4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)の細菌を用いる復帰突然変異試験

Reverse Mutation Test of 4,4-Thiobis(6-*tert*-butyl-*m*-cresol) on Bacteria

要約

既存化学物質安全性調査事業の一環として，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)について，細菌を用いる復帰突然変異試験をプレート法により実施し，陰性の結果を得た．

検定菌として，*Salmonella typhimurium* TA100, TA1535, TA98, TA1537および*Escherichia coli* WP2 *uvrA*の5菌株を用い，S9 mix無添加および添加の条件でプレート法により，用量設定試験を50～5000 μg/プレートの用量で行ったところ，S9 mix無添加試験および添加試験のいずれにおいても*Salmonella*の4検定菌において強い抗菌性が認められたが，WP2 *uvrA*では抗菌性は認められなかった．

したがって，本試験ではS9 mix無添加試験をTA100, TA1535およびTA1537は0.781～50 μg/プレート，TA98は3.13～200 μg/プレート，WP2 *uvrA*は313～5000 μg/プレートの範囲で，S9 mix添加試験を*S. typhimurium*の4菌株は12.5～800 μg/プレート，WP2 *uvrA*は313～5000 μg/プレートの範囲で用量を設定して実施した

その結果，S9 mix無添加試験では，TA100およびTA1537は，12.5 μg/プレート，TA1535は25 μg/プレート，TA98は100 μg/プレートで抗菌性が認められたが，WP2 *uvrA*では認められなかった．またS9 mix添加試験ではTA100, TA1535, およびTA1537は400 μg/プレート，TA98は800 μg/プレートで抗菌性が認められたが，WP2 *uvrA*では認められなかった．復帰変異コロニー数は，2回の本試験とも，用いた検定菌について，いずれの用量においても復帰変異コロニー数の増加は認められなかったことから，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)は，用いた試験系において変異原性を有しない(陰性)と判定された．

方法

〔検 定 菌〕

Salmonella typhimurium TA100
Salmonella typhimurium TA1535
Escherichia coli WP2 *uvrA*
Salmonella typhimurium TA98
Salmonella typhimurium TA1537

*S. typhimurium*の4菌株[1]は1975年10月31日にアメリカ合衆国，カリフォルニア大学のB.N. Ames博士から分与を受けた．

E. coli WP2 *uvrA*株[2]は1979年5月9日に国立遺伝学研究所の賀田恒夫博士から分与を受けた．

検定菌は-80℃以下で凍結保存したものを用い，ニュートリエントブロスNo. 2(Oxoid)を入れたL字型試験管に解凍した種菌を一定量接種し，37℃で10時間往復振とう培養したものを検定菌液とした．

〔被験物質〕

4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)(CAS No. 96-69-5)は，分子量358.54の白色結晶性粉末である．試験には，住友化学工業㈱製〔ロット番号：40701，純度98％以上(不純物：不明)〕のものを，㈳日本化学工業協会から供与され，使用時まで室温保管し，用いた．

4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)は，ジメチルスルホキシド(DMSO)に溶解性がよいことから，DMSOに50 mg/mlになるように溶解した後，同溶媒で公比約3ないし2で希釈し，速やかに試験に用いた．

試験の開始に先立って，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)のDMSO溶液中での安定性試験および含量測定試験を実施した．安定性試験においては，本試験Ⅱで調製した低濃度(7.81 μg/ml)溶液および高濃度(50.0 mg/ml)溶液について，室温遮光条件下で，安定性を調べた．その結果，調製4時間後における各濃度の平均含量は，それぞれ初期値(0時間)の平均値に対して，100および98.6％であった．

また，含量測定試験を行った結果，調製液の濃度は，高濃度は94.7％，低濃度は98.3％であった．

〔陽性対照物質〕

用いた陽性対照物質およびその溶媒は以下のとおりである．

AF2	：2-(2-フリル)-3-(5-ニトロ-2-フリル)アクリルアミド	(上野製薬㈱)
SA	：アジ化ナトリウム	(和光純薬工業㈱)
9AA	：9-アミノアクリジン	(Sigma Chem. Co.)
2AA	：2-アミノアントラセン	(和光純薬工業㈱)

AF2, 2AAはDMSO(和光純薬工業㈱)に溶解したものを-20℃で凍結保存し，用時解凍した．9AAはDMSOに，SAは純水に溶解し，速やかに試験に用いた．

〔培地およびS9 mixの組成〕

1) トップアガー

下記の水溶液(A)および(B)を容量比10:1の割合で混

合した.

(A)	バクトアガー(Difco)	0.6%
	塩化ナトリウム	0.5%
(B)*	L-ヒスチジン	0.5 mM
	D-ビオチン	0.5 mM

*:WP2 *uvrA* 用には，0.5 mM L-トリプトファン水溶液を用いた.

2) 合成培地

培地は，日清製粉㈱製の最少寒天培地を用いた．なお，培地1 *l*あたりの組成は下記のとおりである.

硫酸マグネシウム・7水和物	0.2 g
クエン酸・1水和物	2 g
リン酸水素二カリウム	10 g
リン酸一アンモニウム	1.92 g
水酸化ナトリウム	0.66 g
グルコース	20 g
バクトアガー(Difco)	15 g

径90 mmのシャーレ1枚あたり30 m*l*を流して固めてある.

3) S9 mix

1 m*l*中下記の成分を含む

S9**	0.1 m*l*
塩化マグネシウム	8 μmol
塩化カリウム	33 μmol
グルコース-6-リン酸	5 μmol
NADH	4 μmol
NADPH	4 μmol
ナトリウム-リン酸緩衝液(pH 7.4)	100 μmol

**:7週齢のSprague-Dawley系雄ラットをフェノバルビタール(PB)および5,6-ベンゾフラボン(BF)の併用投与で酵素誘導して作製したS9を用いた.

〔試験方法〕

プレート法により，S9 mix 無添加試験およびS9 mix添加試験を行った.

小試験管中に，被験物質調製液0.1 m*l*，リン酸緩衝液0.5 m*l*(S9 mix添加試験においてはS9 mix 0.5 m*l*)，検定菌液 0.1 m*l*およびトップアガー2 m*l*を混合したのち合成培地平板上に流して固めた．また，対照群として被験物質調製液の代わりにDMSO，または数種の陽性対照物質溶液を用いた．各検定菌ごとの陽性対照物質の名称および用量は各Table中に示した．培養は37℃で48時間行い，生じた変異コロニー数を算定した．抗菌性の有無については，肉眼的あるいは実体顕微鏡下で，寒天表面の菌膜の状態から判断した.

用いた平板は用量設定試験においては，溶媒および陽性対照群では3枚ずつ，各用量については1枚ずつとした．また，本試験においては両対照群および各用量につき，3枚ずつを用い，それぞれの平均値と標準偏差を求めた．用量設定試験は1回，本試験は同一用量について2回実施し，結果の再現性の確認を行った

〔判定基準〕

用いた5種の検定菌のうち，1種以上の検定菌のS9 mix無添加あるいはS9 mix添加条件において，被験物質を含有する平板上における変異コロニー数の平均値が，溶媒対照のそれに比べて2倍以上に増加し，かつ，その増加に再現性あるいは用量依存性が認められた場合に，当該被験物質は本試験系において変異原性を有する(陽性)と判定することとした.

結果および考察

〔用量設定試験〕

4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)について50～5000 μg/プレートの範囲で公比を約3として，試験を実施したところ，S9 mix無添加試験では，TA100，TA1535およびTA1537はすべての用量で，TA98は150 μg/プレート以上の用量で抗菌性が認められたが，WP2 *uvrA*では抗菌性は認められなかった．また，S9 mix添加試験では*S. typhimurium*の4菌株はすべて500 μg/プレート以上の用量で抗菌性が認められたが，WP2 *uvrA*では抗菌性は認められなかった.

〔本試験〕

結果をそれぞれTable 1, 2に示した．4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)の用量を，S9 mix無添加試験ではTA100, TA1535およびTA1537は0.781～50 μg/プレート，TA98は3.13～200 μg/プレート，WP2 *uvrA*は313～5000 μg/プレートの範囲で，S9 mix添加試験では*S. typhimurium*の4菌株は12.5～800 μg/プレート，WP2 *uvrA*は313～5000 μg/プレートの範囲で公比を2として試験を実施した．その結果，2回の試験のいずれも，用いた5種類の検定菌のS9 mix無添加試験および添加試験において，溶媒対照値の2倍以上となる変異コロニー数の増加は認められなかった.

以上の結果に基づき，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)は，用いた試験系において変異原性を有しないもの(陰性)と判定した.

Table 1-1. Mutagenicity of 4,4'-thiobis(6-*tert*-butyl-*m*-cresol)** in reverse mutation test (I) on bacteria

With(+) or without(−) S9 mix	Test substance dose (μg/plate)	Number of revertants (number of colonies / plate, Mean ± S.D.)				
		Base - pair substitution type			Frameshift type	
		TA100	TA1535		TA98	TA1537
S9mix (−)	0	109 134 119 (121±12.6)	20 22 11 (18± 5.9)		21 21 30 (24± 5.2)	11 6 13 (10± 3.6)
	0.781	104 107 119 (110± 7.9)	16 12 17 (15± 2.6)		ND	7 12 12 (10± 2.9)
	1.56	99 115 109 (108± 8.1)	16 11 14 (14± 2.5)		ND	8 8 5 (7± 1.7)
	3.13	88 111 99 (99±11.5)	14 10 9 (11± 2.6)		22 25 23 (23± 1.5)	7 15 12 (11± 4.0)
	6.25	95 85 101 (94± 8.1)	9 12 14 (12± 2.5)		18 14 25 (19± 5.6)	10 7 6 (8± 2.1)
	12.5	95 97 94 (95± 1.5)	9 9 8 (9± 0.6)		22 22 19 (21± 1.7)	5* 5* 5* (5± 0.0)
	25	88* 84* 89* (87± 2.6)	11* 5* 13* (10± 4.2)		11 25 19 (18± 7.0)	7* 8* 2* (6± 3.2)
	50 #	69* 66* 53* (63± 8.5)	7* 14* 5* (9± 4.7)		13 11 14 (13± 1.5)	3* 1* 5* (3± 2.0)
	100 #				5* 5* 9* (6± 2.3)	
	200 #				0* 2* 8* (3± 4.2)	
S9mix (+)	0	156 139 134 (143±11.5)	13 14 24 (17± 6.1)		32 32 31 (32± 0.6)	12 15 12 (13± 1.7)
	12.5	146 145 161 (151± 9.0)	19 12 18 (16± 3.8)		40 39 40 (40± 0.6)	9 15 13 (12± 3.1)
	25	133 141 140 (138± 4.4)	18 17 19 (18± 1.0)		30 41 37 (36± 5.6)	14 13 6 (11± 4.4)
	50	137 156 128 (140±14.3)	12 8 22 (14± 7.2)		38 32 36 (35± 3.1)	17 6 14 (12± 5.7)
	100	134 125 120 (126± 7.1)	11 16 15 (14± 2.6)		42 32 33 (36± 5.5)	23 14 19 (19± 4.5)
	200	98 99 94 (97± 2.6)	9 20 9 (13± 6.4)		25 17 17 (20± 4.6)	13 19 13 (15± 3.5)
	400 #	60 83 74 (72±11.6)	10* 10* 9* (10± 0.6)		2 5 2 (3± 1.7)	11 9 6 (9± 2.5)
	800 #	46* 43* 36* (42± 5.1)	5* 9* 8* (7± 2.1)		2* 2* 3* (2± 0.6)	1* 1* 2* (1± 0.6)
Positive control S9 mix(−)	Chemical	AF2	SA		AF2	9AA
	Dose(μg/plate)	0.01	0.5		0.1	80
	Number of colonies/plate	868 803 830 (834±32.7)	170 165 175 (170± 5.0)		894 873 913 (893±20.0)	1310 1187 1149 (1215±84.2)
Positive control S9 mix (+)	Chemical	2AA	2AA		2AA	2AA
	Dose(μg/plate)	1	2		0.5	2
	Number of colonies/plate	1352 1209 1238 (1266±75.6)	270 316 300 (295±23.4)		420 373 374 (389±26.9)	243 212 142 (199±51.7)

AF2:2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, SA:Sodium azide, 9AA:9-Aminoacridine, 2AA:2-Aminoanthracene

*:Inhibition was observed against growth of the bacteria. #: Precipitate was observed on the surface of agar plates.

**:Purity was above 98% and impurity was unknown.

ND:Not done

Table 1-2. Mutagenicity of 4,4'-thiobis(6-*tert*-butyl-*m*-cresol)** in reverse mutation test (I) on bacteria

With (+) or without (-) S9 mix	Test substance dose (μg/plate)	Number of revertants (number of colonies / plate, Mean ± S.D.)					
		Base-pair substitution type					
				WP2 *uvrA*			
S9mix (-)	0			20 37 22 (26± 9.3)			
	313 #			14 13 12 (13± 1.0)			
	625 #			16 29 18 (21± 7.0)			
	1250 #			16 22 29 (22± 6.5)			
	2500 #			25 24 19 (23± 3.2)			
	5000 #			22 22 23 (22± 0.6)			
S9mix (+)	0			29 22 24 (25± 3.6)			
	313 #			29 21 18 (23± 5.7)			
	625 #			20 16 16 (17± 2.3)			
	1250 #			24 15 18 (19± 4.6)			
	2500 #			12 11 11 (11± 0.6)			
	5000 #			11 11 9 (10± 1.2)			
Positive control S9 mix (-)	Chemical			AF2			
	Dose (μg/plate)			0.01			
	Number of colonies/plate			146 162 170 (159± 12.2)			
Positive control S9 mix (+)	Chemical			2AA			
	Dose (μg/plate)			10			
	Number of colonies/plate			1214 1213 1269 (1232±32.0)			

AF2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, 2AA: 2-Aminoanthracene
#: Precipitate was observed on the surface of agar plates.
**: Purity was above 98% and impurity was unknown.

