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Modifications in the myeloclastogenic * effect of benzene in mice with toluene, phenobarbital, 3-methylcholanthrene, Aroclor 1254 and SKF-525A **

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Summary

Benzene was studied in its target organ of effect, the bone marrow, with the micronucleus test and metaphase analysis. Male and female CD-1 mice were treated with 2 doses of benzene (440 mg/kg) or toluene (860 or 1720 mg/kg) or both 24 h apart, and sacrificed 30 h (or 54 h) after the first dose. Benzene-treated animals were pretreated with phenobarbital (PB), 3-methylcholanthrene (3-MCA), SKF-525A, or Aroclor 1254. Toluene showed no clastogenic activity and reduced the clastogenic effect of benzene when the mixture was given. None of the pretreatments protected against the clastogenic effect of benzene. 3-MCA pretreatment greatly promoted benzene myeloclastogenicity. Females were consistently more resistant to benzene than males. Dose-response curves in benzene-treated mice were much steeper with 3-MCA induction than without. Chromosomal damage was higher with p.o. than i.p. benzene administration.

* Greek myelos, medulla, marrow; Greek klasma, a fragment, genesis, birth. A myeloclastogen is an agent that causes chromosomal breakage (including SCE) in the bone marrow.

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Benzene has been used in industry since 1888. Currently over four million metric tons are used annually in the U.S.A. by the chemical industry (EPA, 1980). A unique feature of benzene among the simple aromatic hydrocarbons is its capability of producing a variety of blood dyscrasias ranging from mild changes in peripheral blood counts to severe aplastic anemia and leukemia (Snyder and Kocsis, 1975; Laskin and Goldstein, 1977). Yet,

Abbreviations: APM, aberrations per metaphase; DC, damaged cells; Expt., experiment; i.p., intraperitoneal; MA, metaphase analysis; 3-MCA, 3-methylcholanthrene; MNT, micronucleus test; MPCE, micronucleated polychromatic erythrocyte; PB, phenobarbital; PCE, polychromatic erythrocyte; p.o., oral.

no clear evidence for a dose-dependent response in chromosomal damage caused by benzene exposure has been established to support its leukemogenic actions (Wolman, 1977; Snyder et al., 1977). The present study was undertaken to obtain a dose-response relationship for chromosomal aberrations caused by benzene and to explore the relationship between benzene's myeloclastogenicity and metabolism.

In a series of experiments, we investigated the myeloclastogenic effect of benzene in mice with the micronucleus test (MNT) and metaphase analysis (MA). We modified benzene metabolism with toluene, 3-MCA, PB, SKF-525A or Aroclor 1254, and examined sex differences and the effects of the route and timing of benzene administration.

Materials and methods

Swiss mice (CD-1), 7-10 weeks old (Charles River Breeding Laboratories, Wilmington, MA), were used throughout this work. The females weighed 25-29 g and the males 32-37 g. Mice were allowed to acclimatize in cages with water and food (Formulab Chow 5008; Ralston Purina Co., St. Louis, MO) ad libitum, in an artificially ventilated room at 25°C with controlled humidity and time-controlled lighting (12 h of light per day), for 10 days before treatment. Usually 5 males and 5 females were randomly assigned to a treatment dose.

Chemicals

Benzene (thiophene free), toluene (99% purity), and olive oil were obtained from Fisher Scientific Co., Fair Lawn, NJ. We redistilled toluene (boiling point 110.4°C). Cytosan (cyclophosphamide, Mead Johnson Laboratories, Evansville, IN); sodium phenobarbital (St. Baker Chemical Co., Phillipsburg, NJ); SKF-525A (Smith, Kline and French Laboratories, Philadelphia, PA); Aroclor 1254 (Analabs, Inc., New Haven, CT), and 3-methylcholanthrene (Sigma Chemical Co., St. Louis, MO) were used in these experiments.

Pretreatments

PB was either given as 0.1 g/100 ml in drinking water for 7 days (Expt. I) or injected i.p. (80 mg/kg/day in distilled water) (Gibco Laborato-

ries, Grand Island, NY) for 3 days (Expt. II) before treatment. SKF-525A was given i.p. (80 mg/kg in distilled water) 2 h before each treatment dose. Aroclor 1254 was injected i.p. (100 mg/kg in olive oil) once, 5 days before treatment. 3-MCA was injected i.p. (30 mg/kg/day in olive oil) twice, 24 h apart, starting a day before the first treatment dose (Gill et al., 1979; Malaveille et al., 1979). Each pretreatment was formulated such that each mouse received 0.01 ml/g body weight.

Treatment

Solutions of benzene and/or toluene in olive oil were freshly prepared, such that each animal received 0.01 ml/g body weight; cytoxan was dissolved in distilled water. Two doses were administered 24 h apart at 8 a.m. by oral gavage, and mice were sacrificed by cervical dislocation 30 h after the first dose (i.p. route for benzene and 54 h termination time were added to Expt. II (A)). Colchicine (Fisher Scientific Co.) was injected i.p. (1 mg/kg) 2 h before sacrifice. Immediately after sacrifice, one femur was used for the MNT and the other for the MA.

Micronucleus test

The procedure was that of Schmid (1975) with some modifications (Connor et al., 1979). (1) The bone marrow from one femur was flushed into a centrifuge tube containing a minimal amount of fetal calf serum (Gibco Laboratories). (2) Two slides were prepared from each cell button, and stained with Wright and Giemsa stains (Fisher Scientific Co.); the latter was diluted with pH 6.0 phosphate buffer. 1000 polychromatic erythrocytes (PCE) were scored and the number of the micronucleated ones (MPCE) recorded as per thousand of PCEs counted. The number of micronucleated oxyphilic erythrocytes (MOE) per 1000 PCEs was also recorded in animals sacrificed at 54 h (Von Ledeber and Schmid, 1973).

Bone marrow metaphase analysis

The bone marrow was flushed into a centrifuge tube containing 5 ml Eagle's minimal essential medium (Gibco Laboratories), and centrifuged at $112 \times g$ for 10 min. The cells were subjected to a 25-min exposure to 0.075 M KCl, followed by fixation in 3 : 1 methanol : acetic acid. Slides were

prepared by flame drying and stained with Giemsa (Connor et al., 1979). 50 metaphases were scored per animal. The various chromosomal lesions were defined according to the 'Glossary of Genetics and Cytogenetics' (Rieger et al., 1976). Cells with at least one gap or break were considered to be damaged (DC) (Comings, 1974; Goetz et al., 1975; Au et al., 1978; Anderson and Richardson, 1981). Metaphases with >10 aberrations were considered severely damaged cells (SDC), and pulverized

cells (PC) were those with >20 aberrations — mostly cells with very few undamaged chromosomes. Exchanges included intra- and inter-exchanges and Robertsonian translocations. For statistical purposes the recorded chromosomal aberrations were converted into 'aberrations (or lesions) per metaphase' (APM), which provide information about the 'severity' of the damage per cell (Au et al., 1978; Sandberg, 1980; Preston et al., 1981). Chromatid gaps and breaks were calculated as 1

TABLE 1

EFFECT OF BENZENE ON THE MICRONUCLEUS TEST AFTER PHENOBARBITAL, 3-METHYLCHOLANTHRENE AND SKF-525A PRETREATMENTS, AND WITH CO-ADMINISTRATION OF TOLUENE

Dosage mg/kg b.w. x 2	Number and sex of mice	$\bar{X} \pm S.D.$ of MPCE per 1000 PCE	$G \cdot \bar{X} \pm S.D.$ of MPCE per 1000 PCE	Range of MPCE per 1000 PCE
Control	5(M)	2.4 ± 0.5	2.4 ± 1.3	2-4
(olive oil)	5(F)	1.2 ± 1.3	1.4 ± 1.7	0-3
Benzene 440	5(M)	24.4 ± 27.9	16.7 ± 2.4	7-74 ^b
	5(F)	8.2 ± 8.1	5.8 ± 2.5	2-22 ^a
Toluene 1720	5(M)	2.0 ± 0.7	1.9 ± 1.4	1-3 ^c
	5(F)	3.0 ± 0.7	2.9 ± 1.3	2-4
Benzene 440 + toluene 1720	4 ^e (M)	8.3 ± 4.4	7.2 ± 1.8	4-13 ^a
	5(F)	4.2 ± 0.8	4.2 ± 1.2	3-5 ^a
Benzene 440 + toluene 860	5(M)	5.6 ± 4.2	4.2 ± 2.6	1-12 ^a
	5(F)	4.4 ± 1.6	4.5 ± 1.4	2-6 ^a
PB	5(M)	4.0 ± 2.2	3.4 ± 2.0	1-7
	5(F)	3.6 ± 0.8	3.5 ± 1.3	3-5
PB + benzene 440	5(M)	20.6 ± 7.1	19.6 ± 1.5	12-28 ^{bd}
	4 ^f (F)	13.8 ± 10.0	11.7 ± 1.8	8-29 ^b
3-MCA	4(M)	4.5 ± 2.5	4.0 ± 1.8	2-8
	5(F)	4.0 ± 1.5	3.9 ± 1.7	2-6
3-MCA + benzene 440	5(M)	58.6 ± 16.4	56.8 ± 1.3	45-84 ^b
	5(F)	23.0 ± 12.4	23.0 ± 1.5	12-46 ^b
SKF-525A	4(M)	3.0 ± 0.8	2.9 ± 1.3	2-4
	5(F)	2.2 ± 1.3	1.9 ± 1.9	1-4
SKF-525A + benzene 440	5(M)	22.8 ± 15.7	18.0 ± 2.3	5-46 ^{bd}
	6(F)	6.3 ± 4	4.9 ± 2.4	1-11 ^a
Cytosax 50	5(M)	35.6 ± 7.6	34.9 ± 1.3	24-38 ^b
	5(F)	33.0 ± 9.4	31.8 ± 1.3	26-49 ^{bd}

$\bar{X} \pm S.D.$, Sample mean and standard deviation; $G \cdot \bar{X}$, geometric mean; M, male; F, female.

