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EFFECTS OF METYRAPONE ON MICROSOMAL-DEPENDENT *SALMONELLA* MUTAGENESIS

STUDIES WITH CHLOROALLYL ETHERS AND MODEL COMPOUNDS

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Abstract—Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, MTP) is used as an inhibitor of cytochrome P-450 enzymes, particularly those induced by phenobarbital (PB). We examined the effects of MTP on the microsomal dependent mutagenesis of a newly isolated promutagen, 3-(2-chloroethoxy)-1,2-dichloropropene (CP), three S-chloroallyl thiocarbamate herbicides, and four model promutagens aflatoxin B_1 (AFB), 2-acetylaminofluorene (2AAF), 2-aminoanthracene (2AA) and benzo[*a*]pyrene (BP). *Samonella* tester strains TA98, TA100 and TA1535 and liver microsomal preparations (S9) from rats induced with PB or Aroclor 1254 (PCB) were employed. For statistical analysis, mutagenesis data were transformed and subjected to two-way analysis of variance. Metyrapone alone was not mutagenic in the absence or presence of S9. In a dose-dependent manner, MTP inhibited mutagenesis of AFB for strains TA98 and TA100 and enhanced mutagenesis of 2AAF, 2AA and BP for these strains. 3-(2-Chloroethoxy)-1,2-dichloropropene and the herbicides diallate, triallate and sulfallate are all chloroallyl ethers. They are similar in their mutagenesis for *Salmonella* with respect to strain specificity, mutagenic potency, and requirement for activation by specifically-induced microsomes. Metyrapone inhibited the mutagenesis of CP, triallate and sulfallate for strain TA100 in the presence of either PB- or PCB-induced S9, and had no apparent effect on diallate mutagenesis; the same results were obtained for TA1535 with PCB-induced S9. On this basis, the mutagenic activation of diallate appears to be different from that of the other chloroallyl ethers tested. Our results indicate that MTP can inhibit as well as enhance microsomal dependent mutagenesis for *Salmonella*. We conclude that MTP may be a useful tool in characterizing pathways for promutagen activation.

For many xenobiotic compounds, metabolic transformation proceeds via the cellular cytochrome P-450 systems, and manipulation of these monooxygenase systems either by induction or by the use of inhibitors has been a major tool in characterizing such metabolism. In the *in vitro Salmonella* mutagenesis assay [1], microsomal cytochrome P-450 enzymes are utilized to provide for biotransformation. Compounds that lack direct-acting mutagenesis may be metabolized into active mutagens, or into compounds of increased or decreased bacterial toxicity. Although microsomes prepared from organs of differentially induced animals have been used extensively [2], there has been relatively little application of cytochrome P-450 inhibitors in mutagenesis assays for the manipulation of *in vitro* metabolism. We report here that one such inhibitor, metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone, MTP§), can enhance, inhibit or fail to influence microsomal dependent mutagenesis.

Metyrapone is a widely used inhibitor which preferentially blocks enzyme activities associated with phenobarbital (PB)-induced forms of cytochrome P-450 [3]. Moreover, its effects have been shown to include the inhibition of microsomal dependent mutagenesis [4–6], particularly when activation was provided by PB-induced microsomes [7]; such inhibition was less effective when activation was provided by 3-methylcholanthrene (3MC)-induced microsomes [7]. We turned to MTP in order to characterize further the metabolic activation of a new, recently identified promutagen, 3-(2-chloroethoxy)-1,2-dichloropropene, CP [8–10].

Detection of CP in the *Salmonella* mutagenesis assay was entirely dependent upon *in vitro* activation. Liver microsomal preparations from rats (S9) induced by PB or Aroclor 1254 (PCB) were most effective, with little or no mutagenic activation provided by 3MC-induced or uninduced S9 [11]. Among the five *Salmonella* tester stains, mutagenic activity of CP was limited to TA100 and TA1535 [9]. With these strains, CP can be activated *in vitro* to become a potent mutagen: given optimal PB- or PCB-induced S9 activation, mutagenic specific activity with TA100 was ≥ 75 revertants/nmole [9, 10].

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§ Abbreviations: MTP, metyrapone; CP, 3-(2-chloroethoxy)-1,2-dichloropropene; PB, phenobarbital; PCB, Aroclor 1254; 3MC, 3-methylcholanthrene; 2AA, 2-aminoanthracene; 2AAF, 2-acteylaminofluorene; AFB, aflatoxin B_1; BP, benzo[*a*]pyrene; MNNG, methyl-*N*-nitroso-*N*-nitro-guanidine; MMS, methylmethanesulfonate; ACNA, 1-amino-2-carboxy-4-nitro-anthraquinone; DMSO, dimethyl sulfoxide; and S9, 9000 *g* rat liver supernatant fraction. The three S-chloroallyl thiocarbamate herbicides are: diallate, *S*-2,3-dichloroallyl diisopropylthiocarbamate; triallate, *S*-2,3,3-trichloroallyl diisopropylthiocarbamate; and sulfallate, *S*-2-chloroallyl diethyldithiocarbamate.

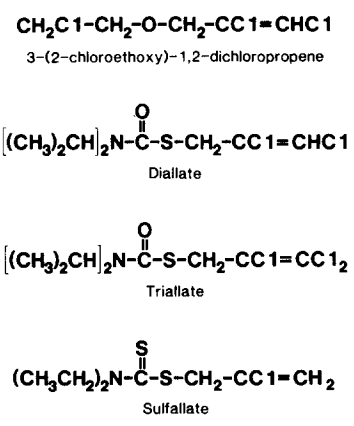

Fig. 1. Structures of the isolated promutagen CP and the S-chloroallyl thiocarbamate herbicides.

The promutagen CP, which contains both chloroethoxy and chloropropenyl moieties, is structurally similar to several compounds that have been identified previously as *Salmonella* mutagens. In contrast to CP, several in this group such as bis(2-chloroethyl)ether [12] and the chlorinated propenes [13] are active in the absence of S9 for both tester strains TA100 and TA98, and they are much less potent mutagens. More comparable to CP in mutagenicity are the S-chloroallyl thiocarbamate herbicides diallate, triallate and sulfallate. As S-chloroallyl thioethers, these herbicides are structurally related to CP, a chloroallyl oxygen ether (Fig. 1). The mutagenic properties of diallate, triallate and sulfallate toward *Salmonella* are similar to those of CP with respect to activation dependence, strain specificity, and mutagenic potency [14–17]. Additionally, they too responded to activation by PB- or PCB-induced microsomal preparations [11]. Accordingly, studies of the effects of MTP on the mutagenic activation of CP were extended to include diallate, triallate and sulfallate.

