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Genotoxicity of aniline derivatives in various short-term tests

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Summary

Various substituted aniline derivatives were tested for genotoxicity in several short-term tests in order to examine the hypothesis that a substitution at both *ortho* positions (2,6-disubstitution) could prevent genotoxicity due to steric hindrance of an enzymatic activation to electrophilic intermediates. In the Salmonella/microsome assay, 2,6-dialkylsubstituted anilines and 2,4,6-trimethylaniline (2,4,6-TMA) were weakly mutagenic in strain TA100 when 20% S9 mix was used, although effects were small compared to those of 2,4-dimethylaniline and 2,4,5-trimethylaniline (2,4,5-TMA). In *Drosophila melanogaster*, however, 2,4,6-TMA and 2,4,6-trichloroaniline (TCA) were mutagenic in the wing spot test at 2-3 times lower doses than 2,4,5-TMA. In the 6-thioguanine resistance test in cultured fibroblasts, 2,4,6-TMA was again mutagenic at lower doses than 2,4,5-TMA. Two methylene-bis-aniline derivatives were also tested with the above methods: 4,4'-methylene-bis-(2-chloroaniline) (MOCA) was moderately genotoxic in all 3 test systems whereas 4,4'-methylene-bis-(2-ethyl-6-methylaniline) (MMEA) showed no genotoxicity at all. DNA binding studies in rats, however, revealed that both MOCA and MMEA produced DNA adducts in the liver at levels typically found for moderately strong genotoxic carcinogens. These results indicate that the predictive value of the *in vitro* test systems and particularly the Salmonella/microsome assay is inadequate to detect genotoxicity in aromatic amines. Genotoxicity seems to be a general property of aniline derivatives and does not seem to be greatly influenced by substitution at both *ortho* positions.

Ortho,ortho-dialkylated anilines, in general, are reported to be non-genotoxic (Zimmer et al., 1980; Mulholland et al., 1983). The hypothesis was that a substitution at both *ortho* positions could prevent genotoxicity due to steric hindrance of the enzymatic oxidation of the aromatic amino group. This observation had been made earlier by various

authors (Holland et al., 1974; Garner et al., 1975; Ashby, et al., 1982). Benzidine, for example, is genotoxic in most short-term tests (Ashby, 1981) and carcinogenic in mammals (Vesselinovitch et al., 1975; Nelson et al., 1982) while its analogue 3,3',5,5'-tetramethylbenzidine was not mutagenic in Salmonella, in yeast, or in higher eukaryotes *in vivo* and *in vitro*. In mammals, the latter was not carcinogenic at doses 100-1000 times higher than benzidine (Holland et al., 1974). It was suggested that this lack of genotoxicity was a result of steric hindrance by the 2 alkyl groups *ortho* to the amine

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group, as primary aromatic amines are known to need enzymatic activation to the corresponding hydroxylamine derivatives to express genotoxic activity. In contradiction to this hypothesis, however, 2,4,6-trimethylaniline (2,4,6-TMA) and 2,4,6-trichloroaniline (2,4,6-TCA) are carcinogenic in mammals (Russfield et al., 1973; Weisburger et al., 1978). The aim of this study was therefore to examine more closely the genotoxic properties of *ortho,ortho*-disubstituted aniline derivatives. If such compounds were indeed non-genotoxic, they would be of great interest for replacing genotoxic aniline derivatives, as aniline derivatives are nowadays widely used chemicals. This paper describes the results of several anilines tested in the *Salmonella*/microsome assay, in the somatic mutation and recombination test in *Drosophila melanogaster*, and in the 6-thioguanine resistance test in rat fibroblast cultures. Furthermore, the DNA binding of the 2 methylenedianilines 4,4'-methylene-bis-(2-chloroaniline) (MOCA) and 4,4'-methylene-bis-(2-ethyl-6-methylaniline) (MMEA) in rats was determined.

Materials and methods

Test compounds

2,4-Dimethylaniline and 2,6-dimethylaniline were from Fluka, Buchs (Switzerland). 2-Ethyl-6-methylaniline, 2,6-diethylaniline, 2-methyl-6-isopropylaniline, 2,6-diisopropylaniline, MMEA and MOCA were from Lonza AG, Basel (Switzerland). 2,4,5-Trimethylaniline (2,4,5-TMA) was from K + K Labs, Plainview, NY (U.S.A.), 2,4,6-TMA and 2,4,6-TCA were from EGA Chemie, Stammheim (F.R.G.). The purity of all compounds was checked by gas chromatography, and was in all cases > 99%. [Methylene-¹⁴C]MOCA, with a specific activity of 216 μ Ci/mg, was purchased from Amersham (U.K.). Radiochemical purity, checked by thin-layer chromatography (TLC, cyclohexane + diethylether, 1 + 1), was > 98%.

