecules, etc. We therefore examined whether toxic potency in bacteria was related in any way to toxicity data in experimental animals. The comparison of absolute toxicity in bacteria and in animals must be excluded. However, by arbitrarily defining the toxic potency of a certain compound in bacteria as unity, it is possible to calculate the relative toxicity of other compounds by using a single protocol. Similarly, it is possible to calculate relative toxicity in animals by using LD_{50} data (obtained in the same animal by the same route of administration).

PH was chosen as the standard compound, and Table 2 shows that the relative toxicities of hydrazines in bacteria and in animals were strongly correlated. Figure 2 shows that the regression line obtained for six different hydrazines, which were tested for toxicity in strain TA1538 and for which LD_{50} values were obtained in two closely related sytems (6), has a slope of 1.12 and a correlation coefficient of 0.95. It should be noted that this relationship was independent of the mutagenicity of the compounds in question.

DISCUSSION

We have shown here that several hydrazine derivatives are frameshift mutagens. The mutagenic potency varies as a function of the nature of the chemical substitution of the aromatic ring. Aromatic hydrazines are potent and direct frameshift mutagens; the addition of electron-attracting functional groups such as $—NO_2$ increases mutagenicity. The decrease in pK suggests that protons more easily dissociate from the nitrogen atom since it is more positively charged and thus more electrophilic. The hydrazine derivatives TH (pK = 5.4), PH (pK = 5.2), 4NPH (pK = 3.95), and 2,4DNPH (pK ~ 3.0)

TABLE 2. Toxicity and mutagenicity of hydrazines: comparison between relative toxic potencies in *Salmonella* and in rodent systems[a]

Compound	Bacterial system			Rodent systems			
	Mutagenicity[b]	Toxicity[c]	Relative toxicity	System	Toxicity[d]	Relative toxicity	Benefit/risk ratio[e]
PH	81	0.156	1.00	Rat, oral	0.0053	1.00	1.9
BH	25	0.469	3.00	Rat, oral	0.0200	3.76	18.8
4NPH	710	0.058	0.37	Mouse, i.p.[f]	0.0040	0.75	0.08
2,4DNPH	2,952	0.031	0.20	Mouse, i.p.	0.0022	0.41	0.01
INH	76	0.047	0.30	Rat, oral	0.0070	1.32	0.62
4NA	12	0.047	0.30	Rat, oral	0.0013	0.25	3.92
TH	—	0.151	0.97		NA[g]		High
MHBH	31	0.237	1.52		NA		7.7
HBA	77	0.067	0.43		NA		0.87
2NPH	342	0.032	0.21		NA		0.09
THD	—	0.022	0.14		NA		High
ABHD	—	0.049	0.31		NA		High

[a] The toxic and mutagenic potencies of hydrazines were calculated as described in the text. Values for toxicity and mutagenicity in *Salmonella* were calculated as described in the text. Toxic potencies in rodents were calculated from published LD_{50} values (32, 20) treated as described in the text. The relative toxicity of PH in each system was arbitrarily defined as unity.

[b] Induced revertants per milliliter at one lethal hit (pH 7.4 in the presence of microsomes). —, Negative.

[c] (Hydrazine, millimolar at one lethal hit)$^{-1}$ at pH 7.4 in the presence of rat liver microsomes.

[d] Reciprocal of LD_{50} (milligrams per kilogram).

[e] Ratio between toxic potency and mutagenic potency in *Salmonella*. High, Value could not be determined due to lack of mutagenicity as defined in the text.

[f] i.p., Intraperitoneal.

[g] NA, Data not available.

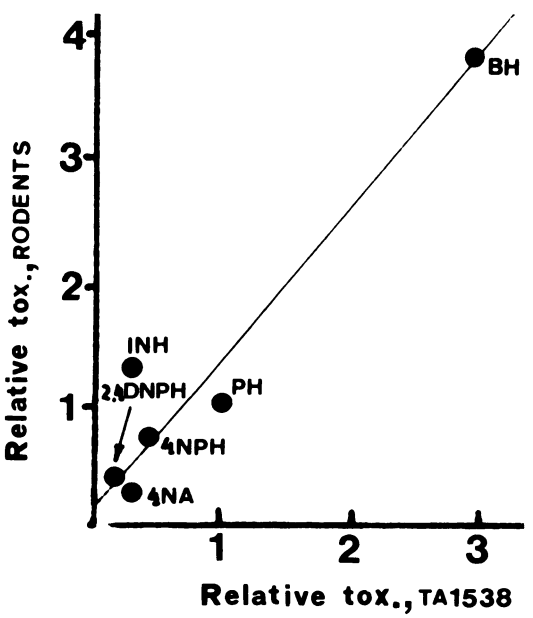


FIG. 2. Correlation between relative toxic potency in animals and relative toxic potency in *S. typhimurium* TA1538 of hydrazine derivatives. The figure is based on relative toxic potencies of hydrazines presented in Table 2. The linear regression line has a slope of 1.12 and a correlation coefficient of 0.950. Abbreviations are defined in the text.

are arranged in the order of increasing mutagenicity. Hydrazinehydrate (pK = 8.07; 7) is less mutagenic than PH (pK = 5.2; 19), and aniline (pK = 4.6) is not known as a mutagen, whereas 4NA (pK = 1.1) is. Addition of a methylene group between the hydrazine and benzene moieties makes the chemical properties of the hydrazine moiety of the resultant compound, BH, resemble those of aliphatic hydrazines and causes an increase in pK of the hydrazine moiety. Thus, PH (pK = 5.2) is mutagenic whereas BH (pK = 8 to 8.2) and MHBH (pK = 7.12) are not.

No correlation was found between mutagenicity of hydrazine derivatives and the concentration of the non-ionized species as a function of pH. The pattern of increased toxicity of almost all compounds at pH 8.0 versus pH 7.4 suggests that the pH effect may be attributed to changes occurring at the cell membrane rather than to the properties of the hydrazines, most of which are protonated at pH 7.4 and 8.0. Increase in medium alkalinity is known to increase the net negative charge of neutral amphoteric phospholipids (e.g., phosphatidylethanolamine), resulting in higher conductivity of the membranes (17, 24) and conformational changes in membrane proteins (mainly due to histidine residues) which could lead to increased membrane permeability.

Although the Ames system does detect mu-

tagenicity of carcinogenic hydrazines, there is an inconsistency between the carcinogenic and mutagenic potencies of these compounds (19) which is also manifested by the lack of quantitative correlation between results concerning DNA-damaging capacity of hydrazines as judged by inactivation of *Bacillus subtilis* transforming DNA (9) and as judged by the DNA alkaline elution technique (19); the DNA-damaging capacity observed by the latter technique is closely related to carcinogenicity.

Regardless of mutagenicity, the relative toxic potency of hydrazines to bacterial cells is quantitatively associated with their toxic potency to animals. It would be of importance, in our opinion, to test whether such relationships exist among classes of chemicals other than hydrazines, because if similar results are obtained, it would be feasible to incorporate the methodology presented here as an inexpensive, rapid prescreen of other chemicals potentially useful as drugs.

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