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Use of 4-(Nitrobenzyl)Pyridine (4-NBP) To Test Mutagenic Potential of Slow-Reacting Epoxides, Their Corresponding Olefins, and Other Alkylating Agents

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Olefins and their corresponding epoxide derivatives are extensively used in industrial processes. Many epoxides are used as intermediates in organic synthesis as well as in surfactants, fumigants, industrial sterilants, cosmetics, and pharmaceuticals. These compounds pose potential hazards for biological systems where 1,2-epoxides are able to cause adverse biological effects through genetic interactions (Ames 1975; Hemminki 1979; Ehrenberg and Hussain 1981; Turchi et al 1983).

Although there are some reports that aliphatic epoxides are mutagenic agents, sufficient evidence of carcinogenicity is still lacking. However, most of bacterial mutagenicity tests have shown that aliphatic epoxides are mutagenic. Trials showing possible carcinogenicity for ethylene oxide, propylene oxide, styrene oxide, 1,2-epoxybutane have been described (Manson 1980; ICPEMC 1984; Hemminki and Hesso 1984). Propylene oxide was found to have weak mutagenicity in bacterial mutagenicity test (Voogd et al 1981; Migliore et al 1982; Bootman et al 1979; Hemminki and Falck 1979). Studies on the mutagenicity of styrene oxide and styrene have been controversial. However, the mutagenicity of styrene oxide was found to be positive in bacteria (Sugiura and Goto 1981). Epoxy butane was shown to be mutagenic (Hardin et al 1983; Voogd et al 1981), but there exists some disagreement concerning its carcinogenicity and mutagenicity.

Eugene et al (1963) first used a 4-NBP (4-(4-Nitrobenzyl)pyridine) to determine the presence of alkylating agents. Later Hammock et al (1974) and Agarwal et al (1979) studied chemical reactivity of epoxide derivatives, demonstrating that the aliphatic epoxides showed strong reactivity to 4-NBP. Nelis et al (1982) have used a standard in vitro procedure for comparing alkylating agents of bionucleophiles based on their reaction with 4-NBP.

An understanding of the biochemistry of genotoxins has led to the preliminary development of a modified NBP test system (Archer and Eng 1981). However, this test system depended on the use of gaseous oxygen which made it difficult and time consuming to employ. A modified chemical activation system (CAS) employing hydrogen peroxide in place of oxygen and designed to mimic the mammalian mixed oxidase enzymes, was developed and tested in this laboratory (Mauro 1982). A mixture of hydrogen peroxide, ascorbic acid, ferrous ion, EDTA, and hydrazine in phosphate buffer was found to activate

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target compounds in reactive electrophile (Fenton's reagent in Fe^{++} and H_2O_2). By applying the chemical activation system to olefins, genetic risks may be quickly and reliably estimated. Also, the use of a chemical approach by the chemical activation system prove to be helpful in detecting and elucidating the structure-activity relationships of carcinogenic and mutagenic alkylating agents in environmental samples without employing complex and expensive biological systems.

MATERIALS AND METHODS

To a reaction flask was added 3.0 mL of 0.2M sodium acetate-acetic acid buffer (pH 4.0), the test compound in 3.0 mL of water (or solvent), and 1.0 mL of 5% (w/w) 4-(4-nitrobenzyl) pyridine in acetone. Sufficient water was added to produce a total volume of 7.0 mL. The reaction flask was sealed and placed in a boiling water bath. After 20 minutes, the solution was chilled in ice and 0.6 mL of ethel acetate/acetone (5:2, v/v) added, followed by 1.0 mL 5 N sodium hydroxide. After mixing in a vortex mixer for about 30 seconds, the organic phase was separated in a separatory funnel, removed, and the absorbance read at 540 nm. Following the addition of base, all the remaining procedures were carried out exactly 1.75 minutes after the addition of NaOH. All the reactions were performed at least in duplicate.

To 3.0 mL of 0.1 M potassium phosphate buffer (monobasic, pH 4.5) containing 10 mM EDT was added 0.1 mL of 0.15 M ferrous sulfate and 0.1 mL of 1.0 M ascorbic acid. The test compound in 2.0 mL of acetone (solvent) and 1.0 mL of 0.5 M hydrazine solution was then added. Finally, 30% (9.79M) hydrogen peroxide was added to make 0.8 M. The reaction flask was then sealed with Teflon tape and its contents mixed by inverting twice.