Influences of MTP upon microsomal dependent mutagenesis were also examined using four well-known promutagens as model compounds: 2-acetylaminofluorene (2AAF), aflatoxin B_1 (AFB), 2-aminoanthracene (2AA) and benzo[*a*]pyrene (BP). Our results suggest that MTP is a useful tool for the differentiation of metabolic pathways in microsomal dependent mutagenesis.

MATERIALS AND METHODS

Materials. 3-(2-Chloroethoxy)-1,2-dichloropropene was prepared as previously described [9, 10]. Diallate, triallate and sulfallate were obtained through the analytical standards program of the U.S. Environmental Protection Agency [18]. Metyrapone, 2AAF, 2AA, methyl-*N*-nitroso-*N'*-nitroguanidine (MNNG) and BP were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Aflatoxin B_1 was purchased from CalBiochem (San Diego, CA), and methylmethanesulfonate (MMS) from Eastman Kodak (Rochester, NY). 1-Amino-2-carboxy-4-nitro-anthraquinone (ACNA) was a gift from Dr. J. P. Brown (Zoecon, Palo Alto, CA). Glass-distilled dimethyl sulfoxide (DMSO) was purchased from Burdick Jackson Laboratories (Muskegon, MI). All other chemicals were reagent-grade, obtained from commercial sources, and used without further purification. Rat liver 9000 *g* supernatant fractions (S9), PCB- or PB-induced, were commercial preparations from Litton Bionetics (Kensington, MD). *Salmonella typhimurium* tester strains TA98, TA100 and TA1535 were provided by Dr. Bruce Ames, Universtiy of California (Berkeley, CA).

Mutagenesis assays. The *Salmonella*/mammalian microsome mutagenesis assay of Ames *et al.* [1] was carried out as previously described [19] using procedures consistent with the recommendations of de Serres and Shelby [20]. Experimental promutagens were dissolved in DMSO and added as 10–100 μl aliquots to the top agar. For each promutagen, initial dose response data were obtained with two strains, and the optimal concentrations of S9 were determined. Metyrapone (25, 250, 2500, or 5000 nmoles/plate) was usually incorporated in the S9 mix; in a few cases, equivalent amounts were added from aqueous solution directly to the top agar. All test promutagens were assayed with PCB-induced S9; experiments with AFB and the chloroallyl ethers were also conducted using PB-induced S9. For each separate assay, experimental and spontaneous plates were prepared in duplicate and triplicate respectively. Assays included positive controls, with and without S9, for each tester strain. The resulting control values are presented in Table 1.

Statistical analysis. Salmonella mutagenesis data were transformed by the Box-Cox power transformation $Y = (X + A)^{\lambda}$, where Y = transformed data, X = net revertant colonies/plate, $A = 1$, and $\lambda = 0.2$ as recommended by Snee and Irr [21] for statistical analysis of *Salmonella* data. This transformation procedure normalizes the distribution of experimental errors and satisfies the statistical assumption of homogeneous variance, which is necessary in order to properly use analysis of variance and other statistical procedures. Transformed data were treated by two-way analysis of variance to determine the significance of the two main effects on mutagenesis, effect of promutagen dose and effect of MTP on promutagen-induced response, and the significance of the interaction between these two main effects [22]. By this analysis, a significant effect of promutagen dose levels demonstrates a dose-dependent mutagenesis. A significant effect of MTP dose levels on promutagen-induced tester cell response indicates that MTP increases or decreases mutagenesis. The analysis may also show a significant interaction between the dose levels of both the promutagen and MTP. Such interactions mean that the effects of MTP are not the same at all dose levels of the promutagen. Conversely, the absence of an interaction between promutagen and MTP dose levels indicates that the incremental effect of MTP on S9-dependent mutagenesis is the same among all dose levels of the promutagen. Significance of the effect of each MTP dose level on the promutagen-induced response also was determined by Scheffé comparisons of group means [23].

Table 1. Revertant colonies per plate for control promutagens*

Salmonella tester strain	2AA (10 nmoles) +S9 PCB	PB	MMS (1 μl)	ACNA (160 nmoles)	MMNG (crystal)
TA100	1544 ± 198 (14)	1848 ± 116 (4)	1274 ± 166 (18)		
TA98	1603 ± 185 (9)			1718 ± 320 (11)	
TA1535	250 ± 76 (5)	527 (1)			Always positive (5)

* Doses are indicated as quantity in the top agar; MMNG was tested in a spot test in which a ring of revertants surrounding the MNNG crystals is a positive, mutagenic response. For 2AA, S9 from PCB- or PB-induced rat livers was used at 20 μl/plate. Each value is the mean from averages of duplicate colony counts per experiment ± standard deviation; the number of experiments is indicated in parentheses.

RESULTS

Preliminary experiments were conducted to verify the non-mutagenicity of MTP [5] and to provide evidence for the involvement of cytochrome P-450 in the metabolic activation of the chloroallyl ethers. Metyrapone was neither mutagenic nor toxic for strains TA98, TA100 and TA1535 in the absence or presence of PB- or PCB-induced S9 (Table 2). Additionally, MTP (25, 250 or 2500 nmoles/plate) had no effect on the direct-acting (−S9) mutagenesis of MMS (0.2, 0.6 or 1.0 μl/plate) with TA100 and of ACNA (32, 96 or 160 nmoles/plate) with TA98 (data not shown). In characterizing the metabolic activation of CP, diallate and triallate, we satisfied the following criteria used to indicate involvement of cytochrome P-450 in the metabolism of xenobiotics [24, 25]: (1) activation by specifically-induced S9 (PB- or PCB-induced); (2) NADPH-dependent mutagenesis; and (3) activation preferentially catalyzed by the 100,000 *g* pellet obtained from S9 [11]. The data with sulfallate fulfilled the same criteria except that activation required both the 100,000 *g* microsomal pellet and the supernatant fraction.

Effects of MTP upon the S9-dependent mutagenesis of four chloroallyl ethers and of AFB, BP, 2AA and 2AAF were determined in thirty separate assays which incorporated various combinations of *Salmonella* strains TA100, TA98 and TA1535 with PCB- and PB-induced rat liver S9. Results of the twenty-five experiments not involving diallate are presented in Table 3. This table shows the maximum observed effects of MTP on mutagenesis, whether inhibitory or enhancing, and the statistical significance of the effects of MTP on S9-dependent mutagenesis, including dependence of these effects on promutagen dose. Also presented in Figs. 2–6 are data from one or more assays conducted with MTP for each promutagen, including diallate.