[Methylene-¹⁴C]MMEA was synthesized by condensation of 2-ethyl-6-methylaniline (154 μ mole) with radiolabelled paraformaldehyde (73 μ mole) in 2 ml xylene solution containing 2 drops of H₂SO₄ 25% at 145°C for 23 h. After cooling the solution to 90°C, 3 drops of NaOH 50% were added to pH 7.4, and the mixture was extracted

twice with water to remove unreacted paraformaldehyde. The yield of radioactive MMEA was 73%. Its radiochemical purity, checked by TLC (toluene + acetone, 3 + 1), was > 98%.

Salmonella / microsome assays

Salmonella typhimurium strains TA98 and TA100 were provided by Dr. B.N. Ames (University of California, Berkeley, CA, U.S.A.). The S9 fraction (9000 \times g supernatant of liver homogenate of aroclor 1254-induced SD rats; 10–40% in the S9 mix) was prepared according to Ames et al. (1975). The liver S9 fraction of aroclor-induced or non-induced Syrian hamsters was kindly supplied by Dr. S. Albertini, Hoffmann-LaRoche AG, Basel (Switzerland). The solvent for all test compounds was dimethyl sulfoxide (DMSO, 50 μ l/plate). Tests were performed without preincubation following the methods of Ames et al. (1975), as pilot experiments with 2,4-dimethylaniline had shown that preincubation reduced the number of induced revertants in comparison to the standard plate incorporation protocol. The following modification was used: the ingredients for 3 replicate plates were mixed in the given order in a test tube (6.0 ml top agar, 1.5 ml S9 mix, 0.15 ml DMSO containing the test compound, and 0.3 ml bacteria); the suspension was vigorously shaken and 3 equal parts of the mixture were added on each of 3 replicate plates. Compounds were tested at various dose levels, the limiting factor in all cases was their solubility in the aqueous top agar. The presence of a background lawn of bacteria on the minimal medium plates was taken as an indicator of no gross toxic effects. Plates were used in triplicate to determine the number of revertants. For a comparison of the compounds, the relative mutagenicity was calculated: total number of revertants on treated plates divided by the number of revertants on the plates with solvent only.

Somatic mutation and recombination test in *Drosophila melanogaster*

72-h-old larvae of *Drosophila melanogaster*, trans-heterozygous for the mutations multiple wing hair (mwh, 3-0.0) and flare (flr, 3-38.8), were fed the test compounds for 48 h. Test compounds were dissolved in an aqueous solution containing

3% absolute ethanol and 1% Tween 80 and used to prepare *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply Company, Burlington, NC, U.S.A.). In order to determine the spontaneous mutation frequency, additional larvae were simultaneously exposed to the solvent only. The larvae were treated, and the wings prepared and scored for induced spots according to Graf et al. (1984). Statistical analysis of the data was carried out as described by Frei and Würzler (1988).

6-Thioguanine resistance test in cultured rat fibroblasts

Fibroblasts were isolated from the upper inside of a subcutaneous air pouch (granuloma pouch assay, Maier, 1984), 4 days after the subcutaneous injection of 25 ml of germ-free air into the loose connective tissue between the shoulder blades of 230–260-g (54 ± 4 days old) male rats (Sprague–Dawley [Iva:SIV-50.SD], Ivanovas, Kissleg, F.R.G.) (Maier et al., 1987). Isolated cells were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum and 0.5% gentamycin for 3 days, subsequently trypsinized, seeded, and exposed to the test agents after another 24 h in culture (pilot experiments) or they were exposed immediately after a 24-h culture period following isolation (subsequent experiments). Exposure intervals chosen were 7 h in the pilot experiments and 24 h in the subsequent experiments. After a 24-h recovery period, cells were trypsinized, and subjected to a 6-day expression period. Mutant clones were recovered after a 7-day growth period in selective 6-thioguanine medium as described earlier (Maier et al., 1987). The solvents used were acetone for the 3 chemicals 2,4,5-TMA, 2,4,6-TMA and MOCA (1% in the medium), and methanol for MMEA (1% in the medium). Significance of the results was tested by the method of Kastenbaum and Bowman.

