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Biological Actions of Nitroarenes in Short-Term Tests on *Salmonella*, Cultured Mammalian Cells and Cultured Human Tracheal Tissues: Possible Basis for Regulatory Control

by Takashi Sugimura* and Shozo Takayama†

Pure synthetic nitropyrene compounds were subjected to a mutation test using *Salmonella typhimurium* TA 98 and TA 100 with and without S9 mix, a metabolic activation system. Dinitropyrenes were highly mutagenic. Among them, 1,8-dinitropyrene was the most potent mutagen, producing 940,000 revertants of TA 98/μg. 1,3,6-Trinitropyrene and 1,3,6,8-tetranitropyrene were also highly mutagenic, producing 708,000 and 221,000 revertants/μg, respectively. 1-Nitropyrene was weakly mutagenic. All nitropyrenes were more mutagenic towards TA 98 than TA 100, and all mutagenic activities were abolished by the presence of S9 mix. Di- and trinitropyrenes were demonstrated to be mutagenic to Chinese hamster lung cells without metabolic activation, by using diphtheria toxin resistancy as a marker. The range of mutagenic potential of nitropyrenes was much narrower with cultured mammalian cells than with Salmonella. 1-Nitropyrene was not mutagenic. 1,6-Dinitropyrene and 1-nitropyrene induced unscheduled DNA synthesis in epithelial cells of *in vitro* cultured human bronchi, as did diol-epoxides of benzo[a]pyrene, while benzo[a]pyrene itself was inert. 1-Nitropyrene and 3-nitrofluoranthene produced subcutaneous fibrosarcomas at the loci of injections in the backs of rats. Tumors were found in 47% and 40% of animals with total doses of 40 mg of 1-nitropyrene and 30 mg of 3-nitrofluoranthene, respectively. The biomedical significance of nitroarenes is discussed.

Introduction

Nitroarenes have been reported to show fairly strong mutagenicity in *Salmonella typhimurium*, and their mutagenic activities have been demonstrated without addition of the metabolic activation system, S9 mix. The specific mutagenic activities of some nitroarenes are very high, and these compounds have been named supermutagens (*1*). Some nitroarenes are air pollutants (*2*) and are present in automobile exhausts (*3*) and carbon blacks (*4*). Thus the biomedical impact, and especially the significance for human carcinogenesis, of these nitroarenes is being seriously considered. However, it is impossible to quantitate their health hazard precisely from data on *Salmonella typhimurium* only.

This paper describes recent results on the mutagenicities of nitroarenes on *Salmonella typhimurium* and on cultured Chinese hamster lung cells. In addition, data on their induction of unscheduled DNA synthesis *in vitro* in cultured human tracheal tissue and on their *in vivo* carcinogenicity are reported. Data obtained in short-term tests are also discussed, on the basis of previous finding on other environmental chemicals of a quantitative relation between carcinogenicity *in vivo* in rodents and mutagenicity in *Salmonella typhimurium*. On the basis of results of short-term tests, the regulatory control of these chemicals is also considered. The experimental results reported were mainly obtained

*National Cancer Center Research Institute, Tokyo, Japan.
†Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan

in our own laboratories: no comprehensive survey of the literature is given.

Microbial Mutation Tests

The preincubation method (*5*), which is a minor modification of Ames' method (*6*), was used throughout the work reported here. The TA 98 and TA 100 strains of *Salmonella typhimurium* were used. S9 mix was prepared from the liver of rats that had been treated with PCB.

The chemicals used and their sources were as follows. 1-Nitropyrene was a generous gift from Dr. K. Shudo, University of Tokyo, and Dr. H. Tokiwa, Fukuoka Environmental Research Center. 1,3-, 1,6- and 1,8-Dinitropyrenes, 1,3,6-trinitropyrene and 1,3,6,8-tetranitropyrene were synthesized as described previously (*1*) and were generous gifts from Dr. R. Mermelstein, Xerox Corporation. 3-Nitrofluoranthene was a gift from Dr. H. Tokiwa. 6-Nitrochrysene, 2-nitrofluorene, 9-nitrophenanthrene, 1-nitrotriphenylene and 2-nitrotriphenylene were synthesized and provided by Prof. T. Kosuge, Shizuoka College of Pharmacy. The purities of all these chemicals were confirmed by HPLC, TLC, NMR and mass spectroscopy.