Table 2-1. Mutagenicity of 4,4'-thiobis(6-*tert*-butyl-*m*-cresol)** in reverse mutation test (I) on bacteria

With (+) or without (−) S9 mix	Test substance dose (μg/plate)	Number of revertants (number of colonies / plate, Mean ± S.D.)			
		Base - pair substitution type		Frameshift type	
		TA100	TA1535	TA98	TA1537
S9mix (−)	0	120 130 141 (130±10.5)	11 12 18 (14± 3.8)	29 18 26 (24± 5.7)	7 12 5 (8± 3.6)
	0.781	124 135 149 (136±12.5)	12 10 14 (12± 2.0)	ND	8 12 7 (9± 2.6)
	1.56	151 112 105 (123±24.8)	20 14 12 (15± 4.2)	ND	6 9 11 (9± 2.5)
	3.13	129 104 132 (122±15.4)	8 17 19 (15± 5.9)	23 25 29 (26± 3.1)	12 7 5 (8± 3.6)
	6.25	114 91 112 (106±12.7)	15 10 13 (13± 2.5)	12 24 24 (20± 6.9)	5 5 6 (5± 0.6)
	12.5	107* 106* 84* (99±13.0)	15 15 6 (12± 5.2)	25 19 21 (22± 3.1)	10* 8* 12* (10± 2.0)
	25	64* 84* 106* (85±21.0)	6* 11* 14* (10± 4.0)	17 23 20 (20± 3.0)	5* 6* 6* (6± 0.6)
	50 #	53* 63* 48* (55± 7.6)	9* 6* 9* (8± 1.7)	8 14 17 (13± 4.6)	0* 0* 2* (1± 1.2)
	100 #			9* 5* 6* (7± 2.1)	
	200 #			10* 4* 6* (7± 3.1)	
S9mix (+)	0	129 137 129 (132± 4.6)	15 16 20 (17± 2.6)	30 36 36 (34± 3.5)	14 13 14 (14± 0.6)
	12.5	172 182 166 (173± 8.1)	20 20 30 (23± 5.8)	33 46 35 (38± 7.0)	8 13 9 (10± 2.6)
	25	160 162 174 (165± 7.6)	33 26 23 (27± 5.1)	45 38 39 (41± 3.8)	12 22 8 (14± 7.2)
	50	166 168 146 (160±12.2)	24 15 20 (20± 4.5)	42 41 39 (41± 1.5)	11 10 9 (10± 1.0)
	100	120 146 118 (128±15.6)	17 26 12 (18± 7.1)	29 30 38 (32± 4.9)	14 15 18 (16± 2.1)
	200 #	117 114 122 (118± 4.0)	11 16 11 (13± 2.9)	19 14 17 (17± 2.5)	8 12 4 (8± 4.0)
	400 #	82* 95* 80* (86± 8.1)	15 9 10 (11± 3.2)	9 5 7 (7± 2.0)	2* 9* 14* (8± 6.0)
	800 #	45* 47* 46* (46± 1.0)	9* 8* 5* (7± 2.1)	3* 3* 4* (3± 0.6)	0* 0* 0* (0± 0.0)
Positive control S9 mix (−)	Chemical	AF2	SA	AF2	9AA
	Dose (μg/plate)	0.01	0.5	0.1	80
	Number of colonies/plate	794 787 840 (807±28.8)	172 181 159 (171±11.1)	932 901 970 (934±34.6)	942 913 905 (920±19.5)
Positive control S9 mix (+)	Chemical	2AA	2AA	2AA	2AA
	Dose (μg/plate)	1	2	0.5	2
	Number of colonies/plate	1418 1526 1543 (1496±67.8)	299 297 298 (298± 1.0)	321 299 319 (313±12.2)	256 270 235 (254±17.6)

AF2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, SA: Sodium azide, 9AA: 9-Aminoacridine, 2AA: 2-Aminoanthracene

*: Inhibition was observed against growth of the bacteria. #: Precipitate was observed on the surface of agar plates.

**: Purity was above 98% and impurity was unknown.

ND: Not done.

Table 2-2. Mutagenicity of 4,4'-thiobis(6-*tert*-butyl-*m*-cresol)** in reverse mutation test(I)on bacteria

With(+)or without(-) S9 mix	Test substance dose (μg/plate)	Number of revertants (number of colonies/plate, Mean ± S.D.)					
		Base-pair substitution type					
				WP2 *uvrA*			
S9mix (-)	0			27 24 18 (23± 4.6)			
	313 #			16 25 17 (19± 4.9)			
	625 #			18 22 22 (21± 2.3)			
	1250 #			23 24 17 (21± 3.8)			
	2500 #			15 22 14 (17± 4.4)			
	5000 #			8 5 10 (8± 2.5)			
S9mix (+)	0			32 26 39 (32± 6.5)			
	313 #			27 33 19 (26± 7.0)			
	625 #			26 17 17 (20± 5.2)			
	1250 #			22 21 20 (21± 1.0)			
	2500 #			13 13 12 (13± 0.6)			
	5000 #			12 12 15 (13± 1.7)			
Positive control S9 mix(-)	Chemical			AF2			
	Dose(μg/plate)			0.01			
	Number of colonies/plate			217 195 204 (205±11.1)			
Positive control S9 mix(+)	Chemical			2AA			
	Dose(μg/plate)			10			
	Number of colonies/plate			1569 1540 1512 (1540±28.5)			

AF2:2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, 2AA:2-Aminoanthracene
#:Precipitate was observed on the surface of agar plates.
**:Purity was above 98% and impurity was unknown.

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4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)の チャイニーズ・ハムスター培養細胞を用いる染色体異常試験

In Vitro Chromosomal Aberration Test of 4,4'-Thiobis(6-*tert*-butyl-*m*-cresol) on Cultured Chinese Hamster Cells

要約

既存化学物質安全性点検に係る毒性調査事業の一環として，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)の培養細胞に及ぼす細胞遺伝学的影響を評価するため，チャイニーズ・ハムスター培養細胞(CHL/IU)を用いて試験管内染色体異常試験を実施した．

連続処理(48時間)においては，50%を明らかに越える増殖抑制濃度，すなわち0.002 mg/mlの濃度を最高処理濃度とした．また，短時間処理の(6時間)S9 mix存在下および非存在下においてはそれぞれ50%を越える増殖抑制濃度，すなわち0.02 mg/mlおよび0.0009 mg/mlの濃度を最高処理濃度とした．最高処理濃度の1/2および1/4をそれぞれ中濃度，低濃度として設定した．連続処理では，S9 mix非存在下における24時間および48時間連続処理後，短時間処理ではS9 mix存在下および非存在下で6時間処理(18時間の回復時間)後，標本を作製し，検鏡することにより染色体異常誘発性を検討した．

CHL/IU細胞を24時間連続処理したいずれの処理群においても，染色体の構造異常や倍数性細胞の誘発作用は認められなかった．48時間連続処理したいずれの濃度群でも，染色体の構造異常は認められなかった．また，高濃度群(0.002 mg/ml)細胞毒性では，十分な細胞数が分析できなかったが，その他の濃度群においては倍数性細胞の誘発作用は認められなかった．短時間処理では，S9 mix存在下および非存在下で6時間処理したいずれの処理群においても，染色体の構造異常や倍数性細胞の誘発作用は認められなかった．

以上の結果より，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)は，上記の試験条件下で染色体異常を誘発しないと結論した．

方法

1. 使用した細胞

リサーチ・リソースバンク(JCRB)から入手(1988年2月，入手時：継代4代，現在12代)したチャイニーズ・ハムスター由来のCHL/IU細胞を，解凍後継代10代以内で試験に用いた．

2. 培養液の調製

培養には，牛胎児血清(FCS：Biocell)を10%添加したイーグルMEM(日水製薬㈱)培養液を用いた．

3. 培養条件

2×10^4個のCHL/IU細胞を，培養液5 mlを入れたディッシュ(径6 cm，Corning)に播き，37℃のCO_2インキュベーター(5% CO_2)内で培養した．連続処理では，細胞播種3日目に被験物質を加え，24時間および48時間処理した．また，短時間処理では，細胞播種3日目にS9 mix存在下および非存在下で6時間処理し，処理終了後新鮮な培養液でさらに18時間培養した．

4. 被験物質

4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)(略号：TBBC，CAS No.:96-69-5，ロット番号：40701，住友化学工業㈱製造，㈳日本化学工業協会提供)は，白色結晶性粉末で，水に対しては不溶，アセトンおよびメタノールには可溶であり，融点160～165℃，分子式$C_{22}H_{30}O_2S$，分子量358.54，純度98%(不純物は不明)の物質である．

被験物質原体の安定性に関する情報は得られなかったが，溶媒中(DMSO)では，7.81 μg/ml～50.0 mg/mlの濃度範囲で4時間安定であった．

5. 被験物質の調製

被験物質の調製は，使用のつど行った．溶媒はDMSO(和光純薬工業㈱)を用いた．原体を溶媒に溶解して原液を調製し，ついで原液を溶媒で順次希釈して所定の濃度の被験物質調製液を作製した．被験物質調製液は，すべての試験において培養液1%(v/v)になるように加えた．染色体異常試験に用いた被験物質調製液の濃度は，許容範囲内(溶媒中での平均含量が添加量の90.0～110%)の値であった．なお濃度の記載について，純度換算は行わなかった．

6. 細胞増殖抑制試験による処理濃度の決定

染色体異常試験に用いる被験物質の処理濃度を決定するため，被験物質の細胞増殖に及ぼす影響を調べた．被験物質のCHL/IU細胞に対する増殖抑制作用は，単層培養細胞密度計(Monocellater™，オリンパス光学工業㈱)を用いて各群の増殖度を計測し，被験物質処理群の溶媒対照群に対する細胞増殖の比をもって指標とした．

その結果，連続処理における50%の増殖抑制濃度を明らかに超える濃度(約60%の増殖抑制濃度)を，60%増殖抑制濃度をはさむ2濃度より算出したところ，0.002 mg/mlであった(Fig. 1)．一方，短時間処理のS9 mix存在下および非存在下では，それぞれ0.02 mg/ml(Fig. 2)および0.0009 mg/mlであった(Fig. 1)．

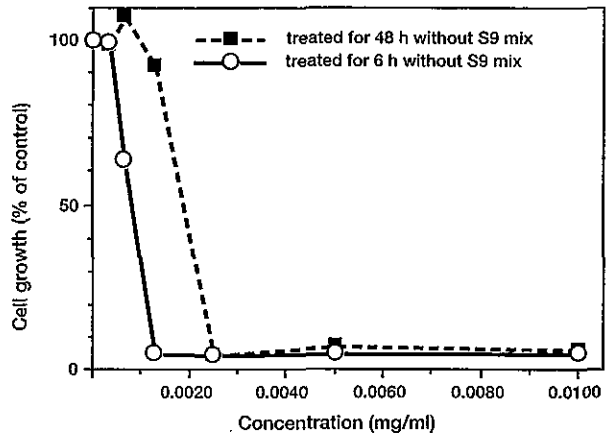

Fig. 1 Growth inhibition of CHL/IU cells treated with 4,4'-thiobis(6-*tert*-butyl-*m*-cresol) without S9 mix

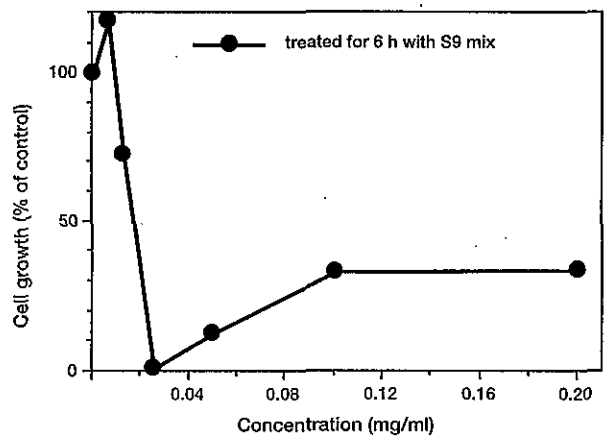

Fig. 2 Growth inhibition of CHL/IU cells treated with 4,4'-thiobis(6-*tert*-butyl-*m*-cresol) with S9 mix

7. 実験群の設定

細胞増殖抑制試験の結果より，染色体異常試験で用いる被験物質の高濃度群を，連続処理では0.002 mg/ml，短時間処理S9 mix 存在下および非存在下では，それぞれ0.02 mg/mlおよび0.0009 mg/mlとし，それぞれ高濃度群の1/2の濃度を中濃度，1/4の濃度を低濃度とした．陽性対照物質として用いたマイトマイシンC(MC，協和醗酵工業㈱)およびシクロホスファミド(CPA，Sigma Chemical Co.)は，注射用水(㈱大塚製薬工場)に溶解して調製した．それぞれ染色体異常を誘発することが知られている濃度を適用した．

8. 染色体標本作製法

培養終了の2時間前に，コルセミドを最終濃度が約0.1 μg/mlになるように培養液に加えた．染色体標本の作製は常法に従って行った．スライド標本は各ディッシュにつき6枚作製した．作製した標本を3%ギムザ溶液で染色した．

9. 染色体分析

作製したスライド標本のうち，1つのディッシュから得られた異なるスライドを，4名の観察者がそれぞれ処理条件が分からないようにコード化した状態で分析した．染色体の分析は，日本環境変異原学会，哺乳動物試験(MMS)分科会[1]による分類法に基づいて行い，染色体型あるいは染色分体型のギャップ，切断，交換などの構造異常の有無と倍数性細胞(polyploid)の有無について観察した．また構造異常については1群200個，倍数性細胞については1群800個の分裂中期細胞を分析した．

10. 記録と判定

無処理対照，溶媒および陽性対照群と被験物質処理群についての分析結果は，観察した細胞数，構造異常の種類と数，倍数性細胞の数について集計し，各群の値を記録用紙に記入した．

染色体異常を有する細胞の出現頻度について，林[2]の方法を参考にして，溶媒の背景データと被験物質処理群間でフィッシャーの直接確率法[3](多重性を考慮してfamilywiseの有意水準を5%とした)により，有意差検定を実施した．また，フィッシャーの直接確率法で有意差が認められた場合には，用量依存性に関してコクラン・アーミテッジの傾向性検定[4](p<0.05)を行った．原則として以上2回の検定でともに有意差が認められた場合を陽性とした．傾向性検定で有意差が認められない場合には疑陽性とした．観察細胞数が，構造異常については100個未満，倍数性細胞については400個未満の場合を細胞毒性のため判定不能とした．

結果および考察

連続処理による染色体分析の結果をTable 1に示した．4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)を加えて24時間処理したいずれの処理群においても，染色体の構造異常および倍数性細胞の誘発は認められなかった．また，48時間連続処理したいずれの処理群においても染色体の構造異常は誘発されなかった．倍数性細胞の誘発作用に関して，高濃度群(0.002 mg/ml)では，細胞毒性のため倍数性細胞の誘発に関しては十分な細胞数が分析できなかったが，その他の処理群では倍数性細胞は誘発されなかった．

短時間処理による染色体分析の結果をTable 2に示した．4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)を加えてS9 mix存在下および非存在下で6時間処理したいずれの処理群においても，染色体の構造異常および倍数性細胞の誘発作用は認められなかった．

従って，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)は，上記の試験条件下で，試験管内のCHL/IU細胞に染色体異常を誘発しないと結論した．

Table 1 Chromosome analysis of Chinese hamster cells (CHL/IU) continuously treated with 4,4'-thiobis (6-*tert*-butyl-*m*-cresol) (TBBC)* without S9 mix

Group	Concentration (mg/ml)	Time of exposure (h)	No. of cells analysed	No. of structural aberrations							Others[3]	No. of cells with aberrations				Polyploid[4] (%)	Trend test[5]	
				gap	ctb	cte	csb	cse	mul[2]	total		TAG	(%)	TA	(%)		SA	NA
Control			200	0	1	0	6	0	0	7	0	2	(1.0)	2	(1.0)	0.13		
Solvent[1]	0	24	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
TBBC	0.00050	24	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.13		
TBBC	0.0010	24	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.38	NT	NT
TBBC	0.0020	24	200	0	0	1	0	0	0	1	0	1	(0.5)	1	(0.5)	0.00		
MC	0.00005	24	200	0	16	58	0	0	0	74	1	62	(31.0)	62	(31.0)	0.13		
Solvent[1]	0	48	200	0	0	0	0	1	0	1	2	1	(0.5)	1	(0.5)	0.50		
TBBC	0.00050	48	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.13		
TBBC	0.0010	48	200	1	1	0	3	0	0	5	0	3	(1.5)	2	(1.0)	0.13	NT	NT
TBBC	0.0020	48	140	0	0	1	2	1	0	4	0	2	(1.4)	2	(1.4)	0.42[6]T		
MC	0.00005	48	200	3	31	67	3	6	20	130	5	70	(35.0)	68	(34.0)	0.13		

Abbreviations: gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring etc.), mul: multiple aberrations, TAG: total no. of cells with aberrations, TA: total no. of cells with aberrations except gap, SA: structural aberration, NA: numerical aberration, MC: mitomycin C, NT: not tested, T: Toxic; this group was excluded from judgement in case of less than one hundred cells for structural aberration analysed or less than four hundred cells for polyploid cells analysed. 1) Dimethyl sulfoxide was used as solvent. 2) More than ten aberrations in a cell were scored as 10. 3) Others, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations. 4) Eight hundred cells were analysed in each group. 5) Cochran - Armitage's trend test was done at $p<0.05$ when the incidence of TAG and polyploid in the treatment groups was significantly defferent from historical solvent control at $p<0.05$ by Fisher's exact test. 6) Two hundred and thirty-eight cells were analysed. *: Purity was more than 98%.