^{a,b} Values significantly higher than control (olive oil), $P < 0.05$ and $P < 0.01$, respectively (Mann-Whitney test statistics).

^c Values statistically significant from the group receiving benzene alone ($P < 0.05$).

^d Values significantly lower than the 3-MCA + benzene 440 treated group ($P < 0.05$).

^e The fifth animal deceased.

^f One animal did not yield a scorable preparation.

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TABLE 2

FREQUENCY OF CHROMOSOMAL ABERRATIONS WITH BENZENE AFTER PHENOBARBITAL, 3-METHYLCHOLANTHRENE AND SKF-525A PRE-TREATMENTS, AND WITH THE CO-ADMINISTRATION OF TOLUENE

Dosage mg/kg b.w. x 2	Number and sex of mice	APM	Damaged cells Percentage	Range	SBC	SPC	Breaks		Gaps		Each.
							Chrd.	Chrom.	Chrd.	Chrom.	
Control (olive oil)	5(M) 5(F)	0.08 0.06	6.4 (4.0) ^a 4.0 (2.8)	1-5 0-4	- -	- -	11(2.6) ^b 7(2.4)	- 2(0.8) ^b	7(2.4) ^b 3(1.2)	- 1(0.4) ^b	1(0.4) ^b -
Benzene 440	5(M) 5(F)	0.53 ^{d,e} 0.22	17.6 (13.6) ^{d,e} 8.4 (7.2)	6-13 1-7	- 0.4	1.2 -	67(11.6) 38(7.6)	8(1.6) 5(1.6)	13(4.4) 4(1.6)	- -	3(0.8) 1(0.4)
Toluene 1720	5(M) 5(F)	0.03 ^f 0.02 ^f	2.0 (1.8) ^f 1.6 (1.2)	0-2 0-2	- -	- -	1(0.4) 3(0.2)	2(0.8) -	1(0.4) -	- -	1(0.4) 1(0.4)
Benzene 440 + toluene 1720	4(M) ^h 5(F)	0.14 ^f 0.03	10.0 (8.0) ^f 2.4 (1.6) ^f	3-7 0-3	- -	- -	18(7.0) 5(1.6)	1(0.5) -	3(1.5) 2(0.8)	1(0.5) -	1(0.5) -
Benzene 440 + toluene 860	5(M) 5(F)	0.10 ^f 0.02 ^f	4.4 (4.4) ^f 1.6 (1.2) ^f	0-5 0-2	- -	- -	16(3.6) 2(0.8)	2(0.8) -	- 1(0.4)	- -	2(0.8) 1(0.4)
PB	5(M) 5(F)	0.08 0.04	5.6 (4.8) 2.8 (0.4)	2-4 0-3	- -	- -	9(3.6) 1(0.4)	4(1.2) -	2(0.8) 7(2.0)	- 1(0.4)	- -
PB + benzene 440	5(M) 5(F)	0.52 ^{e,g} 0.22	18.4 (16.4) ^{e,g} 9.6 (8.8)	4-20 1-9	0.4 -	- -	46(13.2) 30(6.8)	31(7.2) 7(2.4)	13(3.6) 4(1.6)	- -	5(2.0) 3(0.8)
3-MCA	4(M) 5(F)	0.06 0.10	4.0 (4.0) 5.2 (4.8)	1-5 0-5	- -	- -	5(2.0) 9(3.6)	3(2.5) 7(2.0)	- 2(0.8)	- -	- -
3-MCA + benzene 440	5(M) 5(F)	2.38 ^{d,f} 0.28 ^e	43.2 (41.2) ^{d,f} 17.6 (16.0) ^e	12-27 1-13	7.2 -	4.8 -	452(32.4) 57(17.3)	125(17.6) 8(2.0)	17(5.2) 11(4.0)	3(1.2) -	26(4.4) -
SKF-525A	4(M) 5(F)	0.04 0.01	2.0 (1.5) 0.4 (0.4)	0-3 0-1	- -	- -	2(1.0) -	- 1(0.4)	1(0.5) -	- -	2(1.0) -
SKF-525A + benzene 440	5(M) 6(F)	0.88 ^e 0.07 ^g	19.2 (16.8) ^{e,g} 4.3 (3.7)	2-21 0-5	4.0 -	0.8 -	193(14.4) 12(3.3)	47(7.2) -	7(2.8) 4(1.3)	- 1(0.3)	4(1.6) 1(0.3)
Cytosine 50	5(M) 5(F)	3.62 ^{d,f} 3.31 ^{d,f}	50.4 (46.8) ^{d,f} 46.4 (44.8) ^{d,f}	15-35 9-35	7.2 6.0	23.2 22.0	320(20.4) 259(22.0)	87(12.0) 60(9.2)	16(6.8) 7(2.8)	2(0.8) 4(1.6)	14(3.2) 26(5.6)

SBC, severely damaged cells; PC, pulverized cells; Chrd., chromatid; Chrom., chromosome; Each., exchanges; M, Male; F, Female.

^a Numbers in parentheses, percentage of damaged cells when gaps are excluded.^b Numbers in parentheses, percentage of cells per aberration type.^{c,d} Values significantly higher than control (olive oil), $P < 0.05$ and $P < 0.01$, respectively.^e Values significantly higher than the respective control, PB, SKF-525A ($P < 0.05$).^f Values statistically significant from the group receiving benzene alone ($P < 0.05$).^g Values significantly lower than the 3-MCA + benzene 440 treated group ($P < 0.05$).

lesion each, and all other lesions (chromosome gaps or breaks, rings, exchanges, etc) as 2 lesions each. All metaphases with > 10 breaks (SDC and PC) were considered as having 10 lesions each (Au et al., 1978).

Statistical analysis

The Mann-Whitney *U* test was used to analyse the data; *P* values were calculated for a two-tailed test. Values < 0.05 were considered to be statistically significant. Statistical metaphase analysis of both DC and APM were performed. In addition, the MNT and APM data were presented with both the arithmetic mean ($\bar{X}$) and the geometric mean ($\bar{G}\bar{X}$) — the latter being more representative of a highly skewed group (Snedcor and Cochran, 1980).

The following 3 experiments were performed:

Expt. I. 12 groups of male and female mice were used: they received benzene (440 mg/kg × 2), toluene (1720 mg/kg × 2), or 2 benzene-toluene mixtures (440 + 1720 and 440 + 860 mg/kg × 2,

i.e. 1 : 4 and 1 : 2, respectively); others were pretreated with PB, 3-MCA or SKF-525A before being given benzene. Appropriate control groups and a positive cytoxan group (50 mg/kg × 2) were included.

Expt. II. (A) 4 groups of 10 male mice were injected i.p. with benzene (440 mg/kg × 2), with or without 3-MCA pretreatment, and sacrificed at 30 or 54 h. 4 corresponding 5-male groups were given benzene p.o.

(B) We compared the effects of Aroclor 1254, PB and 3-MCA pretreatments on benzene (440 mg/kg × 2) clastogenicity, and 3-MCA pretreatment on toluene (1720 mg/kg × 2) and a benzene-toluene mixture (1 : 4).

Expt. III. Groups of 5 male mice were given benzene p.o. in doses ranging from 8.8 to 880 mg/kg × 2, with or without 3-MCA pretreatments; in one group, 3-MCA induction was initiated 48 h, instead of 24 h, before the first benzene dose.

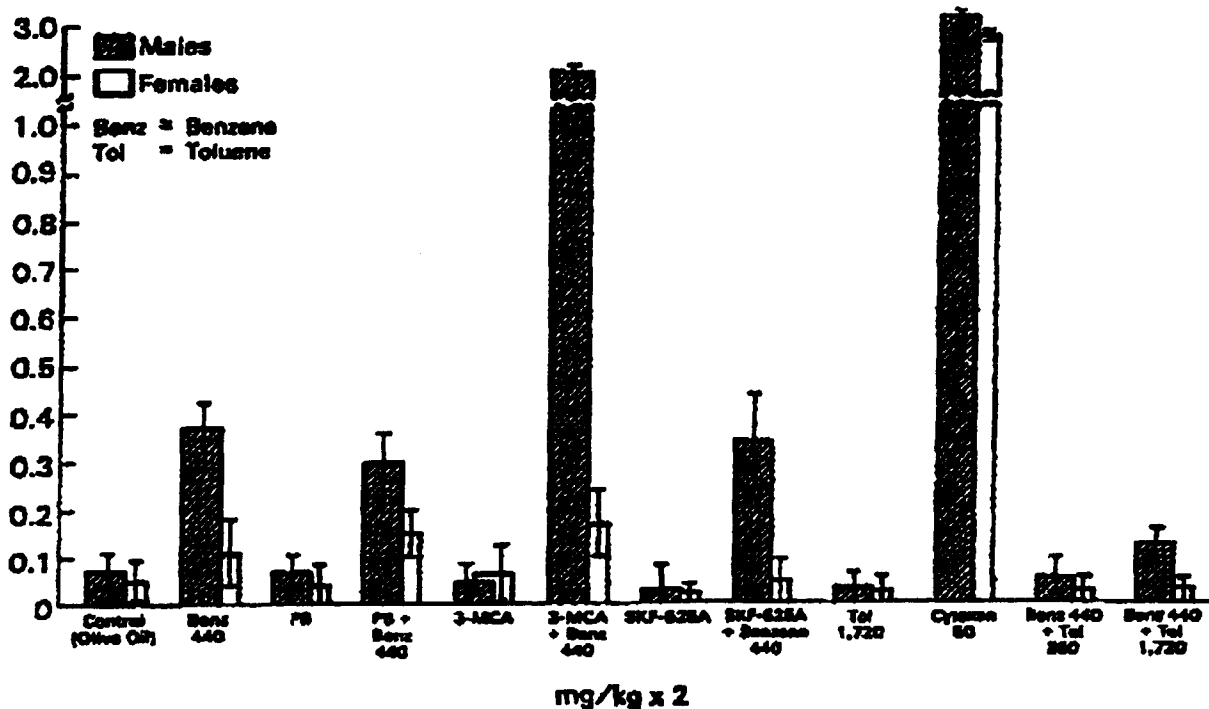


Fig. 1. Effect of modification of benzene metabolism with PB, 3-MCA, SKF-525A and toluene on the aberrations per metaphase. Geometric mean $\pm$ S.D. Ordinate: APM.