The specific mutagenicities of these nitroarenes are shown in Tables 1 and 2. All nitropyrenes except 1-nitropyrene showed very high specific mutagenic activities on the basis of unit weight and molar concentration. The mutagenicities of nitropyrenes were abolished almost completely by addition of S9 mix to the preincubation medium, as reported previously by Rosenkrantz et al. (*1*) and Tokiwa et al. (*7*). Mononitroarenes other than 1-nitropyrene were also mutagenic without S9 mix, as shown in Table 2. Of these compounds, 2-nitrotriphenylene and 3-nitrofluoranthene showed especially high specific mutagenic activities.

Table 1. Mutagenicity of nitropyrenes on *Salmonella typhimurium*.

	Mutagenicity, revertants/μg			
	TA 98		TA 100	
	–S9 mix	+S9 mix[a]	–S9 mix	+S9 mix[a]
1-Nitropyrene	2,000	71	390	169
1,3-Dinitropyrene	561,000	0[b]	126,000	0[b]
1,6-Dinitropyrene	230,000	0[b]	35,000	0[b]
1,8-Dinitropyrene	940,000	0[c]	115,000	0[c]
1,3,6-Trinitropyrene	708,000	0[b]	119,000	0[b]
1,3,6,8-Tetranitropyrene	221,000	0[d]	34,000	0[d]
Benzo(a)pyrene	0	320	0	660
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	0	0	870	0

[a]10 μL of S9 used for benzo(a)pyrene and 100 μL of S9 for all other compounds.
[b]Up to 0.1 μg/plate was tested.
[c]Up to 0.02 μg/plate was tested.
[d]Up to 2 μg/plate was tested.

Table 2. Mutagenicities of nitroarenes on *Salmonella typhimurium*.

	Mutagenicity, revertants/μg			
	TA 98		TA 100	
	–S9 mix	+S9 mix[a]	–S9 mix	+S9 mix[a]
3-Nitrofluoranthene	55,900	179	23,600	174
6-Nitrochrysene	69	145	231	443
2-Nitrofluorene	104	42	186	127
9-Nitrophenanthrene	1,300	197	4,400	97
1-Nitrotriphenylene	1,600	36	434	144
2-Nitrotriphenylene	32,400	450	12,900	695
Benzo(a)pyrene	0	320	0	660
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	0	0	870	0

[a]10 μL of S9 used for benzo(a)pyrene and 100 μL of S9 for other compounds.

Mutagenicity with Cultured Chinese Hamster Lung Cells

Induction of resistance to diphtheria toxin has been used in several laboratories (*8,9*) as a marker of mutation of cultured mammalian cells. We also established a system to detect induction of resistance to diphtheria toxin using Chinese hamster lung cells (*10*). Diphtheria toxin catalyzes ADP-ribosylation of a particular amino acid, diphthamide in elongation factor 2, with NAD as a donor of ADP-ribose. Diphthamide is formed by a post-translational modification of histidine in the peptide chain of elongation factor 2. ADP-ribosylated elongation factor 2 does not show the catalytic property necessary for protein synthesis and this toxin eventually kills the cell. Mutant cells resistant to a high concentration of diphtheria toxin are resistant to ADP-ribosylation of elongation factor 2 by diphtheria toxin. Post-translational modification of histidine to diphthamide is probably blocked in these diphtheria toxin-resistant cells. In this system, an expression time of 7 days was optimal for various mutagens. Table 3 shows the mutagenic activities of 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene, 1,8-dinitropyrene, 1,3,6-trinitropyrene and 1,3,6,8-tetranitropyrene observed in the absence of metabolic activation. 1,6-Dinitropyrene and 1,8-dinitropyrene were the most mutagenic, and 1-nitropyrene and 1,3,6,8-tetranitropyrene showed scarcely any mutagenicity. As shown in Figure 1, there is no apparent correlation between the specific mutagenic potentials, expressed in equimolar terms, on Chinese hamster lung cells and *Salmonella typhimurium* TA 98. Tables 1 and 3 show the mutagenicities of nitropyrenes, benzo[a]pyrene and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine with *Salmonella typhimurium* and with Chinese hamster lung cells, respectively. With *Salmonella typhimurium*, nitropyrenes, especially 1,3-dinitropyrene, 1,8-dinitropyrene and 1,3,6-trinitropyrene are much stronger mutagens than benzo(a)pyrene and (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. But in the system of forward mutation of Chinese hamster lung cells, these nitropyrenes are only moderately mutagenic.

