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Experimental study on the enhancement of the neurotoxicity of methyl n-butyl ketone by non-neurotoxic aliphatic monoketones

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ABSTRACT The neurotoxicity of methyl n-butyl ketone is known to be enhanced by combination with methyl ethyl ketone. This study was conducted to clarify the potentiating effect of aliphatic monoketones on the neurotoxicity of methyl n-butyl ketone. Rats were subcutaneously injected in the back with 4 mmol/kg/day of methyl ethyl ketone, methyl n-propyl ketone, methyl n-amyl ketone, or methyl n-hexyl ketone mixed with an equimolar dose of methyl n-butyl ketone five days a week for 20 weeks. The maximum motor fibre conduction velocity and the distal latency were measured every two weeks in the tail nerves of the treated animals and controls. All the monoketones tested enhanced the neurotoxicity of methyl n-butyl ketone. Of the compounds tested, methyl n-hexyl ketone, which had the longest carbon chain, enhanced the neurotoxicity of methyl n-butyl ketone most strongly. These results suggest that the length of the carbon chain of the aliphatic monoketones combined with methyl n-butyl ketone was related to the enhancement of the neurotoxicity of the neurotoxic compound.

Industrial workers are often exposed to n-hexane or methyl n-butyl ketone (MnBK) in combination with aliphatic monoketones. It has been reported that methyl ethyl ketone (MEK) greatly enhances the neurotoxicity of MnBK[1–5] or n-hexane,[4 6] although the detailed mechanism of the potentiating effect is not clear. Abdel-Rahman *et al* and Couri *et al* have reported that animals exposed the MnBK/MEK in combination showed a significant decrease in sleep time and, further, that the reduction and oxidation of MnBK were carried out in the cytosol and in the microsomal fraction of the liver.[1 7] These results have implied that exposure of animals to the combined vapours resulted in the induction of the common enzyme required for their metabolism, and that subsequent biotransformations might result in the enchancement of the neurotoxicity of MnBK or n-hexane. The similarity of the metabolic processes of aliphatic monoketones suggests that not only MEK but also other non-neurotoxic aliphatic monoketones[8] may enhance the neurotoxicity of MnBK or n-hexane. The investigation of this hypothesis is of great importance in industrial hygiene, especially as regards the establishment of threshold limit values for n-hexane or MnBK. There have been no reports, however, about any possible potentiating effects of aliphatic monoketones other than MEK on the neurotoxicity of MnBK.

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In the experiments described here the potentiating effects on the neurotoxicity of MnBK by aliphatic monoketones, which have a carbonyl group at the second position in their carbon chains with four to eight carbons, and the comparative neurotoxicity of MnBK alone or in combination with other solvents was investigated using an electrodiagnostic technique which we have described previously.[9 10]

Materials and methods

Chemicals—The test compounds were MEK; methyl n-propyl ketone (MnPK); MnBK; methyl n-amyl ketone (MnAK); and methyl n-hexyl ketone (MnHK); the molecular and structural formulas are shown in table 1. MEK (99 V/V%), MnPK (95), MnBK (95), MnAK (98), and MnHK (95) were all obtained from the Wako company, Osaka.

Animals and treatment—Forty eight male Donryu strain rats (248 ± 21 g) were assigned to five test groups and a control group of eight animals each.

Table 1 *Chemical substances tested*

Chemical substance	*Molecular formula*	*Structural formula*
Methyl ethyl ketone (MEK, 2-butanone)	C_4H_8O	$CH_3-C(=O)-CH_2-CH_3$
Methyl n-propyl ketone (MnPK, 2-pentanone)	$C_5H_{10}O$	$CH_3-C(=O)-CH_2-CH_2-CH_3$
Methyl n-butyl ketone (MnBK, 2-hexanone)	$C_6H_{12}O$	$CH_3-C(=O)-CH_2-CH_2-CH_2-CH_3$
Methyl n-amyl ketone (MnAK, 2-heptanone)	$C_7H_{14}O$	$CH_3-C(=O)-CH_2-CH_2-CH_2-CH_2-CH_3$
Methyl n-hexyl ketone (MnHK, 2-octanone)	$C_8H_{16}O$	$CH_3-C(=O)-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3$

The five test groups were exposed to MnBK alone or to combinations of MnBK/MEK, MnBK/MnPK, MnBK/MnAK, or MnBK/MnHK groups. Each compound was administered in equimolar doses of 4 mMol/kg body weight by subcutaneous injections into the back of the animals, once daily, five days a week, for 20 weeks.

