8 mating periods in the phenylbutazone series (50 and 100 mg/kg).

Discussion. With regard to post-implantation loss, the results we obtained with 5 mg/kg of Thio-TEPA are very similar to those reported by EPSTEIN and SHAFNER[7]. Higher doses were not employed by these authors, and the effect of Thio-TEPA in their experiments was limited to the post-meiotic stages. In our investigations, Thio-TEPA in a dosage of 10 mg/kg also affected meiotic stages of spermatogenesis.

The action of MMS with regard to post-implantation loss was evidently confined to the postmeiotic stages, i.e. in keeping with the findings of other authors[8], it was predominantly spermatozoa and to a slight extent spermatids that were affected.

No evidence of induced lethal mutations due to phenylbutazone in i.p. doses of 50 and 100 mg/kg could be detected, so that on the basis of the present in vivo studies the compound cannot be classified as potentially mutagenic.

Analysis of the pre-implantation loss also revealed lethal effects of Thio-TEPA and MMS, but not of phenylbutazone. In this context, the results paralleled the findings with regard to post-implantation loss, in that the various stages of the spermatogenesis cycle were equally susceptible. This finding may indicate that the greater pre-implantation loss observed after the administration of Thio-TEPA and MMS is at least in part attributable to genetic effects.

Nevertheless, pre-implantation loss can only be taken with certain reservations as a criterion in assessing mutagenic effects. In evaluating pre-implantation loss, there is no means of discriminating between genetically and non-genetically induced embryonal loss, whereas this fact appears to be of far less, if not negligible, importance in connection with post-implantation loss[9]. The factors that are of significance in this respect, include paucity of spermatozoa[9], induced impairment of spermatozoal motility, infertility of the oocytes, disturbances of implantation, and other non-genetic causes of damage to the zygote[10]. In principle, therefore, pre-implantation loss by no means is a relevant measure of induced mutations, as other authors have already stressed[3,4]. In fact, if this parameter is to be used at all in assessing mutagenicity, then it should be regarded as being of only subordinate importance.

In our experiments, we found that pre-implantation loss could best be estimated on the basis of the total implantation rates in the control and test groups and not by reference to the difference between the number of corpora lutea and the number of implantations[11]. On the one hand, this is justified by the unreliability of corpus luteum counts in the mouse; on the other, there seems to be a high degree of natural variability in the extent of pre-implantation loss. BATEMAN[3] reports a spontaneous variation of 2.3 to 15.9% ([RCL × NF] F_1-mice) and we ourselves found a variation of 5.0 to 17.5% in the CFLP strain. Evidently, pre-implantation loss displays a lack of statistical homogeneity, as regards both successive mating periods in one and the same dominant lethal study and comparable mating periods in different studies. This has also been described by ROEHRBORN[4]. On the basis of corpus luteum counts none of the statistical analyses of this parameter could therefore be considered as pertinent.

Zusammenfassung. Im Gegensatz zu Thio-TEPA (N, N′, N″-Triaethylen-thiophosphoramid) und MMS (Methansulfonsäuremethylester) führten i.p. Dosen von Phenylbutazon (50 und 100 mg/kg) bei der Maus, unter Berücksichtigung von post- und prae-implantativen Gestationsstadien, zu keinen genetischen Effekten im Sinne der Induktion von dominanten Letalfaktoren.

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Additive and Synergistic Inhibition of Mammalian Microsomal Enzyme Functions by Piperonyl Butoxide, Safrole and Other Methylenedioxyphenyl Derivatives

Piperonyl butoxide (Pib.) and related synthetic methylenedioxyphenyl (MDP) derivatives are widely used as pesticidal synergists. These compounds act, putatively, by inhibiting microsomal enzyme function in insects. MDP synergists and other naturally occurring MDP derivatives inhibit mammalian hepatic microsomal enzyme systems in vitro and in vivo[1–6]. Pib. produces synergistic acute toxicity in mice with certain Freons, griseofulvin and benzo[α]pyrene[3,4]; Pib also produces synergistic hepatocarcinogenicity with Freons[4]

