Comparative Dominant Lethal Studies with Phenylbutazone, Thio-TEPA and MMS in the Mouse

Of the various methods used to find out whether a drug is potentially mutagenic, in vivo experiments in mammals are currently considered the most reliable[1]. In vitro studies are no doubt useful in assessing cytopathic activity, including chromosomal changes, but the results obtained are not necessarily applicable to the intact animal or man.

The present studies were undertaken as a result of findings published by WISSMÜLLER and GEBHART[2] on cytogenetic effects of phenylbutazone (Butazolidin®) on human leucocytes in vitro. In order to determine whether phenylbutazone might possibly possess mutagenic properties, the activity of the compound was compared with that of the known mutagens Thio-TEPA and MMS in the dominant lethal test[3] in the mouse. The validity of this method has been confirmed by various authors, including particularly ROEHRBORN[4], as well as by ourselves[5].

Method. Nine-week-old male mice of the CFLP strain which were kept under standardized conditions were given single i.p. doses of 50 and 100 mg/kg of the sodium salt of phenylbutazone dissolved in distilled water. These doses correspond to $^1/_6$ and $^1/_3$ of the parenteral LD_{50} in this species. The volume of the dose was 1 ml in each case. Each dose was given to 20 mice, which were mated weekly with 60 untreated virgin females (3 per male) for fractionated studies of spermatogenesis. The experiment extended over 8 consecutive mating periods, beginning on the day of administration.

Under the same experimental conditions, the polyfunctional alkylating agent triethylenethiophosphoramide (Thio-TEPA), and the monofunctional alkylating agent methyl methanesulfonate (MMS) were tested over 5 mating periods, to serve as positive controls to phenylbutazone. Thio-TEPA was administered i.p. in doses of 5 and 10 mg/kg and MMS by the same route in doses of 50 and 100 mg/kg, in physiological saline. At each dose-level Thio-TEPA was given to 20 and MMS to 10 males, each of which was mated with 3 females per mating period.

A group of 50 males, each of which was given 1 ml of the solvent by i.p. injection and was mated with 3 females each week, served as negative controls.

On the 15th day of gestation (the day on which spermatozoa were found in the vaginal smear being taken as the first day) the dams were autopsied and the rate of post-implantation loss (deciduomata and dead embryos) was calculated. This is given as a percentage of all implantations, and constitutes the main criterion of evaluation. Pre-implantation loss, i.e. zygotes that had died before implantation, was likewise calculated by reference to the rate of implantation.

The statistical evaluations were based on the relevant control values for each mating period. The results were first tested to ascertain whether there was any 'positive evidence' of induced dominant lethal factors[6]. For this purpose, the numbers of living and dead implants were submitted to the χ^2-test. To verify the results, a *t*-test for 'negative evidence' was performed[6], in which the rates of living implants in the control and treated groups were compared with one another. Furthermore, in order to ascertain whether the test substances had any influence on the pre-implantation stages of gestation, the implantation rates in the control and treatment groups were compared (*t*-test).

Results. Post-implantation loss: The test for positive evidence of induced dominant lethal factors (χ^2-test, probable error $p \leq 0.01$) showed that doses of 5 and 10 mg/kg of Thio-TEPA were highly active on the post-meiotic stages of spermatogenesis; the dose of 10 mg/kg also exerted a weak effect on meiotic stages (Table I).

MMS in doses of 50 and 100 mg/kg exerted a highly lethal effect in mating periods I and II, so that predominantly the epididymal and testicular spermatozoa were affected; the higher dose also had a slight effect on spermatids (mating period III). Meiotic stages were not influenced by MMS (Table II).

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Table I. Thio-TEPA: effect of single i.p. injection in dominant lethal test in the CFLP-mouse

Mating period	Dose (mg/kg i.p.)	No. of fertilized ♀♀	No. of implantations total	X̄	s(±)	No. of live implants total	X̄	s(±)	No. of dead implants total	%
I	TT 5	51	456	8,94[a]	4.13	275	5.39[a]	4.55	181	39.7[b]
	10	39	264	6.77[b]	4.50	131	3.51[a]	3.94	133	50.4[b]
	Controls	128	1488	11.63	2.98	1361	10.67	3.23	127	8.5
II	TT 5	46	212	4.61[a]	2.79	15	0.33[a]	0.89	197	92.9[b]
	10	18	24	1.33[a]	0.97	0	– [a]	–	24	100.0[b]
	Controls	132	1548	11.83	2.91	1430	10.83	3.20	118	7.6
III	TT 5	51	517	10.14	3.64	385	7.55	3.83	132	25.5[b]
	10	50	369	7.32[a]	3.88	144	2.88[a]	2.89	225	61.0[b]
	Controls	140	1687	12.05	2.59	1542	11.06	2.93	145	8.6
IV	TT 5	55	638	11.60	3.39	573	10.42	3.38	65	10.2
	10	44	400	9.67[a]	4.97	326	7.58	4.83	74	18.5[b]
	Controls	134	1669	12.46	2.72	1529	11.40	2.78	140	8.4
V	TT 5	48	520	10.83	4.04	470	9.79	3.93	50	9.6
	10	39	328	8.63[a]	4.75	284	7.47[a]	4.51	44	13.4[b]
	Controls	143	1676	11.72	2.81	1548	10.83	3.02	128	7.6

TT = Thio-TEPA. a, b = significant difference from controls. [a] = *t*-test, $p \leq$ p.05; [b] = χ^2-test, $p \leq 0.01$

Table II. MMS: Effect of single i.p. injection in dominant lethal test in the CFLP mouse

