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Neurotoxicity of Acrylamide and Related Compounds and Their Effects on Male Gonads in Mice

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Abstract. Neurotoxicity of acrylamide and related compounds and their effects on the testis after repeated oral doses were studied in mice. Of fourteen analogues tested, five produced neuropathy. In decreasing order of potency as assessed by the rotarod performance test, these were as follows: acrylamide > N-isopropylacrylamide > N-methylacrylamide = methacrylamide > N-hydroxymethylacrylamide. The development of neurotoxicity was either greatly reduced or delayed by phenobarbital treatment. Acrylamide, N-hydroxymethylacrylamide, N-isopropylacrylamide, N-methylacrylamide and N,N′-methylene-bis-acrylamide produced testicular atrophy. Atrophy was either prevented by phenobarbital treatment, as in the cases of acrylamide and N-isopropylacrylamide, or reduced, as in the case of N-hydroxymethylacrylamide. Histological changes in the testis produced by the active compounds were degenerations of the epithelial cells of the seminiferous tubules, with the interstitial cells being normal.

Key words: Acrylamide analogues – Acrylamide – N-*tert*-Butylacrylamide – Crotonamide – Diacetone acrylamide – N,N-Diethylacrylamide – N,N-Dimethylacrylamide – N-Hydroxymethylacrylamide – Iodoacetamide – N-Isobutoxymethylacrylamide – N-Isopropylacrylamide – Methacrylamide – N-Methylacrylamide – N,N′-Methylene-bis-acrylamide – N-*tert*-Octylacrylamide – Neuropathy – Testicular damage – Phenobarbital – Mice

Introduction

Of many acrylamide derivatives prepared so far, about ten compounds are produced in commercial quantities for the synthesis of various polymers (SRI, 1975), and demand for these compounds has been growing. The parent compound, acrylamide, being by far the most important product, produces peripheral neuropathy in various experimental animals and in humans (Spencer and Schaumburg 1974a, b). Some compounds related to acrylamide also cause

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neuropathy, as shown with N,N-diethylacrylamide, N-hydroxymethylacrylamide and N-methylacrylamide in rats (Edwards 1975a) and with methacrylamide in rabbits (Drees et al. 1976), although the relative potencies of their effects are lower when compared to acrylamide. Besides the neurotoxic effect, acrylamide produces degeneration of the testicular tubules of rats (McCollister et al. 1964). The present authors have found that N-hydroxymethylacrylamide, as well as acrylamide, causes both neuropathy and testicular atrophy in mice (Hashimoto et al. 1979).

This paper describes further studies on the comparative neurotoxicity of acrylamide and thirteen related compounds and their effects on the testis after repeated oral doses in mice. For the comparison of neurotoxic potency, the rotarod performance test developed by Dunham and Miya (1957) was used. The effect on the testis was examined by both gross and microscopic observations. The effect of metabolic activation by phenobarbital on the neuropathy and on testicular damage caused by the test compounds was also investigated. The structure-activity relationships of the analogues are briefly discussed.

Materials and Methods

Specific Chemicals. Acrylamide, dimethylsulfoxide (DMSO), N-hydroxymethylacrylamide, iodoacetamide, methacrylamide and N,N'-methylene-bis-acrylamide were obtained from Wako Pure Chemical Industries (Osaka, Japan); N,N-diethylacrylamide, N-isobutoxymethylacrylamide, N-methylacrylamide, and N-*tert*-octylacrylamide from Polyscience Inc. (Warrington, PA, USA); N-isopropylacrylamide from ICN Pharmaceutical Inc. (Plainview, NY, USA); N-*tert*-butylacrylamide, diacetone acrylamide and N,N-dimethylacrylamide from Tokyo Kasei Co. (Tokyo, Japan); crotonamide from Ishizu Seiyaku LTD (Osaka, Japan); phenobarbital from Sankyo Co. (Tokyo, Japan). All test compounds had a purity of greater than 95% in gas chromatography. The other chemicals were of reagent grade.

Animals. Male mice of ddY strain, 5–6 weeks of age and 29 ± 2.2 g body weight at the beginning of the experiments, were randomly placed in plastic cages (5–7 per cage) containing wooden flakes. They were fed laboratory chow and water ad libitum.

Determination of LD_{50}. The acute toxicity of the test compounds by oral administration in mice was determined according to Weil (1952), using four animals per dosage level and four different doses.