Table 3. Mutagenicities of nitropyrenes on Chinese hamster lung cells.

Compound	Mutagenicity[a]
1-Nitropyrene	< 2.0
1,3-Dinitropyrene	74
1,6-Dinitropyrene	210
1,8-Dinitropyrene	152
1,3,6-Trinitropyrene	65
1,3,6,8-Tetranitropyrene	< 1.5
Benzo(a)pyrene	23[b]
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	590

[a]DT^r cells/10^6 survivors induced by a concentration of 1 μM.
[b]Mutagenicity was assayed with 12.5 μL/mL of S9, 0.75*mM* NADP and 4.27*mM* G6P.

Induction of Unscheduled DNA Synthesis in Cultured Human Trachea

We carried out preliminary studies on unscheduled DNA synthesis in human bronchial epithelium. Human tissues were obtained surgically from patients with lung cancer. The primary or secondary trachea was cut into small pieces and promptly incubated *in vitro* for 6 hr at 37°C in medium containing nitropyrenes and tritiated thymidine. Then the tissues were fixed, embedded in paraffin and cut into sections, and sections were dip-covered with autoradiographic emulsion. Autoradiographic examination revealed the presence of many epithelial cells undergoing unscheduled DNA synthesis. Table 4 gives results on induction of unscheduled DNA synthesis in human tracheal epithelium treated with nitropyrenes and other carcinogens. Human tracheal epithelium showed active unsched-

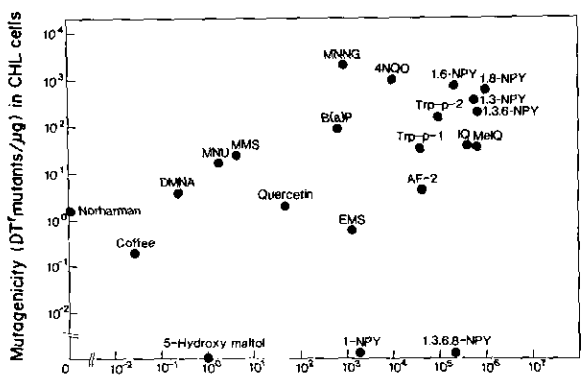

FIGURE 1. Relationship between mutagenicity in *Salmonella typhimurium* and mutagenicity in Chinese hamster lung cells. Abbreviations used: AF-2, 2-(2-furyl-3-(5-nitro-2-furyl)acrylamide; B(a)P, benzo[a]pyrene; DMNA, *N,N*-dimethylnitrosoamine; EMS, ethyl methanesulfonate; IQ, 2-amino-3-methylimidazo[4,5-f]-quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline; MMS, methyl methanesulfonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, methylnitrosourea; 1-NPY, 1-nitropyrene; 1,3-NPY, 1,3-dinitropyrene; 1,6-NPY, 1,6-dinitropyrene; 1,8-NPY, 1,8-dinitropyrene; 1,3,6-NPY, 1,3,6-trinitropyrene; 1,3,6,8-NPY, 1,3,6,8-tetranitropyrene; Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole.

Table 4. Induction of unscheduled DNA synthesis in human tracheal epithelial cells by nitropyrenes and other carcinogens.

Compound	Dose of compound, *M*	Average no. of grains/ nucleus
1-Nitropyrene	10^{-4}	3
1,6-Dinitropyrene	10^{-4}	14
BP	4×10^{-5}	0.5
BPDE I	3×10^{-5}	25
BPDE II	3×10^{-5}	23
ENU	3.2×10^{-3}	9
4NQO	3.2×10^{-3}	13
Control	—	0.3–0.5

uled DNA synthesis in response to nitropyrenes, but 1,6-dinitropyrene was more effective than 1-nitropyrene. In this system, benzo(a)pyrene (BP) was not effective, but ultimate reactive forms of benzo(a)pyrene, namely (±)-(7β,8α)-dihydroxy-(9α, 10α)-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BP DE I) and (±)-(7β, 8α)-dihydroxy-(9β,10β)-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE II), were very effective in inducing unscheduled DNA synthesis, being more effective than nitropyrenes and other representative carcinogens [ethylnitrosourea (ENU) and 4-nitroquinoline 1-oxide (4NQO)].