Observations of neurological signs—The animals ability to walk, their reactions on being picked up by their bodies or tails, and their ability to hang on the edge of a steel cage were examined twice a week.

Electrophysiological examinations—The maximum motor fibre conduction velocity (MCV) and the distal latency (6 cm) were adopted as measures for estimating the effects on peripheral nerve function. The measurements of conduction velocity were undertaken every two weeks during the experiment; the electrophysiological technique used has been described in detail in previous reports.[9 10] Figure 1 shows a schematic illustration of an electrode arrangement for conduction velocity measurement in vivo. The tail nerve was stimulated by a square pulse of 0·3 msec duration, one Herz and supramaximal strength with an electrostimulator (MNS 1101, Nihon Kohden); the biopotentials were observed with an Addscope (ATAC-250, Nihon Kohden) and recorded on graph paper with an X-Y

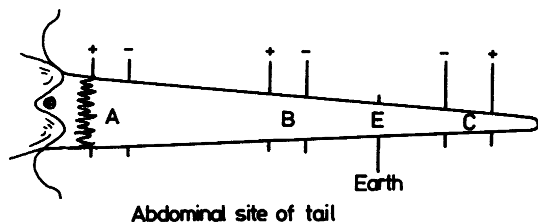

Fig 1 *Schematic illustration of electrode arrangement for measuring maximum motor fibre conduction velocity in tail nerve of rat.*

recorder (Yokogawa Electric Company). The test areas for the MCV and distal latency were sections A-B and B-C in fig 1, respectively. The body weight, neurological signs, and general condition were examined every third day throughout the experiment. The room temperature was contolled at 29 ± 0·5°C, and the skin temperature of the tail was also controlled at about 34°C at the middle point of the tail during the measurement of MCV.

Determination of cytochrome P-450—Fifteen Donryu strain rats weighing 480 g were used for this study. MnBK, MnBK/MEK, or MnBK/MnHK were subcutaneously administered to the rats for six days a week for three weeks.

Preparations of microsomes—Rats were decapitated 24 hours after the last treatment with the chemicals, and their livers were homogenised in 5 vol of 20 mM Tris-1·15% KC1 (PH 7·4). The homogenate was centrifuged at 12 000 × g for 15 min at 4°C, and the supernatant fraction was centrifuged at 78 000 × g for 110 min at 4°C. The resultant microsomal pellet was suspended in the same volume of 20 mM Tris-1·15% KC1 (PH 7·4) and recentrifuged at 78 000 × g for 110 minutes at 4°C.

Assays of the enzyme—The microsomal cytochrome P-450 was determined by the methods of Omura and Sato.[11] Protein measurement was carried out using the method of Lowry *et al*[12] with bovine serum albumin as a standard.

The significance of the difference between the mean values in the treated and control groups was evaluated by Student's *t* test.

Results

BODY WEIGHT AND GENERAL CONDITIONS

As shown in fig 2, all the treated groups showed a significant decrease in body weight as compared with the controls. The time of onset of a significant decrease in body weight (by comparison with the

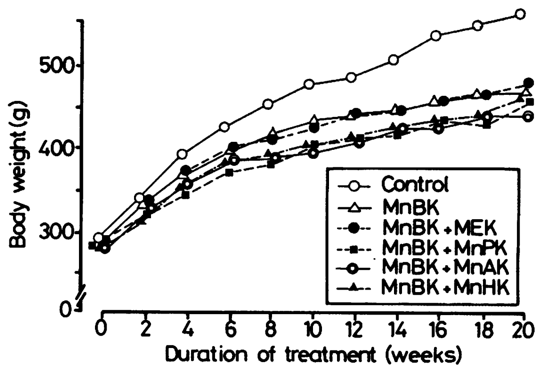

Fig 2 *Changes in mean body weight of all groups. MnBK = Methyl n-butyl ketone, MEK = methyl ethyl ketone, MnHK = methyl n-hexyl ketone, MnPK = methyl n-propyl ketone, MnAK = methyl n-amyl ketone.*