DNA binding in rat liver, lung, kidney, and bladder

Both MOCA and MMEA were dissolved in 0.05 N HCl. In the case of MOCA, 2 application solutions were prepared that differed 10-fold in specific radioactivity, while MMEA was tested at only one dose level. Both compounds were administered to male 210–240-g Sprague–Dawley rats [Iva:SIV-50.SD] (Ivanovas, Kissleg, F.R.G.) by

oral gavage. Chemical and radioactivity doses are presented in Table 4. Urine was collected for 4 h. 20 h after administration, the animals were killed by heart puncture under ether anesthesia, and liver and lung were excised. The carcasses were frozen at -20°C . Later they were thawed to remove kidneys and bladder. Isolation and purification of DNA was performed essentially according to Sagelsdorff et al. (1983): preparation of a chromatin pellet by precipitation with a detergent, then homogenizing in lysing medium, deproteinating with phenol/chloroform/isoamyl alcohol, and extraction with ether. DNA was then purified by adsorption on a hydroxylapatite column, dialysed, and precipitated with ethanol. After dissolving pure DNA in buffer, the concentration was determined spectrophotometrically, the radioactivity of an aliquot was measured by scintillation counting, and the specific radioactivity calculated. If the DNA was radiolabelled, another purification round was performed involving deproteination, ether extraction, dialysis, precipitation with ethanol, and dissolving in buffer, to again determine the specific activity. Those DNA samples which contained more than a total of 50 cpm were subjected to nucleotide analysis according to Sagelsdorff et al. (1988). DNA was enzymatically hydrolyzed to the 3'-deoxynucleotides which were separated by high-performance liquid chromatography (HPLC). This made it possible to distinguish between radioactivity coeluting with the natural DNA constituents (i.e., radioactivity which had been biosynthetically incorporated into the nucleotides) and radioactivity eluting apart from natural DNA constituents (indicating covalently bound test compound).

In order to determine the possible contribution of radiolabelled protein to the radioactivity on the DNA (protein contamination can amount to 0.2% in DNA with the described DNA isolation method, as shown by Caviezel et al., 1984), radioactivity bound to protein was determined. This involved repeated precipitation of chromatin protein (from the phenol/chloroform/isoamyl alcohol extract) with acetone and dissolving in a detergent to constant radioactivity (Sagelsdorff et al., 1983). The specific activity was determined by scintillation counting of an aliquot after determination of the protein concentration by the Lowry method.

DNA was isolated from a control rat which did not receive radioactivity in order to show that no radioactive contamination occurred during DNA isolation. The specific DNA activity attributable to covalent nucleotide-test compound adducts was expressed per administered dose and converted to the molar units of the 'covalent binding index' (CBI) (Lutz, 1979) as μ mole chemical bound per mole DNA nucleotide/mole chemical administered per kg body weight.

Results

Salmonella/microsome assays

Experiments with 2,6-dimethylaniline in TA100 showed an increasing mutagenicity when the amount of rat liver homogenate in the S9 mix was elevated from 10% to 20%. Therefore, all tests were carried out with 20% S9 mix. 2,6-Dimethylaniline, 2-ethyl-6-methylaniline, 2,6-diethylaniline, and 2-isopropyl-6-methylaniline were all weakly mutagenic in strain TA100. The small dose-related increase in the relative mutagenicity was in all cases below a factor of 2. With increasing chain length from ethyl to isopropyl a decrease in mutagenicity can be noted; 2,6-diisopropyl aniline was no longer mutagenic. Results are presented in Table 1. None of these compounds showed mutagenicity in strain TA98. Results with trisubstituted anilines are presented in Fig. 1a. 2,4,6-TMA gave significant dose-related mutagenic effects in TA100 which were, however, very weak compared to those of 2,4,5-TMA. Of the dianilines MMEA failed to

give a positive result in TA98 or TA100 although various amounts of rat or Syrian hamster liver homogenate were used in the S9 mix, whereas the simultaneously tested MOCA was positive in all experiments (Fig. 1b). Activation of MOCA by hamster instead of rat liver homogenate increased the mutagenicity.

Somatic mutation and recombination test in Drosophila melanogaster

Three trisubstituted anilines (2,4,5- and 2,4,6-TMA and 2,4,6-TCA) as well as MOCA and MMEA, but not disubstituted derivatives, were investigated for genotoxicity in *Drosophila*. The trimethylanilines were both tested at 3 different concentrations. The results of all tested compounds are presented in Table 2. As 2,4,6-TMA was 2-3 times more toxic than 2,4,5-TMA for the larvae, it had to be tested at lower doses. Both compounds were positive for small single spots; large single and twin spots were not significantly different from controls. At 4 mM, 2,4,6-TMA showed a comparable genotoxicity to 2,4,5-TMA at 15 mM while 2,4,6-TCA at 5 mM was slightly more genotoxic than 2,4,6-TMA. MOCA and MMEA were both tested at 5 mM as higher doses were insoluble in the solvent. MOCA was weakly positive for small single spots but negative for large single spots and twin spots (Fig. 2a). MMEA was negative for all spot sizes at 5 mM (Fig. 2b). As the twin spots were not different from controls, the figures show only the results of the single spots.