Results

Expt. 1. Modification of benzene metabolism with PB (p.o.), 3-MCA, SKF-525A and toluene (Tables 1 and 2 and Fig. 1)

Benzene increased the MPCE, DC and APM in both sexes above those of the control and toluene groups; the differences were statistically significant for males ($P < 0.01$) but in females only for the APM between the benzene and toluene groups ($P < 0.05$). Toluene groups showed virtually no

difference from untreated controls. Both benzene-toluene mixtures resulted in lower MPCE, DC and APM than benzene alone; the difference was statistically significant for DC and APM in both sexes, and for MPCE in the male (1 : 2) group ($P < 0.05$). The two mixtures, however, resulted in statistically higher MPCE than control ($P < 0.05$) except for males in the (1 : 2) group ($P > 0.05$); the differences in APM and the percentage of DC between either mixture and the controls were statistically insignificant ($P > 0.05$).

TABLE 3

EFFECT OF THE ROUTE OF BENZENE ADMINISTRATION, AND TIMING ON THE MICRONUCLEUS TEST

Dose/mg/kg b.w. x 2	Number and sex of mice	$\bar{X} \pm S.D.$ of MPCE per 1000 PCE	G. $\bar{X} \pm S.D.$ of MPCE per 1000 PCE	Range of MPCE per 1000 PCE	$\bar{X} \pm S.D.$ of MOE per 1000 PCE	$\bar{X} \pm S.D.$ of all micronucleated cells (MPCE + MOE) per 1000 PCE	G. $\bar{X} \pm S.D.$ of all micronucleated cells per 1000 PCE
Benzene 440 (i.p.; 30 h)	10 (M)	11.9 $\pm$ 3.8	11.4 $\pm$ 1.4	6-17 ^a	1.7 $\pm$ 1.8	13.6 $\pm$ 3.5	13.2 $\pm$ 1.3
3-MCA + Benzene 440 (i.p.; 30 h)	10 (M)	33.8 $\pm$ 14.0	31.8 $\pm$ 1.4	22-69 ^{ab}	0.8 $\pm$ 1.3	34.6 $\pm$ 13.7	32.7 $\pm$ 1.4
Benzene 440 (i.p.; 54 h)	10 (M)	9.2 $\pm$ 4.0	8.4 $\pm$ 1.6	4-17 ^a	2.5 $\pm$ 2.3	11.7 $\pm$ 5.8	10.4 $\pm$ 1.7
3-MCA + Benzene (i.p.; 54 h)	10 (M)	29.0 $\pm$ 20.0	23.0 $\pm$ 2.2	4-80 ^{ab}	12.0 $\pm$ 8.0	40.5 $\pm$ 25.2	33.7 $\pm$ 2.0
Benzene 440 (p.o.; 30 h)	5 (M)	46.0 $\pm$ 18.6	43.2 $\pm$ 1.5	29-70 ^{ac}	2.0 $\pm$ 1.0	47.8 $\pm$ 19.4	44.9 $\pm$ 1.5
3-MCA + Benzene 440 (p.o.; 30 h)	5 (M)	107.8 $\pm$ 25.8	105.0 $\pm$ 1.3	80-149 ^{abc}	5.6 $\pm$ 2.6	113.4 $\pm$ 25.0	111.3 $\pm$ 1.2
Benzene 440 (p.o.; 54 h)	5 (M)	54.0 $\pm$ 13.0	52.7 $\pm$ 1.3	33-67 ^{ac}	28.0 $\pm$ 11.0	81.2 $\pm$ 19.7	79.0 $\pm$ 1.3
3-MCA + Benzene 440 (p.o.; 54 h)	5 (M)	109.2 $\pm$ 24.0	107.0 $\pm$ 1.3	83-138 ^{abc}	51.0 $\pm$ 15.5	160.6 $\pm$ 27.9	158.7 $\pm$ 1.2
3-MCA (54 h)	5 (M)	3.0 $\pm$ 1.2	2.8 $\pm$ 1.5	2-5	-	3.0 $\pm$ 1.2	2.8 $\pm$ 1.5
Control (olive oil)	10 (M)	2.4 $\pm$ 1.0	2.2 $\pm$ 1.5	1-4	-	2.4 $\pm$ 1.0	2.2 $\pm$ 1.5

MOE, Micronucleated oxyphilic erythrocyte.

^a Values significantly higher than control (olive oil) ($P < 0.01$).^b Values significantly higher than the corresponding non-pretreated group ($P < 0.01$).^c Values significantly higher than the corresponding group treated with benzene i.p. ($P < 0.01$).

TABLE 4
EFFECT OF THE ROUTE OF BENZENE ADMINISTRATION AND TIMING ON THE FREQUENCY OF CHROMOSOMAL ABERRATIONS

Dose mg/kg b.w. x 2	Number and sex of mice	APM	Damaged cells		Range	RSDC ^c	SFC	Breaks		Gaps		Exch.
			Percentage					Chrid.	Chroma.	Chrid.	Chrom.	
Benzene 440 (i.p.; 30 h)	10(M)	0.42 ^c	23.4(21.4) ^{ac}		7-17	-	-	167(20.4) ^b	12(2.2) ^b	19(3.6) ^b	-	-
3-MCA + benzene 440 (i.p.; 30 h)	10(M)	0.29 ^c	17.6(15.4) ^c		4-18	-	-	105(14.4)	5(1.0)	25(4.0)	-	3(0.6) ^b
Benzene 440 (i.p.; 54 h)	10(M)	0.07	5.0(4.2) ^c		0-10	-	-	19(3.4)	2(0.4)	5(0.8)	-	4(0.6)
Benzene 440 (p.o.; 30 h)	5(M)	0.54 ^c	16.0(15.2) ^c		5-15	0.8	-	118(15.2)	15(2.4)	3(1.2)	-	-
3-MCA + benzene 440 (p.o.; 30 h)	5(M)	1.56 ^c	32.4(30.8) ^c		10-24	2.4	6.0	186(23.6)	54(10.0)	8(2.8)	-	3(1.2)
Benzene 440 (p.o.; 54 h)	5(M)	0.08	3.2(3.2) ^c		0-6	-	-	11(2.0)	3(0.8)	-	-	2(0.8)
3-MCA + benzene 440 (p.o.; 54 h)	5(M)	0.96 ^d	18.4(17.6) ^d		1-18	4.0	3.6	180(14.4)	67(7.2)	1(0.4)	-	13(2.0)
3-MCA (54 h)	5(M)	0.03	2.4(2.4)		0-2	-	-	5(2.0)	-	-	-	1(0.4)
Control (olive oil)	10(M)	0.02	1.4(1.4)		0-2	-	-	7(1.0)	-	-	-	1(0.2)

^a Number in parentheses, percentage of damaged cells when gaps are excluded.

^b Numbers in parentheses, percentage of cells per aberration type.

^{c,d} Values significantly higher than control (olive oil); $P < 0.01$ and $P < 0.05$ respectively.

^e Values significantly lower ($P < 0.05$) at 54 h time 30 h, with the same treatment.

Mice pretreated with PB or SKF-525A before receiving benzene exhibited no statistically significant difference from those given benzene alone ($P > 0.05$). Pretreatment with 3-MCA, however, raised the MPCE, DC and APM above all the other benzene-treated groups; these differences were statistically significant for males ($P < 0.05$). An increase in exchange figures was seen in the groups showing the most overall damage, namely the cytoxan-treated groups and 3-MCA-benzene-treated males.

Expt. II. (A) Effect of the route of benzene administration, induction with 3-MCA, and timing (Tables 3 and 4 and Fig. 2)

(i) *p.o. vs. i.p.* Mice receiving benzene orally without pretreatment and sacrificed at 30 or 54 h, scored higher MPCE than those injected i.p. ($P < 0.01$), whereas the differences in the DC and APM were statistically insignificant ($P > 0.05$). Pretreatment with 3-MCA resulted in higher MPCE with

benzene p.o. than i.p. at both termination times ($P < 0.01$); the DC and APM at 30 h were higher with the p.o. than i.p. route, and the difference was statistically significant in APM ($P < 0.05$). (Note: the group 3-MCA-benzene i.p. at 54 h did not yield scorable metaphases.)

(ii) *Induction with 3-MCA vs. none.* Whether p.o. or i.p., for the same route of benzene administration and treatment period, 3-MCA pretreatment resulted in higher MPCE ($P < 0.01$). Higher DC and APM due to 3-MCA pretreatment were seen with the oral ($P < 0.05$) but not the i.p. route ($P > 0.05$).