After the reaction mixture was incubated in a shaker bath at 37°C for 15 minutes, it was removed and 1.0 mL of 5% 4-(4-nitrobenzyl) pyridine (NBP) in acetone was added and placed in a boiling water bath. After 20 minutes, the reaction mixture was chilled on ice and 0.6 mL of ethyl acetate/acetone (5:2) added, followed by 1.0 mL of 5 M sodium hydroxide. Following mixing in a vortex mixer for about 30 seconds, the organic phase was separated in a separatory funnel, removed, and its absorbance read at 540 nm. From the addition of base on, additional handling was carried out rapidly in the dark. Absorbance was read exactly 1.75 minutes after the addition of NaOH. Blank solutions contained all components except the test compound.

It was particularly important that the reaction components be added in the exact order given, that the total volume in the reaction flask be constant initially for all tests, and that the boiling temperature be constant (97°C- 100°C). Heating at lower temperature gave lower absorbances. Test tubes (16x150nm) with Teflon lined screw caps were suitable as reaction flasks. All glassware was washed in warm soapy water, rinsed, and then placed in diluted chromic acid for at least 24 hours. Following the acid soak, glassware was rinsed thoroughly with distilled water, then placed in an oven at 450°C for a minimum of 90 minutes.

It was also important that solutions be made daily or before each test. The purity of the hydrogen peroxide used was maintained by the storing of the H_2O_2 solution at 4°C, and minimizing the handling of the stock bottle.

This system was used for aliphatic olefins and dieldrin that did not show any activities with test system 1. The same experimental procedure described in (B) was used, with the exception of changing incubation time from 15 minutes to 24 hours.

RESULTS AND DISCUSSION

All the epoxides tested produced a significant dose-related increase of alkylating activities, with no requirement for activation: as expected, the oxirane ring leads to a direct genotoxic activity (Table 1). 4-NBP reacts with epoxides to form a violent dye in an alkaline or acid medium according to the following mechanism (Figure 1). The epoxide reacts with the unpaired electrons in the pyridine ring which acts as a nucleophilic agent. The postulated chromophore is thought to be formed for the inductive effect of a NO_2 group. Epoxides exhibited good linear relationships between doses and responses in the absence or present of activation in the concentration of 0-10 mM.

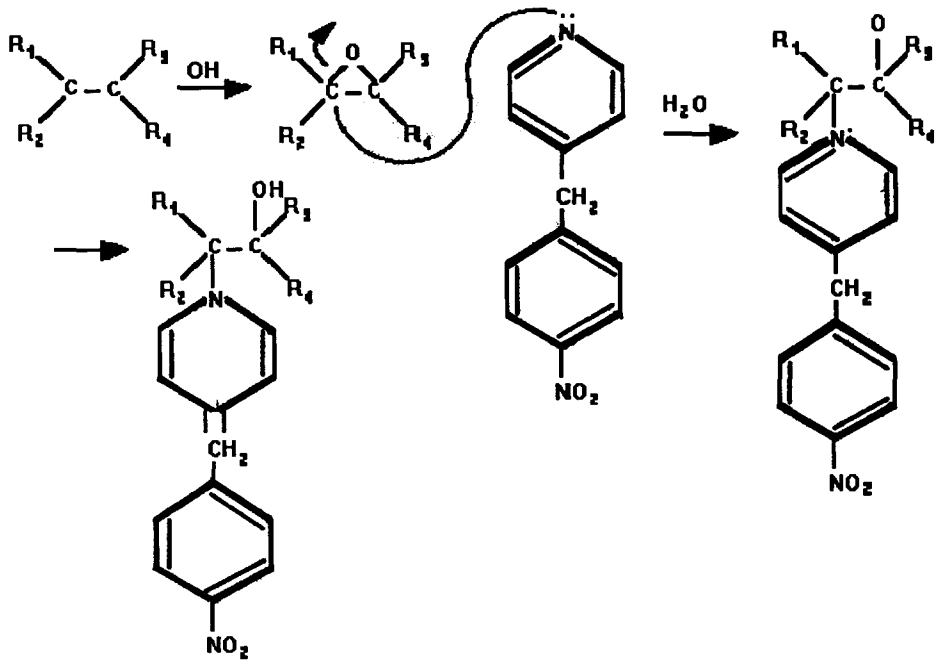

Figure 1. Postulated chromophone

Tables 2 and 3 allow absorption sensitivity comparisons of moles vs. grams. Tables 2 and 3 show the strong electrophilic activity of epoxides in the absence of a CAS, but much reduced reactivity following chemical activation. There was a particularly strong decrease