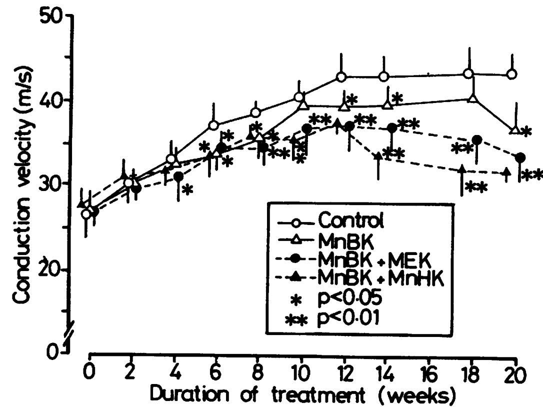

Fig 3 *Changes in maximum motor fibre conduction velocity in proximal part (A-B) of tail nerve of rat. MnBK = Methyl n-butyl ketone, MEK = methyl ethyl ketone, MnHK = methyl n-hexyl ketone.*

controls) was in the fourth week of the experiment in the MnBK/MnHK group, the sixth week in the MnBK/MnPK group, the eigth week in the MnBK/MnAK group, the tenth week MnBK/MEK, and the fourteenth week in the MnBK alone group. The groups treated with a mixture of MnBK/MnPK, MnBK/MnAK, or MnBK/MnHK seemed to show greater decreases in body wieght than the MnBK alone group, but these differences were not significant.

Weakness in the hind legs was more severe in the combined solvent groups than in the MnBK alone group and the MnBK/MnHK group seemed to be especially severely affected. Animals in this group first showed disturbances of gait in the hind legs in the sixth week of treatment, at which time they could not hang on to the edge of a steel chamber. Furthermore, when they were picked up they showed little resistance but none of them had developed paralysis of the hind legs by the end of the experiment. Animals in the other combined solvent groups were less alert and resistant on handling than those of the MBK alone group, but differences among the combined groups, except the MnBK/MnHK group, were not clear because non-quantitative tests were used.

NEUROPHYSIOLOGICAL FINDINGS

The changes in the MCVs in the MnBK, the MnBK/MEK, the MnBK/MnHK, and the control groups are illustrated in fig 3 and the mean values are summarised in table 2. The MCVs began to decrease, compared with the controls, in the sixth week of treatment in all the treated groups. The degree of decrease in the values was in the order: MnBK/MnHK > MnBK/MnAK = MnBK/MnPK = MnBK/MEK > MnBK alone group, with the MnBK/MnHK group showing the greatest decrease.

In addition, when the MCV values of the combined solvent groups were compared with those of

Table 2 *Changes in maximal motor fibre conduction velocity of the groups exposed to MBK alone, MnBK/MEK, MnBK/MnPK, MnBK/MnAK, and MnBK/MnHK*

Groups	*Before*	*Weeks* 2	4	6	8	10	12	14	18	20
Control (n = 8)	26·3	30·1	33·1	37·4	38·4	40·5	43·0	43·0	43·4	43·3
	2·6	2·1	2·1	2·5	1·3	1·9	2·4	2·2	3·0	2·3
MnBK alone (n = 8)	26·8	30·4	32·5	33·5*	35·5*	39·5	39·3*	39·5*	40·2*	36·1**
	2·5	1·3	1·5	1·7	2·7	1·3	2·1	2·5	4·0	4·0
MnBK/MEK (n = 8)	26·6	29·6	30·2*	34·4*	34·4**	36·7*	37·1**	36·9**	35·3**	33·4**
	1·6	1·6	2·3	2·1	2·2	3·9	3·0	2·7	3·8	4·8
MnBK/MnPK (n = 8)	28·3	33·4	33·6	33·1*	35·8**	39·0	38·5**	38·4*	36·8**	35·0**
	2·3	2·7	3·2	3·2	0·9	1·6	1·7	3·1	1·7	3·8
MnBK/MnAK (n = 8)	27·1	31·6	33·8	33·8*	35·9*	36·9*	38·4*	38·4*	32·3**	35·5**
	2·3	1·2	1·9	2·2	2·0	3·5	1·0	3·0	2·5	3·2
MnBK/MnHK (n = 8)	27·9	31·1	31·5	33·4*	35·7*	35·2**	38·3*	33·4**	31·9**	30·7**
	1·5	2·0	1·1	3·0	1·4	2·6	2·1	3·8	4·0	1·7

Values indicate mean ± SD (m/sec). Significance level, *$p < 0·05$, **$p < 0·01$, as compared with the control values by *t* test.