The effects of Pib. on mammalian microsomal enzyme function suggest the possibility of potential human hazards due to inhibition of detoxification of environmental pollutants and drugs. Tolerances for post-harvest applications of 8–20 ppm have been established for Pib. It is thus unlikely that such low dietary levels of MDP synergists will produce significant microsomal enzyme inhibition. The widespread distribution of naturally occurring MDP derivatives in foods, flavoring agents and spices is well recognized[1]. A wide range of these compounds has been shown to produce inhibition of microsomal enzyme function[6]. Thus, synthetic MDP derivatives may interact with naturally occurring derivatives in foods, flavoring agents and spices to produce interactive inhibi-

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tion of microsomal enzyme function. The preliminary studies reported here were designed to test these possibilities.

Piperonyl butoxide (Pib.), methyl eugenol (ME), safrole (SAF), methylenedioxyaniline (MDA), piperonylic acid (PA) and vanillylamine (VA) were tested (Figure 1). Pib., ME, SAF, and MDA were dissolved in corn oil and PA and VA were suspended in corn oil; compounds were injected i.p. either alone or in combination in single doses of 0.1 ml volume in groups of 4–6 mice. All tests were replicated on 3 occasions.

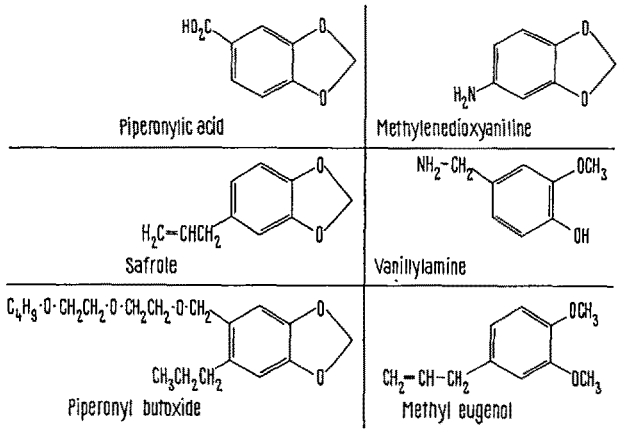

Fig. 1. Structures of methylenedioxyphenyl derivatives.

Male Swiss Albino mice (ICR/Ha), weighing between 20 and 25 g, were maintained on Purina chow and water ad libitum. Mice were killed by cervical dislocation 1 h after i.p. injection with compounds under test. In some specified experiments, livers from 2 animals were pooled. Microsomal suspensions were prepared as described previously[2]. The standard incubation mixture for all enzyme assays contained 75 μmoles nicotinamide, 4.5 nmoles NADP, 15 μmoles $MgCl_2$, 30 μmoles $MnCl_2$, 15 μmoles D,L-isocitrate and 75 μg isocitric dehydrogenase in a final volume of 3 ml. In assaying for aminopyrine demethylase, 30 μmoles aminopyrine and microsomes from 37.5 mg of liver were added to the incubation mixture. Following deproteination with 10% TCA, either aminoantipyrine[2] or formaldehyde[7] was determined. When formaldehyde was determined, 45 μmoles of neutralized semi-carbazide were added to the incubation mixture. For biphenyl hydroxylase assay, 7.5 μmoles of biphenyl were added as substrate along with microsomes from 250 mg of liver; 4-hydroxybiphenyl was determined as described previously[8]. One unit of biphenyl hydroxylase represents 1 mmole of biphenyl hydroxylated per gram liver per h at 37 °C.