in the activity of ethylene oxide in the presence of CAS. These results can be explained by oxidative destruction of the epoxide by the CAS system. Bootman et al (1979), Migliore et al (1982), Watanabe et al (1982) and Hardin et al (1983) investigated a similar result observed when direct activity was reduced due to the fact that metabolic enzymes might plan an active detoxifying role.

Table 1. Linear dose-response relationships of alkylating agents in the NBP-CAS assay.

Compound	r^2	Linear Regression	
Ethylene oxide	0.996	Y=0.95 X	- 0.18
Propylene oxide	0.998	Y=0.28 X	+ 0.14
1,2-Epoxy butane	0.998	Y=0.20 X	+ 0.03
1,2-Epoxy hexane	0.998	Y=0.12 X	+ 0.00
1,2-Epoxy octane	0.992	Y=0.08 X	+ 0.03
Styrene oxide	0.984	Y=0.05 X	+ 0.00
Styrene	0.997	Y=0.00045 X	+ 0.0291
1-Butene	0.963	Y=0.00020 X	+ 0.0027
1-Hexene	0.999	Y=0.00007 X	+ 0.0169
1-Octene	0.992	Y=0.00007 X	+ 0.0055
œ-Chloroacetophenone	0.770	Y=3.00 X	+ 0.78
p-Chloroacetophenone	0.990	Y=0.0006 X	+ 0.0170
Busulfan	0.960	Y=0.46 X	+0.23
Dieldrin	1.000	Y=0.0013 X	+ 0.0019

Table 2. Results of assay of compounds with the CAS-NBP system (alkylation of NBP with and without chemical activation). The figures represent absorbance/mmol.

Compound	Absorbance/mmol without CAS x	Absorbance/mmol with CAS $x \pm s_m$	
Ethylene oxide	6.35	7.27×10^{-1}	$\pm 8.87 \times 10^{-2}$
Proylene oxide	6.25×10^{-1}	3.18×10^{-1}	$\pm 3.60 \times 10^{-2}$
1,2-Epoxy butane	3.60×10^{-1}	2.10×10^{-1}	$\pm 4.71 \times 10^{-3}$
1,2-Epoxy hexane	2.09×10^{-1}	1.23×10^{-1}	$\pm 2.17 \times 10^{-3}$
1,2-Epoxy octane	1.59×10^{-1}	8.86×10^{-2}	$\pm 4.00 \times 10^{-3}$
Styrene oxide	8.50×10^{-2}	4.64×10^{-2}	$\pm 2.60 \times 10^{-3}$
Styrene	0.00	5.50×10^{-4}	$\pm 1.90 \times 10^{-5}$
1-Butene	0.00	1.70×10^{-4}	$\pm 7.20 \times 10^{-6}$
1-Hexene	0.00	1.19×10^{-4}	$\pm 8.16 \times 10^{-7}$
1-Octene	0.00	7.34×10^{-5}	$\pm 1.07 \times 10^{-6}$
œ-Chloroacetophenone	8.55	2.53×10	± 6.88
p-Chloroacetophenone	1.00×10^{-4}	4.80×10^{-4}	$\pm 3.40 \times 10^{-5}$
Busulfan	2.52×10^{-1}	7.93	± 4.94
Dieldrin	2.65×10^{-3}	1.27×10^{-3}	$\pm 5.00 \times 10^{-6}$

The rate of alkylation depends on the molecular structure of the electrophile. The following rules regarding the mutagenicity of aliphatic epoxides have been postulated by many authors (Jones and Mackrodt 1983; Voogd et al 1981; Mason 1980; Sinsheimer et al 1987). (1) The mutagenicity of the aliphatic epoxides decreases with increasing length of the carbon chain. (2) The mutagenic activity increases with withdrawal of electrons from the epoxide group. (3) Polar epoxides are more mutagenic. These results can be best explained on the basis of solubility and distribution coefficient considerations.

Table 3. Results of CAS-NBP assays with alkylating agents. (Alkylation of NBP with and without chemical activation.) The figures represent absorbance/gram (g).