Treatment of Animals. Groups of five to seven animals were dosed with the test compounds, which were dissolved in either 0.9% saline solution, olive oil or DMSO, with a blunt tip metal intubation needle. Doses were given twice weekly, at levels, ranging from $^1/_2$ to $^1/_5$ of their LD_{50} for 8–10 weeks. Dose levels were so chosen, by preliminary experiments, that they produce either the least acute general symptoms of poisoning or no such symptoms at all. Control animals received a comparable volume of the vehicle. To examine the effect of metabolic activation, sodium phenobarbital (PB), which was prepared from phenobarbital before use, was given intraperitoneally at 50 mg/kg for five successive days per week, from one week before, up until the last week of treatment with the test compounds.

Evaluation of Neurotoxicity in Mice. For the test experiments, only those animals in which rotarod performance was able to be carried out were preliminarily selected. A modified apparatus of Dunham and Miya (1957), which consisted of a 5 cm diameter, roughly surfaced PVC rod, rotated at 3 revolutions per minute, was used. Arithmetic means of the longest performance periods, in five successive 30 s trials in each rat, were calculated for every test group. For the comparison of

neurotoxic potencies among compounds, ID_{50}, a half maximal inhibition dose of the walking performance, was estimated from a plot of time course vs. response as follows: days to half maximal inhibition $\times {}^{2}/_{7} \times$ single oral dose (mmol/kg).

Histopathological study of the testis and examination of blood: After treatment with the test compounds for 8–10 weeks, mice were killed under ether anesthesia for histology and blood examination. The testis was weighed and fixed in 10% neutral formalin, processed, and embedded in paraffin. Ten-micron sections were stained with hematoxylin and eosin. Blood was taken from the right atrium with a heparinized syringe. Measurements of red and white blood cell counts, hemoglobin concentration, and hematocrit value, and differentiation of white blood cells, were conducted by routine methods.

Statistical Analysis. Intergroup comparison was conducted by the Student's *t*-test.

Results

Acute Toxicity

The oral LD_{50} values of 14 test compounds administered to the mice are presented in Table 1. Iodoacetamide, chosen for comparison, was the most toxic, followed by acrylamide and N,N-dimethylacrylamide. Crotonamide and N,N-diethylacrylamide had the largest values.

Body Weight after Repeated Dose

Results are shown in Table 2, together with testicular weights. No significant change was seen in any of the treated groups when compared to control.

Neurotoxicity

Of the 14 test compounds, acrylamide, N-hydroxymethylacrylamide, N-isopropylacrylamide, methacrylamide, and N-methylacrylamide produced neurotoxic effects. Mice treated with these compounds gradually showed signs of weakness and ataxia of hindlimbs, with symptoms of slight behavioral changes such as aggressiveness and alertness in some cases. Otherwise, they appeared normal in all groups. Figure 1 illustrates rotarod performance results. The coefficient of variance (C.V.) for each point was rather large, ranging from about 10–100%. When the repeated treatment began with the five active compounds, performance on the rod gradually diminished with time. Table 3 shows the ID_{50} of these compounds and their approximate relative neurotoxic potency as a percentage of acrylamide potency. The ID_{50} value of each compound, however, seemed to be not absolute but variable, dependent upon the dosing conditions. In the case of N-isopropylacrylamide, for example, the ID_{50} value was altered from 5.8 to 9mmol/kg when the dose level was lowered from ${}^{1}/_{3}$ to ${}^{1}/_{5}$ LD_{50}. Therefore, the relative neurotoxicity indicates only an approximate value, valid only for the dosing conditions used in the present study.