In preliminary studies, 5 MDP compounds were tested alone and in combination to determine whether combined treatment at low doses produced significant inhibition of

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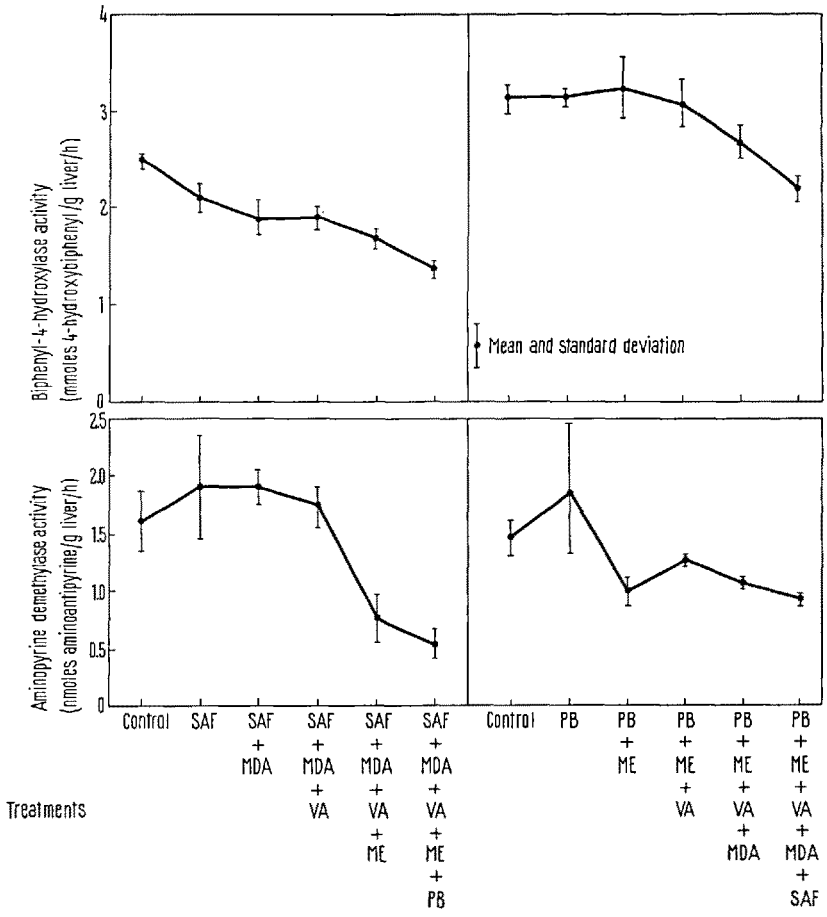

Fig. 2. Additive effects of low doses of methylenedioxyphenyl derivatives. Groups of 6 mice were treated with safrole (SAF), methylenedioxyaniline (MDA), vanillylamine (VA), methyl eugenol (ME) and piperonyl butoxide (PB) at doses of 10 mg/kg singly and in various combinations. PB = Pib.

microsomal enzyme activity. Pib., ME, SAF, MDA, and VA were administered to groups of mice alone at a dose of 10 mg/kg and in various combinations at the same dose (Figure 1); aminopyrine demethylase and biphenyl-4-hydroxylase activities were assayed from pooled homogenates in duplicate. Compounds were tested in a simple cumulative pattern and also in reverse sequence (Figure 1).

Experiments were then designed to elucidate the nature of interactive effects observed at low dose levels in the previous experiment. Interactions of Pib. and SAF were tested on groups of mice at dosages of 50 and 10 mg/kg, respectively. Formaldehyde was determined as the product of aminopyrine demethylase; enzyme assays were performed on livers from single animals.

Experiments were also designed to test possible interactions between Pib. and the structurally similar but inactive PA on aminopyrine demethylase activity. Pib. and PA, at doses of 64 and 640 mg/kg, respectively, were tested alone and in combination. Aminopyrine demethylase assays were performed on individual livers.