Table 2 Chromosome analysis of Chinese hamster cells (CHL/IU) treated with 4,4'-thiobis (6-*tert*-butyl-*m*-cresol) (TBBC)* with and without S9 mix

Group	Concentration (mg/ml)	S9 mix	Time of exposure (h)	No. of cells analysed	No. of structural aberrations							Others[3]	No. of cells with aberrations				Polyploid[4] (%)	Trend test[5]	
					gap	ctb	cte	csb	cse	mul[2]	total		TAG	(%)	TA	(%)		SA	NA
Control				200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.13		
Solvent[1]	0	-	6-(18)	200	0	1	0	0	0	0	1	0	1	(0.5)	1	(0.5)	0.13		
TBBC	0.00023	-	6-(18)	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.25		
TBBC	0.00045	-	6-(18)	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.25	NT	NT
TBBC	0.00090	-	6-(18)	200	0	1	0	0	0	0	1	0	1	(0.5)	1	(0.5)	0.38		
CPA	0.005	-	6-(18)	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.00		
Solvent[1]	0	+	6-(18)	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
TBBC	0.0050	+	6-(18)	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.25		
TBBC	0.010	+	6-(18)	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.13	NT	NT
TBBC	0.020	+	6-(18)	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
CPA	0.005	+	6-(18)	200	1	26	112	0	1	40	180	0	90	(45.0)	89	(44.5)	0.00		

Abbreviations: gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring etc.), mul: multiple aberrations, TAG: total no. of cells with aberrations, TA: total no. of cells with aberrations except gap, SA: structural aberration, NA: numerical aberration, CPA: cyclophosphamide, NT: not tested. 1) Dimenthyl sulfoxide was used as solvent. 2) More than ten aberrations in a cell were scored as 10. 3) OThers, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations. 4) Eight hundred cells were analysed in each group. 5) Cochran - Armitage's trend test was done at $p<0.05$ when the incidence of TAG and polyploid in the treatment groups was significantly different from historical solvent control at $p<0.05$ by Fisher's exact test. *: Purity was more than 98%.

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STUDIES OF 4,4′-THIOBIS(6-*t*-BUTYL-*m*-CRESOL)

(CAS NO. 96-69-5)

IN F344/N RATS AND B6C3F$_1$ MICE

(FEED STUDIES)

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this Technical Report were performed under the direction of the NIEHS and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The prechronic and chronic studies were conducted in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations, and all aspects of the chronic studies were subjected to retrospective quality assurance audits before being presented for public review.

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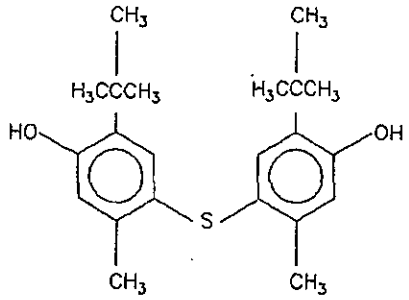
ABSTRACT



4,4′-THIOBIS(6-*t*-BUTYL-*m*-CRESOL)

CAS No. 96-69-5

Chemical Formula: $C_{22}H_{30}SO_2$ Molecular Weight: 358.52

Synonyms: 4,4′-Thiobis(6-*t*-butyl-3-cresol); bis(3-*t*-butyl-4-hydroxy-6-methylphenyl)sulfide
Trade names: Santonox; Santowhite Crystals; Sumilizer; Thioalkofen; Yoshinox

4,4′-Thiobis(6-*t*-butyl-*m*-cresol) (TBBC) is used in the rubber and plastics industries as an antioxidant. TBBC is also used as a stabilizer in polyethylene and polyolefin packaging materials for foodstuffs. Toxicology and carcinogenesis studies were conducted by administering TBBC (99% pure) in feed to groups of male and female F344/N rats and B6C3F$_1$ mice for 15 days, 13 weeks, and 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium* and cultured Chinese hamster ovary cells.

15-DAY STUDY IN RATS

Groups of 10 male and 10 female F344/N rats were fed diets containing 0, 1,000, 2,500, 5,000, 10,000 or 25,000 ppm TBBC for 15 days. Rats given to 1,000, 2,500, 5,000, or 10,000 ppm received approximate doses of 95, 235, 335, or 365 mg TBBC per kilogram body weight per day (males) or 85, 220, 325, or 270 mg/kg per day (females). Approximate doses for rats receiving 25,000 ppm could not be calculated due to early deaths. All 25,000 ppm rats and three male and four female 10,000 ppm rats died. Surviving rats in the 10,000 ppm groups had a significant weight loss and the final mean body weights of 5,000 and 10,000 ppm male and female rats were significantly lower than those of the controls. Male and female rats exposed to 5,000, 10,000, or 25,000 ppm TBBC consumed markedly less feed than the controls.

Diarrhea occurred in 5,000, 10,000, and 25,000 ppm males and females. The principal lesions attributed to the administration of TBBC were renal papillary and tubule necroses which occurred in 10,000 ppm rats. Focal necrosis or erosions of the glandular stomach also occurred in some 10,000 ppm rats. Changes observed in the thymus and spleen were attributed to debilitation or stress; bone marrow depletion was attributed to nutrient deficiency accompanying weight loss.

15-DAY STUDY IN MICE

Groups of 10 male and 10 female B6C3F$_1$ mice were fed diets containing 0, 1,000, 2,500, 5,000, 10,000, or 25,000 ppm TBBC for 15 days. Mice given 1,000, 2,500, or 5,000 ppm received approximate doses of 285, 585, or 475 mg TBBC per kilogram body weight per day (males) or 360, 950, or 1,030 mg/kg per day (females). Approximate doses for mice given 10,000 or 25,000 ppm could not be calculated due to early deaths. All 10,000 and 25,000 ppm mice died, as did eight males and eight females given 5,000 ppm. A

significant weight loss occurred in surviving 5,000 ppm males and females and the final mean body weights of 2,500 ppm females and 5,000 ppm males and females were significantly lower than those of the controls. Feed consumption by mice given 5,000, 10,000, or 25,000 ppm was markedly reduced. Diarrhea occurred in all 25,000 ppm mice and in most male and female mice given 5,000 or 10,000 ppm. Renal tubule necrosis occurred in eight males and three females in the 5,000 ppm groups. Lymphocytic depletion of lymphoid tissues in many 5,000 ppm males and females was attributed to debilitation and stress or to nutrient deficiency accompanying weight loss.

13-Week Study in Rats

Groups of 10 male and 10 female F344/N rats were fed diets containing 0, 250, 500, 1,000, 2,500, or 5,000 ppm TBBC for 13 weeks. These exposure levels delivered approximate doses of 15, 30, 60, 165, or 315 mg TBBC per kilogram body weight per day (males) or 15, 35, 70, 170, or 325 mg/kg per day (females). All rats survived to the end of the study. The final mean body weight of 5,000 ppm males was 40% lower than that of the controls; the final mean body weight of 5,000 ppm females was 27% lower than that of the controls. Feed consumption by male and female rats exposed to 5,000 ppm TBBC was markedly lower than that by the controls throughout the study. The absolute and relative liver weights of 5,000 ppm females were significantly greater than those of the controls.

Serum alkaline phosphatase (ALP) levels were significantly higher in 2,500 and 5,000 ppm males and slightly higher in 5,000 ppm females. Serum alanine aminotransferase levels were significantly higher in 2,500 and 5,000 ppm males and females. Hematocrit and hemoglobin concentrations and mean erythrocyte volume (MCV) values were significantly lower in 1,000, 2,500, and 5,000 ppm males than in controls; MCV values were also significantly lower in 5,000 ppm females. A dose-related significant increase in forelimb and hindlimb grip strength was observed in exposed male and female rats.

Histopathologic findings in the liver of 2,500 and 5,000 ppm males and females included hypertrophy of Kupffer cells, bile duct hyperplasia, and individual cell necrosis of hepatocytes; centrilobular hepatocyte hypertrophy also occurred in males and females exposed to 5,000 ppm TBBC. Macrophages were increased in size and number in the mesenteric lymph nodes of males and females exposed to 5,000 ppm, and to a lesser extent in 2,500 ppm male and female rats. Pigmentation and degeneration of the renal cortical tubule epithelial cells was also present in males and females in the 2,500 and 5,000 ppm groups; cortical tubule necrosis occurred in 5,000 ppm males and females.

13-Week Study in Mice

Groups of up to 10 male and 10 female B6C3F$_1$ mice were fed diets containing 0, 100, 250, 500, 1,000, or 2,500 ppm TBBC for 13 weeks. These exposure levels delivered approximate doses of 15, 30, 65, 145, or 345 mg TBBC per kilogram body weight per day (males) or 10, 35, 60, 165, or 340 mg/kg per day (females). All mice survived to the end of the study. The final mean body weights of 2,500 ppm males and of 500, 1,000, or 2,500 ppm females were significantly lower than those of the controls. Feed consumption by 2,500 ppm males averaged 24% lower than that by controls through week 3 and was similar to that by controls for the remainder of the study. Feed consumption by females receiving 2,500 ppm averaged 27% less than that by the controls during most of the study. The absolute and relative liver weights of males and females exposed to 2,500 ppm TBBC were slightly but significantly greater than those of the controls. Males exposed to 500, 1,000, or 2,500 ppm and females exposed to 2,500 ppm had significantly increased absolute and relative spleen weights. No clinical findings in mice were considered chemical related.

Hematocrit concentrations and erythrocyte counts of males receiving 1,000 or 2,500 ppm were significantly less than those of the controls; hemoglobin concentration in males receiving 2,500 ppm was significantly less and mean erythrocyte volume was significantly less in males receiving 2,500 ppm. Females in the 1,000 and 2,500 ppm groups had significantly decreased hematocrit concentrations and erythrocyte counts; 2,500 ppm females also had significantly decreased hemoglobin concentrations and mean erythrocyte volumes.

Kupffer cell hypertrophy, bile duct hyperplasia, and an increase in size and number of macrophages in mesenteric lymph nodes were present in 2,500 ppm male and female mice.

2-YEAR STUDY IN RATS

Doses selected for the 2-year study of TBBC were based on the lower body weights and liver and kidney toxicity observed at 5,000 ppm in the 13-week study.

Groups of 115 male and 75 female F344/N rats were fed diets containing 0, 500, 1,000, or 2,500 ppm TBBC for 2 years. Based on average daily feed consumption, these exposure levels resulted in a daily ingestion of TBBC of approximately 20, 40, or 100 mg/kg body weight for males and 20, 45, or 120 mg/kg body weight for females. Hematology, clinical chemistry, and urinalysis evaluations were performed on 15 male and 15 female rats from each group at 3, 9, and 15 months. Also at 15 months, an additional 10 male and 10 female rats from each group were evaluated for histopathology, hematology, and clinical chemistry. Forty male rats per group were evaluated for neurotoxic effects.

Survival, Body Weights, Feed Consumption, and Clinical Findings

Two-year survival rates and mean body weights of exposed male and female rats were generally similar to those of the controls. The mean body weights of 2,500 ppm male rats were slightly lower than those of the controls throughout the study. At week 65, the mean body weight of 2,500 ppm females was 14% lower than that of the controls, but the final mean body weight of this group was 6% lower than that of the control group. Feed consumption, behavior, and general health and appearance of exposed male and female rats were similar to those of the controls.

Hematology and Clinical Chemistry

Results of the hematology evaluation were not uniformly consistent at 3, 9, and 15 months in one set of rats, nor were they consistent between the two sets of rats evaluated at 15 months. Slight but significant decreases in hematocrit levels, hemoglobin concentrations, and erythrocyte counts were observed in the 1,000 and 2,500 ppm groups in one set of males at 15 months. Similar significant decreases in hematocrit level and hemoglobin concentration occurred in 2,500 ppm females at 9 months. Mean erythrocyte hemoglobin and mean erythrocyte hemoglobin concentration of 2,500 ppm females were also significantly lower than those of controls at 9 months and in both sets of female rats evaluated at 15 months. Platelet counts of 2,500 ppm male and female rats were slightly but significantly higher than those of controls at 3 and 9 months. Platelet counts were also slightly but significantly increased in 2,500 ppm males of one set evaluated at 15 months, and in 2,500 ppm females of the second set evaluated at 15 months.

Serum activities of alkaline phosphatase, alanine aminotransferase, and sorbitol dehydrogenase in 2,500 ppm males were significantly greater than those in the controls at 3, 9, and 15 months. Alkaline phosphatase activities in both sets of 1,000 ppm males evaluated at 15 months were also significantly greater than those of controls. Serum activities of alanine aminotransferase and sorbitol dehydrogenase in 2,500 ppm females were also significantly greater than those in controls at 3, 9, and 15 months.

Neurotoxicity Findings

There were no significant inhibitory effects of TBBC on motor nerve excitability or conduction, neuromuscular transmission, or muscle contractility. There were no microscopic lesions in the sciatic nerve, quadriceps muscle, or teased nerve preparations of sciatic nerve that could be attributed to TBBC administration.

Pathology Findings

At the 15-month interim evaluation, the absolute and relative liver weights of 2,500 ppm female rats were significantly greater than those of controls; at 15 months and at the end of the study, the incidences of Kupffer cell hypertrophy, hepatocyte cytoplasmic vacuolization, and mixed cell foci were also significantly increased. At the end of the study, the incidence of hepatocellular fatty change was significantly increased in 2,500 ppm females. The incidence of Kupffer cell hypertrophy was significantly increased in 2,500 ppm males at 15 months and at 2 years; the incidence of cytoplasmic vacuolization was significantly increased in all exposed males at 15 months but only moderately increased in 1,000 and 2,500 ppm males at 2 years; the incidence of basophilic foci was significantly increased in 2,500 ppm males at 15 months and the incidence of mixed cell foci was significantly increased in 1,000 and 2,500 ppm male rats at 2 years. The incidences of hepatocellular adenoma or carcinoma (combined)

in exposed male rats were not significantly greater than that in the controls (0 ppm, 1/50; 500 ppm, 3/50; 1,000 ppm, 3/50; 2,500 ppm, 5/49), were within the historical control range, and were not considered chemical related. The severity of nephropathy was significantly increased in 2,500 ppm female rats.

There was a significant negative trend in the incidence of mammary gland fibroadenoma, adenoma, or carcinoma (combined) in female rats (32/50, 24/50, 11/50, 16/50), and the incidences of fibroadenoma in 1,000 and 2,500 ppm females were significantly less than that of the controls.

2-YEAR STUDY IN MICE

Because of the reduction in body weights, the increase in liver and spleen weights, and the accompanying histopathologic changes in the liver of 2,500 ppm male and female mice in the 13-week study, the doses selected for the 2-year study were 250, 500, and 1,000 ppm.

Groups of 80 male and 80 female mice were fed diets containing 0, 250, 500, or 1,000 ppm TBBC for 2 years. Based on average daily feed consumption, these exposure levels resulted in the daily ingestion of approximately 30, 60, or 145 mg TBBC/kg body weight for males and 45, 110, or 255 mg TBBC/kg body weight for females. Nine or 10 animals from each exposure group were evaluated at 3, 9, and 15 months.