TABLE 1

MUTAGENICITY OF 2,6-DISUBSTITUTED ANILINES IN *Salmonella typhimurium* TA100 AFTER METABOLIC ACTIVATION WITH 20% RAT LIVER HOMOGENATE IN THE S9 MIX

To allow comparison, the results are presented as relative mutagenicity (total revertants of triplicate plates divided by spontaneous revertants). nd, no data. t, toxic for bacteria.

Dose (μ mole/plate)	Relative mutagenicity				
	Dimethyl- aniline	Methylethyl- aniline	Diethyl- aniline	Methylisopropyl- aniline	Diisopropyl- aniline
4.1	1.21	1.02	1.30	1.15	0.97
8.3	1.26	1.23	1.32	1.12	0.95
12.4	1.36	nd	1.32	1.10	t
16.5	1.39	1.51	t	t	
24.6	1.48	1.52			
33.1	1.43	t			

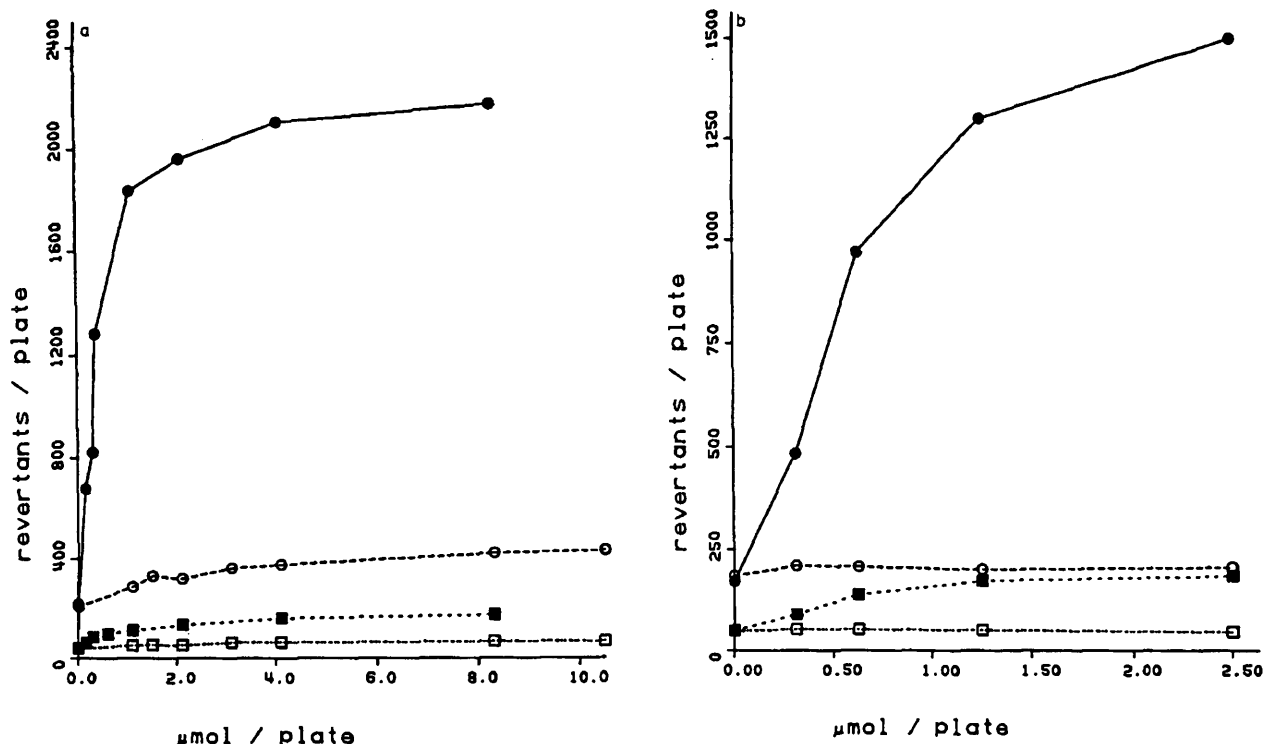


Fig. 1. (a) Mutagenicity of 2,4,5- and 2,4,6-trimethylaniline (TMA) in *Salmonella typhimurium* TA98 and TA100 after metabolic activation with 20% rat liver homogenate in the S9 mix. Total number of revertants per plate as mean of triplicates. ●—● 2,4,5-TMA in TA100, ○—○ 2,4,6-TMA in TA100, ■—■ 2,4,5-TMA in TA98, □—□ 2,4,6-TMA in TA98. (b) Mutagenicity of MOCA and MMEA in *Salmonella typhimurium* TA98 and TA100 after metabolic activation with 20% rat liver homogenate in the S9 mix. Total number of revertants per plate as mean of triplicates. ●—● MOCA in TA100, ○—○ MMEA in TA100, ■—■ MOCA in TA98, □—□ MMEA in TA98.