(iii) *Sacrifice at 30 h vs. 54 h.* Whether benzene was given p.o. or i.p., for the same route the difference in MPCE was not statistically significant between the two termination times, but the DC and APM were much lower at 54 h than at 30 h ($P < 0.05$). When 3-MCA pretreatment was added, for the same route difference in MPCE as well as DC and APM were not statistically signifi-

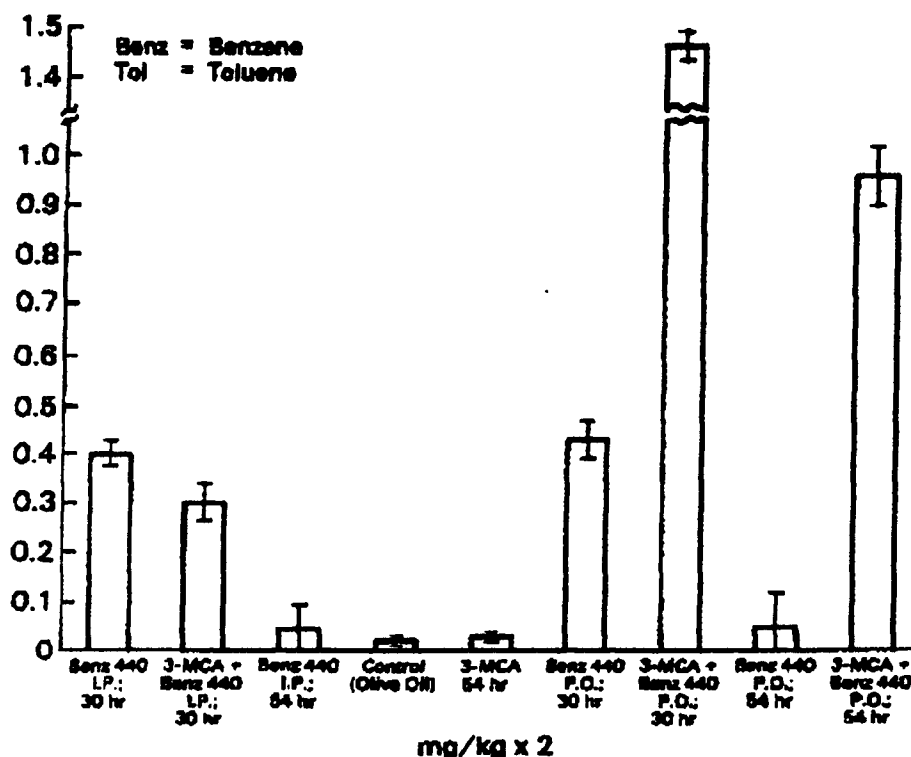


Fig. 2. Effect of the route of benzene administration, the timing and 3-MCA induction on the aberrations per metaphase in male mice. Geometric mean $\pm$ S.D. Ordinate: APM. L.P., intraperitoneal; P.O., oral.

TABLE 5

EFFECT ON THE MICRONUCLEUS TEST OF PHENOBARBITAL OR AROCLOR-1254 PRETREATMENT ON BENZENE, AND OF 3-METHYLCHOLANTHRENE ON BENZENE, TOLUENE, OR BOTH

Dosage mg/kg b.w. x 2	Number and sex of mice	$\bar{X} \pm S.D.$ of MPCE per 1000 PCE	$G \cdot \bar{X} \pm S.D.$ of MPCE per 1000 PCE	Range of MPCE per 1000 PCE
Benzene 440	5 (M)	46.0 ± 18.6	43.2 ± 1.5	29 - 70 ^a
	5 (F)	17.6 ± 14.0	11.0 ± 3.6	2 - 33 ^a
3-MCA + benzene 440	5 (M)	107.8 ± 25.8	105.0 ± 1.3	80 - 149 ^{ab}
Aroclor-1254	5 (M)	32.8 ± 6.0	32.4 ± 1.2	27 - 41 ^a
+ benzene 440	5 (F)	9.2 ± 3.6	8.3 ± 1.5	4 - 13 ^a
PB +	5 (M)	38.0 ± 19.0	34.4 ± 1.7	20 - 62 ^a
benzene 440	5 (F)	8.8 ± 5.0	7.6 ± 2.0	4 - 16 ^a
Benzene 440	5 (M)	6.4 ± 1.7	6.2 ± 2.0	5 - 9 ^a
+ toluene 1720	5 (F)	2.4 ± 0.5	3.0 ± 1.3	2 - 3
3-MCA	4 ^c (M)	3.5 ± 1.7	3.0 ± 2.1	1 - 5
+ toluene 1720	4 ^c (F)	2.8 ± 1.0	2.6 ± 1.4	2 - 4
3-MCA	5 (M)	31.4 ± 12.0	29.5 ± 1.5	19 - 49 ^a
+ benzene 440	6 (F)	9.7 ± 5.0	8.4 ± 2.0	3 - 16 ^a
+ toluene 1720				
Aroclor-1254	6 (M)	2.2 ± 1.0	2.0 ± 1.6	1 - 4
Control	10 (M)	2.4 ± 1.0	2.2 ± 1.5	1 - 4
(olive oil)	5 (F)	1.8 ± 1.3	1.8 ± 1.7	0 - 3

^a Values significantly higher than control (olive oil), ($P < 0.01$).

^b Values significantly higher than any treated group ($P < 0.01$).

^c The fifth animal did not yield a scoreable preparation.

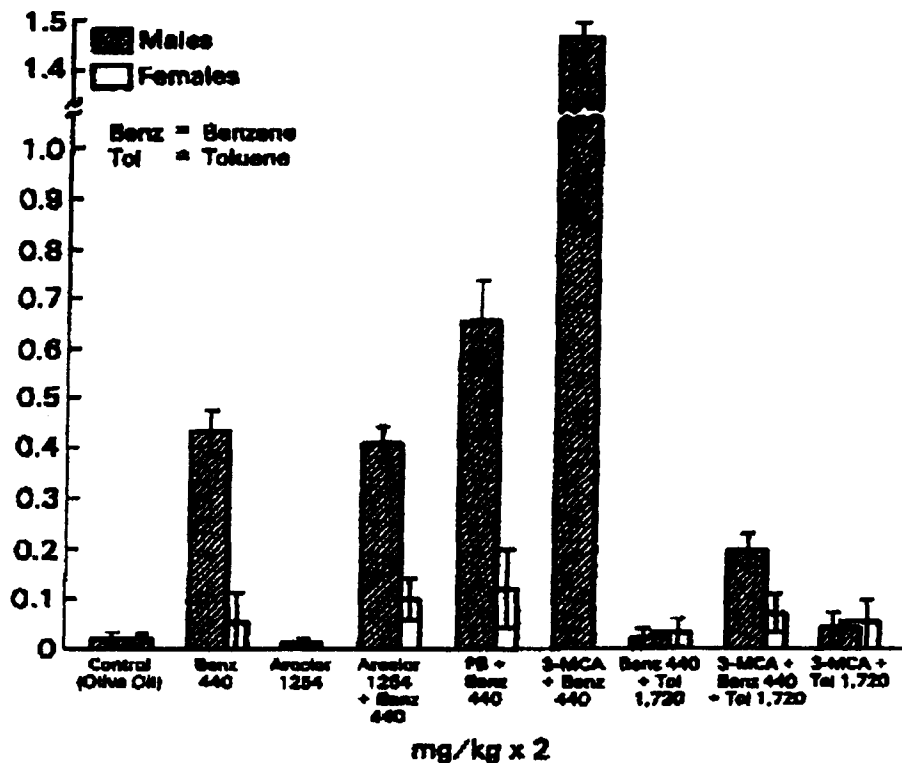


Fig. 3. Effect of Aroclor 1254, phenobarbital, and 3-MCA on toluene and benzene-toluene mixture. Geometric mean $\pm$ S.D. Ordinate: APM.

TABLE 6

FREQUENCY OF CHROMOSOMAL ABERRATIONS WITH BENZENE AFTER PHENOBARBITAL OR AROCLOR-1254, AND WITH BENZENE, TOLUENE, OR BOTH AFTER 3-METHYLCHOLANTHRENE PRETREATMENT

Dose mg/kg b.w. x 2	Number and sex of mice	APM	Damaged cells Percentage	Range	%SDC	%PC	Breaths		Gaps		Each.
							Chrid.	Chrom.	Chrid.	Chrom.	
Benzene 440	5 (M)	0.58 ^c	16.0(15.2) ^{a,d}	5-15	0.8	-	118(15.8) ^b	15 (2.4) ^b	3(1.2) ^b	-	-
5 (F)	0.07	4.4 (3.2)	0-6	-	-	-	9 (2.4)	1 (0.4)	3(1.2)	-	2 (0.8) ^b
5 (M)	1.56 ^c	32.4(30.8) ^{a,d}	10-24	2.4	6.0	-	186(23.6)	54(10.0)	8(2.8)	-	3(1.2)
3-MCA + benzene 440											
Aroclor-1254	5 (M)	0.46 ^c	16.8(16.0) ^c	5-12	-	-	90(15.2)	19 (4.4)	7(2.8)	-	-
+ benzene 440	5 (F)	0.13 ^d	10.0 (6.8) ^d	2-10	-	-	17 (2.8)	2 (0.8)	11(3.6)	-	-
PB	5 (M)	0.95 ^c	23.6(20.8) ^c	5-20	4.0	1.2	178(17.2)	44 (7.6)	11(4.0)	-	8(1.6)
+ benzene 440	5 (F)	0.25 ^d	10.8(10.0)	0-13	0.8	-	59(10.0)	10 (1.2)	4(1.6)	-	-
Benzene 440	5 (M)	0.01	0.8 (0.8)	0-2	-	-	2 (0.8)	-	-	-	-
+ toluene 1720	5 (F)	0.03	2.8 (1.6)	1-2	-	-	3 (1.2)	-	1 (0.4)	-	1 (0.4)
3-MCA	5 (M)	0.04	3.2 (2.0)	0-3	-	-	3 (1.2)	-	3(1.2)	-	2 (0.8)
+ toluene 1720	5 (F)	0.06	4.4 (2.4)	0-4	-	-	7 (2.8)	2 (2.0)	5(2.0)	-	-
3-MCA	4 ^f (M)	0.21 ^c	13.0(12.0) ^c	4-10	-	-	33(11.0)	1 (0.5)	5(2.5)	-	1 (0.5)
+ benzene 440											
+ toluene 1720	5 (F)	0.07	4.4 (3.6)	0-4	-	-	11 (3.2)	1 (0.4)	2(0.8)	-	1 (0.4)
Aroclor-1254	6 (M)	0.01	1.0 (0.7)	0-3	-	-	2 (0.7)	-	1(0.3)	-	-
Control	10 (M)	0.02	1.4 (1.4)	0-2	-	-	7 (1.0)	-	-	-	1 (0.2)
(olive oil)	5 (F)	0.02	1.6 (1.2)	0-2	-	-	3 (1.2)	-	-	-	1 (0.4)

^a Numbers in parentheses, percentages of damaged cells when gaps are excluded.