Survival, Body Weights, Feed Consumption, and Clinical Findings

Two-year survival rates of exposed male and female mice were similar to those of the controls. The final mean body weights of male and female mice exposed to 1,000 ppm were 8% and 18% lower than those of the controls, respectively. The final mean body weights of females exposed to 250 or 500 ppm were 8% to 9% lower than that of the controls. Feed consumption by exposed males was similar to that by controls, and there were no clinical findings attributed to TBBC administration.

Hematology and Clinical Chemistry

Hematocrit level, hemoglobin concentration, and erythrocyte count in 1,000 ppm male mice were significantly lower than those in controls at the 15-month interim evaluation. Serum alkaline phosphatase activities in 1,000 ppm males were slightly but significantly greater than those in controls at 3 and 9 months, as was the serum alkaline phosphatase activity in 1,000 ppm females at 9 months. Serum levels of total bilirubin in all exposed groups of males were significantly greater than those in controls at 9 and 15 months.

Pathology Findings

In the liver of male mice, negative trends in the incidences of fatty change, clear cell foci, and adenoma or carcinoma combined occurred at the end of the 2-year study. There were no compound-related increased incidences of neoplasms or non-neoplastic lesions in mice receiving TBBC for 2 years. A negative trend in the incidence of fatty change in the liver of male mice also occurred at 15 months.

GENETIC TOXICOLOGY

4,4'-Thiobis(6-*t*-butyl-*m*-cresol) was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 with or without exogenous metabolic activation (S9). Sister chromatid exchanges were induced in cultured Chinese hamster ovary cells treated with TBBC, with and without S9, but no increases in chromosomal aberrations were noted in cultured Chinese hamster ovary cells after treatment with TBBC.

CONCLUSIONS

Under the conditions of these 2-year feed studies, there was *no evidence of carcinogenic activity** of 4,4'-thiobis(6-*t*-butyl-*m*-cresol) in male or female F344/N rats administered 500, 1,000, or 2,500 ppm or in male or female B6C3F$_1$ mice administered 250, 500, or 1,000 ppm.

Nonneoplastic lesions associated with exposure to TBBC included: Kupffer cell hypertrophy, cytoplasmic vacuolization, and mixed cell foci in the liver of male and female rats, fatty change in the liver of female rats, and an increase in the severity of nephropathy in the kidney of female rats. In addition, decreased incidences of fibroadenoma, adenoma, or carcinoma (combined) were observed in the mammary gland of female rats. Decreases also occurred in the incidences of fatty change, clear cell foci, and adenoma or carcinoma (combined) in the liver of male mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 11. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 13.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of 4,4'-Thiobis(6-*t*-Butyl-*m*-Cresol)

	Male F344/N Rats	Female F344/N Rats	Male $B6C3F_1$ Mice	Female $B6C3F_1$ Mice
Doses	0, 500, 1,000, or 2,500 ppm in feed (approximately 20, 40, or 100 mg/kg/day)	0, 500, 1,000, or 2,500 ppm in feed (approximately 20, 45, or 120 mg/kg/day)	0, 250, 500, or 1,000 ppm in feed (approximately 30, 60, or 145 mg/kg/day)	0, 250, 500, or 1,000 ppm in feed (approximately 45, 110, or 255 mg/kg/day)
Body weights	Exposed groups lower than controls	2,500 ppm group lower than controls	1,000 ppm group lower than controls	Exposed groups lower than controls
2-Year survival rates	18/50, 28/50, 22/50, 18/50	34/50, 31/50, 32/50, 28/50	42/50, 42/50, 49/50, 45/50	40/51, 38/50, 36/50, 35/50
Nonneoplastic effects	Liver: Kupffer cell hypertrophy: 2/50, 3/50, 2/50, 31/49; cytoplasmic vacuolization: 13/50, 11/50, 19/50, 18/49; mixed cell foci: 6/50, 14/50, 18/50, 15/49	Liver: Kupffer cell hypertrophy: 11/50, 10/50, 9/50, 42/50; cytoplasmic vacuolization: 12/50, 10/50, 20/50, 34/50; fatty change: 9/50, 8/50, 15/50, 19/50; mixed cell foci: 5/50, 4/50, 14/50, 34/50 Kidney: nephropathy severity (1.4, 1.4, 1.6, 2.3)	None	None
Neoplastic effects	None	None	None	None
Other findings	None	Mammary gland: fibroadenoma, adenoma, or carcinoma (combined): 32/50, 24/50, 11/50, 16/50	Liver: fatty change: 19/50, 17/50, 5/50, 6/50; clear cell foci: 6/50, 5/50, 2/50, 0/50; adenoma or carcinoma (combined): 25/50, 30/50, 27/50, 16/50	None
Level of evidence of carcinogenic activity	No evidence	No evidence	No evidence	No evidence

Genetic toxicology

Salmonella typhimurium gene mutation: Negative in strains TA98, TA100, TA1535, and TA1537 with and without S9
Chinese hamster ovary cells *in vitro*
Sister chromatid exchanges: Positive with and without S9
Chromosomal aberrations: Negative with and without S9

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence** and **some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on 4,4'-thiobis(6-*t*-butyl-*m*-cresol) on June 22, 1993, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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* Did not attend

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On June 22, 1993, the draft Technical Report on the toxicology and carcinogenesis studies of 4,4′-thiobis(6-*t*-butyl-*m*-cresol) (TBBC) received public review by the National Toxicology Program Board of Scientific Counselors Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Mr. J.D. Cirvello, NIEHS, introduced the toxicology and carcinogenesis studies of TBBC by discussing the uses of the chemical and rationale for study, describing the experimental design, reporting on survival and body weight effects, and commenting on compound-related nonneoplastic lesions in rats and mice. The proposed conclusions were *no evidence of carcinogenic activity* of 4,4′-thiobis(6-*t*-butyl-*m*-cresol) in male or female F344/N rats or male or female B6C3F$_1$ mice.

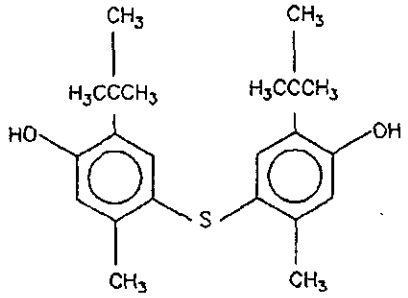
Mr. Beliczky, a principal reviewer, agreed with the proposed conclusions. He asked if the literature had been reviewed as most of the references were from the 1950's. Mr. Cirvello said a literature search had been done in 1992. Mr. Beliczky questioned the reference to the NIOSH Permissible Exposure Limit because the levels that were mentioned as either total dust or respirable dust are generally referred to as nuisance dust, those dusts which are physiologically inactive or inert. He did not think one could call TBBC inert or physiologically inactive. He commented that the nomination for review by the NTP was referenced to a 1978 study at Harvard and wanted to note that this epidemiological study had been funded by the United Rubber Worker's Joint Occupational Health Program.

Dr. Zeise, the second principal reviewer, agreed in principle with the proposed conclusions. She pointed out that, while the liver in male rats is clearly a target organ for toxicity, the data are unclear as to whether or not the liver is a target organ for carcinogenicity. She said the incidence of hepatocellular adenoma would be statistically significant if the historical control incidence at the study laboratory were used instead of the concurrent controls. She said there should be consideration given to changing the conclusion in male rats to "equivocal evidence of carcinogenic activity." Mr. Cirvello commented that if one looks at the overall historical control database, there were three studies from other laboratories with control values as high as those recorded in male rats in the high-dose group in the present study.

Dr. Ward, the third principal reviewer, agreed in principle with the proposed conclusions. He said it should be noted that the degree of nephropathy was increased in female rats and there should be a statement that male rats may have been able to tolerate a slightly higher dose. Mr. Cirvello said a statement about the nephropathy should have been included. He said that toxicity and reduction in body weight gain in the prechronic and 2-year studies indicated that the high dose was correct in male rats. Dr. Ward agreed with Dr. Zeise as to the uncertain significance of the liver neoplasms in male rats. Since mixed cell foci were increased more in exposed animals, Dr. Ward said it would be useful to have a morphologic description and an assessment as to whether they are preneoplastic lesions. Dr. S.L. Eustis, NIEHS, said a description would be added to the report, but it was difficult to say whether the foci were preneoplastic. There was no atypia reported, a finding often found in foci induced by hepatocarcinogens.

Mr. Beliczky moved that the Technical Report on 4,4′-thiobis(6-*t*-butyl-*m*-cresol) be accepted with the revisions discussed and with the conclusions as written for male and female rats and mice, *no evidence of carcinogenic activity*. Dr. Bailey seconded the motion, which was accepted unanimously with ten votes.

INTRODUCTION



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CHEMICAL AND PHYSICAL PROPERTIES

4,4′-Thiobis(6-*t*-butyl-*m*-cresol) (TBBC) is a fine, white crystalline powder with a melting point of 161° C and specific gravity of 1.10. This chemical is very soluble in methanol (79 g/mL), soluble in acetone (20 g/mL), less soluble in benzene (5.0 g/mL), and slightly soluble in water (0.08 g/mL) (Lefaux, 1968).

USE AND HUMAN EXPOSURE

TBBC is widely used in the rubber and plastics industries as an antioxidant for polyolefins, polyethylenes, polypropylenes, natural rubber, and latex. TBBC is approved by the U.S. Food and Drug Administration as a constituent of high-pressure polyethylene packaging for foodstuffs, excluding fats, and as a component of polyolefin film packaging in contact with meat or meat food products (Lefaux, 1968). Although the potential exists for the general population to be exposed through contact with polymer products or leaching of TBBC from such products into food, two studies investigating the migration of TBBC from plastic packaging materials indicated no significant exposure from this source (Udhe and Woggon, 1971; Ruedt and Herbolzheimer, 1976). Exposure is also possible via surface water contamination resulting from releases through manufacturing or use operations. No data were found on the environmental occurrence of TBBC.

TBBC reportedly has potential uses as a fungicide against such molds as *Aspergillus niger*, *Penicillium citrinum*, and *Rhizopus nigricans*, and as a preservative for paints, paper, fiber, and leather (Umekawa *et al.*, 1972). However, Hejtmankova *et al.* (1979) found that TBBC did not inhibit *A. niger* or *A. fumigatus*, and only weakly to moderately inhibited seven other strains of fungi.

No recent annual TBBC production or use data were found. Based on a survey conducted by NIOSH from 1981 to 1983, an estimated 12,349 workers are potentially exposed to TBBC in the workplace (NIOSH, 1991). The current Permissible Exposure Limits established by NIOSH for TBBC (as an 8-hour time-weighted average) are 15 mg/m^3 for total dust and 5 mg/m^3 for the respirable fraction.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

The disposition of [^{14}C]-labeled TBBC was studied in male F344/N rats (Birnbaum *et al.*, 1983). TBBC was administered by single oral gavage doses of 5, 50, or

500 mg TBBC/kg body weight in corn oil or in Emulphor:ethanol and by intravenous injection of 5 mg/kg in Emulphor:ethanol:water. Following oral exposure, TBBC was incompletely absorbed (the percentage absorbed was not determined) and there was a dose-related decrease in the rate of absorption. When administered *in situ* via luminal perfusion of 4, 49, or 500 mg/kg body weight, TBBC absorption in the small intestine was directly proportional to dose, suggesting that retention of the compound in the stomach was responsible for the apparent dose-related decline in absorption. Following intravenous administration of 5 mg/kg, very low percentages of total dose administered were detected rapidly in liver, adipose tissue, skin, muscle, and blood. The highest percentage of total dose was found in the liver, which had 2% after 15 minutes, 0.5% after 2 hours, and 0.4% after 1 day. The initial rate of clearance from liver and skin was very rapid, followed by a slower terminal decay phase. A slow rate of clearance was also observed in adipose tissue. Twenty-four hours after treatment, the parent compound accounted for most of the residual radioactivity in liver and adipose tissue; chronic exposure to TBBC could result in some accumulation of unmetabolized compound at these sites. More than half of the administered compound was excreted the first day, primarily through the bile into the feces; less than 2% was excreted into the urine. All radioactivity in the bile was in the form of metabolites of TBBC, the major metabolite being a glucuronide conjugate. A later study (Smith *et al.*, 1985) identified the major metabolite of TBBC in bile as the monoglucuronic acid conjugate.

To evaluate the effects of age on the glucuronidation of TBBC, male F344 rats 2.5, 16, and 26 months old were administered 5 mg [^{14}C]-labeled TBBC/kg intravenously. Urine and feces were collected for 3 days (Borghoff *et al.*, 1988). Bile was also collected for 6 hours after intravenous doses of 5 or 25 mg/kg. The 26-month-old animals excreted significantly less TBBC-derived radioactivity in bile, feces, and urine than both of the younger groups. The percentage of the dose eliminated in bile as a glucuronide also decreased with age. After 30 minutes of bile collection following a 5 mg/kg dose, 8% had been eliminated as a glucuronide by the 2.5-month-old group, 5.6% by the 16-month-old group, and 4.4% by the 26-month-old group. When the 26-month-old animals were given 25 mg/kg, elimination as glucuronide was only 2% of the dose. *In vitro* studies using TBBC as a substrate demonstrated that hepatic uridine diphosphate glucuronyl transferase activity decreased in aging animals. Further, the hepatic concentration of uridine diphosphate glucuronyl acid (UDPGA) also decreased in animals from 2.5 to 28 months of age. Thus, the decrease in the ability of the aging rats to conjugate and excrete TBBC may be caused by a decrease in both the activity of the conjugating enzyme and the availability of UDPGA.

Humans

No information on the absorption, distribution, metabolism, or excretion of TBBC in humans was found in the literature.

TOXICITY

Experimental Animals

Few published studies on the toxicity of TBBC exist. In acute oral toxicity studies in rats, the LD_{50} varies from 5,000 to 7,000 mg/kg depending on the purity of the test material (personal communication cited in Birnbaum *et al.*, 1983). Details are not given except that rats exhibited severe diarrhea preceding death. In the previously discussed disposition studies (Birnbaum *et al.*, 1983), TBBC administered by gavage in either Emulphor:ethanol or corn oil (5, 50, or 500 mg/kg) caused mild inflammation, congestion, hemorrhage, and mucosal erosion of the stomach in rats. These findings were dose related and detectable as early as 1 hour after administration of 500 mg/kg. Studies in which rats ingested TBBC in feed for 30 or 90 days were performed by E.I. du Pont de Nemours & Co. and the results are summarized briefly by Lefaux (1968). In the 30-day study, groups of six male and six female rats were fed diets containing 500 or 2,500 ppm TBBC. The 500 ppm group displayed no signs of toxicity, whereas at 2,500 ppm, rats exhibited growth retardation and increased liver weights. In the 90-day study, rats were fed diets containing 50 or 500 ppm TBBC, and the only effects noted were decreased feed consumption and slight growth retardation in 500 ppm males. Monsanto Chemical Company conducted 3-month feed studies using the same doses of TBBC (50 or 500 ppm) and obtained similar results; the only sign of toxicity was growth retardation in animals receiving 500 ppm (McCormick, 1972).