Aminopyrine demethylase and biphenyl-4-hydroxylase activities were progressively inhibited by the combined effects of 5 MDP compounds tested at levels which by themselves produced either no or negligible inhibition (Figure 1). For example, SAF alone inhibited biphenyl-4-hydroxylase activity from 2.47 to 2.11 units. Addition of MDA, ME and Pib. produced progressive inhibition to 1.88, 1.65 and 1.38 units, respectively. When the compounds were tested in reverse sequence, Pib., ME and VA did not produce significant inhibition, but further addition of MDA and SAF each produced marked inhibition. Inhibition of aminopyrine demethylase activity also increased with increasing number of MDP compounds. In all experiments, the response to all compounds was significantly greater than the response to the first alone ($p < 0.01$).

Table I. Synergistic effects of piperonyl butoxide and safrole on aminopyrine demethylase activity

Treatment [a]	Aminopyrine demethylase activity [b] (Mean ± S.E.)
Control	8.00 ± 0.35
Piperonyl butoxide (50 mg/kg)	7.66 ± 0.35
Safrole (10 mg/kg)	8.83 ± 0.44
Piperonyl butoxide and safrole	6.32 ± 0.25 [c]

[a] 16 mice treated in each group. [b] Expressed as μmoles formaldehyde/g liver/h. [c] Significant synergistic interaction, $p < 0.01$.

Table II. Interaction of piperonyl butoxide and piperonylic acid

Treatment [a]	Aminopyrine demethylase [b] (Mean ± S.E.)
Control	12.0 ± 0.4
Piperonyl butoxide (64 mg/kg)	10.1 ± 0.4
Piperonylic acid (640 mg/kg)	10.5 ± 0.2
Piperonyl butoxide and piperonylic acid	9.7 ± 0.6

[a] 12 mice treated in each group. [b] Expressed as μmoles formaldehyde g liver/h.

Under conditions of test, treatment with Pib. at 50 mg/kg or SAF at 10 mg/kg alone had no effect on aminopyrine demethylase activity (Table I); however, combined treatment with Pib. and SAF produced a 20% inhibition of aminopyrine demethylase activity. Analysis of variance reveals a highly significant synergistic interaction between Pib. and SAF.

No synergism between Pib and PA was noted with respect to aminopyrine demethylase activity (Table II). Pib. and PA, both alone and in combination, produced slight inhibition of aminopyrine demethylase activities. However, analysis of variance showed that there were no statistically significant treatment effects, and no interactions.

The data presented here indicate that combined administration of low levels of MDP derivatives produced significant cumulative inhibition of hepatic microsomal enzyme activity. When very low doses (10 mg/kg) of Pib., ME, SAF, MDA and VA were injected into mice, the level of inhibition of biphenyl-4-hydroxylase activity increased linearly with increasing numbers of compounds tested; cumulative inhibition was less consistent with aminopyrine demethylase. Pib. and SAF induced synergistic inhibition of aminopyrine demethylase. However, Pib. induced no synergistic inhibition when tested in combination with large doses of the structurally related but inactive PA. These interactions thus appear functional rather than structural in nature.

MDP derivatives are extensively distributed as natural products in foods, flavoring agents and spices[6]. A wide range of structurally related MDP derivatives has been shown to induce inhibition of microsomal enzyme activity in mice[6]. The data presented here raise the possibility that Pib. and other related pesticidal synergists may interact with low levels of MDP derivatives present as natural products in foods to produce additive or synergistic inhibition of hepatic microsomal enzyme function. Such interactive inhibitions may have significant implications with regard to detoxification of a wide range of environmental pollutants and drugs.

Zusammenfassung. Piperonylbutoxyd, Methylendioxylanilin, Methyleugenol, Safranol und Vanillylamin bewirken eine additive Hemmung der Funktion mikrosomaler Enzyme in Mäusen, wenn sie zusammen in Dosen verabreicht werden, die einzeln inaktiv sind. Piperonylbutoxyd und Safranol induzieren synergistisch die Hemmung von Aminopyrenedemethylase-Aktivität. Es erfolgte keine synergistische Hemmung nach kombinierter Behandlung mit Pib. und hohen Dosen der strukturell verwandten, aber inaktiven Piperonylsäure.

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