Apparently human tracheal tissues *in vitro* cannot activate benzo(a)pyrene, but can activate nitropyrenes in the absence of the S9 mix metabolic activation system, as has been observed with Chinese hamster lung cells. Human tracheal epithelial cells should have nitroreductase(s), which converts nitropyrenes to ultimate reactive forms. This experiment suggests that nitropyrenes could be more important than benzo(a)pyrene as a cause of human cancer.

In Vivo Carcinogenicity in Rats

Since only limited amounts of the test compounds were available, 1-nitropyrene and 3-nitrofluoranthene were injected subcutaneously into rats.

Male 8-week-old F344/DuCrj Fischer rats were given 2 mg of 1-nitropyrene twice weekly 20 times or 2 mg of 3-nitrofluoranthene twice weekly 15 times. These chemicals were given as solutions in dimethyl sulfoxide at a concentration of 10 mg/mL. Control rats received 0.2 ml of dimethyl sulfoxide twice weekly 20 times. During the experimental period of 12 months, the incidences of tumors at the site of injection were 47% with 1-nitropyrene and 40% with 3-nitrofluoranthene, as shown in Table 5. Histologically, most of the tumors were malignant fibrous histiocytomas. Some tumors were transplantable in the same strain of rats (*11*). The carcinogenic potencies of chemicals cannot be determined from data on the effect of their subcutaneous injection at one dose level. However, considering the total dose injected, and the fact that the first tumor was found on the 162nd experimental day with 1-nitropyrene and the 277th day with 3-nitrofluoranthene, it may be concluded that these two compounds are not very potent carcinogens.

Quantitative Relation between Carcinogenic and Mutagenic Potencies

Most typical carcinogens have been shown to be mutagenic. Some, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-nitroso-ethylurea, are direct acting chemicals, while others, such as 4-nitroquinoline 1-oxide and nitrofurylfurylacrylamide, are activated by enzymes present in most microbes and cultured mammalian cells. A third class of compounds, including benzo(a)pyrene and acetylaminofluorene, has to be metabolically activated by microsomal enzymes before conversion to ultimate forms.

Many compounds have been newly identified as mutagens by the Salmonella test. These include about 10 heterocyclic amines isolated from pyrolysates of amino acids and proteins. Some of these have been shown to be carcinogenic (*12*, *13*). In early studies a fairly good overlap was found between bacterial mutagenicity and carcinogenicity (*14–16*). It was once claimed that the mutagenic potency was also quantitatively correlated with the carcinogenic potency (*17*). However, based on our findings, as shown in Figure 2, the carcinogenic potency, expressed as the TD_{50}, and the mutagenic potency in *Salmonella typhimurium* are not correlated linearly. Trp-P-1 (3-amino-1,4-dimethyl-5H-pyrido-[4,3-b]indole) and Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3-b]-indole), obtained from the pyrolysate of tryptophan, and Glu-P-1 (2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole) and Glu-P-2 (2-aminodipyrido[1,2-a:3',2'-d]imidazole), obtained from the pyrolysate of glutamic acid, are almost equally as mutagenic to *Salmonella typhimurium*, as aflatoxin B_1, but their carcinogenicities are about the same as that of o-aminoazotoluene, which is about one thousandth as mutagenic as aflatoxin B_1.

A serious discrepancy between the results of short-term assay tests and carcinogenicity is seen in the case of the flavonoid, quercetin: the mutagenicity of quercetin is well established (*18*, *19*), but its carcinogenicity *in vivo* could not be demonstrated in mice (*20*), rats (*21*) or hamsters (*22*), and

Table 5. Inductions of tumors by 1-nitropyrene and 3-nitrofluoranthene in F344/DuCrj rats.

Group	Carcinogen	Total dose, mg/rat	Effective no. of rats[a]	No. of rats with tumors, %	Day when tumors detected, no. of days after first injection
I	1-Nitropyrene	40	17	8 (47)	162, 186, 200, 221, 269, 269, 330, 333
II	3-Nitrofluoranthene	30	10	4 (40)	277, 277, 298, 319
I	—	0	20	0 (0)	

[a]Effective rats are those that survived beyond day 162, when the first tumor was found in a rat treated with 1-nitropyrene.