TBBC toxicity was also studied in adult female B6C3F$_1$ mice by administering 10, 100, or 200 mg/kg daily in corn oil by gavage for 14 consecutive days (Munson *et al.*, 1988). No overt toxicity was observed and no marked effects on serum enzymes occurred. The highest exposure group had a 41% increase in total leukocytes with a 31% increase in lymphocytes and a 177% increase in neutrophils. Bone marrow studies revealed a significant (30%) increase in the number of cells/femur in 200 mg/kg mice; macrophage progenitors were significantly increased by 28% and granulocyte-monocyte progenitors were increased by 20%. A dose-related increase occurred in absolute weights of both the spleen and liver, although the histopathology of the spleens of TBBC-treated mice was not different from that of the controls. The livers of mice in the high-dose group had changes described as mild focal hydropic degeneration, mild hepatitis, and a slight increase in the number of Kupffer cells. Hepatic cytochrome P-450 and microsomal protein levels exhibited a dose-related increase, as did enzyme activities of aminopyrine demethylase and aniline hydroxylase.

Immunotoxicologic studies were conducted after administering TBBC in corn oil by gavage at doses of 10, 100, or 200 mg/kg to B6C3F$_1$ mice daily for 14 consecutive days (Holsapple *et al.*, 1988). A 200 mg/kg dose produced a decrease in the peak IgM (44%) and peak IgG (48%) antibody response to *in vivo* challenge with sheep erythrocytes, but had no effect on the delayed hypersensitivity response to challenge with keyhole limpet hemocyanin. At 10 and 200 mg/kg, a significant decrease in the mixed lymphocyte response (MLR) occurred, but doses of 10, 100, or 200 mg/kg produced no effects on the *in vitro* lymphoproliferative responses of spleen cells to optimal concentrations of concanavalin A, phytohemagglutinin, or lipopolysaccharide. A dose-related increase in the basal (unstimulated) DNA synthesis of the spleen cells occurred in both the MLR and the mitogen assays. A significant increase in natural killer cell and serum complement activity was also observed. The increase in natural killer cell activity was significant in mice administered 100 and 200 mg/kg, with the greatest increase at the 100 mg/kg dose; 10 mg/kg TBBC produced a significant (35%) increase in CH50 and at 100 mg/kg a significant (54%) increase occurred. Effects on macrophage function were complex; either an increase or no effect was observed, depending on the parameter measured. Exposure to 10, 100, or 200 mg/kg caused a dose-related increased resistance to challenge with *Streptococcus pneumoniae* and B16F10 melanoma, a decreased resistance to challenge with PYB$_6$ neoplasms, and no effect on the resistance to HSV-2, *Listeria*, or *Plasmodium*. Thus, several parameters reflecting immune function were altered following 14-day gavage exposure to TBBC.

Humans

Two patients with allergic contact dermatitis were found to be patch-test positive to latex gloves made by the same manufacturer. TBBC was the antioxidant used in making the gloves and both patients had a positive patch test reaction to the TBBC itself (Rich *et al.*, 1991). No other information on the toxicity of TBBC in humans was found in the literature.

Reproductive and Developmental Toxicity

Experimental Animals

In a study to evaluate the effects of TBBC on reproduction in female Swiss mice, 485 mg/kg was administered daily by gavage to 50 pregnant mice on days 6 through 15 of gestation (EHRT, 1989). TBBC caused maternal mortality and a decreased rate of survival of pups, but had no effect on the number of viable litters, litter size, pup birth weight, or pup weight gain.

Humans

No information on the reproductive and developmental toxicity of TBBC in humans was found in the literature.

Carcinogenicity

Experimental Animals

A report by Draganov *et al.* (1974) suggests that TBBC may be a neoplasm promoter. When Yoshida sarcomas were transplanted to rats, neoplasm development was enhanced if TBBC was administered orally for 10 days at a dose of 80 mg/kg daily, beginning 5 days after transplantation. No other data were provided in the report.

Humans

No information on the potential carcinogenicity of TBBC in humans was found in the literature.

GENETIC TOXICITY

TBBC was tested for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 with a preincubation protocol in the presence and absence of S9; no mutagenic activity was observed in any of these four strains (Zeiger *et al.*, 1987). There are no other published data on the genotoxicity of this compound.

STUDY RATIONALE

The National Cancer Institute nominated TBBC for study as a representative of the sulfur-containing class of antioxidants used in rubber processing. A study that was recent at the time of nomination demonstrated an excess of several types of cancer among a cohort of 13,570 rubber workers (Monson and Fine, 1978). In addition, the presence of TBBC in plastic food wraps and containers was viewed as a possible hazard to the general population.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF 4,4′-THIOBIS(6-*T*-BUTYL-*M*-CRESOL)

4,4′-Thiobis(6-*t*-butyl-*m*-cresol) was obtained in one lot (12) from Monsanto Industrial Chemical Company (Akron, OH). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO), (Appendix I).

The chemical, a white powdered solid, was identified as 4,4′-thiobis(6-*t*-butyl-*m*-cresol) (TBBC) by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. Purity was determined by elemental analyses, Karl Fischer water analysis, functional group titration, thin-layer chromatography, and gas chromatography. Analyses of the chemical for carbon, hydrogen and sulfur were in agreement with theoretical values for TBBC. Functional group titration indicated a purity of 100% ± 3%. Thin-layer chromatography using two systems indicated a major spot and two trace impurities. Gas chromatography using one system indicated two impurities with a total area of 0.7% relative to the major peak area that eluted before the major peak. A second system indicated one impurity that eluted before the major peak and had an area of 0.39% relative to the major peak. The overall purity was determined to be approximately 99%. Subsequent analysis by the analytical chemistry laboratory indicated a purity of approximately 99%.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared weekly by mixing 4,4′-thiobis(6-*t*-butyl-*m*-cresol) with feed (Table I1). Homogeneity and stability studies of the 250 and 25,000 ppm dose formulations were performed by the analytical chemistry laboratory. For the homogeneity and stability studies, dose formulations were analyzed by high performance liquid chromatography. Homogeneity was confirmed at the 100 and 10,000 ppm concentrations, and stability was established at these concentrations for at least 3 weeks at –20° C when stored in the dark and for 3 days when exposed to air and light.

Periodic analyses of the dose formulations of TBBC were conducted at the study laboratory and analytical chemistry laboratory using high-performance liquid chromatography. During the 15-day studies, only the initial formulation was analyzed (Table I2). During the 13-week and the 2-year studies, the dose formulations were analyzed every 6 to 10 weeks (Tables I3 and I4). In the 2-year studies, 93% (86/92) of the formulations were within 10% of the target concentrations. Results of the periodic referee analyses performed by the analytical chemistry laboratory were in good agreement with the results obtained by the study laboratory (Table I5).

15-DAY STUDIES

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Frederick Cancer Research Center (Frederick, MD). At receipt, the rats and mice were 6 weeks old. Animals were quarantined for 13 to 15 days before exposure began. At this time, two males and two females of each species were randomly selected and evaluated for evidence of disease. Groups of 10 male and 10 female rats and mice were fed diets containing 0, 1,000, 2,500, 5,000, 10,000, or 25,000 ppm TBBC. Feed and water were available *ad libitum*. Rats and mice were housed five per cage. Clinical findings were recorded daily for rats and mice. Feed consumption was recorded daily by cage. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

At the end of the 15-day studies, blood was collected from all animals by cardiac puncture for hematology analyses. The parameters measured are listed in Table 1. A necropsy was performed on all rats and mice. The brain, gastrointestinal tract, heart, right kidney, liver, lung, spleen, right testis, and thymus were weighed. Tissues for microscopic examination were embedded in paraffin, sectioned to a thickness of 4 to 6 μm, and stained with hematoxylin and eosin. Histopathologic examinations were performed on 0, 2,500, 5,000, and 10,000 ppm rats and 0, 2,500, and 5,000 ppm mice. Table 1 lists the tissues and organs examined microscopically.

13-WEEK STUDIES

The 13-week studies were conducted to evaluate the cumulative toxic effects of repeated exposure to TBBC and to determine the appropriate exposure levels to be used in the 2-year studies.

Male and female F344/N rats and B6C3F$_1$ mice were obtained from the Frederick Cancer Research Center (Frederick, MD). On receipt, the rats and mice were 29 days old. The rats were quarantined for 15 days and the mice for 22 days before exposure began. Before initiation of the studies, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At the end of the studies, serologic analyses were performed on five male and five female control rats and mice using the protocols of the NTP Sentinel Animal Program (Appendix L).

Groups of 10 male and 10 female rats were fed diets containing 0, 250, 500, 1,000 2,500, or 5,000 ppm TBBC. Groups of 10 male and 10 female mice were fed diets containing 0, 100, 250, 500, 1,000, or 2,500 ppm TBBC. Feed and water were available *ad libitum*. Rats were housed five per cage and mice were housed individually. Clinical findings were recorded weekly. Feed consumption was recorded daily by cage for rats and daily by animal for mice. The animals were weighed initially, weekly, and at the end of the studies. Further details of study design and animal maintenance are summarized in Table 1.

During the final eight days of the 13-week study in rats, males and females receiving 0, 1,000, and 2,500 ppm were tested for forelimb and hindlimb grip strength, startle response, tail flick, and foot splay. See Appendix H for detailed methods.

Two days before the end of the 13-week studies, blood was collected from the orbital sinus of all rats and mice for hematology analyses. At the end of the 13-week studies, blood was collected from all rats by cardiac puncture for clinical chemistry analyses. The hematology and clinical chemistry parameters measured are listed in Table 1. A necropsy was performed on all animals. The brain, heart, right kidney, liver, lung, spleen, right testicle, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin. A complete histopathologic examination was performed on 0, 1,000, 2,500, and 5,000 ppm rats and 0, 1,000, and 2,500 ppm mice. Table 1 lists the tissues and organs routinely examined.

2-YEAR STUDIES

Study Design

Groups of 115 male and 75 female rats were fed diets containing 0, 500, 1,000, or 2,500 ppm TBBC (Table 1). Fifteen male and 15 female rats from each group were evaluated at 3, 9, and 15 months for alterations in hematology, clinical chemistry, and urinalysis parameters and then discarded. An additional 10 male and 10 female rats from each group were also evaluated at 15 months for alterations in hematology and clinical chemistry parameters; these animals received complete necropsy and histopathology examinations.

Forty of the 115 male rats in each exposure group were designated for neurotoxicity evaluation at 3 and 6 months (Appendix H). At 3 months, startle reflex and fore- and hindlimb grip strength were measured in all 40 animals. Ten males per group received electrophysiologic evaluations, including measurements of sciatic nerve conduction time following various frequencies of electrical stimulation and contractile tension of the gastrocnemius muscle following various frequencies of electrical stimulation or following graded electrical stimulation. An additional 10 males per group received whole body perfusion for histopathologic examination of the left quadriceps muscle and left sciatic nerve and of teased nerve preparations of the sciatic nerve. The remaining 20 male rats in each group were fed the control diet for 13 additional weeks to determine the reversibility of TBBC-induced changes. At 6 months, grip strength tests were repeated in all 20 rats per group. These 20 rats were then split into two groups of 10 and given electrophysiologic and neuropathologic evaluations as described above.

Groups of 80 male and 80 female mice were fed diets containing 0, 250, 500, or 1,000 ppm TBBC. At 3, 9, and 15 months, groups of 10 male and 10 female mice per group were killed and evaluated for alterations in hematology and clinical chemistry parameters. The 10 male and 10 female mice per group killed at 15 months also received a complete necropsy and histopathologic evaluation.

Source and Specification of Animals

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Farms (Germantown, NY) for use in the 2-year studies. Rats and mice were quarantined for 11 days before the beginning of the studies. Five male and five female rats and mice were selected for parasite evaluation and gross observation of disease. Serology samples were collected for viral screening. Rats were approximately 43 days old and mice approximately 39 days old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix L).

Animal Maintenance

Rats were housed five per cage and mice were housed individually. Feed and water were available *ad libitum*. Feed consumption was measured twice weekly by cage. Cages and racks were rotated biweekly. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix K.

Clinical Examinations and Pathology

All animals were observed twice daily. Clinical findings and body weights were recorded at the beginning of the studies, weekly for 13 weeks, and monthly thereafter. A complete necropsy and microscopic examination were performed on all rats and mice except: the 15 male and 15 female rats per group designated for hematology, clinical chemistry, and urinalysis evaluations at 3, 9, and 15 months; the 10 male and 10 female mice per group designated for hematology and clinical chemistry at 3 and 9 months; and the 40 male rats per group designated for neurotoxicity and neuropathologic evaluations. At the 15-month interim evaluation, the brain, gastrointestinal tract, right kidney, liver, and spleen of rats and mice were weighed. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin for microscopic examination. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The microscopic slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist reviewed the liver of male and female rats, neoplasms of the thyroid gland, mammary gland, and uterus of female rats, neoplasms of the skin, bone, and nose of male rats, the liver of female mice, and neoplasms of the ovary of female mice.

The quality assessment report and slides were submitted to the NTP Pathology Working Group (PWG) chair, who reviewed the selected tissues and any other tissues for which a disagreement in diagnosis between the laboratory and quality assessment pathologists existed. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologist, or lesions of general interest were presented by the chair to the PWG for review. Tissues examined included the skin, bone, and nose of male rats, the liver of male and female rats, the mammary gland, thyroid gland, and uterus of female rats, and the liver and ovary of female mice. The PWG consisted of the quality assessment pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of exposure groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Thus, the final diagnoses represent a consensus of contractor pathologists and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the diagnosed lesions for each tissue type were evaluated separately or combined according to the guidelines of McConnell *et al.* (1986).

Statistical Methods

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing

were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions as presented in Tables A1, A5, B1, B5, C1, C5, D1, and D4 are given as the number of animals bearing such lesions at a specific anatomic site and the number of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A3, B3, C3, and D3) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., skin, intestine, harderian gland, and mammary gland) before microscopic evaluation, or when neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed.

Analysis of Neoplasm Incidences

The majority of neoplasms in these studies were considered to be incidental to the cause of death or not rapidly lethal. Thus, the primary statistical method used was logistic regression analysis, which assumed that the diagnosed neoplasms were discovered as the result of death from an unrelated cause and thus did not affect the risk of death. In this approach, neoplasm prevalence was modeled as a logistic function of chemical exposure and time. Both linear and quadratic terms in time were incorporated initially, and the quadratic term was eliminated if the fit of the model was not significantly enhanced. The neoplasm incidences of exposed and control groups were compared on the basis of the likelihood score test for the regression coefficient of dose. This method of adjusting for intercurrent mortality is the prevalence analysis of Dinse and Lagakos (1983), further described and illustrated by Dinse and Haseman (1986). When neoplasms are incidental, this comparison of the time-specific neoplasm prevalences also provides a comparison of the time-specific neoplasm incidences (McKnight and Crowley, 1984).

In addition to logistic regression, other methods of statistical analysis were used, and the results of these tests are summarized in the appendixes. These methods include the life table test (Cox, 1972; Tarone, 1975), appropriate for rapidly lethal neoplasms, and the Fisher exact test and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979), procedures based on the overall proportion of neoplasm-bearing animals.

Tests of significance included pairwise comparisons of each exposed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of neoplasm incidence, and reported P values are one sided. The procedures described in the preceding paragraphs were also used to evaluate selected nonneoplastic lesions. For further discussion of these statistical methods, see Haseman (1984).

Analysis of Nonneoplastic Lesion Incidences

Because all nonneoplastic lesions in this study were considered to be incidental to the cause of death or not rapidly lethal, the primary statistical analysis used was a logistic regression analysis in which nonneoplastic lesion prevalence was modeled as a logistic function of chemical exposure and time. For lesions detected at the interim evaluation, the Fisher exact test was used, a procedure based on the overall proportion of affected animals.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed using the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Clinical chemistry and hematology data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Average severity values were analyzed for significance using the Mann-Whitney U test (Hollander and Wolfe, 1973).