Aflatoxin B_1 was selected to test the model of MTP inhibition of mutagenesis because its metabolic activation had been shown previously to be associated with PB-induced microsomal enzyme activities [26]. Moreover, MTP had been shown to inhibit the *in vitro* activation of AFB in assays measuring variables other than mutagenesis [27]. In the current study, MTP was examined for its effects on AFB mutagenesis in seven assays using strains TA100 or TA98 in the presence of PB- or PCB-induced S9. In all cases, MTP significantly inhibited AFB mutagenesis in a dose-dependent manner (Table 3). Data for TA100 plus PB-induced S9 are shown in Fig. 2.

For CP, MTP showed a pattern of inhibition similar to that observed for AFB. A statistically significant inhibition of CP mutagenesis by MTP was observed in the presence of PB-induced S9 for strain TA100, and in the presence of PCB-induced S9 for both TA100 and TA1535 (Table 3). Data from an assay with TA100 and PB-induced S9 are presented in Fig. 3.

To compare CP with other mutagenic chloroallyl ethers, MTP was examined for its effects on the mutagenesis of the S-chloroallyl thiocarbamate herbicides triallate, sulfallate and diallate. These compounds were recognized to be similar to CP in several respects. They had been shown to be dependent

Table 2. Non-mutagenicity of metyrapone*

MTP (nmoles/plate)	Revertant colonies/plate TA98 −S9	TA98 +S9 PCB	TA100 −S9	TA100 +S9 PB	TA100 +S9 PCB	TA1535 −S9	TA1535 +S9 PB	TA1535 +S9 PCB
0	15	21	113	141	119	21	13	16
25	22	26	134	128	118	22	15	16
250	20	27	132	134	127	10	11	10
2500	14	16	119	137	134	18	14	12
5000	18	21	130	ND†	115	ND	ND	ND

* Each value is the mean colony count of two or more plates. The activation mix provided 20 μl of PB- or PCB-induced rat liver S9 per plate.

† Not determined.

Table 3. Inhibition and enhancement by MTP of S9-dependent chemical mutagenesis*

Promutagen	Strain and S9	Maximum observed effect on mutagenic activity (% of control)	Statistical significance of: Effect of each MTP dose (nmoles/plate) 25	250	2500	5000†	Dependence of MTP effects on promutagen dose
AFB	TA100, PB	21	NS‡	§	§		§
	TA100, PCB (3)	20	NS	‖	§		NS
	TA98, PCB (3)	35	NS	NS	§		NS
CP	TA100, PB	8	‖	§	§		NS
	TA100, PCB	22	NS	§	§		NS
	TA1535, PCB	6	‖	§	§		NS
Triallate	TA100, PCB (2)	14	NS	§	§		NS
	TA100, PB	22	‖	§	§		NS
	TA1535, PCB	11	§	§	§		NS
Sulfallate	TA100, PCB	24	§	§	§		§
	TA100, PB	17	NS	§	§		NS
	TA1535, PCB	14	§	§	§		§
BP	TA98, PCB (2)	260	NS	§	§	§	NS
	TA100, PCB (2)	180	NS	‖	§	§	‖
2AA	TA98, PCB	910	NS	§	§		§
	TA100, PCB	550	‖	§	§		§
2AAF	TA98, PCB	370	NS	§	§		‖
	TA100, PCB	430	NS	§	§		§

* Diallate is not listed since its mutagenic activity was not affected significantly by MTP in each of the five assays conducted. One assay representing this lack of effect with dialate is presented in Fig. 5. Data from the underlined assays are depicted in Figs 2–4 and 6; other assays for each promutagen were conducted similarly. Results presented in this table were from single experiments or from multiple experiments as indicated by the number in parentheses. Percent of control = (net revertant colonies with promutagen in the presence of MTP/net revertant colonies with promutagen in the absence of MTP) × 100. Mutagenesis data were transformed and statistically analyzed by two-way analysis of variance and Sheffé comparisons as described in Material and Methods.

† Effect of the 5000 nmole MTP dose was only examined in assays with BP.

‡,§,‖ The effect of each MTP dose, as compared to control activity with each promutagen (no MTP), and the extent of interaction between the MTP and promutagen dose levels were determined to be: significant at $P < 0.01$ (§), significant at $P < 0.05$ (‖), or not significant ($P > 0.05$, NS).

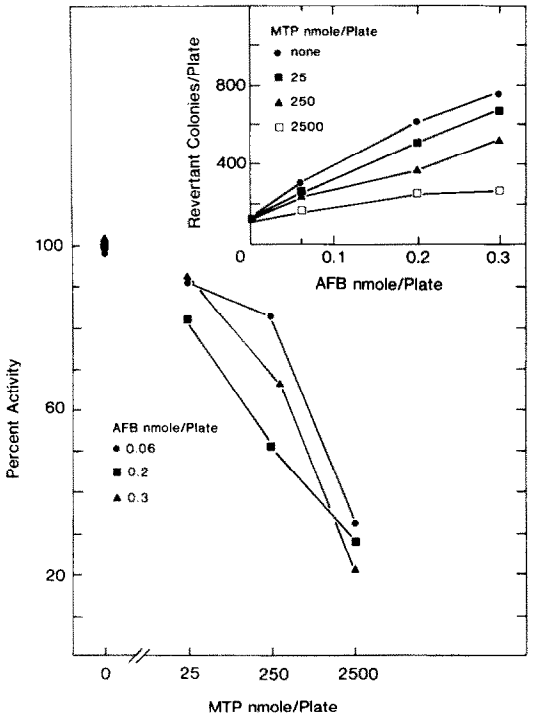

Fig. 2. Inhibition of AFB mutagenesis for TA100 by MTP with PB-induced S9. Top graph: Dose response of TA100 to AFB in the absence or presence of MTP. Each data point represents the mean of triplicate colony counts from one experiment. Each plate contained 20 μl of rat liver S9. Bottom graph: Percent activity of TA100 with AFB as a function of the MTP dose. Dose–response data in the top graph were converted to express the response of TA100 to AFB in the presence of MTP relative to the response to AFB observed in the absence of MTP. Percent activity = [net revertant colonies in the presence of MTP/ control net revertant colonies] × 100.