^b Numbers in parentheses, percentage of cells per aberration type.

^{c,d} Values significantly higher than control (olive oil), $P < 0.01$ and $P < 0.05$, respectively.

^e Values significantly higher than benzene treatment alone ($P < 0.05$).

^f The fifth animal did not yield a scorable preparation.

TABLE 7

EFFECT OF BENZENE, WITH AND WITHOUT 3-METHYLCHOLANTHRENE PRETREATMENT, ON THE MICRO-NUCLEUS TEST: DOSE-RESPONSE

Dosage mg/kg b.w. $\times 2$	Number and sex of mice	$\bar{X} \pm S.D.$ of MPCE per 1000 PCE	$G \cdot \bar{X} \pm S.D.$ of MPCE per 1000 PCE	Range of MPCE per 1000 PCE
Benzene 880	5(M)	28.8 ± 3.0	28.7 ± 1.1	24 - 32 ^a
Benzene 440	5(M)	30.4 ± 18.1	26.7 ± 1.8	14 - 60 ^a
Benzene 88	5(M)	7.0 ± 4.2	6.0 ± 2.0	3 - 12 ^b
Benzene 8.8	5(M)	4.6 ± 3.0	4.0 ± 1.8	2 - 10
Control (olive oil)	5(M)	2.6 ± 1.1	2.4 ± 1.7	1 - 4
3-MCA + benzene 8.8	5(M)	8.8 ± 4.2	7.9 ± 1.7	4 - 14 ^b
3-MCA + benzene 44	5(M)	11.2 ± 4.0	10.6 ± 1.5	6 - 15 ^a
3-MCA + benzene 88	4 ^c (M)	14.0 ± 7.5	11.9 ± 2.1	4 - 22 ^b
3-MCA + benzene 220	5(M)	34.8 ± 11.7	33.7 ± 1.3	25 - 55 ^a
3-MCA + benzene 440	5(M)	56.8 ± 22.8	51.5 ± 1.7	20 - 81 ^a
3-MCA + benzene 880	5(M)	80.0 ± 32.4	75.2 ± 1.5	48 - 130 ^a
3-MCA (delayed) + benzene 440 ^d	5(M)	62.6 ± 12.2	61.7 ± 1.2	51 - 82 ^a

^{a,b} Values significantly higher than control (olive oil), $P < 0.01$ and $P < 0.05$, respectively.^c The fifth animal did not yield a scorable preparation.^d 3-Methylcholanthrene was started two days, instead of one, before the first benzene dose for this group.

cant between the two time periods ($P > 0.05$). The rates of micronucleated oxyphilic erythrocytes (MOE) were higher at 54 h than 30 h, for the p.o. but not i.p. route, and were higher with 3-MCA

induction than without; the highest rate was in the group 3-MCA-benzene (p.o.) at 54 h ($\bar{X} = 51 \pm 15.5$).

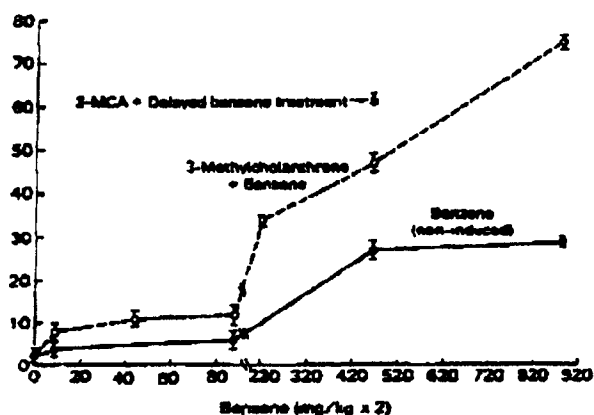


Fig. 4. Micronucleus test. Dose response with benzene (non-induced) (●) and 3-MCA induction (○). Geometric mean $\pm$ S.D. Ordinate: micronucleated polychromatic erythrocytes (MPCE) per 1000 PCE.

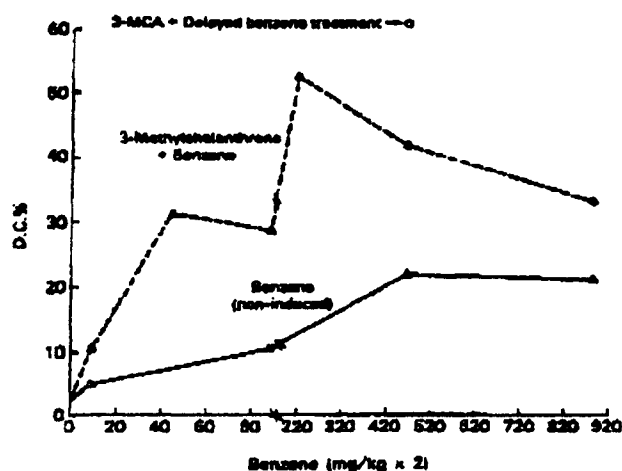


Fig. 5. Metaphase analysis. Dose response in damaged cells (%DC) with benzene (non-induced) (●) and 3-MCA induction (○). Ordinate: percentage of damaged cells.

TABLE 8
EFFECT OF BENZENE, WITH AND WITHOUT 3-METHYLCHOLANTHRENE PRETREATMENT, ON THE FREQUENCY OF CHROMOSOMAL ABERRATIONS: DOSE-RESPONSE

Dose/ mg/kg b.w. X 2	Number and sex of mice	APM	Damaged cells		Range	SSDC	SPC	Breasts		Gaps		Exch.
			Percentage					Chrid.	Chrom.	Chrid.	Chrom.	
Benzene 880	5 (M)	1.10 ^d	21.6(20.8) ^d		5-17	4.4	-	265(19.6) ^b	55(10.4) ^b	3(1.2) ^b	-	2(0.8) ^b
Benzene 440	5 (M)	0.76 ^d	22.4(20.8) ^d		7-18	2.0	-	180(20.4)	24(4.8)	4(1.6)	1(0.4) ^b	1(0.4)
Benzene 88	5 (M)	0.16 ^d	10.8(10) ^d		3-9	-	-	23(8.4)	5(1.6)	3(1.2)	-	2(0.8)
Benzene 8.8	5 (M)	0.06	5.2(4.8)		0-5	-	-	12(4.4)	1(0.4)	1(0.4)	-	-
Control (olive oil)	5 (M)	0.03	2.4(2.4)		0-2	-	-	5(2.0)	-	-	-	1(0.4)
3-MCA + benzene 8.8	5 (M)	0.14 ^d	10.8(9.6) ^d		3-9	-	-	29(8.8)	1(0.4)	3(0.4)	-	-
3-MCA + benzene 44	5 (M)	0.56 ^d	31.6(31.2) ^d		13-18	-	-	115(29.2)	10(2.8)	4(1.2)	-	1(0.4)
3-MCA + benzene 88	4 ^c (M)	0.82 ^d	29.0(28.5) ^d		5-23	-	-	119(27.5)	16(6.5)	4(2.0)	-	4(2.0)
3-MCA + benzene 220	5 (M)	1.22 ^d	52.8(51.6) ^d		21-34	-	-	238(44.8)	25(9.6)	17(5.2)	-	-
3-MCA + benzene 440	5 (M)	1.04 ^d	42.0(40.0) ^d		14-25	0.4	-	235(39.6)	16(4.8)	8(2.8)	-	-
3-MCA + benzene 880	5 (M)	0.77 ^d	33.6(31.6) ^d		14-20	-	-	148(30.4)	11(3.2)	11(3.2)	2(0.8)	3(0.8)
3-MCA + benzene 440 (delayed) ^c	5 (M)	4.39 ^d	61.6(60.8) ^d		20-47	14.0	20.4	648(38.4)	204(23.2)	5(2.0)	1(0.6)	14(4.8)

^a Numbers in parentheses, percentage of damaged cells when gaps are excluded.

^b Numbers in parentheses, percentage of cells per aberration type.

^c 3-MCA was started two days, instead of one, before the first benzene dose for this group.

^d Values significantly higher than control (olive oil), ($P < 0.01$).

^e The fifth animal did not yield a scorable preparation.

Expt. II. (B) Effect of Aroclor 1254, PB (i.p.) pretreatments on benzene, and of 3-MCA on benzene, toluene, or both (Tables 5 and 6 and Fig. 3)

Pretreatment with Aroclor 1254 or PB showed no statistically significant differences from benzene treatment alone in both sexes ($P > 0.05$); whereas, 3-MCA-pretreated males scored significantly higher MPCE, DC, and APM ($P < 0.01$). 3-MCA pretreatment of benzene-toluene groups resulted in differences statistically insignificant from benzene treatment alone (both sexes) ($P > 0.05$), but in higher MPCE, DC and APM than male groups receiving the mixture without induction ($P < 0.01$). 3-MCA induction had no effect on toluene-treated mice.