Table 3 *Changes in maximal motor fibre conduction velocity (MCV) and motor distal latency (DL) compared with the control or MBK only group*

Groups		*With control Weeks* 4	6	8	10	12	14	16	18	20	*With MnBK only Weeks* 4	6	8	10	12	14	16	18	20
MnBK	MCV	—	↓↓	↓	—	↓	↓		—	↓									
	DL	—	—	↑	—	↑	—		↑↑	↑↑									
MnBK + MEK	MCV	↓	↓	↓↓	—	↓↓	↓↓		↓↓	↓↓	—	—	—	—	—	—		↓↓	—
	DL	—	—	↑	—	↑	↑		↑	↑↑	—	—	—	↑↑	—	—		—	—
MnBK + MnPK	MCV	—	↓	↓↓	—	↓↓	↓		↓↓	↓↓	—	—	—	—	—	—		—	—
	DL	—	—	↑	—	↑	↑↑		↑↑	↑↑	—	—	—	↑	—	—		—	↑
MnBK + MnAK	MCV	—	↓	↓	—	↓	↓		↓↓	↓↓	—	—	—	—	—	—		↓↓	—
	DL	—	—	—	—	↑	↑↑		↑↑	↑↑	—	—	—	↑↑	—	↑↑		—	↑↑
MnBK + MnHK	MCV	—	↓	↓	↓↓	↓	↓↓		↓↓	↓↓	—	—	—	↓↓	—	↓↓		↓	↓↓
	DL	—	↑↑	↑↑	↑	↑↑	↑↑		↑↑	↑↑	—	—	—	↑	↑	↑		↑	↑↑

— Not significant, ↑, ↓ $p < 0.05$, ↑↑, ↓↓ $p < 0.01$, as compared with the control or MnBK alone groups by t test.

Table 4 *Changes in motor distal latency of the MBK, MnBK/MEK, MnBK/MnPK, MnBK/MnAK, and MnBK/MnHK groups*

Groups	*Before*	*Weeks* 2	4	6	8	10	12	14	18	20
Control (n = 8)	3·23	2·68	2·65	2·36	2·17	2·23	2·07	2·22	2·25	2·03
	0·54	0·27	0·18	0·16	0·17	0·23	0·11	0·10	0·13	0·15
MnBK only (n = 8)	3·37	2·58	2·54	2·56	2·50*	2·16	2·24*	2·37	2·73**	2·74**
	0·54	0·20	0·07	0·21	0·24	0·07	0·13	0·19	0·33	0·24
MnBK/MEK (n = 8)	3·19	2·90	2·77	2·43	2·53**	2·39**	2·42*	2·60*	3·00*	2·96**
	0·15	0·20	0·19	0·13	0·14	0·15	0·28	0·29	0·69	0·43
MnBK/MnPK (n = 8)	3·08	2·58	2·69	2·39	2·42*	2·34**	2·19**	2·55**	3·00**	3·37**
	0·14	0·11	0·13	0·16	0·20	0·15	0·17	0·17	0·38	0·45
MnBK/MnAK (n = 8)	3·10	2·65	2·61	2·56	2·33	2·47	2·36**	2·96**	3·00**	3·69**
	0·23	0·24	0·12	0·30	0·14	0·23	0·12	0·08	0·10	0·22
MnBK/MnHK (n = 8)	3·09	2·88	2·62	2·75**	2·51**	2·67**	2·59**	2·76**	3·50**	3·50**
	0·32	0·20	0·18	0·15	0·11	0·28	0·19	0·18	0·66	0·56

Values indicate mean ± SD (msec). Significance level, $^*p < 0.05$, $^{**}p < 0.01$ as compared with the control values by t test.