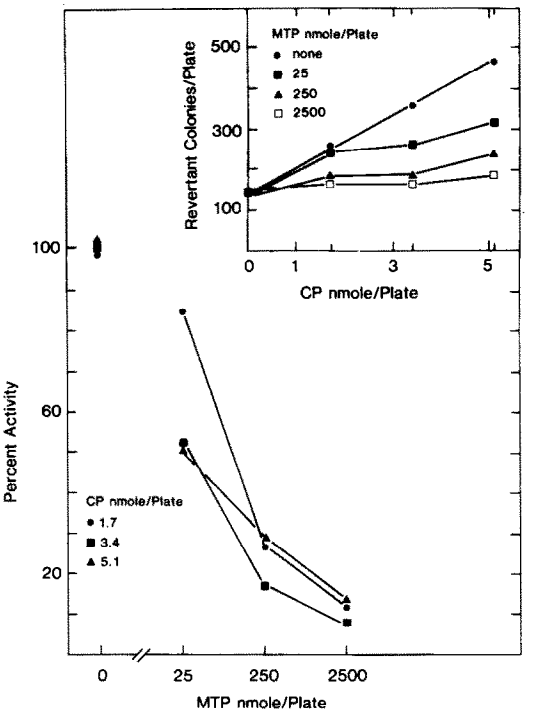

Fig. 3. Inhibition of CP mutagenesis for TA100 by MTP with PB-induced S9. Top graph: Dose response of TA100 to CP in the absence or presence of MTP. Each data point represents the mean of duplicate colony counts from one experiment. Each plate contained 20 μl of rat liver S9. Bottom graph: Percent activity of TA100 with CP as a function of the MTP dose. Percent activity was calculated as described in the legend of Fig. 2.

upon activation by PB- or PCB-induced S9, and to exhibit specificity for strains TA100 and TA1535 among the five standard tester strains [14–17]. In our assays, specific mutagenic activities (net revertants/ nmole) of triallate, sulfallate and diallate for TA100 with PCB-induced S9 were 6, 11 and 37 respectively [11]. By comparison, the value for CP was ≥ 75 revertants/nmole. The effects of MTP on the mutagenesis of triallate were determined in four assays using strain TA100 with PB- or PCB-induced S9 and strain TA1535 with PCB-induced S9. Metyrapone showed a significant dose-dependent inhibition of triallate mutagenesis (Table 3). Data combined from two experiments with strain TA100 and PCB-induced S9 are shown in Fig. 4A.

Sulfallate mutagenesis was examined in the presence of MTP in three assays using strain TA100 with PB- or PCB-induced S9 and strain TA1535 with PCB-induced S9. A pattern of MTP inhibition similar to that obtained with triallate was observed (Table 3). Data for strain TA100 with PCB-induced S9 are presented in Fig. 4B.

For the third S-chloroallyl herbicide, diallate, the effects of MTP on mutagenesis were determined in five assays using strain TA100 with PB- or PCB-induced S9 and strain TA1535 with PB- or PCB-induced S9. In contrast to the inhibitory effects of MTP on the mutagenesis of the other S-chloroallyl herbicides, MTP did not significantly inhibit diallate mutagenesis under any of the assay conditions examined. Data combined from two assays with strain TA100 in the presence of PCB-induced S9 from two experiments are shown in Fig. 5.

Also examined were the effects of MTP on the mutagenesis of BP, 2AA and 2AAF for TA98 and TA100 with activation by PCB-induced S9. For all three compounds, mutagenesis for both strains was not inhibited but rather enhanced by MTP in a dose-dependent manner (Table 3). Data showing this statistically significant enhancement with TA98 are presented in Fig. 6, A–C.

With several of the promutagens, the effect of MTP in some assays was dependent upon the dose level of the promutagen. Statistically, this effect is demonstrated by a significant interaction between MTP and promutagen dose levels in a two-way analysis of variance. The results of this analysis are summarized in the last column of Table 3. Such interactions due to promutagen dose occurred in assays with AFB, sulfallate, BP, 2AA and 2AAF. Among the multiple assays for each compound, however, this dependence upon promutagen dose was not

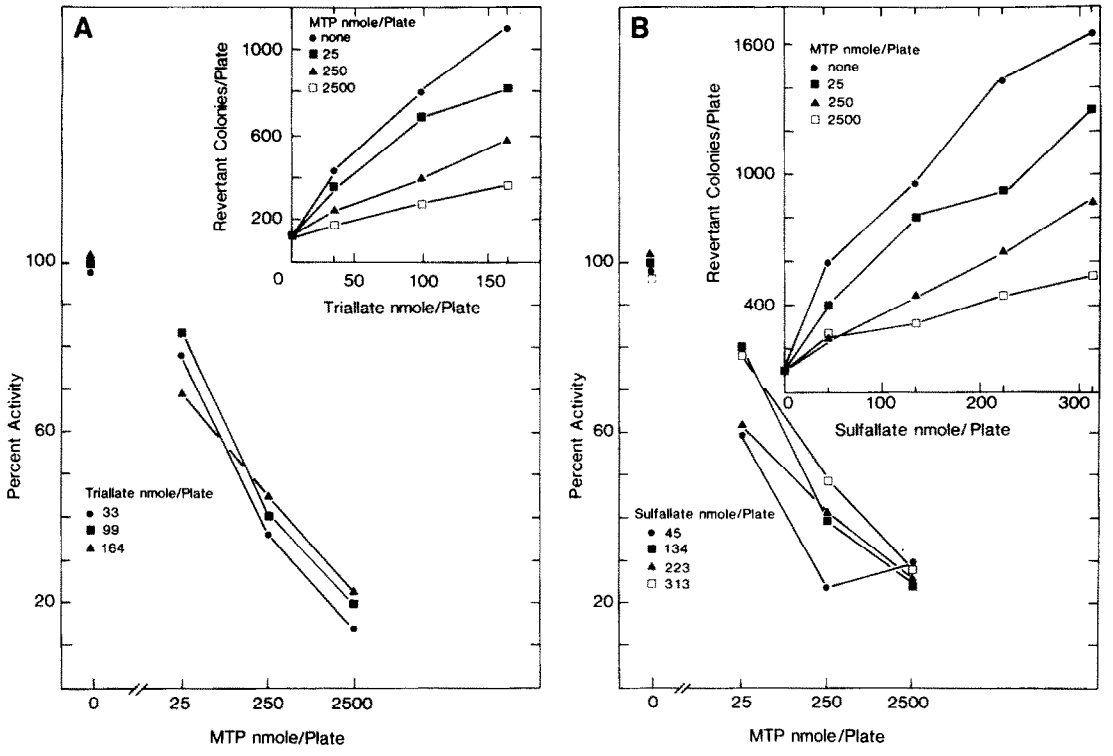

Fig. 4. MTP inhibition of the mutagenesis of triallate (A) and sulfallate (B) for TA100 with PCB-induced S9. Top graphs: Dose response of TA100 to triallate or sulfallate in the absence or presence of MTP. Each data point for triallate represents the mean of duplicate colony counts from two experiments; each data point for sulfallate represents the mean of duplicate plate counts from one experiment. Both compounds were assayed in the presence of 20 μl of rat liver S9 per plate. Bottom graphs: Percent activity of TA100 with triallate or sulfallate as a function of the MTP dose. Percent activity was calculated as described in the legend of Fig. 2.

observed for both strains in the case of BP or for both S9 preparations when AFB and sulfallate were examined. Furthermore, dependence upon promutagen dose was not strictly associated only with enhancement (e.g. with 2AA or 2AAF) or inhibition (e.g. with AFB or sulfallate) of mutagenesis by MTP. For the remaining promutagens, CP and triallate, inhibition of mutagenesis by MTP was not dependent upon promutagen dose under any of the assay conditions examined.