Expt. III. Dose response (Tables 7 and 8 and Figs. 4-6)

When the non-induced benzene-treated groups were ranked together and compared with the corresponding and similarly ranked 3-MCA-induced animals, the latter exhibited significantly higher MPCE and DC ($P < 0.05$); the difference in APM was significant ($P < 0.05$) only when the highest

dose group (880 mg/kg) which presumably showed cytotoxicity was excluded from the comparison. The group (440 mg/kg) induced for 48 h prior to the first benzene dose — labeled 'Delayed' — showed a clastogenic response dramatically elevated over the corresponding 3-MCA-induced group. Thus, overall 3-MCA pretreatment increased the parameters we examined, and these effects were dose-related to benzene.

Discussion

We have studied benzene with the MNT and MA for the following reasons: first, the bone marrow is a 'target organ' of benzene effect (Laskin and Goldstein, 1977; Snyder et al., 1977); the MNT and MA which were positive with benzene in mice (Diaz et al., 1980; Hite et al., 1980; Meyne and Legator, 1980; Siou et al., 1981) are performed on cells originating in the marrow (Kligisch et al., 1981). Second, the MNT reveals early insult to erythropoiesis reflected in the maturing erythrocytes (Matter et al., 1974; Schmid, 1975). Third, the spontaneous rate of micronuclei in the bone marrow is low and consistent — about 3 MPCE per 1000 PCE (Schmid, 1975) — and since the PCE is easily identifiable, both the numerator and denominator of this ratio are exceptionally accurate. Fourth, the MA provides information about the chromosomal damage sustained by all nucleated bone marrow cells i.e. both erythrocytic and leukocytic series.

Chromosomal aberrations following prolonged benzene exposure have been reported in humans and animals (Tough et al., 1970; Forni et al., 1971; Kissling and Speck, 1972 a, b; Picciano, 1979). In addition, benzene suppressed radioactive incorporation of iron (^{59}Fe) in maturing erythrocytes (Lee et al., 1974). Benzene inhibited the incorporation of [^3H]thymidine into DNA (Moeschlin and Speck, 1967) as well as [^3H]cytidine into RNA (Kissling and Speck, 1972 a, b) of bone marrow cells in rabbits; the authors interpreted this as inhibition of synthesis of both DNA and RNA by benzene. However, these studies do not prove that the 'primary' toxic event was indeed inhibition of DNA synthesis, as these studies were performed 'after' toxicity (pancytopenia) had occurred (Freedman, 1977). Binding of labeled benzene and

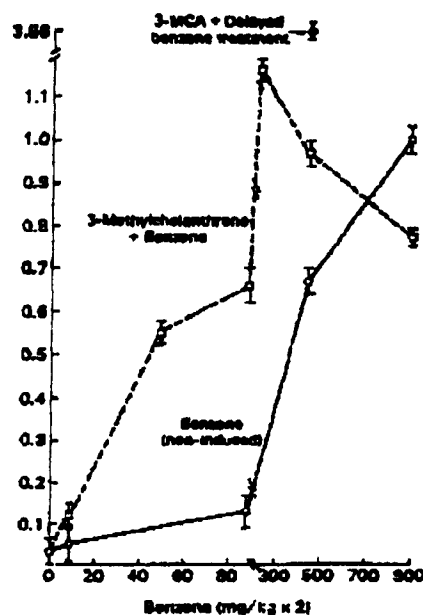


Fig. 6. Metaphase analysis. Dose response in aberrations per metaphase with benzene (non-induced) (O), and 3-MCA induction (□). Geometric mean $\pm$ S.D. Ordinate: APM.

metabolites to nucleic acids in rat liver (Lutz and Schlatter, 1977) as well as mouse bone marrow (Snyder et al., 1978; Gill and Ahmed, 1981) has been reported.

We have generated a dose-response relationship for benzene-induced myeloclastogenicity (Tables 7 and 8 and Figs. 4-6). Compared to the micronucleus test, the metaphase analysis was relatively more sensitive to very low benzene dosage and the chromosomal aberrations expressed per metaphase (APM) yielded the most sensitive index for clastogenicity; thus, at 8.8, 88, 440 and 880 mg/kg $\times$ 2 the APM were twice (0.06), 5 (0.16), 25 (0.76) and 37 (1.10) times, respectively, higher than the untreated controls (0.03). Severely damaged cells were found at the high benzene doses (440 and 880 mg/kg). In our study, biological variability was observed with benzene treatment, e.g. in Table 1, one animal had 74/1000 MPCE while the geometric mean ($\bar{G}\bar{X}$) was 16.7. Interestingly, this mouse also displayed more severe chromosomal damage than the rest of the group (Table 2).

Sex difference with benzene

Male mice have previously been found to be more sensitive to the chromosome-breaking effect of benzene than females (Meyne and Legator, 1980; Siou et al., 1981). Castrated males, as sensitive as females, regained their original sensitivity when treated with testosterone (Siou et al., 1981). We have observed the same sex difference in the clastogenic response to benzene whether its metabolism was unaltered modified by co-administered toluene or 3-MCA pretreatment. We have gavaged pregnant female CD-1 mice with doses of benzene ranging from 440 to 4400 mg/kg by the 16 gestational day — hemopoiesis being maximal in the fetal liver between the 12th and 16th gestational days (Cole et al., 1979) — and the rate of MPCE was similar to that of untreated controls in both maternal femur bone marrow and fetal liver (Gad-El-Karim and Legator, unpublished results). These results reinforce hormonal influence as an etiological factor in female resistance to benzene clastogenicity.

Toluene

Conflicting results have been reported on the effects of toluene in mammalian chromosomes

(Dean, 1978). The purity of toluene was not mentioned in those studies which reported toluene-induced myelotoxicity (Cohr and Stockholm, 1979). Using re-distilled toluene, we have demonstrated it to be entirely devoid of detectable clastogenic activity (1720 mg/kg $\times$ 2). Higher and lower doses p.o. and i.p. used in our laboratory yielded similar results (Gad-El-Karim and Legator, unpublished results). Moreover, pretreatment with 3-MCA had no effect on toluene-treated mice (Tables 5 and 6).

It was demonstrated *in vitro*, using liver microsomes, that toluene is a competitive inhibitor of benzene metabolism (Mikulski et al., 1979; Sato and Nakajima, 1979). Toluene protected against benzene-induced depression of red cell ^{59}Fe uptake in mice (Andrews et al., 1979). Toluene significantly alleviated the adverse effects of benzene on granulopoietic stem cells (CFU per tibia) and on tibial bone marrow cellularity in mice (Tunek et al., 1981). However, the clastogenic effects of toluene and benzene were reported to be additive in rats when simultaneously inhaled (Dobrokhotov, 1972; Dobrokhotov and Enikeev, 1976). We found that toluene antagonized the clastogenic activity of benzene when the mixture was administered p.o. in CD-1 mice (Tables 1 and 2).

Modifiers of mixed-function oxidase activities

Various inhibitors or inducers of mixed-function oxidase activities have been used previously to elucidate the role of metabolism in the expression of benzene toxicity. Pretreatment of rats with 3-amino-1,2,4-triazole or piperonyl butoxide, inhibitors of hepatic microsomal monooxygenases, protected against benzene toxicity (Snyder and Kocsis, 1975; Laskin and Goldstein, 1977). Benzene-associated leukopenia was also alleviated by pretreating rats with phenobarbital, an inducer of cytochrome P-450 (Ikeda and Ohtsui, 1971; Drew et al., 1973; Gill et al., 1979). However, pretreatment with 3-methylcholanthrene, a prototype polycyclic aromatic hydrocarbon inducing a profile of hepatic microsomal activities different from that induced by phenobarbital (Meyer et al., 1980), did not prevent benzene-induced leukopenia (Gill et al., 1979). A paradox, hence, existed: Both inhibitors and inducers of benzene metabolism protected against toxicity. Thus, it has been postulated that these agents elicit different responses

in the liver (the major site of detoxication) and the bone-marrow (the major site of benzene toxicity): inducers stimulate hepatic detoxication, whereas inhibitors prevent the formation of reactive metabolites in the bone marrow (Snyder et al., 1977).

PB pretreatment in our study did not protect the mice against benzene-induced myeloclastogenicity; nevertheless, animals of the same group exhibited a wide range of MPCE and chromosomal aberrations (Tables 5 and 6). This is in agreement with the previous finding that PB did not exert a protective effect against benzene-induced depression of ^{59}Fe uptake into red cells in the mouse (Snyder, 1973) even though it protected against leukopenia in rats. In contrast, it has been reported that phenobarbital increased the frequency of sister-chromatid exchange in female DBA/2 mice exposed to benzene but not in males; chromosomal aberrations were increased in both sexes, but more in males (Tice et al., 1980).

Aroclor 1254 protected rats from benzene-induced lymphocytopenia (Greenlee et al., 1981 a, b; Greenlee and Irons, 1981), and neither SKF-525A nor 3-MCA had an effect on benzene leukopenia in rats (Gill et al., 1979). We found, in CD-1 mice, that neither Aroclor 1254 nor SKF-525A had an effect on the myeloclastogenicity of benzene. Pretreatment of mice with 3-MCA, an inducer of P-448 monooxygenase, greatly promoted the clastogenic activity of benzene. In the dose-response curve for benzene with 3-MCA pretreatment, every point — geometric mean — on the curve was higher than the corresponding point for animals given benzene alone (Figs. 4–6). The MNT showed differences between induced and non-induced animals at all benzene doses (the former group exhibited higher MPCE), and while the magnitude of that difference was small at the low doses, it increased at the high doses 440 and 880 mg/kg (Table 7, Fig. 4). At the highest benzene dose (880 mg/kg), however, there was a tendency for chromosomal aberrations to decrease in the metaphases (Figs. 5 and 6), presumably due to cytotoxicity.