Historical Control Data

Although the concurrent control group is always the first and most appropriate control group used for evaluation, historical control data can be helpful in the overall assessment of neoplasm incidence in certain instances. Consequently, neoplasm incidences from the NTP historical control database (Haseman *et al.*, 1984, 1985) are included in the NTP reports for neoplasms appearing to show compound-related effects.

Quality Assurance Methods

The 13-week and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covering completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and preliminary review draft of this NTP Technical Report were conducted. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, so all comments had been resolved or were otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of TBBC was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium* and chromosomal aberrations in cultured Chinese hamster ovary cells. The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies of TBBC are part of a larger effort by the NTP to develop a database that would permit the evaluation of carcinogenicity in experimental animals from the structure and responses of the chemical in short-term *in vitro* and *in vivo* genetic toxicity tests. These genetic toxicity tests were originally developed to study mechanisms of chemically induced DNA damage and to predict carcinogenicity in animals, based on the electrophilic theory of chemical carcinogenesis and the somatic mutation theory (Miller and Miller, 1977; Straus, 1981; Crawford, 1985).

There is a strong correlation between a chemical's potential electrophilicity (structural alert to DNA reactivity), mutagenicity in *Salmonella*, and carcinogenicity in rodents. The combination of electrophilicity and *Salmonella* mutagenicity is highly correlated with the induction of carcinogenicity in rats and mice and/or at multiple tissue sites (Ashby and Tennant, 1991). Other *in vitro* genetic toxicity tests do not correlate well with rodent carcinogenicity (Tennant *et al.*, 1987; Zeiger *et al.*, 1990), although these other tests can provide information on the types of DNA and chromosome effects that can be induced by the chemical being investigated. Data from NTP studies show that a positive response in *Salmonella* is currently the most predictive *in vitro* test for rodent carcinogenicity (89% of the *Salmonella* mutagens were rodent carcinogens), and that there is no complementarity among the *in vitro* genetic toxicity tests. That is, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. The predictivity for carcinogenicity of a positive response in bone marrow chromosome aberration or micronucleus tests is not yet defined.

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

15-Day Studies	13-Week Studies	2-Year Studies
Study Laboratory		
American Biogenics Corporation (Woburn, MA)	American Biogenics Corporation (Woburn, MA)	Battelle Columbus Laboratories (Columbus, OH)
Strain and Species		
Rats: F344/N Mice: B6C3F$_1$	Rats: F344/N Mice: B6C3F$_1$	Rats: F344/N Mice: B6C3F$_1$
Animal Source		
Frederick Cancer Research Center (Frederick, MD)	Frederick Cancer Research Center (Frederick, MD)	Taconic Farms (Germantown, NY)
Time Held Before Studies		
Rats: 14 days (males) or 15 days (females) Mice: 13 days (males) or 14 days (females)	Rats: 15 days Mice: 22 days	11 days
Average Age When Studies Began		
Rats: 44 days Mice: 43 days	Rats: 43 days Mice: 50 days	Rats: 43 days Mice: 39 days
Date of First Dose		
Rats: 29 December (males) or 30 December (females) 1983 Mice: 3 January (males) or 4 January (females) 1984	Rats: 1 August 1984 Mice: 15 August 1984	Rats: 29 December 1986 (special studies and 15-month interim) or 22 December 1986 (2-year study) Mice: 19 January 1987
Duration of Dosing		
15 days	92-94 days	104 weeks
Date of Last Dose		
Rats: 12 January (males) or 13 January (females) 1984 Mice: 17 January (males) or 18 January (females) 1984	Rats: 2 November 1984 Mice: November 1984	Rats: 12 December 1988 Mice: 9 January 1989
Necropsy Dates		
Rats: 12 January (males) or 13 January (females) 1984 Mice: 17 January (males) or 18 January (females) 1984	Rats: 31 October to 2 November 1984 Mice: 14 to 16 November 1984	Rats: 15-Month interim evaluation and clinical pathology - 21-22 March 1988 Terminal - 19-21 December 1988 Mice: 15-Month interim - 18-19 April 1988 Terminal - 16-20 January 1989

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) (continued)

15-Day Studies	13-Week Studies	2-Year Studies
Average Age at Necropsy		
Rats: 59 days Mice: 57 days	Rats: 135 days Mice: 141 days	15-Month interim evaluation and clinical pathology - 71 weeks Terminal - 111 weeks
Size of Study Groups		
10 males and 10 females	Same as 15-day studies	Rats: 115 males and 75 females Mice 80 males and 80 females
Method of Distribution		
Animals randomized from weight classes into cage groups using a computer-generated list of random numbers; cages randomized into test groups from another computer-generated list of random numbers	Same as 15-day studies	Animals randomized from weight classes into cage groups and dose groups using a partitioning algorithm
Animals per Cage		
5	Rats: 5 Mice: 1	Rats: 5 Mice: 1
Method of Animal Identification		
Ear punch	Same as 15-day studies	Rats: Neurological - ear tag Clinical pathology - toe clip Terminal - toe clip Mice: Toe clip
Diet		
NIH-07 open formula meal diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed daily	Same as 15-day studies	Same as 15-day studies, changed twice weekly
Maximum Storage Time for Feed		
108 days post-milling	120 days post-milling	Same as 13-week studies
Water Distribution		
Tap water (Woburn municipal supply) via automatic watering system (Hardco, Cincinnati, OH), available *ad libitum*	Same as 15-day studies	Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)
(continued)

15-Day Studies	13-Week Studies	2-Year Studies
Cages		
Polycarbonate, (Suburban Surgical Co., Inc., Wheeling, IL), changed twice weekly	Same as 15-day studies except cages were changed twice weekly for rats.	Polycarbonate (Lab Products, Inc., Garfield, NJ), changed twice weekly (rats) or weekly (mice)
Bedding		
SaniChip® hardwood chips (P.J. Murphy Forest Products Corp., Rochelle Park, NJ), changed twice weekly	Same as 15-day studies	BetaChip® hardwood chips (Northeastern Products, Inc., Warrensburg, NY) until 22 May 1988; SaniChip® (P.J. Murphy Forest Products Corp., Montville, NJ) thereafter; changed twice weekly (rats) or weekly (mice)
Cage Filters		
Nonwoven filter sheets, DuPont (Snow Filtration Co., Cincinnati, OH), changed biweekly	Same as 15-day studies	Spun-bonded polyester, DuPont 2024 (Snow Filtration Co., Cincinnati, OH), changed biweekly
Racks		
Stainless steel, changed biweekly	Stainless steel, changed biweekly	Stainless steel (Lab Products, Inc., Maywood, NJ), changed biweekly
Animal Room Environment		
Average temperature: 18.6° C (male rats), 18.5° C (female rats), 18.4° C (mice) Relative humidity: 35% to 51% Fluorescent light: 12 hours/day Room air: 12 to 16 changes/hour	Average temperature: 21.7° C (rats), 17.8° C (mice) Relative humidity: 41% to 60% Fluorescent light: 12 hours/day Room air: 12 changes/hour	Average temperature: 22.5° C (rats), 22.2° C (mice) Relative humidity: 40% to 56% (rats), 45% to 58% (mice) Fluorescent light: 12 hours/day Room air: minimum of 10 changes/hour
Doses		
0, 1,000, 2,500, 5,000, 10,000, or 25,000 ppm in feed, available *ad libitum*	Rats: 0, 250, 500, 1,000, 2,500, or 5,000 ppm in feed, available *ad libitum* Mice: 0, 100, 250, 500, 1,000, or 2,500 ppm in feed, available *ad libitum*	Rats: 0, 500, 1,000, or 2,500 ppm in feed, available *ad libitum* Mice: 0, 250, 500, or 1,000 ppm in feed, available *ad libitum*

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)
(continued)

15-Day Studies	13-Week Studies	2-Year Studies
Type and Frequency of Observation		
Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; and clinical observations were recorded daily. Feed consumption was recorded daily by cage.	Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; clinical observations were recorded weekly. Feed consumption was recorded daily by cage (rats) and daily by animal (mice).	Observed twice daily; animals were weighed and clinical observations were recorded initially, weekly for 13 weeks, monthly thereafter, and at the end of the studies. Feed consumption was recorded monthly by cage (rats) or by animal (mice).
Method of Sacrifice		
Anesthesia with methoxyflurane followed by exsanguination by cardiac puncture	Same as 15-day studies	Carbon dioxide asphyxiation or pentobarbital anesthesia with exsanguination and transcardial perfusion (neurotoxicity evaluation rats)
Necropsy		
Necropsy performed on all animals. Organs weighed were brain, gastrointestinal tract, heart, right kidney, liver, lung, spleen, right testis, and thymus.	Necropsy performed on all animals. Organs weighed were brain, heart, right kidney, liver, lung, spleen, right testis, and thymus.	Necropsy performed on all animals. Organs weighed were brain, gastrointestinal tract, right kidney, liver, and spleen.
Clinical Pathology		
Blood was collected from all animals surviving to the end of the studies by cardiac puncture for hematology. *Hematology:* hematocrit, hemoglobin, erythrocytes, mean erythrocyte volume, mean erythrocyte hemoglobin, mean erythrocyte hemoglobin concentration, reticulocytes, leukocyte counts, and nucleated erythrocytes	Blood was collected from all animals from the orbital sinus for hematology and by cardiac puncture from rats for clinical chemistry. *Hematology:* hematocrit, hemoglobin, erythrocytes, mean erythrocyte volume, reticulocytes, leukocyte differentials, and nucleated erythrocytes *Clinical chemistry:* (rats) urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase, and γ-glutamyltranspeptidase	Blood was collected from the orbital sinus and urine was collected from up to 15 male and female rats per group (slated only for clinical pathology evaluation). Blood was also collected from the orbital sinus of 10 male and female rats and mice at 3, 9, and 15 months into the 2-year study. *Hematology:* hematocrit, hemoglobin, erythrocytes, mean erythrocyte volume, mean erythrocyte hemoglobin, mean erythrocyte hemoglobin concentration, platelets, reticulocytes, leukocyte differentials, and nucleated erythrocytes *Clinical chemistry:* urea nitrogen, creatinine, sodium, potassium, chloride, calcium, direct bilirubin (15-month rats and mice), total bilirubin, alkaline phosphatase, alanine aminotransferase, sorbitol dehydrogenase, and bile salts (rats and 15-month mice) *Urinalysis:* creatinine, alkaline phosphatase, lactate dehydrogenase, *N*-acetyl-β-*D*-glucosaminidase, volume, and β-galactosidase

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) (continued)

15-Day Studies	13-Week Studies	2-Year Studies
Histopathology		
Histopathology was performed on 0, 2,500, 5,000, and 10,000 ppm rats and 0, 2,500, and 5,000 ppm mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, bone and marrow, large intestine (cecum, colon, rectum), mandibular or mesenteric lymph node, small intestine (duodenum, jejunum, ileum), spleen, stomach (forestomach and glandular), and thymus. The following tissues were examined only from the 10,000 ppm rats and 5,000 ppm mice: brain, clitoral gland (rats), esophagus, gallbladder (mice), heart, kidney, liver, lung, mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland (rats), prostate gland, salivary gland, skin, testis with epididymis and seminal vesicle, thyroid gland, trachea, urinary bladder, and uterus.	Complete histopathology was performed on 0, 1,000, 2,500, and 5,000 ppm rats and 0, 1,000 and 2,500 ppm mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, brain, clitoral gland (rats), esophagus, gallbladder (mice), heart, kidney, large intestine (cecum, colon, rectum), liver, lung, mammary gland, mandibular or mesenteric lymph node, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland (rats), prostate gland, salivary gland, skin, small intestine (duodenum, jejunum, ileum), spleen, sternum and vertebra (including marrow), stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thyroid gland, thymus, trachea, urinary bladder, and uterus. Only the following tissues were examined from the 1,000 and 2,500 ppm rats and 1,000 ppm mice: liver and mandibular or mesenteric lymph node. The kidney from the 2,500 ppm rats was also examined.	Complete histopathology was performed on all rats and mice. No histopathology was performed on the clinical pathology group rats or mice or the neurotoxicity group male rats. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, bone (including marrow), brain, clitoral gland (rats), esophagus, gallbladder (mice), heart, kidney, large intestine (cecum, colon, rectum), liver, lung, mammary gland with surface skin, mandibular or mesenteric lymph node, nose, ovary, pancreas, parathyroid gland, pharynx, pituitary gland, preputial gland (rats), prostate gland, salivary gland, skeletal muscle, skin, small intestine (duodenum, jejunum, and ileum), spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thyroid gland, thymus, trachea, urinary bladder, and uterus.
Neurotoxicity Evaluations		
None	Male and female 0, 1,000, and 2,500 ppm rats were tested for forelimb and hindlimb grip strength, tail flick, startle response, and foot splay.	Forty male rats per group were designated for neurotoxicity studies. After 3 months of exposure, startle reflex and forelimb and hindlimb grip strength were measured in all 40 animals. Ten males per group were killed and given electrophysiological evaluations; another ten males per group were killed and given whole body perfusion for histopathologic examination. The remaining 20 males per group were fed the control diet for an additional 14-16 weeks to determine the reversibility of TBBC-induced changes. At 6 months, grip strength tests were repeated in all 20 rats per group; these 20 were then split into two groups of ten and given electrophysiologic and neuropathologic evaluations.

RESULTS

RATS

15-DAY STUDY

All male and female rats receiving diets containing 25,000 ppm 4,4′-thiobis(6-*t*-butyl-*m*-cresol) (TBBC), and three males and four females receiving 10,000 ppm died before the end of the study (Table 2). The majority of these deaths occurred during the second week of the study. The seven surviving 10,000 ppm males had a mean body weight loss of 29% and a final mean body weight 51% lower than those of the controls. The mean body weight gain of the 5,000 ppm males was 71% lower than that of the controls, and the final mean body weight was 22% lower than that of the controls. Surviving females in the 10,000 ppm group had a 27% mean body weight loss and a final mean body weight 43% lower than those of the controls. The 5,000 ppm females had a mean body weight gain 77% lower than that of the controls and a final mean body weight 18% lower than that of the controls. Mean body weight gains, final mean body weights, and feed consumption by males and females receiving 1,000 and 2,500 ppm were generally similar to those of the controls. All rats exposed to 5,000, 10,000, or

TABLE 2
Survival, Body Weights, and Feed Consumption of Rats in the 15-Day Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 2
Male							
0	10/10	145 ± 2	212 ± 4	67 ± 4		15.8	16.2
1,000	10/10	149 ± 3	224 ± 3	75 ± 2	106	16.0	18.8
2,500	10/10	147 ± 4	222 ± 5	74 ± 2	105	15.2	19.6
5,000	10/10	146 ± 2	165 ± 3**	19 ± 5**	78	8.8	11.9
10,000	7/10[d]	145 ± 3	103 ± 4**	–44 ± 3**	49	3.1	6.0
25,000	0/10[e]	149 ± 2	–	–	–	3.4	5.4
Female							
0	10/10	118 ± 3	154 ± 3	36 ± 1		11.9	12.1
1,000	10/10	120 ± 2	156 ± 2	36 ± 2	101	12.3	10.9
2,500	10/10	118 ± 2	157 ± 2	39 ± 1	102	11.9	12.3
5,000	10/10	118 ± 2	127 ± 1**	8 ± 2**	82	7.8	8.1
10,000	6/10[f]	121 ± 2	88 ± 4**	–35 ± 4**	57	2.2	3.4
25,000	0/10[g]	117 ± 2	–	–	–	1.1	4.8

** Significantly different (P≤0.01) from the control group by Williams' or Dunnett's test
[a] Number of animals surviving at 15 days/number initially in group
[b] Weights are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study. No final mean body weights were calculated for groups with 100% mortality.
[c] Feed consumption is expressed as grams per animal per day.
[d] Day of death: 11, 14, 14
[e] Day of death: 9, 9, 9, 10, 11, 11, 11, 12, 12, 13
[f] Day of death: 12, 13, 15, 15
[g] Day of death: 7, 8, 8, 9, 10, 11, 11, 11, 15, 15

25,000 ppm TBBC consumed markedly less feed than did the control groups. Rats exposed to 1,000, 2,500, 5,000, or 10,000 ppm received approximate doses of 95, 235, 335, or 365 mg TBBC per kilogram body weight per day (males) and 85, 220, 325, or 270 mg per kg per day (females). Approximate doses for rats exposed to 25,000 ppm cannot be calculated due to early deaths. Since the reduction in feed consumption was evident from the beginning of the study when no signs of toxicity were apparent, reduced feed consumption appeared to be due to poor feed palatability.