6-Thioguanine resistance test in rat fibroblast cultures

Pilot experiments showed that freshly isolated (as opposed to subcultured) fibroblasts, incubated at 19% O₂ (as opposed to 5% O₂), were most sensitive for testing aniline derivatives. Treatment of the cells with the test compound for 24 rather

than 7 h further increased the number of mutants. Experiments were carried out in a dose range of 74–740 μmole test compound per ml medium for 2,4,5-TMA, and of 37–148 μmole/ml for 2,4,6-TMA, as 2,4,6-TMA was rather toxic for the cells. 2,4,6-TMA showed the maximum rate of mutants at 74 μmole/ml whereas for 2,4,5-TMA the maxi-

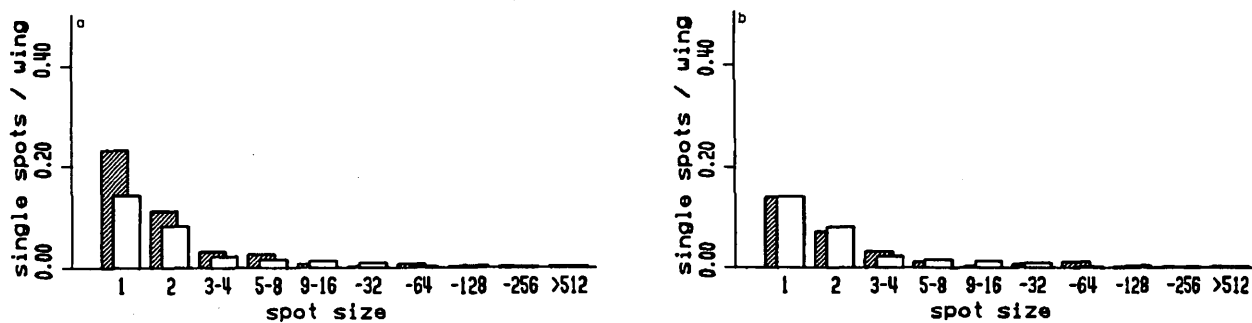


Fig. 2. Single wing spots induced by 48 h treatment of 72-h-old trans-heterozygous larvae of *Drosophila melanogaster* with 5 mM MOCA (a) and 5 mM MMEA (b). Open bars: controls (442 wings); hatched bars: treated series (200 wings).

TABLE 2

WING SPOTS INDUCED BY 48-h TREATMENT OF 72-h-old TRANS-HETEROZYGOUS LARVAE OF *Drosophila melanogaster*

Compound concentration	Number of wings	Spots per wing and statistical diagnoses ^a			
		small single spots m = 2.0	large single spots m = 5.0	twin spots m = 5.0	total spots m = 2.0
Control	442	0.22	0.05	0.01	0.28
2,4,5-TMA					
10 mM	136	0.33 +	0.06 -	0.01 i	0.40 +
15 mM	160	0.41 +	0.08 -	0.03 i	0.52 +
20 mM	184	0.57 +	0.07 -	0.03 i	0.66 +
2,4,6-TMA					
2 mM	90	0.21 -	0.08 -	0.01 i	0.30 -
4 mM	120	0.37 +	0.10 w	0.01 i	0.47 +
6 mM	160	0.37 +	0.09 -	0.03 i	0.48 +
2,4,6-TCA					
5 mM	80	0.45 +	0.08 -	0.01 i	0.54 +
MOCA					
5 mM	200	0.34 +	0.06 -	0.01 -	0.42 w
MMEA					
5 mM	240	0.21 -	0.05 -	0.00 -	0.26 -

^a Statistical diagnoses according to Frei and Würzler (1988). +, positive; -, negative; w, weak; i, inconclusive. Probability levels $\alpha = \beta = 0.05$. One-sided statistical tests. Small single spots, 1-2 cells; large single spots, > 2 cells. m, multiplication factor.

mum rate was at 148 μ mole/ml. No dose response was obtained, which is considered to be due mainly to a toxic or growth-inhibiting activity of the chemicals. Results are summarized in Table 3.