Benzene p.o. vs. i.p.

In mice, higher rates of MPCE were reported with the p.o. than i.p. route of benzene administration; such a route difference was not found in the MA (Meyne and Legator, 1980), nor was it seen in

rats with single 2-h inhalation vs. i.p. exposure (Anderson and Richardson, 1981). We have witnessed the same findings, but also found that after 3-MCA induction the clastogenic effect of benzene was much more pronounced (by both MNT and MA) when it was administered p.o. instead of i.p. (Tables 3 and 4). Diaz et al. (1980) found a higher rate of MPCE at 54 h than 30 h in mice given benzene (s.c.); they ascribed the difference to a cell-cycle delay caused by benzene. We found no statistically significant difference — i.p. or p.o. — in MPCE at 30 h vs. 54 h in Expt. II with or without 3-MCA pretreatment (Table 3). To further examine this route difference, we performed an experiment in which germ-free male CD-1 mice were given benzene p.o. or i.p., and compared them with groups of 'conventional', i.e., non-germ-free males receiving the same treatment (Gad-El-Karim and Legator, unpublished results). The oral route remained superior to the intraperitoneal route in induction of MPCE in the bone marrow. Thus, the possibility that the gut flora (Gabridge et al., 1969; Batzinger et al., 1978; Tunek et al., 1978) played a major role in enhancing benzene clastogenicity was ruled out. The differences observed between the two routes of administration could be due to more rapid benzene absorption after intraperitoneal injection than oral gavage, resulting in increased loss of unmetabolized benzene via the lungs. The volatility of benzene has been described as a factor which differentiates its metabolic fate from most drugs, which are also lipophilic, but not volatile (Gut, 1976). Therefore, not as many dividing cells would be exposed to the clastogenic effect of benzene due to the limited availability of the compound for biotransformation after i.p. injection. Lukas et al. (1971) have demonstrated that compounds administered i.p. were absorbed primarily through the portal circulation — similar to the p.o. route — but were very rapidly distributed into the entire blood volume. On the other hand, a possible role of small intestine mixed-function oxidases in the biotransformation of orally administered benzene remains undetermined.

Sacrifice at 30 h vs. 54 h

The clastogenic effect of the second benzene dose 6 and 30 h prior to termination, i.e. at 30 and

54 h, respectively, differs in MNT from MA. In the MNT, the PCE are not a target for benzene's clastogenicity since they are non-dividing; instead, they reflect the chromosomal damage sustained by dividing erythroblasts and become micronucleated (MPCE). Cole et al. (1979, 1981) have determined the timing of the final stages of mouse bone marrow erythropoiesis as roughly 10 h from the final mitosis to the expulsion of the nucleus from an orthochromatic erythroblast to produce a PCE. Considering cell-cycle phases G_1 , S, and G_2 of 1.0, 7.5, and 1.5 h respectively, a clastogen acting in S phase, similar to benzene (Irons, 1981), should not show any effect before at least 1.5 ± 10 h after treatment. However, the time necessary for benzene uptake, the formation of the active metabolite(s) as well as a mitotic delay caused by the parent compound, and the need to accumulate a scorable number of MPCE considerably prolongs the period for optimum increase in MPCE. In Table 3, for example, the 3-MCA-benzene p.o. treated group at 54 h maintained a similar rate of MPCE as the 30-h group — approximately 105 per 1000 PCE — due to the second benzene dose. However, the 54-h group exhibited a higher rate of *total* micronucleated cells — about 160 per 1000 PCE — due to the increase in micronucleated oxyphilic erythrocytes which are, presumably a function of the first benzene dose (Von Ledeberg and Schmid, 1973). The contribution of the second benzene treatment 6 h prior to sacrifice is greater in the induction of aberrations in the metaphase chromosomes than the MNT. The metaphases are cells that were themselves exposed to clastogenic benzene metabolite(s) late in their S phase. Thus, the frequency of aberrations becomes lower, as a result of loss at division, at later — 54 h — fixation time; however, 3-MCA induction has significantly increased the aberrations at 54 h (Table 4).

Benzene oxide was found to be very potent in terms of genetic toxicity and DNA damage and was positive in the Ames assay (Kinoshita et al., 1981). Products of benzene metabolism were formed upon the addition of benzene oxide to a microsomal preparation (Jerina et al., 1968). Gonasun et al. (1973) found that treatment of mice with benzene increased the metabolism of benzene in vitro without increasing cytochrome

P-450 concentrations, and that treatment with PB increased cytochrome P-450 but did not increase benzene metabolism; on the other hand, SKF-525A inhibited benzene metabolism. Thus, the likely events involved in the biotransformation of benzene leading to its myeloclastogenicity would entail the reported ability of benzene to induce its own metabolism (Snyder et al., 1967), described as being similar to that of the polycyclic aromatic hydrocarbons (Gonasun et al., 1973; Saito et al., 1973; Tunek and Oesch, 1979; Ungvary et al., 1981). This study confirms that 3-MCA, a polycyclic aromatic hydrocarbon and an inducer of P-448 monooxygenase (Haugen et al., 1976; Tunek and Oesch, 1979; Boyd, 1980; Delaforge et al., 1980; Dresner et al., 1981; Nebert, 1981; Nebert et al., 1981), induces a critical pathway of benzene metabolism in mice that leads to enhanced myeloclastogenicity. It is also possible that 3-MCA induces bone marrow mixed-function oxidase (Andrews et al., 1976, 1979; Boyd, 1980; Irons et al., 1980; Dresner et al., 1981), especially the nuclear aryl hydrocarbon hydroxylase (Jernström et al., 1976) which, due to its proximity to nuclear DNA, could play a significant role in covalent binding of metabolite(s) to DNA (Snyder et al., 1978; Gill and Ahmed, 1981), leading to increased chromosomal damage, especially in a rapidly proliferative organ such as bone marrow. Benzene oxide (Jerina et al., 1968; Snyder and Kocsis, 1975), proven a potent inducer of DNA damage (Kinoshita et al., 1981), and perhaps with catechol (Nomiyama, 1965; Morimoto and Wolff, 1980) or hydroquinone (Tunek et al., 1981), may play the major role in the chemical pathogenesis of benzene-induced chromosomal damage, hence aplastic anemia, preleukemia, and leukemia (Snyder et al., 1977; Hogstedt and Mittleman, 1981). We have not found in CD-1 mice any benzene metabolite (phenol, catechol or hydroquinone) with or without 3-MCA pretreatment to possess any of the potent myeloclastogenicity of the parent compound, benzene. In fact, only hydroquinone (150 mg/kg $\times$ 2) p.o. induced a mild clastogenic response (unpublished results).

In conclusion, we have found benzene induction of chromosomal damage in proliferating bone marrow cells to be dose-dependent. The effects of modifications in benzene metabolism by ap-

appropriate pretreatment, as 3-MCA or co-administration of toluene, are reflected in the extent of chromosomal damage, being increased or decreased respectively. The sex difference, with females consistently more resistant to benzene, is maintained with induction of benzene metabolism by 3-MCA or its inhibition by co-administered toluene. The oral route more than the intraperitoneal route of benzene administration results in greater chromosomal damage which is distinctly increased with 3-MCA induction. PB, Aroclor 1254 or SKF-525A pretreatments have not modified benzene myeloclastogenicity. This study leaves no doubt that a metabolite(s) of benzene is responsible for the myeloclastogenicity of the parent compound. Toluene is not clastogenic, even with 3-MCA pretreatment. The myeloclastogenic effect of benzene appears to be the earliest benzene-induced cellular damage that is readily identifiable (scorable), antedating changes in other hematologic parameters such as peripheral blood counts.