Diarrhea was observed in two 25,000 ppm males on day 3 of the study and in the eight remaining 25,000 ppm males on days 6, 7, or 8. Diarrhea occurred in three 25,000 ppm females on day 2 and was observed in other females exposed to 25,000 ppm from day 6 onward. Male and female rats exposed to 5,000 or 10,000 ppm TBBC began to experience diarrhea midway or late into the study. No clinical signs were observed in male or female rats receiving 1,000 or 2,500 ppm TBBC. Statistically significant changes in absolute or relative organ weights reflected decreased final mean body weights or stress and were not considered to be directly related to chemical administration (Table F1).

Since no 25,000 ppm rats survived, hematology parameters were measured only in rats receiving 10,000 ppm or less (Table G1). Leukocyte counts in all exposed females were slightly but significantly greater than those of the controls. Segmented neutrophil counts were significantly higher in the 10,000 and 25,000 ppm male and female groups. This increase was not accompanied by an increase in immature forms, suggesting that this was not an inflammatory response but rather to a shift in the total blood pool distribution without an absolute increase.

Significantly lower reticulocyte counts occurred in male rats receiving 10,000 and 5,000 ppm TBBC and in females receiving 10,000 ppm. In males, this decrease was accompanied by a decrease in nucleated erythrocytes. The slightly lower reticulocyte counts in rats receiving TBBC were probably related to the debilitation rather than to a primary effect on the bone marrow. Females receiving 5,000 or 10,000 ppm also had a very slight decrease in erythrocyte size compared to controls as indicated by decreased mean erythrocyte volume values. This also was probably related to debilitation.

Microscopic examination was not performed on tissues from 25,000 ppm rats since they died before the end of the study. The principal lesions associated with the ingestion of TBBC occurred in the kidney and glandular stomach of 10,000 ppm rats (Table 3). There was partial to complete necrosis of the tip of the renal papilla in one male and two females and minimal focal or multifocal necrosis of tubule epithelium in the cortex or outer medulla of four males and seven females receiving 10,000 ppm (Plates 1 and 2). Erosion and/or focal necrosis of the mucosal epithelium was also observed in the glandular stomach of several male and female rats in the 10,000 ppm groups. Lymphocyte depletion in the thymus and spleen were also observed in rats receiving 10,000 ppm, but these changes were attributed to severe debilitation and stress. Depletion of hematopoietic cells from the bone marrow was attributed to nutrient deficiency accompanying weight loss.

Because of decreased survival in 10,000 and 25,000 ppm rats in the 15-day study, the high exposure selected for the 13-week study was 5,000 ppm.

TABLE 3
Incidences of Selected Nonneoplastic Lesions in Rats in the 15-Day Feed Study of 4,4'-Thiobis(6-*t*-Butyl-*m*-Cresol)[a]

Dose (ppm)	0	1,000	2,500	5,000	10,000
Male					
Kidney[b]	10	–[d]	–	10	10
Renal Papillary Necrosis[c]	0	–	–	0	1 (4.0)[e]
Renal Tubule Necrosis	0	–	–	0	4* (1.3)
Glandular Stomach	10	–	–	10	10
Erosion	0	–	–	0	1 (3.0)
Necrosis	0	–	–	0	2 (2.0)
Hemorrhage	0	–	–	0	4* (1.8)
Congestion	0	–	–	0	4* (1.8)
Female					
Kidney	10	–	–	10	9
Renal Papillary Necrosis	0	–	–	0	2 (3.5)
Renal Tubule Necrosis	0	–	–	0	7**(1.0)
Glandular Stomach	10	–	–	10	9
Erosion	0	–	–	0	1 (3.0)
Necrosis	0	–	–	0	3 (2.3)
Hemorrhage	0	–	–	0	2 (2.5)
Congestion	0	–	–	0	5* (2.4)

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test
** $P \leq 0.01$
a No histopathology performed on animals receiving 25,000 ppm due to 100% mortality in this group.
b Number of animals with organ examined microscopically
c Number of animals with lesion
d Animals in these groups not examined microscopically
e Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

13-WEEK STUDY

All animals survived to the end of the study (Table 4). The final mean body weights of 5,000 ppm males and females were markedly lower than those of the controls; the mean body weight of males receiving 2,500 ppm was slightly but consistently lower than that of the controls throughout the study. Feed consumption by 5,000 ppm rats was markedly lower than that by controls throughout the study. Feed consumption by 2,500 ppm males was somewhat reduced initially, but was similar to or greater than that by the controls after week 4. Rats exposed to 250, 500, 1,000, 2,500, or 5,000 ppm received approximate doses of 15, 30, 60, 165, or 315 mg TBBC per kilogram body weight per day (males) or 15, 35, 70, 170, or 325 mg/kg per day (females). Since reduction in feed consumption was apparent from the beginning of the study, the reduction would seem more likely to have been caused by decreased feed palatability than by anorexia resulting from toxicity. This conclusion is supported by the fact that diarrhea, the major clinical finding in 5,000 ppm rats, did not appear in the males until day 64 (with the exception of one male in which diarrhea was observed on day 29) or in the females until day 57.

TABLE 4
Survival, Body Weights, and Feed Consumption of Rats in the 13-Week Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 13
Male							
0	10/10	142 ± 4	359 ± 7	220 ± 7		16.3	14.9
250	10/10	140 ± 4	382 ± 6	243 ± 7	107	16.5	15.8
500	10/10	138 ± 5	378 ± 6	240 ± 7	105	16.1	16.1
1,000	10/10	139 ± 3	368 ± 5	230 ± 6	103	15.8	14.1
2,500	10/10	138 ± 4	351 ± 7	213 ± 7	98	15.2	16.7
5,000	10/10	134 ± 5	217 ± 3**	82 ± 3**	60	10.0	12.1
Female							
0	10/10	109 ± 3	209 ± 8	99 ± 7		11.2	9.9
250	10/10	108 ± 3	204 ± 5	96 ± 6	98	11.4	9.0
500	10/10	108 ± 3	200 ± 2	93 ± 3	96	11.5	9.8
1,000	10/10	107 ± 3	201 ± 3	94 ± 4	96	11.8	9.5
2,500	10/10	109 ± 3	200 ± 3	91 ± 3	96	11.9	9.3
5,000	10/10	106 ± 3	153 ± 5**	48 ± 3**	73	8.5	8.3

** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test
[a] Number of animals surviving/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams per animal per day.

A significant increase in absolute and relative liver weights occurred in females that received 5,000 ppm TBBC (Table F2). The relative, but not absolute, liver weight of 2,500 ppm males was significantly increased. As in the 15-day study, other significant differences in absolute or relative organ weights were considered due to much lower final mean body weights and not to organ-specific toxicity.

Serum alkaline phosphatase levels were significantly higher in 2,500 and 5,000 ppm males and were slightly higher in the females exposed to 5,000 ppm (Table G2). Males and females exposed to 2,500 or 5,000 ppm TBBC had significantly higher serum alanine aminotransferase levels. The increased activity of γ-glutamyl transpeptidase in rats exposed to 5,000 ppm was not considered to be biologically significant.

Hematocrit and hemoglobin concentrations in male rats exposed to 1,000, 2,500, and 5,000 ppm were significantly lower than those of the controls; these results suggest a mild anemia. However, considering the diarrhea and unthriftiness that occurred in these animals, possible dehydration could be masking larger decreases, including decreases in erythrocyte counts, or could account for the absence of changes in hematocrit or hemoglobin values in females. Since reticulocyte counts in male rats were not higher than those of the controls, the anemia in the male rats was considered nonresponsive. Mean erythrocyte volume was significantly lower in males that received 1,000 or 2,500 ppm TBBC and in males and females that received 5,000 ppm; this effect is usually associated with a disturbance in hemoglobin production and has commonly been observed with anemias of chronic inflammation or iron deficiency.

Total leukocyte counts were significantly higher in 5,000 ppm females and slightly increased in 5,000 ppm males. Male and female rats that received 5,000 ppm also exhibited significantly higher segmented neutrophil counts. Band neutrophil counts were significantly higher in all exposed female groups than in controls; the largest increase occurred in 5,000 ppm rats. These changes in leukocyte parameters are consistent with an inflammatory response.

Results of three neurotoxicity trials in 0, 1,000, and 2,500 ppm rats demonstrated a significant dose-related increase in forelimb and hindlimb grip strength (Table H1). Foot splay, tail flick, and startle response reflexes were unaffected by exposure to TBBC.

The principal lesions associated with the administration of TBBC for 13 weeks occurred in the liver and kidney, primarily in 2,500 and 5,000 ppm males and females (Table 5). The lesions in the liver consisted of scattered individual cell necrosis, individual or aggregates of enlarged Kupffer cells with abundant yellow-tan pigmented cytoplasm (Kupffer cell hypertrophy), focal accumulations of similar macrophages in or adjacent to the portal areas, and a slight increase in small bile ductules in the portal areas (Plate 3). By electron microscopy, the pigmented material in the cytoplasm of Kupffer cells was amorphous to finely granular and light to moderately electron dense with a scattering of irregular, highly electron-dense bodies. While the more abundant amorphous substance was not membrane bound, many of the smaller electron-dense bodies were partially surrounded by a plasma membrane. The cytoplasm of the Kupffer cells stained strongly positive with PAS, weakly to strongly by the Ziehl-Neelsen method for acid-fast material, and inconsistently weakly positive by Perl's iron method. While not observed by the study pathologist, enlargement of centrilobular hepatocytes, relative to the periportal hepatocytes, in the 5,000 ppm group was also observed by the Pathology Working Group. This finding is consistent with hepatocellular hypertrophy and with the higher activities of serum enzymes in the 2,500 and 5,000 ppm groups.

The kidney lesions consisted of focal, segmental degeneration and necrosis of the proximal tubule epithelium, primarily in the outer stripe of the outer medulla, and extensive pigmentation of the proximal convoluted tubule epithelium (Plate 4). The degeneration and necrosis were characterized by faintly stained, pale cells with little cytoplasmic or nuclear detail, suggestive of cytolysis and karyolysis. The pigmentation was characterized by pale, yellow-red discoloration of the epithelial cytoplasm.

Both the size and number of macrophages were increased in the mesenteric lymph nodes of male and female rats exposed to 2,500 or 5,000 ppm TBBC (Table 5).

Dose selection rationale: The exposure levels selected for the 2-year rat study were 500, 1,000, and 2,500 ppm. A high dose of 5,000 ppm was not included because of reduced body weights and the degree of liver and kidney toxicity observed in 5,000 ppm males and females in the 13-week study.

TABLE 5
Incidences of Selected Nonneoplastic Lesions in Rats in the 13-Week Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Dose (ppm)	0	250	500	1,000		2,500		5,000	
Male									
Liver[a]	10	–[c]	–	10		10		10	
Bile Duct Hyperplasia[b]	0	–	–	1	(1.0)[d]	2	(1.5)	10**	(2.0)
Kupffer Cell Hypertrophy	0	–	–	0		6**	(1.0)	10**	(3.7)
Necrosis	0	–	–	1	(1.0)	3	(1.0)	10**	(1.0)
Lymph Node, Mesenteric	10	–	–	10		10		10	
Macrophage Hyperplasia	0	–	–	1	(2.0)	2	(1.0)	10**	(3.2)
Kidney	10	–	–	10		10		10	
Necrosis	0	–	–	0		0		9**	(1.3)
Pigmentation	0	–	–	0		2	(1.0)	10**	(1.1)
Female									
Liver	10	–	–	10		10		10	
Bile Duct Hyperplasia	0	–	–	0		1	(1.0)	10**	(1.7)
Kupffer Cell Hypertrophy	0	–	–	0		10**	(1.6)	10**	(3.6)
Necrosis	0	–	–	0		1	(1.0)	10**	(1.1)
Lymph Node, Mesenteric	10	–	–	10		10		10	
Macrophage, Hyperplasia	0	–	–	0		3	(1.7)	10**	(2.9)
Kidney	10	–	–	10		10		10	
Necrosis	0	–	–	0		0		9**	(1.8)
Pigmentation	0	–	–	0		3	(1.0)	10**	(1.0)

** Significantly different ($P \leq 0.01$) from the control group by the Fisher exact test
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Animals in these groups not examined microscopically
[d] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

2-YEAR STUDY

Survival

Estimates of survival probabilities for male and female rats receiving TBBC in feed for 2 years are presented in Table 6 and in Kaplan-Meier survival curves (Figure 1). Survival rates of exposed rats were similar to those of the controls.

Body Weights, Feed Consumption, and Clinical Findings

Throughout most of the study, the mean body weights of 2,500 ppm male rats were approximately 3% lower than those of the controls and the final mean body weight was 5% lower than that of the controls. Mean body weights of 500 and 1,000 ppm males were similar to those of the controls during the study, but the final mean body weights of these groups were 5% and 6% lower than that of the controls, respectively. The mean body weights of 2,500 ppm females began to decrease 12 weeks into the study and at week 65 was 14% lower than that of the controls. The final mean body weight, however, was 6% lower than that of the controls (Figure 2 and Tables 7 and 8). Exposure levels of 500, 1,000, or 2,500 ppm TBBC resulted in a daily ingestion of 20, 40, or 100 mg/kg body weight for males or 20, 45, or 120 mg/kg body weight for females. Feed consumption by male and female rats was similar to that by controls (Tables J1 and J2). The behavior and general health and appearance of exposed male and female rats were similar to those of controls.