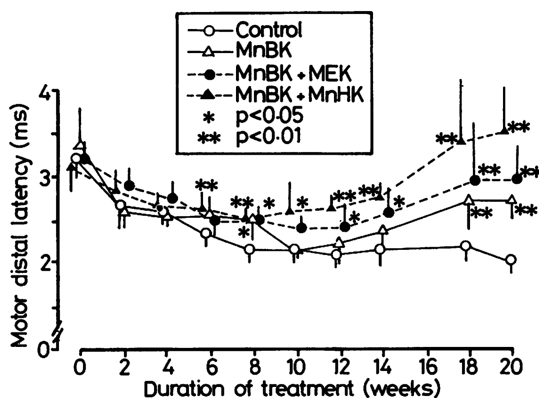

Fig 4 *Changes in motor distal latency of distal part (6 cm) of tail nerve in rat. MnBK = Methyl n-butyl ketone, MEK = methyl ethyl ketone, MnHK = methyl n-hexyl ketone.*

the MnBK alone group, the MnBK/MnHK group began to show a significant decrease in MCV in the tenth week. In the MnBK/MnAK and the MnBK/MEK groups, a significantly greater decrease in the MCV was first observed in the eighteenth week by comparison with the MnBK alone group. These results are summarised in table 3.

DISTAL LATENCY

Table 4 shows the mean values for the distal latency (msec) of the distal part (6 cm) of the tail. The mean values of the distal latency in all the treated groups were significantly greater than the control value. Furthermore, as compared with the MnBK alone group, the distal latencies of all the combined solvent groups became prolonged in the tenth week of the experiment (table 3). Figure 4 shows the mean values of the MnBK alone, the MnBK/MEK,

the MnBK/MnHK, and the control group.

Discussion

It has been reported that animals administered the compounds MEK,[3–6] MnPK,[8 13 14] MnAK,[8 13 15–17] or MnHK[8 13] alone exhibit no clinical, pathological, or neurophysiological evidence of peripheral neuropathy except for increased salivation in the MnPK group.[13] In the present study, however, combinations of MnPK, MnAK, or MnHK, in addition to MEK combined with MnBK, were found to enhance the neurotoxicity of MnBK considerably, judging from both the clinical results and the electrophysiological findings. At the sixth week of the experiment, when neurological signs in all the other groups except for the MnBK/MnHK group were not apparent, retardation of the MCVs in all the groups was observed. As table 3 and fig 3 show, the MCV and DL values in the MnBK/MnHK group moved into the abnormal range. During the eighth to the twelfth weeks of the experiment, clinical signs including dullness gradually became apparent. The non-quantitative observations of the clinical signs were unable to supply accurate information about the nervous function of the rats which suggests that the MCV measurement is valuable for the early detection of neuropathies. Futher studies carried out in our laboratory have reconfirmed the enhancement of the neurotoxicity of MnBK by MnHK.That is, animals treated for three months with the combined MnHK (8 mM/kg)/MnBK (4 mM/kg) showed pronounced paralysis in the hind legs, whereas animals treated with increased daily doses of 10 mM/kg of MnBK alone did not.

This result indicates that the longer the length of the carbon chain of the aliphatic monoketones, the greater the potentiating effect on the neurotoxicity of MnBK.

Until now, there have been no reports of the neurotoxicity of MnBK being enhanced by analogues of 5- to 8-carbon compounds, although MEK and 5-methyl 2-octanone have been reported to enhance the neurotoxicity of MnBK and 5-nonanone,[18] respectively. The neurotoxic effect of 5-nonanone is thought to be produced by the metabolic transformation of the compound to MnBK.[18] It has been reported that MnBK is metabolised by ω- or ω-1 oxidations, or both,[19–21] and that those oxidations are carried out in the microsomal fraction of the liver.[7] Branchflower *et al* have reported that 24 hours after a single dose and three days treatment with MBK (15 mM/kg), the level of microsomal cytochrome P-450 in the rat liver was significantly increased.[22 23] In our experiments the rats treated with MnBK (4 mM/kg) alone showed a significant increase of cytochrome P-450 in the microsomal fraction in the liver as compared with the non-treated group (table 5). Treatment with MEK or MnHK alone also increased the levels of the microsomal cytochromes P-450 in the rat liver.[24] This indicates that microsomal cytochrome P-450 required for the metabolism of MnBK is also necessary for the metabolism of MEK or MnHK. With respect to n-hexane, reports indicated that this compound does not increase cytochrome P-450 levels in rat liver.[25]

As shown in table 5, the rats treated with the combined MnBK/MEK or MnBK/MnHK for three weeks showed a significantly decreased induction of the enxyme as compared with the MnBK alone group. Furthermore, four hours after the last treatment with a greater dose (10 mM/kg) of the above mentioned compounds, the level of the cytochrome P-450 decreased as compared with the values of the non-treated group.[24]