DISCUSSION

Our primary interest in this study was the use of MTP to elucidate the metabolic activation for the newly discovered promutagen, 3-(2-chloroethoxy)-1,2-dichloropropene (CP), and related S-chloroallyl ethers. The effects of MTP on microsomal dependent mutagenesis of four model promutagens were also examined. Metyrapone is generally recognized as a differential inhibitor of PB-induced microsomal enzyme activities mediated by cytochrome P-450 [3]. Additionally, however, it has been shown to enhance certain microsomal enzyme activities: acetanilide hydroxylase [28], benzene monooxygenase [29] and epoxide hydrolase [30].

Aflatoxin B_1 was used as a model promutagen whose activation to a *Salmonella* mutagen had been shown by antibody studies to be preferentially catalyzed by a PB-inducible form of cytochrome P-450 [31, 32]. A role for PB-induced biotransformation had also been indicated by studies of AFB-2,3-oxide formation [27, 33]. The *in vitro* production of this metabolite, measured both as AFB-dihydrodiol formation and as the covalent binding of AFB to DNA, was enhanced by hepatic microsomes from rats pretreated with PB, as compared to control microsomes [27, 33]. By the same criteria, 3MC-induced microsomes decreased the production of AFB-2,3-oxide as compared to control microsomes [27, 33]. Moreover, MTP had been shown to inhibit the binding of AFB to DNA in the presence of liver microsomes from PB-induced rats [27]. The data in this report are consistent with these published observations [27, 31–33]: MTP inhibited AFB mutagenesis for both TA98 and TA100 in the presence of PB- or PCB-induced S9 activation.

In using 2AAF, 2AA and BP as model promutagens, however, the presence of MTP in the activation mixture produced dose-dependent enhancement of their mutagenic activities for both TA98 and TA100 in the presence of PCB-induced S9. Such enhancement had not been reported previously for 2AAF and BP. Concerning 2-AAF, published results of *in vitro* metabolism had shown that MTP inhibited the formation of 5-, 7- and 9-hydroxy-2-AAF by rat liver

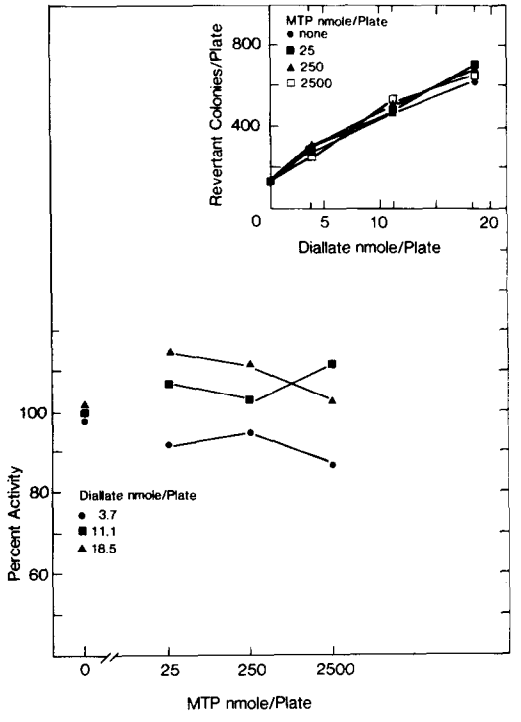

Fig. 5. Effect of MTP on diallate mutagenesis for TA100 with PCB-induced S9. Top graph: Dose response of TA100 to diallate in the absence or presence of MTP. Each data point represents the mean of duplicate plate counts from two experiments. Each plate contained 20 μl of PCB-induced rat liver S9. Bottom graph: Percent activity of TA100 with diallate as a function of the MTP dose. Percent activity was calculated as described in the legend of Fig. 2.

microsomes [34]. However, MTP was found to be only a weak inhibitor of N-hydroxylase activity toward 2-AAF in liver microsomes from control rats or from rats pretreated with 3-methylcholanthrene [35]. Also, the N-hydroxylation of two carcinogenic aminoazo dyes by 3-methylcholanthrene-induced rat liver microsomes was not inhibited by MTP, and a 1.5-fold stimulation of N-hydroxylase activity by MTP was reported with 3'-methyl-*N*,*N*-dimethyl-4-aminoazobenzene [36]. Similarly, MTP had no effect on the *Salmonella* mutagenicity of 2-amino-9*H*-pyrido-[2,3b]indole, which is metabolized by PCB-induced rat liver S9 to N-hydroxy derivatives detectable as *Salmonella* mutagens [37]. Since N-hydroxylation of 2-AAF is considered to be a pathway for its mutagenic and carcinogenic activation [38], MTP might have been expected not to inhibit 2-AAF mutagenesis. The enhancement of 2-AAF mutagenesis by MTP which we observed might have occurred due to the presence of alternate metabolic pathways. Inhibition by MTP of only the detoxicating C-hydroxylation pathways and not the N-hydroxylation steps could result in more 2-AAF available for mutagenic activation via N-hydroxylation. A similar mechanism of enhancement by MTP may be applicable to the mutagenesis of the other aromatic amine examined in this study, 2AA. Enhancement of 2AA mutagenesis by MTP had been reported previously: with activation by PB- or PCB-induced S9, reversion of strain TA1538 was increased 2 to 5-fold [39]. Our results show that such an enhancement also occurs with strains TA98 and TA100.

Inhibition of detoxicating pathways by MTP also might account for MTP-mediated enhancement of BP mutagenesis. In studies with BP in the presence of PB-induced rat liver microsomes, MTP was found to inhibit aryl hydrocarbon hydroxylase, as measured by 3-hydroxy-BP formation [40], and to inhibit the binding of BP metabolites to DNA [27]. Interestingly, MTP has been reported to enhance the mutagenesis of 1-hydroxy-BP for TA98 with activation by uninduced or PB-induced mouse liver S9 [41]; however, the significance of these observations cannot be determined because of the variable and limited data presented in that report. A recent investigation of the mutagenic activation of BP to a *Salmonella* mutagen with purified isozymes of rat liver cytochrome P-450 showed that a major 3MC-inducible form of cytochrome P-450 activated benzo[*a*]pyrene and its 7,8-dihydrodiol derivative to mutagenic products, whereas a major PB-inducible isozyme did not [42]. Since MTP does not bind 3MC-induced forms of cytochrome P-450 [43], it probably would not inhibit mutagenesis of BP mediated by those forms of that enzyme. By inhibiting other enzymes involved in BP detoxication, MTP may have increased the amount of BP available to form highly mutagenic products such as 7,8-dihydrodiol-9,10-epoxide [44].