Treating freshly isolated fibroblasts with 35-364 nmole MMEA per ml medium resulted in no mutant clones in 2 independent experiments. MOCA tested in the same dose range induced 2 mutants per 10^6 cells at 36 nmole/ml medium (not significantly different from control) and 4 mutants per 10^6 cells at 72 nmole/ml medium. In a second identical test no clones could be detected.

DNA binding of MOCA and MMEA

The amount of radioactivity excreted in the urine during 4 h following oral application was 17% in the MOCA high-dosed rats, 10% in the MOCA low-dosed rats, and 8% in the rats treated with MMEA. When the rats were killed after 20 h,

2% of the administered radioactivity was found in the liver, 0.13-0.19% in the blood, 0.10-0.15% in the kidneys, and less than 0.01% in the bladder. In the lung, a marked difference was noted between the MOCA- and the MMEA-treated animals: 0.03-0.09% of labelled MOCA (or metabolites) was found versus 0.016% of labelled MMEA (or metabolites). The results with MOCA agree with those found by Farmer et al. (1981) and Tobes et al. (1983).

From the first to the second DNA purification the specific activity was either constant or not significantly decreased, so that a third purification round was not necessary. In order to distinguish between biosynthetic incorporation of radioactive fragments into the DNA and genuine covalent binding, DNA was enzymatically degraded to the nucleotides which were separated by HPLC. The specific radioactivity of the MOCA liver and lung DNA and MMEA liver DNA was sufficiently high (52-320 dpm/mg) to allow for this analysis.

TABLE 3

6-THIOGUANINE RESISTANCE TEST

Induction of mutations after 24 h in vitro exposure of fibroblasts to 2,4,5- and 2,4,6-trimethylaniline (TMA), and 4,4'-methylene-bis-(2-chloroaniline) (MOCA), and 4,4'-methylene-bis-(2-ethyl-6-methylaniline) (MMEA). nd, no data. -, no clones detected, resulting in a mutant frequency below 1.1-4.4 (depending on the number of cells tested).

Compound concentration ($\mu\text{g}/\text{ml}$ medium)	Cloning efficiency (%)		Clone forming cells tested $\cdot 10^5$		Mutant frequency (mean $\cdot 10^{-6}$)	
	expt. 1	expt. 2	expt. 1	expt. 2	expt. 1	expt. 2
2,4,5-TMA						
74	75	nd	9.00	nd	-	nd
148	74	52	8.88	6.24	11.3	8.0
370	64	46	5.12	5.52	3.9	1.8 ^a
740	63	nd	4.41	nd	-	nd
2,4,6-TMA						
37	69	60	8.28	7.20	1.2 ^a	1.4 ^a
74	62	51	7.44	6.12	6.7	4.9
148	64	56	7.68	6.72	2.6	-
Control	91	72	9.10	8.64	-	1.2
MOCA						
36	56	50	5.04	6.00	2.0 ^a	-
72	47	41	3.76	4.92	4.0	-
181	39	40	3.12	4.80	-	-
362	41	36	4.10	4.32	-	-
MMEA						
36	59	52	4.72	6.24	-	-
72	51	46	4.08	5.52	-	-
177	49	45	4.41	5.40	-	-
364	38	40	2.28	4.80	-	-
Control	63	80	7.56	8.80	-	1.5

^a Not significantly different from control (method of Kastenbaum and Bowman, probability levels $\alpha = \beta = 0.05$, one-sided statistical tests).

In all 3 samples, 2 peaks of radioactivity eluted after the natural nucleotides, at retention times normally seen with lipophilic nucleotide-carcinogen adducts. These putative adducts all had different retention times and were therefore probably all structurally unique. In the liver and in the lung of the MOCA-treated animals 75% and 87% of the radioactivity, respectively, was covalently bound to DNA, and in the liver of the MMEA-treated animals 98% of the radioactivity was covalently bound. In order to compare the DNA binding ability of MOCA and MMEA with other substances, CBI values were calculated. The values ranged from 17 to 35 in liver, 1.4 to 72 in lung, 4 in kidney, and were below the limit of detection in the bladder (Table 4). There was a marked dif-

ference between MOCA and MMEA only in binding to lung DNA: MOCA produced 30-50 times more adducts than did MMEA. In the other organs the effects of the compounds were comparable. With both test compounds, the radioactivity of bladder DNA was not significantly different from control values. Because of the small amount of bladder DNA, the detection limit was at a CBI value of 4.