Mating period	Dose (mg/kg i.p.)	No. of fertilized ♀♀	No. of implantations total	$\bar{X}$	s(±)	No. of live implants total	$\bar{X}$	s(±)	No. of dead implants total	%
I	MMS 50	24	250	10.42	3.18	171	7.13	3.90	79	31.6[b]
	100	22	118	5.36[a]	4.55	50	2.27[a]	3.63	68	57.6[b]
	Controls	128	1488	11.63	2.98	1361	10.67	3.23	127	8.5
II	MMS 50	23	299	9.96	3.00	134	5.83[a]	3.39	95	41.5[b]
	100	18	69	3.83[a]	4.09	25	1.39[a]	2.28	44	63.8[b]
	Controls	132	1548	11.83	2.91	1430	10.83	3.20	118	7.6
III	MMS 50	21	249	11.86	2.83	233	11.10	3,34	16	6.4
	100	29	345	11.90	2.80	299	10.31	3.12	46	13.3[b]
	Controls	140	1687	12.05	2.59	1542	11.06	2.93	145	8.6
IV	MMS 50	24	294	12.25	2.74	274	11.42	2.84	20	6.8
	100	28	340	12.14	3.81	303	10.82	3.69	37	10.9
	Controls	134	1669	12.46	2.72	1529	11.40	2.78	140	8.4
V	MMS 50	24	295	12.29	2.93	359	10.79	3.38	36	12.2
	100	26	318	12.23	2.58	289	11.12	2.68	29	9.1
	Controls	143	1676	11.72	2.81	1548	10.83	3.02	128	7.6

MMS = Methyl methanesulfonate. a, b = significant difference from controls. [a] t-test, $p \leqq 0.05$; [b] χ^2-test, $p \leqq 0.01$.

Table III. Phenylbutazone: Effect of single i.p. injection in dominant lethal test in the CFLP-mouse

Mating period	Dose (mg/kg i.p.)	No. of fertilized ♀♀	No. of implantations total	$\bar{X}$	s(±)	No. of live implants total	$\bar{X}$	s(±)	No. of dead implants total	%
I	PhB 50	52	630	12.12	3.16	565	10.87	3.16	65	10.3
	100	50	617	12.32	2.35	568	11.36	2.85	49	7.9
	Controls	128	1488	11.63	2.98	1361	10.67	3.23	127	8.5
II	PhB 50	54	627	11.61	3.03	579	10.72	3.11	48	7.7
	100	50	602	12.04	3.35	565	11.30	3.40	37	6.1
	Controls	132	1548	11.83	2.91	1430	10.83	3.20	118	7.6
III	PhB 50	55	612	11.13	2.74	569	10.35	2.91	43	7.0
	100	56	657	11.73	3.26	604	10.80	3.37	53	8.1
	Controls	140	1687	12.05	2.59	1542	11.06	2.93	145	8.6
IV	PhB 50	48	562	11.71	2.70	526	10.96	2.88	36	6.4
	100	49	580	11.84	3.20	530	10.82	3.62	50	8.6
	Controls	134	1669	12.46	2.72	1529	11.40	2.78	140	8.4
V	PhB 50	50	575	11.50	2.48	527	10.54	2.76	48	8.3
	100	52	583	11.21	3.18	525	10.10	3.61	58	9.9
	Controls	143	1676	11.72	2.81	1548	10.83	3.02	128	7.6
VI	PhB 50	54	598	11.07	2.30	548	10.15	2.40	50	8.4
	100	50	576	11.52	3.44	515	10.30	3.64	61	10.6
	Controls	54	648	12.00	2.63	604	11.19	2.87	44	6.8
VII	PhB 50	58	658	11.34	3.41	599	10.33	3.73	59	9.0
	100	48	575	11.98	3.26	512	10.67	3.50	63	11.0
	Controls	54	616	11.41	2.96	570	10.56	3.12	46	7.5
VIII	PhB 50	55	668	12.15	3.00	616	11.20	3.06	62	7.8
	100	42	452	10.76	3.33	409	9.74[a]	3.92	43	9.5
	Controls	53	662	12.49	2.86	604	11.40	2.90	58	8.8

PhB = Phenylbutazone. [a] significant difference from controls (t-test, $p \leqq 0.05$).

No increase in the number of dead implants attributable to phenylbutazone was noted over 8 mating periods (Table III).

In the case of Thio-TEPA, a comparison of the ratios of living implants in the control and treated groups by means of the t-test made in the course of an examination for negative evidence of induced lethal factors showed, with a probable error of $p \leqq 0.05$, significant differences from the controls in mating periods I and II (5 and 10 mg/kg), and also in periods III and V (10 mg/kg). This is, by and large, in keeping with the above-mentioned results (Table I).

With MMS, significant differences from the controls (Table II) were found in mating periods I (100 mg/kg) and II (50 and 100 mg/kg).

In the animals treated with phenylbutazone, on the other hand, this analysis merely revealed a slightly significant difference during mating period VIII in the 100 mg/kg-group, which can only be ascribed to the fact that the control group during this period displayed the highest rate of implantations and living embryos observed. This finding is not the result of a greater loss of post-implantation stages (Table III).

Pre-implantation loss: Both doses of Thio-TEPA displayed effects in mating periods I and II, the higher dose also being active in mating periods III to V (Table I). In the case of MMS, only the dose of 100 mg/kg exerted an effect during the first two mating periods (Table II).

With a probable error of $p \leqq 0.05$, no significant differences from the controls were demonstrable (Table III) over

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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