For the chloroallyl ethers, MTP had no effect on diallate mutagenesis, but inhibited the mutagenesis of CP, triallate and sulfallate. All four of these ethers were metabolized by PB- and PCB-induced rat liver microsomal preparations to mutagens for *Salmonella*, but show little or no mutagenesis in the presence of activation systems containing 3MC-induced or uninduced microsomes [11]. Metyrapone, as a differential inhibitor of PB-inducible forms of cytochrome P-450, might have been expected to inhibit the mutagenesis of all of these compounds. The lack of an effect of MTP on diallate mutagenesis is particularly interesting in light of the mechanisms of mutagenic activation for the S-chloroallyl ethers which have been proposed by Casida and co-workers. By one model, mutagenic activation for both diallate and triallate involves the formation of analogous sulfoxide intermediates [16]. Diallate sulfoxide and triallate sulfoxide, or their decomposition products, 2-chloroacrolein and 2-chloroacrylyl chloride, respectively, are possible proximate mutagenic metabolites for diallate and triallate. These sulfoxides and decomposition products have been found to be direct-acting mutagens for TA100 [16]. 2-Chloroacrolein has also been proposed by these authors to be a mutagenic metabolite of sulfallate [45]; its formation apparently would involve an α-carbon hydroxylation step rather than a sulfoxide intermediate. Casida and co-workers have considered that a similar α-carbon hydroxylation step for diallate and triallate would yield 2,3-dichloroacrolein and 2,3,3-trichloroacrolein respectively [46]: these chloroacroleins are also potent direct-acting mutagens for TA100 [47]. Thus, both sulfoxidation and α-carbon hydroxylation are included in the proposed

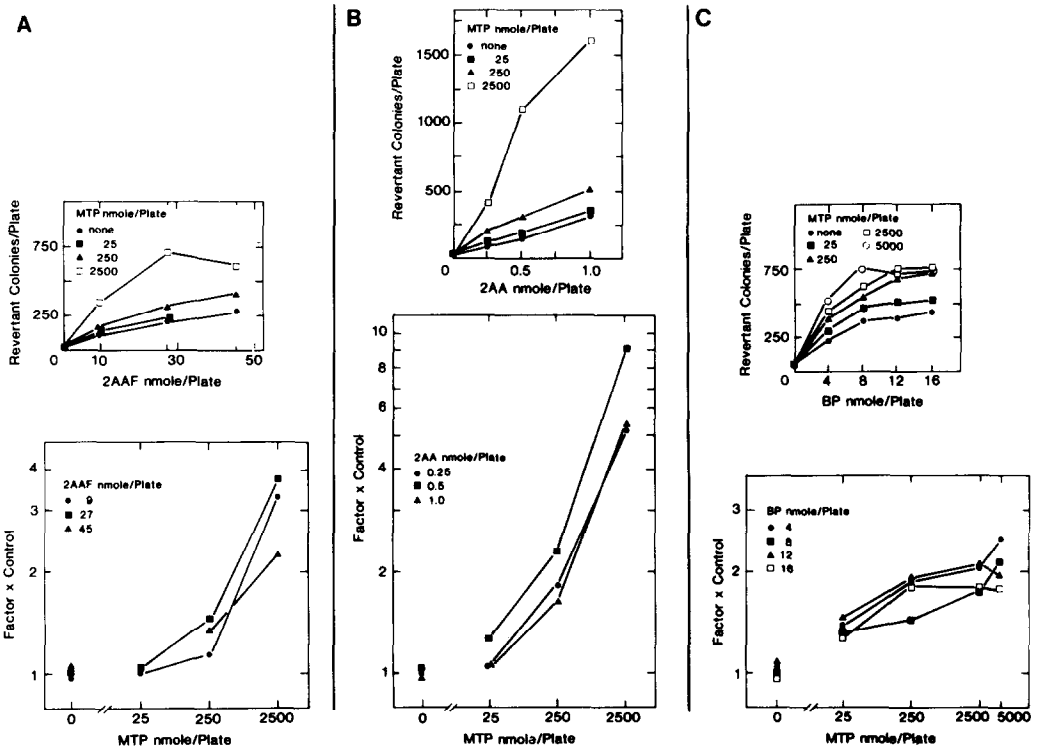

Fig. 6. MTP enhancement of the mutagenesis of 2AAF (A), 2AA (B) and BP (C) for TA98 with PCB-induced S9. Top graphs: Dose response of TA98 to 2AAF, 2AA or BP in the absence or presence of MTP. For 2AAF and 2AA, each data point represents the mean of duplicate colony counts from one experiment; for BP, each data point represents the mean of duplicate colony counts from one (open symbols) or two (closed symbols) experiments. 2AAF and 2AA were assayed in the presence of 20 μl of rat liver S9; each plate for BP contained 50 μl of rat liver S9. Bottom graphs: Relative response of TA98 to 2AAF, 2AA or BP as a function of the MTP dose. Dose–response data in the top graphs were converted to express the response of TA98 to 2AAF, 2AA or BP as a factor of the control response observed in the absence of MTP. Factor × control = net revertant colonies in the presence of MTP/control net revertant colonies.

models of mutagenic activation for the S-chloroallyl herbicides. The studies reported here show that MTP inhibited the mutagenesis of triallate and sulfallate, but not of diallate.

Inability of MTP to inhibit diallate mutagenesis may indicate that: (1) cytochrome P-450 is not responsible for diallate mutagenesis; (2) the concentrations of MTP were too low for inhibition; or (3) the cytochrome P-450 which activates diallate is insensitive to MTP inhibition. Flavin-containing monooxygenase, another NADPH-dependent enzyme found in mammalian microsomes, catalyzes both the nitrogen oxidation of tertiary and secondary amines, and the sulfur oxidation of thiocarbamides, thiocarbamates, thioamides and alkyl sulfides [48]. Also, this enzyme is not sensitive to MTP [49]. Since sulfoxidation has been proposed as a mechanism for diallate activation [16], we considered the possibility that flavin monooxygenase might be the catalyst for formation of diallate sulfoxide. However, this enzyme is not induced by the xenobiotics which induce cytochrome P-450 [48]. Moreover, immunoquantitation studies with antibody raised to the hog flavin monooxygenase have indicated that pretreatment of rats with either PB or 3MC actually lowers the specific content of this hepatic enzyme [50]. Since the metabolic activation capacity of rat liver microsomes toward diallate is induced by PB, flavin monooxygenase is probably not the major enzyme involved in activation of this promutagen.