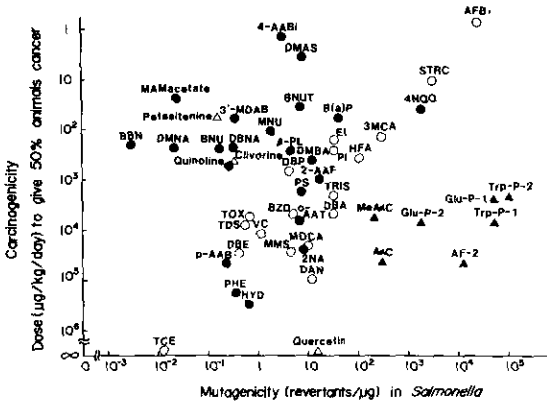

FIGURE 2. Relationship between carcinogenicity and mutagenicity in *Salmonella typhimurium*. Abbreviations used: *p*-AAB, *p*-aminoazobenzene; 4-AABi, 4-acetylaminobiphenyl; 2-AAF, 2-acetylaminofluorene; *o*-AAT, *o*-aminoazotoluene; AαC, 2-amino-9H-pyrido-[2,3-b]indole; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; AFB_1, aflatoxin B_1; BBN, *N*-butyl-*N*-butanolnitrosamine; BNU, *N*-*n*-butyl-*N*-nitrosourea; BNUT, *N*-*n*-butyl-*N*-nitrosourethane; B(a)P, benzo[a]pyrene; BZD, benzidine; DAN, 2,4-diaminoanisole; DBA, dibenz[a,h]anthracene; DBE, 1,2-dibromoethane; DBNA, *N*,*N*,di-*n*-butylnitrosamine; DBP, 1,2-dibromochloropropane; DMAS, 4-dimethylaminostilbene; DMBA, 7,12-dimethylbenz[a]-anthracene; DMNA, *N*,*N*-dimethylnitrosamine; EI, ethyleneimine; Glu-P-1, 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole; Glu-P-2, 2-aminodipyrido[1,2-a:3',2'-d]imidazole; HFA, *N*-hydroxy-2-acetylaminofluorene; HYD, hydralazine; MAM acetate, methylazoxymethanol acetate; 3MCA, 3-methylcholanthrene; 3'-MDAB, 3'-methyl-4-dimethylaminoazobenzene; MeAαC, 2-amino-3-methyl-9H-pyrido[2,3-b]indole; MMS, methyl methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea; MOCA, 4,4'-methylenebis(2-chloro-aniline); 2NA, 2-naphthylamine; 4NQO, 4-nitroquinoline 1-oxide; PHE, phenacetin; PI, propyleneimine; β-PL, β-propiolactone; PS, propane sultone; STRC, sterigmatocystin; TCE, trichloroethylene; TDS, toluenediamino sulfate; TOX, toxaphene; TRIS, tris(2,3-dibromopropylphosphate); Trp-P-1, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole; VC, vinyl chloride.

although it is reported to be carcinogenic in Norwegian rats (*23*), it clearly does not have a carcinogenic potency comparable to its mutagenic potency, which is equivalent to that of benzo[a]pyrene. Some nitroarenes are strongly mutagenic, but this does not necessarily indicate that they are potent carcinogens; some are only weakly mutagenic, but this does not necessarily mean that they are only weakly carcinogenic.

General Comments

The carcinogenic process is divided into two steps, initiation and promotion. Mutagenicity may be closely related to initiation, but may not be directly related to promotion; mutagenicity may indicate a capacity to produce DNA damage. It was recently suggested that DNA damage that results in chromosomal structural change may be more relevant to the initiation step than that yielding point mutations. Little is known about the molecular mechanism of DNA damage caused by nitroarenes, and it is still unknown whether nitroarenes have the tumor promoting activity expected of most typical carcinogens.

Furthermore the nitroarenes present in automobile exhaust should be carefully analyzed, since it is known that automobile exhaust shows definite mutagenic activity and this may well be due to nitroarenes. The incidence of lung cancer has increased greatly in many industrialized countries. This could largely be due to cigarette smoking, but it is noteworthy that many cases of lung cancer can not be explained by active or passive smoking. The information available at present on the relation between data obtained in short-term tests and in long-term *in vivo* animal tests is not yet sufficient to warrant taking any regulatory measures, especially in the case of automobile exhaust and nitroarenes, but the etiological factors responsible for lung cancer should be studied in more detail.

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