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References

- Anderson, D., and C.R. Richardson (1981) Issues relevant to the assessment of chemically induced chromosome damage in vivo and their relationship to chemical mutagenesis, *Mutation Res.* 90, 261-272.
- Andrews, L.S., B.R. Sonawane and S.J. Yaffe (1976) Characterization and induction of aryl hydrocarbon benzo(a)pyrene hydroxylase in rabbit bone marrow, *Res. Commun. Chem. Pathol. Pharmacol.* 15, 319-330.
- Andrews, L.S., E.W. Lee, C.M. Witmer, J.J. Kocsis and R. Snyder (1977) Effects of toluene on the metabolism, disposition and hemopoietic toxicity of [^3H]benzene, *Biochem. Pharmacol.* 26, 293-300.
- Andrews, L.S., H.A. Sasame and J.R. Gillette (1979) [^3H]Benzene metabolism in rabbit bone marrow, *Life Sci.* 25, 567-572.
- Au, W., S. Pathak, C.J. Collie and T.C. Hsu (1978) Cytogenetic toxicity of gentian violet and crystal violet on mammalian cells in vitro, *Mutation Res.* 58, 269-276.
- Babich, H., and D.L. Davis, (1981) Phenol: A review of environmental and health risks, *Regul. Toxicol. Pharmacol.* 1, 90-109.
- Batzinger, R.P., E. Bueding, B.S. Reddy and J.H. Weisburger (1978) Formation of a mutagenic drug metabolite by intestinal microorganisms, *Cancer Res.* 38, 608-612.
- Boyd, M.R. (1980) Effects of inducers and inhibitors on drug-metabolizing enzymes and on drug toxicity in extrahepatic tissues, in: *Environmental Chemicals, Enzyme Function and Human Disease*, Ciba Found. Symp., Vol. 76, pp. 43-60.
- Cobr, K., and J. Stockholm (1979) Toluene, a toxicologic review, *Scand. J. Work Environ. Health* 5, 71-90.
- Cole, R.J., N.A. Taylor, J. Cole and C.F. Arlett (1979) Transplacental effects of chemical mutagens detected by the micronucleus test, *Nature (London)* 277, 317-318.
- Cole, R.J., N.A. Taylor, J. Cole and C.F. Arlett (1981) Short-term test for transplacentally active carcinogens, I. Micronucleus formation in fetal and maternal mouse erythroblasts, *Mutation Res.* 90, 141-157.
- Comings, D.E. (1974) What is a chromosome break? in: J. German (Ed.), *Chromosomes and Cancer*, Wiley, New York, pp. 95-133.
- Connor, T.H., J. Mayne, L. Molina and M.S. Legator (1979) A combined testing protocol approach for mutagenicity testing, *Mutation Res.* 64, 19-26.
- Dean, B.J. (1978) Genetic toxicology of benzene, toluene, xylenes and phenols, *Mutation Res.* 47, 75-97.
- DeLaforge, M., C. Ioannides and D.V. Parke (1980) Inhibition of cytochrome P-448 mixed function oxidase activity following administration of 9-hydroxyellipticine to rats, *Chem.-Biol. Interact.* 32, 101-110.
- Diaz, M., A. Reiser, L. Reiser and J. Diaz (1980) Studies on benzene mutagenesis, I. The micronucleus test, *Experientia* 36, 297-298.
- Dobrokhotoy, V.B. (1972) Mutagenic action of benzene and toluene under experimental conditions, *Gig. Sanit.* 37, 36-39.
- Dobrokhotoy, V.B., and M.J. Enikeev (1976) Mutagenic effect of benzene, toluene, and a mixture of these hydrocarbons in a chronic experiment, *Gig. Sanit.* 41, 32-34.
- Dresser, J.H., N.G. Ibrahim and R.D. Levere (1981) Presence and induction of drug metabolizing enzymes in rat bone marrow, *Res. Commun. Chem. Pathol. Pharmacol.* 32, 281-298.
- Drew, R.T., J.R. Fouts and C. Harper (1973) The influence of certain drugs on the metabolism and toxicity of benzene, in: D. Braun (Ed.), *Symposium on Toxicology of Benzene and Alkylbenzenes*, Industrial Health Foundation, Pittsburgh, PA, pp. 17-31.
- Environmental Protection Agency (1980) *Ambient Water Quality Criteria for Benzene*.
- Forni, A., E. Pacifico and A. Limonta (1971) Chromosome studies in workers exposed to benzene or toluene or both, *Arch. Environ. Health* 22, 373-378.

- Freedman, M.L. (1977) The molecular site of benzene toxicity. *J. Toxicol. Environ. Health*, Suppl. 2, 37-43.
- Gabridge, M.D., A. DeNunzio and M.S. Legator (1969) Cycasin: Detection of associated mutagenic activity in vivo. *Science*, 163, 689-691.
- Gill, D.P., and A.E. Ahmed (1981) Covalent binding of [14 C]benzene to cellular organelles and bone marrow nucleic acids. *Biochem. Pharmacol.*, 30, 1127-1131.
- Gill, D.P., R.R. Kempen, J.B. Nash and S. Ellis (1979) Modifications of benzene myelotoxicity and metabolism by phenobarbital, SKF-525A and 3-methylcholanthrene. *Life Sci.*, 25, 1633-1640.
- Goetz, P., R.J. Sram and J. Dolnikova (1975) Relationship between experimental results in mammals and man. I. Cytogenetic analysis of bone marrow injury induced by a single dose of cyclophosphamide. *Mutation Res.*, 31, 247-254.
- Gonessa, L.M., C. Wimmer, J.J. Kocsis and R. Snyder (1973) Benzene metabolism in mouse liver microsomes. *Toxicol. Appl. Pharmacol.*, 26, 398-406.
- Greenlee, W.F., and R.D. Irons (1981) Modulation of benzene-induced lymphocytopenia in the rat by 2,4,5,2',4',5'-hexachlorobiphenyl and 3,4,3',4'-tetrachlorobiphenyl. *Chem.-Biol. Interact.*, 33, 345-360.
- Greenlee, W.F., E.A. Gross and R.D. Irons (1981a) Relationship between benzene toxicity and the disposition of 14 C-labelled benzene metabolites in the rat. *Chem.-Biol. Interact.*, 33, 285-299.
- Greenlee, W.F., J.D. Sun and J.S. Bus (1981b) A proposed mechanism of benzene toxicity: Formation of reactive intermediates from polyphenol metabolites. *Toxicol. Appl. Pharmacol.*, 59, 187-195.
- Gut, I. (1976) Effect of phenobarbital pretreatment on in vitro enzyme kinetics and in vivo biotransformation of benzene in the rat. *Arch. Toxicol.*, 35, 195-206.
- Gut, I., K. Hudec and L. Ziskova (1981a) Effect of phenobarbital pretreatment on benzene biotransformation in the rat. *Arch. Toxicol.*, 47, 13-24.
- Gut, I., J. Kopecky, J. Nerudova, M. Krivcova and L. Peloch (1981b) Metabolic and toxic interactions of benzene and acrylonitrile with organic solvents. in: J. Gut, M. Cikrt and G.L. Flaa, *Industrial and Environmental Xenobiotics*, Springer, Berlin, pp. 255-262.
- Haugen, D.A., M.J. Coon and D.W. Nebert (1976) Induction of multiple forms of mouse liver cytochrome P-450. Evidence for genetically controlled de novo protein synthesis in response to treatment with β -naphthoflavone or phenobarbital. *J. Biol. Chem.*, 251, 1817-1828.
- Hite, M., M. Pecharo, I. Smith and S. Thornton (1980) The effect of benzene in the micronucleus test. *Mutation Res.*, 77, 149-155.
- Hogstedt, B., and F. Mielman (1981) Brief report. The interrelations of micronuclei, chromosomal instability and mutational activity in acute non-lymphocytic leukemia-A hypothesis. *Hereditas*, 95, 163-167.
- Ikeda, M., and H. Ohtsuiji (1973) Phenobarbital-induced protection against toxicity of toluene and benzene in the rat. *Toxicol. Appl. Pharmacol.*, 20, 30-43.
- Ikeda, M., H. Ohtsuiji and T. Imamura (1972) In vivo suppression of benzene and styrene oxidation by co-administered toluene in rats and effects of phenobarbital. *Xenobiotica*, 2, 101-106.
- Irons, R.D. (1981) Benzene-induced myelotoxicity: Application of flow cytofluorometry for the evaluation of early proliferative change in bone marrow. *Environ. Health Perspect.*, 39, 39-49.
- Irons, R.D., J.G. Dent, T.S. Baker and D.E. Richter (1980) Benzene is metabolized and covalently bound in bone marrow in situ. *Chem.-Biol. Interact.*, 30, 241-245.
- Jarins, D., J. Daly, B. Whitkop, P. Zaitman-Nirenberg and S. Udenfried (1968) Role of the arane-oxide-oxygen system in the metabolism of aromatic substrates. I. In vitro conversion of benzene oxide to a permercapturic acid and a dihydrodiol. *Arch. Biochem. Biophys.*, 128, 176-183.
- Jernström, B., J. Vadi and S. Orranius (1976) Formation in isolated rat liver microsomes and nuclei of benzo(a)pyrene metabolites that bind to DNA. *Cancer Res.*, 36, 4107-4113.
- Kinoshita, T., R. Santella, P. Fuhrabak and A.M. Jeffrey (1981) Benzene oxide: Genetic toxicology. *Mutation Res.*, 91, 99-102.
- Kissling, M., and B. Speck (1972a) Chromosome aberrations in experimental benzene intoxication. *Helv. Med. Acta*, 36, 59-66.
- Kissling, M., and B. Speck (1972b) Further studies on experimental benzene induced aplastic anemia. *Blut*, 25, 97-103.
- Kliesch, U., N. Danford and I.D. Adler (1981) Micronucleus test and bone marrow chromosome analysis. A comparison of 2 methods in vivo for evaluating chemically induced chromosomal alterations. *Mutation Res.*, 80, 321-332.
- Lastin, S., and B.D. Goldstein (1977) Benzene toxicity. a critical evaluation. *J. Toxicol. Environ. Health*, Suppl. 2.
- Lee, E.W., J.J. Kocsis and R. Snyder (1974) Acute effect of benzene on [59 Fe] incorporation into circulating erythrocytes. *Toxicol. Appl. Pharmacol.*, 27, 431-436.
- Loong, B.K.J. (1977) Experimental benzene intoxication. *J. Toxicol. Environ. Health*, Suppl. 2, 45-61.
- Lukat, G., S.D. Brindle and P. Greengard (1971) The route of absorption of intraperitoneally administered compounds. *J. Pharmacol. Exp. Ther.*, 178, 562-566.
- Lutz, W.K., and C.H. Schlatter (1977) Mechanism of the carcinogenic action of benzene: Irreversible binding to rat liver DNA. *Chem.-Biol. Interact.*, 18, 241-245.
- Malaville, C., T. Kuroki, G. Brun, A. Hausenfeulle, A.-M. Camus and H. Baruch (1979) Some factors determining the concentration of liver proteins for optimal mutagenicity of chemicals in the *Salmonella*/microsome assay. *Mutation Res.*, 63, 245-258.
- Matter, B.E., I. Jaeger and J. Grauwiler (1974) The relationship between the doses of various chemical mutagens required to induce micronuclei in mouse bone marrow and their lethal doses. in: *Proc. Eur. Soc. Study Drug Toxicity*, Vol. 14. Excerpta Medica, Amsterdam. ICS 311, pp. 303-307.
- Meyer, U.A., P.J. Meir, H. Hirsiger, U. Oiger and F.R. Althaus (1980) Induction of drug-metabolizing enzymes by phenobarbital: structural and biochemical aspects. in: *Environmental Chemicals. Enzyme Function and Human Disease*, Ciba Found. Symp., Vol. 76, pp. 101-118.