Lack of inhibition of diallate mutagenesis by MTP could have resulted from the use of insufficient concentrations of MTP. This would suggest that, relative to the other chloroallyl ethers whose mutagenesis is inhibited by MTP, diallate has an extremely high affinity for the cytochrome P-450 responsible for its activation. For several reasons, it is unlikely that this can account for the resistance of diallate mutagenesis to MTP inhibition. First, the concentrations of MTP in the S9 mix were 0.05 to 5.0 mM, well within or greater than the range of concentrations of MTP known to inhibit many microsomal enzyme activities [28]. Second, the molar ratios of MTP: diallate used in the *Salmonella* assays extended from 1.3 to 900. Thus, both the absolute concentrations of MTP and its concentration relative to that of diallate were sufficient to obtain an inhibition of diallate mutagenesis even if diallate was a relatively high affinity substrate for the activating cytochrome P-450. Finally, diallate is a much more potent mutagen than

triallate or sulfallate and it could be argued that this greater potency has its basis in a higher affinity of diallate toward the activating cytochrome P-450. By this model, diallate mutagenesis would be least sensitive to MTP inhibition. However, the isolated promutagen CP is at least twice as potent as diallate ($\geq$ 75 TA100 revertants/nmole for CP vs 37 for diallate), and CP mutagenesis, like that of triallate and sulfallate, is readily inhibited by MTP.

A remaining possibility for the differential effects of MTP on chloroallyl ether mutagenesis is that diallate is activated by a MTP-insensitive isozyme of cytochrome P-450, while CP, triallate and sulfallate are activated by a MTP-sensitive isozyme of cytochrome P-450. All the chloroallyl ethers are activated to mutagens by PB-induced rat liver S9, and MTP is a known inhibitor of PB-induced microsomal enzyme activities mediated by cytochrome P-450. However, PB induces multiple isozymes of cytochrome P-450 in rat liver [43, 51, 52], and at least one of these isozymes in purified form is insensitive to MTP [52]. Other PB-inducible isozymes of cytochrome P-450 form spectral complexes with MTP [43, 52] but differ in their sensitivity to MTP with respect to catalytic activity [52]. Thus, it appears most likely that diallate is activated by a MTP-insensitive isozyme of cytochrome P-450 in PB-induced rat liver S9.

Statistical analysis of mutagenesis data by two-way analysis of variance also demonstrated the absence or presence of significant interactions between MTP dose levels and promutagen dose levels. Effects of MTP on the mutagenesis of CP and triallate were independent (no interaction) of promutagen dose levels under all assay conditions examined. In some assays with AFB, sulfallate, BP, 2AA and 2AAF, the extent of inhibition or enhancement of mutagenesis by MTP was dependent (interaction) upon the dose level of the promutagen. However, for AFB, sulfallate and BP, the presence of an interaction between MTP and promutagen dose levels depended upon the tester strain and S9 used in the assay (Table 3). There are several plausible reasons for these different interactions. Variability among assays incorporating tester strains and S9 fractions undoubtedly is a contributing factor. A second consideration is that inhibition or enhancement of S9-dependent mutagenesis by MTP is the net effect of two processes: the interaction between MTP and the enzymes responsible for promutagen activation and/or detoxication, and the biological response of the tester strain cells to various mutagenic metabolites which are present in increased or decreased amounts due to these MTP effects. Mechanisms for the effects of MTP are probably not the same for the eight different promutagens examined in this study. As a heme ligand, MTP can inhibit cytochrome P-450 enzyme activities by interfering with the binding of oxygen, but MTP can also inhibit the binding of certain xenobiotics at the substrate binding site [53]. Moreover, any one or more of cytochrome P-450 isozymes present in the S9 activation system may contribute to the metabolism of the eight promutagens examined in this study, and not all of these isozymes would be expected to interact with MTP. Considering the complexity of activation by S9 systems, it is not surprising that the promutagen dose influences the extent of MTP effects on mutagenesis in a manner dependent upon conditions of assay. Future investigations of the mechanisms of inhibition and enhancement of mutagenesis by MTP, and the role of promutagen dose levels on these effects, will require the use of reconstituted activation systems with purified isozymes of cytochrome P-450.

Other authors have incorporated MTP in mutagenesis assays on the understanding that it can be used as an inhibitor of microsomal activation [4–7]. It is important to note that, from results presented here, MTP can enhance, inhibit or fail to influence the microsomal dependent mutagenesis of promutagens. In the presence of PCB-induced rat liver S9, MTP enhanced the mutagenesis of 2AA, 2AAF and BP, inhibited the mutagenesis of AFB, CP, triallate and sulfallate, and had no effect on diallate mutagenesis. The inhibition or enhancement of mutagenesis by MTP possibly resulted from an alteration of the normal balance of activating and detoxicating enzyme pathways that can be utilized by specific promutagens. The lack of an apparent effect of MTP on diallate mutagenesis can best be explained on the basis of an activating enzyme pathway which is insensitive to MTP.

The ability of PB- or PCB-induced rat liver S9 to activate the eight compounds examined here, as well as a wide variety of other promutagens of diverse chemical structure [54], reflects the heterogeneity of enzyme activities in S9 which can produce mutagenic metabolites. Given this complexity of microsomal activation systems, identification of enzyme activities required for the formation of active metabolites from specific promutagens can be a difficult task. The effects of MTP on mutagenesis in the presence of PB- or PCB-induced S9 provide an additional approach to this problem. Based upon the differential effects of MTP on the mutagenesis of the four chloroallyl ethers and the four model promutagens used in this study, MTP appears to be a useful tool in the characterization of microsomal pathways leading to mutagen activation.

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REFERENCES

1. B. N. Ames, J. McCann and E. Yamasaki, *Mutation Res.* **31**, 347 (1975).
2. H. Bartsch, T. Kuroki, M. Roberfroid and C. Malaveille, in *Chemical Mutagens: Principles and Methods for Their Detection* (Eds. F. J. de Serres and A. Hollaender), Vol. 7, p. 95. Plenum Press, New York (1982).
3. B. Testa and P. Jenner, *Drug Metab. Rev.* **12**, 1 (1981).
4. E. J. Soderlund, S. D. Nelson and E. Dybing, *Acta pharmac. tox.* **45**, 112 (1979).
5. B. F. Hales and R. Jain, *Biochem. Pharmac.* **29**, 256 (1980).
6. E. J. Soderlund, E. Dybing, S. Nordenson and E. Tjelta, *Acta pharmac. tox.* **47**, 175 (1980).