Table 1. Acute oral toxicity of acrylamide analogues in mice

Compound		M.W.	Vehicle	LD_{50} mmol/kg
Acrylamide	$CH_2 = CHCONH_2$	71.08	0.9% saline	1.5 (1.1 – 2.1)[a]
Crotonamide	$CH_3HC = CHCONH_2$	85.11	0.9% saline	32 (27 –38)
Diacetone acrylamide	$CH_2 = CHCONHC(CH_3)_2CH_2COCH_3$	169.22	0.9% saline	7.7 (6.0 –10)
N,N-Dimethylacrylamide	$CH_2 = CHCON(CH_3)_2$	199.13	0.9% saline	3.4 (2.4 – 4.8)
N-Hydroxymethylacrylamide	$CH_2 = CHCONHCH_2OH$	101.01	0.9% saline	5.7 (4.2 – 7.8)
Iodoacetamide	ICH_2CONH_2	184.96	0.9% saline	0.40 (0.34– 0.48)
N-Isopropylacrylamide	$CH_2 = CHCONHCH(CH_3)_2$	113.16	0.9% saline	3.7 (2.0 – 5.3)
Methacrylamide	$CH_2 = CCH_3CONH_2$	85.11	0.9% saline	5.3 (4.2 – 6.7)
N-Methylacrylamide	$CH_2 = CHCONHCH_3$	85.11	0.9% saline	5.6 (3.9 – 7.9)
N,N′-Methylene-bis-acrylamide	$(CH_2 = CHCONH)_2CH_2$	154.17	0.9% saline	2.6 (1.7 – 3.9)
N,N-Diethylacrylamide	$CH_2 = CHCON(C_2H_5)_2$	127.19	Olive oil	11 (7.4 –17)
N-Isobutoxymethylacrylamide	$CH_2 = CHCONHCH_2OCH_2CH(CH_3)_2$	157.20	Olive oil	4.1 (3.5 – 4.7)
N-*tert*-Butylacrylamide	$CH_2 = CHCONHC(CH_3)_3$	127.19	DMSO	7.4 (5.6 – 9.7)
N-*tert*-Octylacrylamide	$CH_2 = CHCONH(CH_2)_4C(CH_3)_3$	183.29	DMSO	6.6 (3.1 – 9.2)

[a] Mean (95% confidence interval)

Table 2. Body and testicular weights after treatment with acrylamide analogues in mice

Compound	Vehicle	No. of mice	Dose[a] mmol/kg (× LD_{50})	Duration week	Body weight g	Relative testicular weight[b]
Control (0.9% saline)	–	6	–	8	34.5 ± 2.2[d]	0.36 ± 0.051[d]
Control (0.9% saline)	–	6	–	10	38.7 ± 4.2	0.34 ± 0.036
Acrylamide	0.9% saline	6	0.5 (1/3)	8	32.4 ± 2.4	0.30 ± 0.016*
Crotonamide	0.9% saline	5	16 (1/2)	10	37.2 ± 1.9	0.35 ± 0.036
Diacetone acrylamide	0.9% saline	6	2.6 (1/3)	10	36.6 ± 2.0	0.33 ± 0.026
N,N-Dimethylacrylamide	0.9% saline	5	1.7 (1/2)	10	35.5 ± 2.3	0.35 ± 0.036
N-Hydroxymethylacrylamide	0.9% saline	5	2.9 (1/2)	8	36.5 ± 6.3	0.20 ± 0.050**
Iodoacetamide	0.9% saline	5	0.2 (1/2)	10	38.4 ± 3.2	0.38 ± 0.050
N-Isopropylacrylamide	0.9% saline	6	1.2 (1/3)	8	37.0 ± 4.3	0.28 ± 0.036*
Methacrylamide	0.9% saline	5	1.8 (1/3)	8	33.1 ± 2.4	0.38 ± 0.065
N-Methylacrylamide	0.9% saline	7	1.9 (1/3)	8	40.0 ± 3.8	0.22 ± 0.045**
N,N′-Methylene-bis-acrylamide	0.9% saline	5	1.3 (1/2)	8	34.8 ± 2.9	0.17 ± 0.028**
Control (olive oil)	–	5	–	10	37.8 ± 3.0	0.38 ± 0.031
N,N-Diethylacrylamide	Olive oil	5	5.5 (1/2)	10	38.6 ± 2.7	0.36 ± 0.048
N-Isobutoxymethylacrylamide	Olive oil	5	1.4 (1/3)	10	40.7 ± 5.8	0.34 ± 0.035
Control (DMSO[c])	–	5	–	10	37.2 ± 2.4	0.36 ± 0.048
N-*tert*-Butylacrylamide	DMSO	6	2.5 (1/3)	10	36.2 ± 2.7	0.37 ± 0.031
N-*tert*-Octylacrylamide	DMSO	6	2.2 (1/3)	10	40.3 ± 3.5	0.34 ± 0.033

[a] Oral dose twice weekly; [b] Testis/body weight × 100; [c] Dimethylsulfoxide 0.1 ml/40 g bw; [d] Mean ± SD; * $p < 0.05$; ** $p < 0.001$