Concerning n-hexane, Iwata *et al* have reported that continuous exposure to a combination of n-hexane and MEK (1000 ppm, each), or to 3000 ppm n-hexane, appreciably decreases metabolite levels, especially the level of 2-hexanol in the urine compared with n-hexane (1000 ppm) alone.[26] No animals treated with MEK/MnBK or MnHK/MnBK (10 mM/kg, each) showed significantly

Table 5 *Effect of treatment of rats with MnBK alone, MnBK/MEK, or MnBK/MnHK on levels of microsomal cytochrome P-450 in rat liver**

Group	*Body weight (g)*	*Liver weight (g)*	*Protein (mg/ml)*	*Cytochrome P-450 (nmol/mg protein)*†
Control	485 ± 23	17·2 ± 1·6	8·6 ± 1·4	0·84 ± 0·03
MnBK alone	487 ± 41	17·0 ± 0·6	9·1 ± 2·1	1·60 ± 0·32‡
MnBK/MEK	463 ± 40	15·6 ± 1·9	9·3 ± 1·3	0·92 ± 0·09§
MnBK/MnHK	480 ± 36	15·4 ± 1·7	8·3 ± 2·2	0·84 ± 0·09§

MnBK = Methyl n-butyl ketone, MEK = methyl ethyl ketone, MnHK = methyl n-hexyl ketone.
*Rats were administered MnBK, MnBK/MEK, or MnBK/MnHK (4 mmol/kg, each, sc) for 31 weeks. Twenty four hours after the last treatment the level of cytochrome P-450 was determined as described (see methods).
†Values indicate means ± SD of the values obtained with three rats (six rats for controls).
‡Significantly different from control values ($p < 0.05$).
§Significantly different MnBK only values ($p < 0.05$) by Student's *t* test.

increased excretions of 2, 5-HDione or 2-hexanol in the urine (using the techniques described by Iwata *et al*[26]) compared with the MBK alone group.[27]

These data suggest that the longer the length of the carbon chain of the aliphatic ketone which is given in combination with MnBK, the greater the amount of cytochrome P-450 required for its hydroxylation. Thus MnHK with a long carbon chain may impede the metabolism of MnBK; differences in the length of the carbon chains of MnHK and MEK might produce different potentiating effects on the neurotoxicity of MnBK. Sato and Nakajima have also observed competitive metabolic interaction between chemical compounds with such similar structures as benzene and toluene.[28]

One third of absorbed MEK (0·3 to 0·4 g/kg), which is a volatile, water soluble liquid and less of a potentiator of MnBk neurotoxicity than MnHK, is eliminated unchanged in the expired air of dogs.[29] The remainder is reduced to 2-butanol and oxidised by ω-1 oxidation to 3-hydroxy 2-butanone.[19] Most of the 2-butanol is probably excreted as CO_2 or n-butanol in the expired air.[30] The metabolism of MnHK, which has a low volatility and is practically insoluble in water, is not well known but it may also have a pattern of metabolism similar to that of MEK, MnPK, and MnBK.[19]

This suggests that a dose of aliphatic monoketones large enough to overload the metabolic capacity of the liver might competitively consume the common metabolic enzymes and prolong their elimination in the urine by the inhibition of hydroxylation.[26] These considerations, however, seem to be inconsistent with the report[1] that, in a combined MEK/MnBK group or an MnBK group pretreated with phenobarbital, a cytochrome P-450 inducer, significant increases in 2, 5-HDione levels were observed in the blood, despite the fact that decreases in the level of cytochrome P-450 would be expected to inhibit the production of 2, 5-hexanedione from MnBK. Decreases of this enzyme, also, seem to be inconsistent with the result[4] that mice exposed to the MEK/MnBK combined vapours showed a significant decrease in sleep time.

It is also thought that the elimination of 2, 5-hexanedione into the urine is delayed by the inhibition of the conjugation of MnBK with glucuronic acid by solvent combinations, since the inhibitory action of primary aliphatic alcohols to uridine diphosphate glucuronic acid transferase increases exponentially with the length of the alkyl chain and linearly with lipid solubility.[31] The detailed mechanism of these potentiating effects on neurotoxicity remains to be found. Since hexacarbon solvents such as n-hexane are often used in combination with other solvents, it is important, from the view point of the establishment of threshold limit values of MBK or n-hexane, to recognise that non-neurotoxic aliphatic monoketones may enhance their neurotoxicity.

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