Compound	Absorbance/mmol without CAS x	Absorbance/mmol with CAS $x \pm s_m$	
Ethylene oxide	1.67×10^{-2}	1.65×10	± 2.01
Proylene oxide	1.08×10	5.47	$\pm 2.28 \times 10^{-1}$
1,2-Epoxy butane	4.99	2.91	$\pm 6.54 \times 10^{-2}$
1,2-Epoxy hexane	2.08	1.22	$\pm 2.16 \times 10^{-2}$
1,2-Epoxy octane	1.24	6.91×10^{-1}	$\pm 3.12 \times 10^{-2}$
Styrene oxide	0.71	3.86×10^{-1}	$\pm 2.17 \times 10^{-2}$
Styrene	0.00	6.54×10^{-4}	$\pm 9.50 \times 10^{-6}$
1-Butene	0.00	5.25×10^{-3}	$\pm 1.83 \times 10^{-4}$
1-Hexene	0.00	2.97×10^{-3}	$\pm 1.28 \times 10^{-4}$
1-Octene	0.00	1.43×10^{-3}	$\pm 1.94 \times 10^{-5}$
œ-Chloroacetophenone	5.53	1.64×10^{2}	$\pm 4.87 \times 10$
p-Chloroacetophenone	6.47×10^{-4}	3.08×10^{-3}	$\pm 2.80 \times 10^{-4}$
Busulfan	1.02	3.22×10	$\pm 1.86 \times 10$
Dieldrin	6.96×10^{-3}	3.45×10^{-3}	$\pm 9.90 \times 10^{-5}$

Simple olefins were found to be weak indirect alkylating agents to 4-NBP in this assay. Alkylation of NBP following chemical activation was observed for the concentration range 0-1 M range. A good correlation coefficient (r^2) indicated close conformity to Beer's law and it appears that the high precision indicated a stable oxidation product (Table 1). CAS activations of olefins were performed to determine reactivity at different temperatures. The reactivity of olefins at 20°C decreases as follows: 1-butene > styrene > 1-hexene > 1-octene. It was found that approximately 1.4% of styrene, 0.1% of 1-butene, 0.1% of 1-hexene and 0.1% of 1-octene was converted to oxides by CAS-NBP alkylation. The reactivity of styrene was almost doubled from 20°C to 37°C after 24 hours of incubation, whereas the reactivity of 1-butene was decreased by half. This finding indicates that styrene produces a more stable alkylating agent than the other aliphatic olefins tested. Activation of the olefins can be best explained by epoxidation of the double bond. Thus, styrene oxide can be presumed to be the oxidation derivative. These results suggest that styrene would be mutagenic and possibly carcinogenic.

Alkylation of NBP with dieldrin following chemical activation was observed for the concentration range 50-300 mM. The Ames' test of dieldrin fails since it is not known to be sensitive enough to detect weak carcinogens. Thorp and Walker (1973) demonstrated that dieldrin may be only a weak carcinogen or, perhaps a promoter. œ and *p*-chloroacetophenone exhibited electrophilic activity with and without CAS indicating that the compound may be both direct and indirect alkylating agents (Table 2). The electron migration in chloroacetophenone appears to be toward the carbonyl group. It is expected that the positively charged chloro ion will oppose the dipole created in the excited state, and thus raise its energy. Electronegativity provides additional stabilization for anion by resonance, thus the *a*-chloroacetophenone will become much more reactive than *p*-chloroacetophenone towards a nucleophile. A linear absorbance relationship was evident with a high degree of correlation (r^2=0.985). An increase in alkylating activity following activation was shown (Table 2 and 3). The alkanesulfonates, such as busulfan, are potent alkylating agents because the sulfonate ion (RSO_3) is a relatively strong leaving group. The reaction mechanism occurs via SN_2 reaction. Busulfan was found to be both a direct and an indirect alkylating agent. The response was linear in the range from 0.01 mM to 2mM (r^2=0.93).

The Chemical Activation System (CAS) proved to be practical in elucidating the reactivity of indirect alkylating agents. Evidence of alkylation is presumed to be evidence of mutagenic risk, and thus the test may prove to be a simple, non-biological indicator of carcinogenic risks. From the results presented herein, it is evident that certain olefins have significant indirect alkylating potential indicating carcinogenic/mutagenic risks.

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