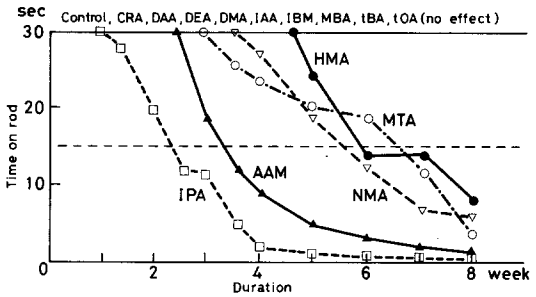

Fig. 1. Rotarod performance of mice after treatment with acrylamide and analogues. Abbreviations: Acrylamide (AAM, ▲), N-*tert*-Butylacrylamide (tBA), Crotonamide (CRA), Diacetone acrylamide (DAA), N,N-Diethylacrylamide (DEA), N,N-Dimethylacrylamide (DMA), N-Hydroxymethylacrylamide (HMA, ●), Iodoacetamide (IAA), N-Isobutoxymethylacrylamide (IBM), N-Isopropylacrylamide (IPA, ■), Methacrylamide (MTA, ○), N-Methylacrylamide (NMA, ▽), N,N′-Methylene-bis-acrylamide (MBA), N-*tert*-Octylacrylamide (tOA). Dose schedule: Oral dose twice weekly. Dose of each compound is shown in Table 2

Table 3. Relative neurotoxic potencies of acrylamide analogues

Compound	ID_{50}[a] mmol/kg bw	Approximate relative neurotoxic potency as percentage of acrylamide potency
Acrylamide	3.4	100
N-Isopropylacrylamide	5.8	59
N-Methylacrylamide	20.5	17
Methacrylamide	21.0	16
N-Hydroxymethylacrylamide	30.0	11

[a] Half maximal inhibition dose of rotarod performance

Effect on Testis

Four neurotoxic compounds, acrylamide, N-hydroxymethylacrylamide, N-isopropylacrylamide, and N-methylacrylamide, and one non-neurotoxic compound, N,N′-methylene-bis-acrylamide, produced both atrophy and significant reduction of weight in testis (Table 2).

Effect of PB Treatment

Seven groups of mice, treated with five neurotoxic compounds and two non-neurotoxic compounds (N,N-diethylacrylamide and N,N-dimethylacrylamide), were given PB (50 mg/kg, 5/week), which has been known to elevate the activity of drug metabolizing enzymes, from one week before, up until the last week of treatment with the test compounds. Neither weakness nor ataxia developed in the groups treated with N,N-diethylacrylamide or N,N-dimethyl-

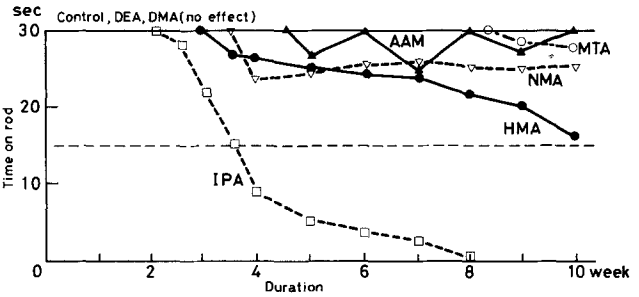

Fig. 2. Effect of phenobarbital on rotarod performance of mice treated with acrylamide and analogues. Abbreviations: Acrylamide (AAM, ▲), N,N-Diethylacrylamide (DEA), N,N-Dimethylacrylamide (DMA), N-Hydroxymethylacrylamide (HMA, ●), N-Isopropylacrylamide (IPA, □), Methacrylamide (MTA, ○), N-Methylacrylamide (NMA, ▽). Dose schedule: Oral dose twice weekly. Dose of each compound is shown in Table 4. Phenobarbital: Intraperitoneal dose, 50 mg/kg, 5/week through the experiment

acrylamide within 10 weeks following treatment. In the groups treated with the four neurotoxic compounds, symptoms of neuropathy were considerably reduced and rotarod performance improved when compared to the same treatment groups without PB. In the mice treated with N-isopropylacrylamide, however, weakness and ataxia of hindlimbs were not as well reduced as in the other groups, although the onset and progress of symptoms were delayed and rotarod performance somewhat improved when compared to the group without the PB treatment. When PB was given to another group of mice treated with $^1/_5$ LD_{50} dose of the compound, their symptoms and rotarod performance were much improved. The effect of PB on rotarod performance is shown in Fig. 2. Table 4 represents the effects of PB on body and testicular weights. No significant changes were seen in body weight. Reltative testicular weights in acrylamide- and N-isopropylacrylamide-treated groups were not different from the control value, while those in N-hydroxymethylacrylamide- and N-methylacrylamide-treated groups were significantly lower.