The specific activity of chromatin protein isolated from the lung was at about the same level as for DNA with both MOCA and MMEA. In liver, kidney, and bladder protein had a 5-10 times higher specific activity. As the maximum possible contamination of the DNA preparations with protein was less than 0.2% (Caviezel et al., 1984), this

TABLE 4

COVALENT BINDING OF [METHYLENE-¹⁴C]LABELLED 4,4'-METHYLENE-BIS-(2-CHLOROANILINE) (MOCA) AND 4,4'-METHYLENE-BIS-(2-ETHYL-6-METHYLANILINE) (MMEA) TO DNA IN MALE SPRAGUE-DAWLEY RATS, 20 h FOLLOWING ORAL GAVAGE

Test compound	MOCA				MMEA	
	Low		High		High	
Chemical dose (mg/kg)	1.43	2.32	28.1	29.6	31.6	29.3
Radioactivity (10 ⁸ dpm/kg)	6.13	11.9	14.5	15.3	16.8	15.5
DNA binding potency (CBI values)						
Liver	20 ^b	17 ^b	33 ^a	23 ^b	34 ^a	35 ^b
Lung	71 ^b	72 ^b	41 ^a	34 ^b	≤ 1.4 ^c	≤ 1.8 ^c
Kidney	nd	≤ 4 ^c	nd	≤ 4 ^c	nd	≤ 4 ^c
Bladder	< 4 ^d		< 4 ^d		< 4 ^d	

nd, no data.

^a On the basis of nucleotide analyses.

^b Percent adduct formation taken from nucleotide analysis a.

^c No nucleotide analysis possible; upper limit value derived from specific activity of DNA.

^d DNA of 2 animals pooled. Specific activity of DNA below limit of detection.

The covalent binding index (CBI) is defined as:

$$\text{CBI} = \frac{\mu\text{mole chemical bound/mole DNA nucleotide}}{\text{mmole chemical applied/kg body weight}}$$

$$\text{CBI} = \frac{\text{dpm/mg DNA}}{\text{dpm/kg body weight}} \times 3.09 \times 10^8$$

contribution to the DNA radioactivity can be ignored.

Discussion

Salmonella/microsome assays

Tests with 2,6-disubstituted compounds showed, in contrast to reports in the literature, weak mutagenicity when 20% instead of 10% rat liver homogenate was used as the activation system. The relative mutagenicity varied between 1.10 and 1.52. These weak effects are interpreted as positive because (1) they were reproducible, (2) the increase could be observed with at least 3 different doses, and (3) at least for 2,6-dimethylaniline and 2-methyl-6-ethylaniline there was a certain dose-effect relationship (weak increase of the relative mutagenicity with increasing dose).

Increase of the length of the side chain in the *ortho* positions resulted in decreased mutagenicity (2,6-diisopropylaniline was negative at the tested

doses). The same effect has been seen in a series of phenylenediamines with alkyl or alkoxy substituents (Shahin et al., 1983). 2,6-Dialkylanilines with longer side chains showed increased bactericidal effects together with a decreased mutagenicity.

*Somatic mutation and recombination test in *Drosophila melanogaster**

In *Drosophila* the test compounds tended to induce mainly small spots, which suggests that the somatic mutations were induced late in the development of the larvae, at a time point where only 1-2 more cell divisions took place. This phenomenon can be explained by the increasing activity of the metabolizing enzymes in the course of the development of the larvae (Hällström et al., 1983). From the absence of induced twin spots it can be concluded that the compounds do not exhibit strong recombinogenic activity. When MOCA and MMEA were tested, problems arose because of their low solubility in the aqueous solvent. The

maximum possible concentration was 5 mM. MOCA was weakly positive at this dose level whereas MMEA was negative.

6-Thioguanine resistance test in rat fibroblast cultures

The experiments with fibroblasts in culture showed the importance of adequate activation of the aniline derivatives to reactive metabolites. Modifications which could improve oxidative activation increased the sensitivity of the test: freshly isolated fibroblasts usually have a higher enzyme activity than subcultured ones, exposure of the fibroblasts to the test compounds for 24 rather than 7 h allow a more complete metabolization, and incubation at 21% instead of 5% O₂ provides more oxygen for oxidative metabolism. It can be concluded that the activation mechanism in mammalian cells for anilines is not stable under *in vitro* conditions. Therefore handling of the freshly isolated cells, culture conditions, and culture time will affect the activation of anilines. This might explain why the positive results of MOCA could not be reproduced in a second experiment. The decrease of mutagenicity at high dose levels in the thioguanine test can be explained by the possible interference of anilines with prostaglandin synthesis which in turn affects DNA synthesis and mutation fixation (Maier and Schawalder, 1988).