7. J. S. Felton and D. W. Nebert, *J. biol. Chem.* **250**, 6769 (1975).
8. J. C. Loper and M. W. Tabor, in *Application of Short-Term Bioassays in the Analysis of Complex Environmental Mixtures* (Eds. M. D. Waters, S. S. Sandhu, J. L. Huisingh, L. Claxton and S. Nesnow), Vol. 2, p. 155. Plenum Press, New York (1980).
9. M. W. Tabor and J. C. Loper, *Int. J. environ. analyt. Chem.* **8**, 197 (1980).
10. M. W. Tabor, *Environ. Sci. Technol.* **17**, 324 (1983).
11. L. M. Distlerath, J. C. Loper and M. W. Tabor, *Environ. Mutagen.* **4**, 302 (1982).
12. V. F. Simmon, K. Kauhanen and R. G. Tardiff, in *Progress in Genetic Toxicology* (Eds. D. Scott, B. A. Bridges and F. H. Sobels), p. 249. Elsevier/North-Holland, New York (1977).
13. S. J. Stolzenberg and C. H. Hine, *Environ. Mutagen.* **2**, 59 (1980).
14. H. C. Sikka and P. Florczyk, *J. agric. Fd Chem.* **26**, 146 (1978).
15. F. De Lorenzo, N. Staiano, L. Silengo and R. Cortese, *Cancer Res.* **38**, 13 (1978).
16. I. Schuphan, J. D. Rosen and J. E. Casida, *Science* **205**, 1013 (1979).
17. G. R. Douglas, E. R. Nestmann, C. E. Grant, R. D. L. Bell, J. M. Wytsma and D. J. Kowbel, *Mutation Res.* **85**, 45 (1981).
18. United States Environmental Protection Agency, *Analytical Reference Standards and Supplemental Data for Pesticides and Other Organic Compounds*, EPA-600/2-81-011 (1981).
19. J. C. Loper, D. R. Lang, R. S. Schoeny, B. B. Richmond, P. M. Gallagher and C. C. Smith, *J. Toxic. environ. Hlth* **4**, 919 (1978).
20. F. J. de Serres and M. D. Shelby, *Science* **203**, 563 (1979).
21. R. D. Snee and J. D. Irr, *Biometrics* **37**, 191 (1981).
22. D. G. Kleinbaum and L. L. Kupper, *Applied Regression Analysis and Other Multivariable Methods*, p. 315. Duxbury Press, North Scituate, MA (1978).
23. H. Scheffé, *Analysis of Variance*. John Wiley, New York (1959).
24. M. D. Burke, *Biochem. Pharmac.* **30**, 181 (1981).
25. P. G. Wislocki, G. T. Miwa and A. Y. H. Lu, in *Enzymatic Basis of Detoxication* (Ed. W. B. Jakoby), Vol. 1, p. 135. Academic Press, New York (1980).
26. B. N. Ames, W. E. Durston, E. Yamasaki and F. D. Lee, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2281 (1973).
27. H. L. Gurtoo and R. P. Dahms, *Biochem. Pharmac.* **28**, 3441 (1979).
28. K. C. Leibman, *Molec. Pharmac.* **5**, 1 (1969).
29. A. Tunek and F. Oesch, *Biochem. Pharmac.* **28**, 3425 (1979).
30. F. Oesch, N. Kaubisch, D. M. Jerina and J. W. Daly, *Biochemistry* **10**, 4858 (1971).
31. K. Kawajiri, H. Yanekawa, N. Harada, M. Noshiro, T. Omura and Y. Tagashira, *Cancer Res.* **40**, 1652 (1980).
32. K. Kawajiri, H. Yonekawa, O. Gotoh, J. Watanabe, S. Igarashi and Y. Tagashira, *Cancer Res.* **43**, 819 (1983).
33. S. A. Metcalfe, P. J. Colley and G. E. Neal, *Chem. Biol. Interact.* **35**, 145 (1981).
34. O. S. Son, J. W. Fowble, D. D. Miller and D. R. Feller, *Toxic. appl. Pharmac.* **51**, 367 (1979).
35. C. Razzouk, M. Mercier and N. Roberfroid, *Cancer Res.* **40**, 3540 (1980).
36. T. Kimura, M. Kodama and C. Nagata, *Gann* **73**, 55 (1982).
37. T. Niwa, Y. Yamazoe and R. Kato, *Mutation Res.* **95**, 159 (1982).
38. E. C. Miller and J. A. Miller, *Cancer, N.Y.* **47**, 2327 (1981).
39. J. C. Kawalek and A. W. Andrews, in *Microsomes, Drug Oxidations and Chemical Carcinogenesis* (Eds. M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gilette and P. J. O'Brien), Vol. 2, p. 1157. Academic Press, New York (1980).
40. P. Lesca, P. LeCointe, C. Paoletti and D. Mansuy, *Biochem. Pharmac.* **27**, 1203 (1978).
41. I. S. Owens, G. M. Koteen and C. Legraverend, *Biochem. Pharmac.* **28**, 1615 (1979).
42. I. G. C. Robertson, E. Zeiger and J. A. Goldstein, *Carcinogenesis* **4**, 93 (1983).
43. D. E. Ryan, P. E. Thomas and W. Levin, *Archo Biochem. Biophys.* **216**, 272 (1982).
44. A. H. Conney, *Cancer Res.* **42**, 4875 (1982).
45. J. D. Rosen, I. Schuphan, Y. Segall and J. E. Casida, *J. agric. Fd Chem.* **28**, 880 (1980).
46. P. J. Marsden and J. E. Casida, *J. agric. Fd Chem.* **30**, 627 (1982).
47. J. D. Rosen, Y. Segall and J. E. Casida, *Mutation Res.* **78**, 113 (1980).
48. D. M. Ziegler, in *Enzymatic Basis of Detoxication* (Ed. W. B. Jakoby), Vol. 1, p. 201. Academic Press, New York (1980).
49. D. J. Waxman, D. R. Light and C. Walsh, *Biochemistry* **21**, 2499 (1982).
50. G. A. Dannan and F. P. Guengerich, *Molec. Pharmac.* **22**, 787 (1982).
51. F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).
52. D. J. Waxman and C. Walsh, *J. biol. Chem.* **257**, 10446 (1982).
53. K. J. Netter, *Pharmac. Ther.* **10**, 515 (1980).
54. J. McCann, E. Choi, E. Yamasaki and B. N. Ames, *Proc. natn. Acad. Sci. U.S.A.* **72**, 5135 (1975).