Histopathology of Testis

Figure 3a–f show light microscopic patterns of the mouse testis. Figure 3a represents a normal pattern, with orderly arrangement of spermatogenic cells in the seminiferous tubules and interstitial cells of Leydig. Figure 3b–f represent patterns obtained from mice treated with acrylamide, N-hydroxymethylacrylamide, N-isopropylacrylamide, N-methylacrylamide and N,N′-methylene-bis-acrylamide respectively. The seminiferous tubules show injuries in their epithelia in all cases. Lesions commonly seen in the epithelia are: degeneration of cells, especially of the spermatids and spermatocytes; reduction of the spermatozoa; and the presence of multinucleate giant cells, specially in case of N-methylacrylamide. Sertoli cells and interstitial cells, however, appeared to be unaffected in all cases. Epididymis seemed histologically normal, although their relative weights were slightly reduced in some cases.

Table 4. Effect of metabolic activation by phenobarbital[a] on body and testicular weights of mice treated with acrylamide analogues

Compound	Vehicle	No. of mice	Dose[b] mmol/kg ($\times$ LD_{50})	Duration week	Body weight g	Relative testicular weight[c]
Control (0.9% saline)	–	7	–	10	36.0 ± 2.7[d]	0.34 ± 0.037[d]
Acrylamide	0.9% saline	7	0.5 (1/3)	10	37.1 ± 2.5	0.31 ± 0.016
N,N-Dimethylacrylamide	0.9% saline	6	1.7 (1/2)	10	36.0 ± 2.4	0.32 ± 0.032
N-Hydroxymethylacrylamide	0.9% saline	6	2.9 (1/2)	10	33.5 ± 2.3	0.26 ± 0.029**
N-Isopropylacrylamide	0.9% saline	6	1.2 (1/3)	10	36.1 ± 3.4	0.31 ± 0.067
Methacrylamide	0.9% saline	6	1.8 (1/3)	10	36.6 ± 0.97	0.32 ± 0.030
N-Methylacrylamide	0.9% saline	6	1.9 (1/3)	10	34.9 ± 4.5	0.25 ± 0.070*
Control (olive oil)	–	6	–	10	33.9 ± 3.4	0.38 ± 0.040
N,N-Diethylacrylamide	Olive oil	6	5.5 (1/2)	10	34.1 ± 2.2	0.39 ± 0.037

[a] Sodium phenobarbital 50 mg/kg, 5/week; [b] Oral dose twice weekly; [c] Testis/body weight $\times$ 100; [d] Mean ± SD; * $p < 0.05$; ** $p < 0.01$