DNA binding in the rat

The DNA binding experiments in rats showed comparable CBI values for MOCA and MMEA in the liver. The differences of the liver CBI between the 2 dose groups of MOCA was in the range of animal-to-animal variations. A liver CBI of 20–30, as was also reported by Silk and Martin (1986) for MOCA, is typically found for weak to moderately strong carcinogens. Using the linear correlation shown by Lutz (1986) between CBI and tumorigenic potency *in vivo* (TD₅₀, Gold et al., 1984), this CBI level would correspond to a TD₅₀ of about 0.2 mmole/kg body weight/day, which correlates well with carcinogenicity data for MOCA (10 mg/kg/day to induce a 50% liver tumor incidence in CD rats; Gold et al., 1984). In the lung there was a significant difference in DNA binding between MOCA and MMEA: MOCA was 30–50 times stronger than MMEA. This suggests that

MMEA (which has not been tested for carcinogenicity) might be less likely than MOCA to cause lung tumors. In MOCA, lung DNA binding was higher than that of liver, in agreement with the finding that MOCA effectively induces lung tumors (Kommineni et al., 1978). These authors judged the lung to be the most sensitive organ for tumor formation by MOCA. The activation of MOCA to DNA reactive metabolites probably takes place in the lung and is saturable in this tissue, as the CBI is markedly lower in the lung at the high dose level. The DNA binding in kidney and bladder was very low or not detectable for both MOCA and MMEA so that significant tumor formation in these organs appears unlikely.

Structure-activity relationships

The hypothesis that aniline derivatives could be rendered innocuous by substituting both *ortho* positions was not confirmed by this work. Eight out of 9 investigated compounds tested were genotoxic in one or more systems. In addition, recent data from the National Toxicology Program showed that 2,6-dimethylaniline induced tumors in rats at about the same dose level as does 2,4,5-TMA. This is in strong contrast to the data with *Salmonella*, where 2,6-dimethylaniline was about 20 times less mutagenic than 2,4,5-TMA.

The above quantitative differences between the *Salmonella*/microsome assay and the other tests used in the present studies are illustrated even better with 2,4,5-TMA and 2,4,6-TMA. 2,4,6-TMA was about 20 times less mutagenic than 2,4,5-TMA, although it was 5–10 times more potent in evoking tumors. It seems, therefore, that the *Salmonella*/microsome assay underestimates the genotoxicity of di-*ortho*-substituted compounds in mammals. The data obtained with *Drosophila* and fibroblasts in culture, on the other hand, agree with the potency ranking seen in the carcinogenicity bioassay in rodents. The similarity of the results of tests using *Drosophila* and fibroblasts in culture had not been expected, because in the latter the compounds are probably activated via prostaglandin synthase pathways (Maier, 1984), while in *Drosophila* the activation depends mainly on cytochrome P-450 (Baars et al., 1977). While the *Drosophila* data with 2,4,5- and 2,4,6-TMA seem to correlate with tumorigenic potency in a

quantitative manner, this test system ranked 2,4,6-TCA about equally with 2,4,6-TMA. The carcinogenicity bioassay data, however, showed that 2,4,6-TCA was about 5 times less potent than the methyl analogue. These results indicate that one must be careful in attempting quantitative extrapolations.

MMEA was an even more extreme case: MMEA was negative not only in the *Salmonella*/microsome assay, but also in the somatic mutation and recombination test in *Drosophila* and in the 6-thioguanine resistance test in rat fibroblast cultures. However, it produced covalent binding to rat liver DNA at about the same level as the proven carcinogen MOCA.

A literature survey (Kugler-Steigmeier, 1988, available upon request) showed that di- and tri-substituted aniline derivatives with one free *ortho* position are generally mutagenic and carcinogenic. Negative results in the *Salmonella*/microsome assay were found for monosubstituted as well as for 2,6-di- and 2,4,6-tri-substituted compounds. In the case of the monosubstituted anilines, the results were somewhat confusing, as a few authors reported a weak mutagenicity in substances that were classified as negative by others. Therefore, several conclusions published in the past appear to be unreliable.

In considering the present experiments as well as the reports in the literature, one must conclude that no class of aniline derivatives can be considered non-genotoxic. A quantitative prediction was poor with the *Salmonella*/microsome assay, more satisfactory with *Drosophila* and rat fibroblasts in vitro. A DNA binding study in an intact mammalian organism probably reflects the complicated enzymatic activation and inactivation processes most closely (Lutz, 1986). Since genotoxicity appears to be a general characteristic of aniline derivatives, and hence quantitative data are needed for risk assessment, such quantitative in vivo covalent binding studies might therefore be preferred over qualitative results from other short-term tests.

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