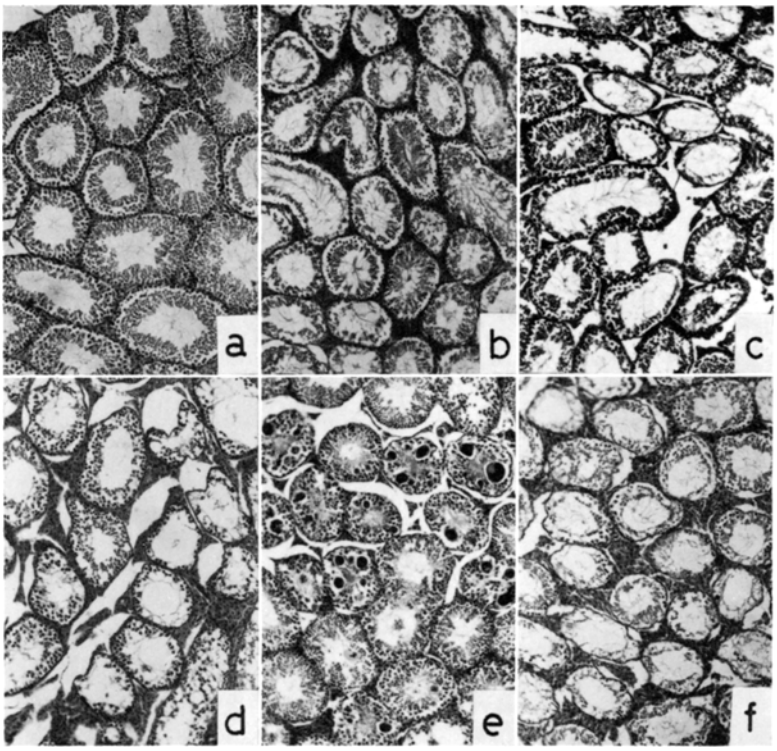

Fig. 3a–f. Seminiferous tubules of mice after treatment with acrylamide analogues for 8 weeks. (**a**) Normal control, (**b**) Acrylamide, (**c**) N-Hydroxymethylacrylamide, (**d**) N-Isopropylacrylamide, (**e**) N-Methylacrylamide, (**f**) N,N′-Methylene-bis-acrylamide (× 50). Dose schedule; oral dose twice weekly. Dose of each compound is shown in Table 2

Blood Study

Effects of test compounds on the blood were examined after the last treatment. Only one compound, N,N′-methylene-bis-acrylamide, produced marked effects on red and white blood cell counts, hemoglobin concentration and hematocrit value. The hematological as well as testicular effects of this compound will be reported elsewhere.

Discussion

The results of the present study indicate that acrylamide and four related compounds, i.e., N-hydroxymethylacrylamide, N-isopropylacrylamide, N-methylacrylamide and methacrylamide, the first three being N-monoalkyl- and the last one β-alkyl-substitute, produce neuropathy in mice after repeated oral treatment. No other test derivatives showed this effect. N,N-diethylacrylamide, which has been shown to be neurotoxic in rats (Edwards 1975a), was not active

in mice in the present study. It is not clear whether the inactivity of this compound in mice is due to species differences in susceptibility to the compound or to differences in treatment schedule between the two studies: 11,200 mg/kg cumulative dose in 8 weeks (oral administration, twice weekly) in our study, compared to 9,400 mg/kg in 10 weeks (feeding administration) in Edwards's. When the approximate relative neurotoxic effects of the analogues were compared to acrylamide by use of ID_{50}, N-isopropylacrylamide showed a potency about 60% that of acrylamide. The other analogues were weaker, as already reported for rats (Edwards 1975a; Drees et al. 1976).

It is known that most acrylamide analogues react with reduced glutathione in vitro and in vivo (Hashimoto and Aldridge 1970; Edwards 1975a, b), and the reactivity of acrylamide has been suggested to play an important role in the development of tissue damage in chick ganglia culture (Sharma and Obersteiner 1977). On the other hand, Edwards (1975a) found that the neurotoxic potency of the analogues bore no relation to the reactivity in vitro. Iodoacetamide, which is known to be a strong sulphydryl reagent (Smythe 1936), was not neurotoxic in the present study. The structure-neurotoxicity relationship of these analogues is now under investigation.

In this study, N-isopropylacrylamide, N-methylacrylamide and N,N′-methylene-bis-acrylamide all produced testicular damage, in addition to acrylamide and N-hydroxymethylacrylamide, two compounds already known to be toxic to the organ (McCollister et al. 1964; Hashimoto et al. 1979). Another neurotoxic compounds, methacrylamide, did not produce the effect, which may imply the existence of a different mechanism of action between the neurotoxic and testicular effects of the analogues.

In regard to the effect of hepatic microsomal inducers on the development of acrylamide neuropathy, Kaplan et al. (1973) reported a significantly delayed onset of the deficit after treatment with DDT or PB in rats, while Edwards (1975a) failed to obtain the effect in the same animal. The present study in mice has indicated that PB treatment reduces neurotoxic symptoms due to acrylamide and analogues. Testicular damage was completely prevented by PB in mice treated with acrylamide or N-isopropylacrylamide and in other groups to a lesser extent. These results indicate that the biotransformation of the former two compounds into some inactive metabolites, which have been shown by Langvardt et al. (1979) for acrylamide in rats, might have been enhanced by the metabolic inducer in mice as well. This could also be the same for the other analogues.

A study of the in vitro metabolism of the present test analogues, done for better understanding of the relationship between their biological effect and biotransformation, is being prepared for publication.

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