TABLE 6
Survival of Rats in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

	0 ppm	500 ppm	1,000 ppm	2,500 ppm
Male				
Animals initially in study	60	60	60	60
15-month interim evaluation[a]	10	10	10[e]	10
Natural deaths	9	8	6	9
Moribund	23	14	22	23
Animals surviving to study termination	18	28	22	18
Percent probability of survival at end of study[b]	36	56	42	36
Mean survival (days)[c]	614	637	633	620
Survival analysis[d]	P=0.506	P=0.049N	P=0.540N	P=1.000N
Female				
Animals initially in study	60	60	60	60
15-month interim evaluation[a]	10	10	10	10
Natural deaths	5	5	2	6
Moribund	11	14	16	16
Animals surviving to study termination	34	31[f]	32	28
Percent probability of survival at end of study	68	62	64	56
Mean survival (days)	663	651	645	644
Survival analysis	P=0.202	P=0.559	P=0.711	P=0.195

[a] Censored from survival analyses
[b] Kaplan-Meier determinations based on the number of animals alive on the first day of terminal sacrifice
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice)
[d] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed columns. A lower mortality in an exposure group is indicated by N.
[e] Three male rats exposed to 1,000 ppm were killed moribund prior to the 15-month interim evaluation.
[f] Includes one animal that died the last week of the study

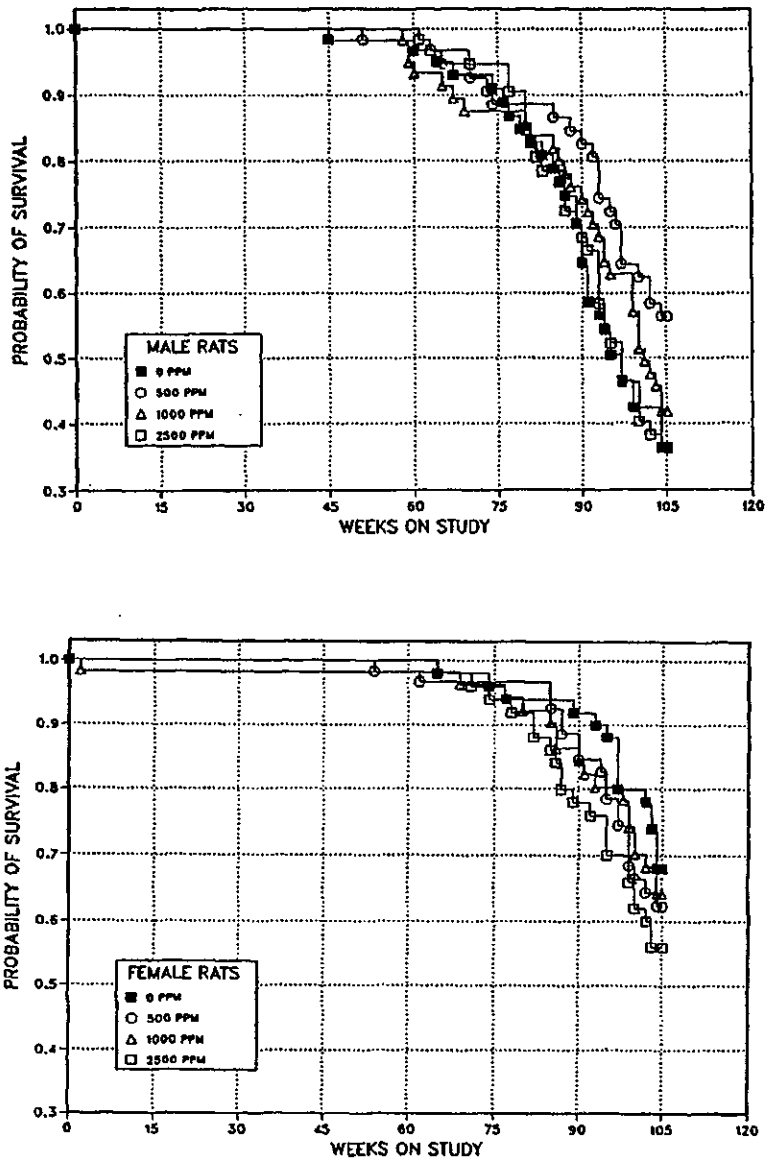


FIGURE 1
Kaplan-Meier Survival Curves for Male and Female Rats Administered 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) in Feed for 2 Years

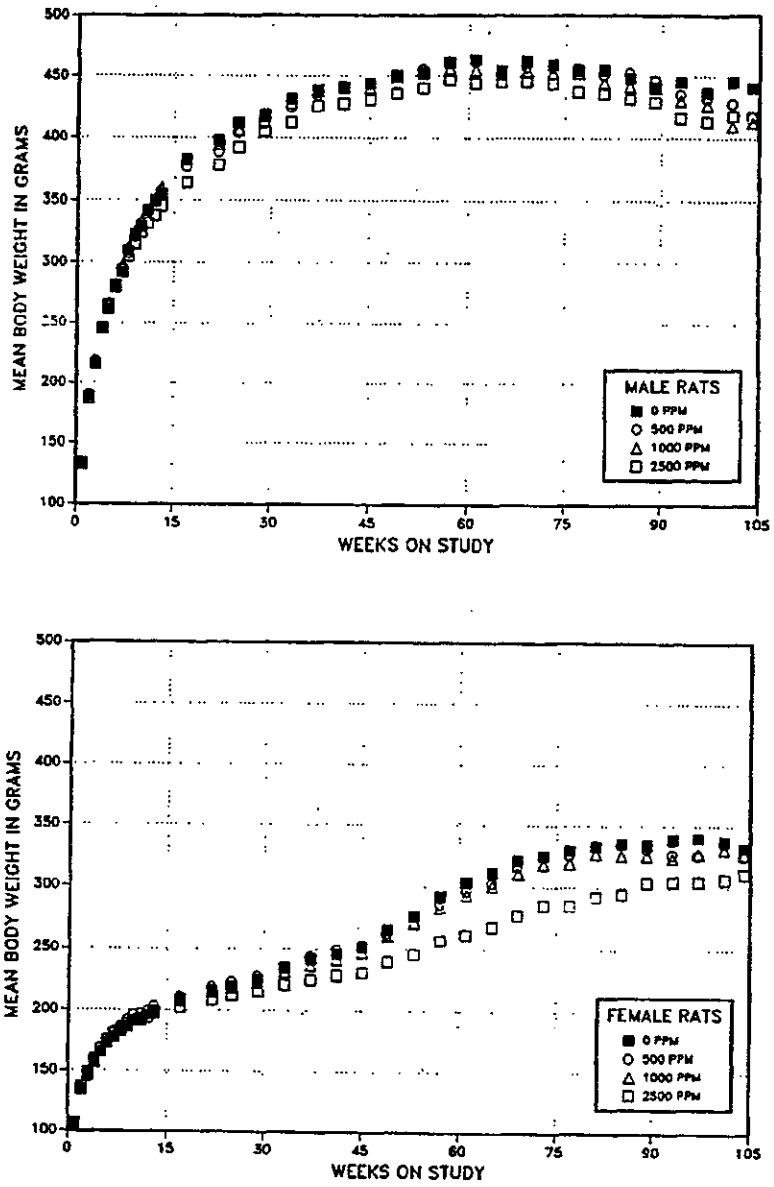


FIGURE 2
Growth Curves for Male and Female Rats
Administered 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) in Feed for 2 Years

TABLE 7
Mean Body Weights and Survival of Male Rats in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Weeks on Study	0 ppm		500 ppm			1,000 ppm			2,500 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	134	60	135	100	60	134	100	60	132	99	60
2	188	60	190	101	60	189	100	60	186	99	60
3	215	60	218	101	60	218	101	60	216	100	60
4	245	60	246	100	60	246	100	60	246	100	60
5	261	60	264	101	60	266	102	60	265	102	60
6	281	60	280	100	60	282	100	60	280	100	60
7	292	60	295	101	60	298	102	60	294	101	60
8	310	60	307	99	60	312	101	60	305	98	60
9	323	60	321	100	60	325	101	60	315	98	60
10	329	60	332	101	60	335	102	60	325	99	60
11	342	60	341	100	60	343	100	60	332	97	60
12	350	60	349	100	60	351	100	60	338	97	60
13	355	60	358	101	60	360	102	60	346	97	60
17	383	60	377	99	60	383	100	60	365	95	60
22	397	60	389	98	60	395	99	60	382	95	60
25	412	60	405	98	60	406	99	60	392	95	60
29	419	60	412	98	60	417	100	60	405	97	60
33	431	60	425	99	60	428	99	60	413	96	60
37	438	60	434	99	60	437	100	60	425	97	60
41	441	60	442	100	60	440	100	60	428	97	60
45	444	59	444	100	60	440	99	60	431	97	60
49	451	59	451	100	60	449	100	60	436	97	60
53	453	59	455	101	59	453	100	60	440	97	60
57	461	59	462	100	59	456	99	60	447	97	60
61	464	58	462	100	58	455	98	56	445	96	59
65[a]	454	47	453	100	47	452	100	48	445	98	48
69	462	46	458	99	47	455	98	46	446	96	48
73	459	46	458	100	44	453	99	46	444	97	48
77	455	43	457	100	44	453	100	46	437	96	46
81	455	41	451	99	44	445	98	44	436	96	42
85	448	39	453	101	43	442	99	43	431	96	39
89	442	35	447	101	42	441	100	40	429	97	35
93	445	28	435	98	40	430	97	37	416	94	32
97	437	24	432	99	33	427	98	33	413	95	25
101	446	21	428	96	31	410	92	27	418	94	20
104	441	20	417	95	29	413	94	23	417	95	18
Mean for weeks											
1-13	279		280	100		281	101		275	99	
14-52	427		424	99		422	100		409	96	
53-104	451		452	99		442	98		433	96	

[a] Interim evaluation occurred.

TABLE 8
Mean Body Weights and Survival of Female Rats in the 2-Year Feed Study of 4,4'-Thiobis(6-*t*-Butyl-*m*-Cresol)

Weeks on Study	0 ppm		500 ppm			1,000 ppm			2,500 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	107	60	107	100	60	107	100	60	107	100	60
2	134	60	137	102	60	136	101	60	137	102	60
3	146	60	148	102	60	147	101	59	149	102	60
4	156	60	159	102	60	158	101	59	160	102	60
5	165	60	169	102	60	166	101	59	168	102	60
6	173	60	176	102	60	174	101	59	175	101	60
7	178	60	182	103	60	178	101	59	180	101	60
8	182	60	186	102	60	184	101	59	184	101	60
9	186	60	192	103	60	188	101	59	189	102	60
10	192	60	196	103	60	192	100	59	191	100	60
11	192	60	197	103	59	194	101	59	193	101	60
12	196	60	200	102	60	196	100	59	193	99	60
13	199	60	203	102	60	198	100	59	197	99	60
17	209	60	211	101	60	208	99	59	202	97	60
22	216	60	219	101	60[b]	215	100	59	209	97	60
25	219	60	223	102	60	218	100	59	212	97	60
29	225	60	228	101	60	223	99	59	216	96	60
33	235	60	236	100	60	232	99	59	221	94	60
41	246	60	249	101	60	241	98	59	228	93	60
45	252	60	253	100	60	248	98	59	231	92	60
49	266	60	263	100	60	261	98	59	240	90	60
53	277	60	277	100	60	272	98	59	246	89	60
57	293	60	287	98	59	284	97	59	257	88	60
61	304	60	297	98	59	294	97	59	262	86	60
65[a]	312	49	303	97	48	301	97	49	268	86	49
69	322	49	316	98	48	312	97	48	278	86	49
73	326	49	324	100	48	319	98	48	286	88	48
77	330	47	327	99	48	320	97	48	286	87	47
81	334	47	335	100	48	328	98	46	293	88	46
85	336	47	335	100	46	326	97	45	295	88	43
89	335	47	331	99	44	327	98	43	304	91	39
93	339	46	327	96	42	324	96	41	305	90	38
97	341	40	327	96	38	328	96	40	305	90	35
101	338	40	336	100	33	331	98	35	307	91	31
104	333	37	326	98	32	327	98	32	311	94	28
Mean for weeks											
1-13	170		173	102		171	101		171	101	
14-52	234		236	101		231	99		220	94	
53-104	323		318	98		314	97		286	89	

[a] Interim evaluation occurred.
[b] The number of animals weighed for this week is less than the number of animals surviving.

Hematology, Clinical Chemistry, and Urinalysis

Results of hematology evaluations at 3, 9, and 15 months are presented in Tables G3 through G6. Slight but significant decreases in hematocrit levels, hemoglobin concentrations, and erythrocyte counts were observed in one set of 1,000 and 2,500 ppm males at 15 months, but not in the other set. These differences were not observed in males at 3 or 9 months. Similar significant decreases in hematocrit level and hemoglobin concentration occurred in 2,500 ppm females at 9 months; hemoglobin concentrations of 2,500 ppm females were significantly decreased in both sets evaluated at 15 months, but hematocrit levels were similar to those of the controls. Mean erythrocyte hemoglobin counts and concentration in the 2,500 female group were significantly lower than those of the controls at 9 months and in both sets of animals evaluated at 15 months. Platelet counts in 2,500 ppm males and females were slightly but significantly higher than those of the controls at 3 and 9 months, as were the platelet counts of 2,500 ppm males in one set of animals evaluated at 15 months and of 2,500 ppm females in the other set. While the results of the hematology evaluations were somewhat variable, they do suggest a slight chemical-related effect. It is not clear, however, if these differences indicate a direct effect on stem cells in the bone marrow or on circulating erythrocytes, or if they are secondary to other physiological alterations caused by TBBC.

Clinical chemistry results for rats evaluated at 3 and 9 months and for the two sets of rats evaluated at 15 months were generally similar (Tables G3, G4, G5, and G6). Serum activities of alkaline phosphatase, alanine aminotransferase, and sorbitol dehydrogenase in 2,500 ppm males were significantly greater that those of the controls at each evaluation. Alkaline phosphatase activities in both sets of 1,000 ppm males evaluated at 15 months were also significantly greater than those of controls. Serum activities of alanine aminotransferase and sorbitol dehydrogenase in 2,500 ppm females were also significantly greater than those in the controls at each evaluation. These results are consistent with hepatocellular damage caused by TBBC.

Urine volumes of all exposed groups of males and females were significantly lower than those of the controls at 3 months, but not at later evaluations. This is consistent with decreased water or feed intake in the exposed groups, but it is not considered a direct chemical effect. Elevated urine creatinine concentrations at the 3-month evaluation, particularly in exposed groups of male rats, indicate that the urine constituents were more highly concentrated in these groups and are consistent with the volume measurements. Urine specific gravity was not measured, however. The urinary activity of *N*-acetyl-ß-D-glucosaminidase was mildly increased at all evaluations in 2,500 ppm females in comparison to controls. Differences in other urine enzyme activities between exposed and control rats were variable and not considered chemical related.

Neurotoxicity Evaluation

At 3 months, there was no difference in startle reflex between exposed and control male groups and, in contrast to the findings in the 13-week study, there were no differences in forelimb or hindlimb grip strength between exposed and control groups in the first three trials (Table H2). The standard methodology for measuring grip strength consists of three trials. However, eight trials were used in the chronic study, and the grip strength of control groups decreased with subsequent trials, apparently due to fatigue or habituation. Although the grip strength of exposed groups also decreased with repeated trials, the decrement was less than that of the controls. Thus, grip strength in later trials (particularly that of the forelimbs) of each exposed group was significantly greater than controls. The electrophysiologic evaluation revealed no significant inhibitory effects of TBBC on motor nerve excitability or conduction, neuromuscular transmission, or muscle contractility (Tables H4, H5, and H6). Further, there were no microscopic lesions that could be attributed to TBBC observed in the sciatic nerve, quadriceps muscle, or teased nerve preparations of the sciatic nerve.

In the reversibility study, the effects on grip strength observed at 3 months were no longer evident at the 6 month evaluation (Table H3). The results of the remaining neurotoxicity studies at 6 months were similar to those at 3 months (Tables H4, H5, and H6), and there were no significant effects of TBBC on motor nerve excitability or conduction, neuromuscular transmission, muscle contractility, or pathology.

Pathology and Statistical Evaluation

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and nonneoplastic lesions in the liver, kidney, thyroid gland, uterus, and mammary gland. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix A for male rats and Appendix B for female rats.

Liver: At the 15-month interim evaluation, both the absolute and relative liver weights of 2,500 ppm females were significantly greater than those of the controls (Table F3). Relative liver weights of 2,500 ppm males and 1,000 ppm females were also significantly greater than those of the controls.

The incidence of Kuppfer cell hypertrophy was significantly increased in 2,500 ppm males and females at the 15-month interim evaluation and at the end of the 2-year study (Tables 9, A5, and B5). At 15 months, the incidence of cytoplasmic vacuolization was significantly increased in all exposed groups of males and in 2,500 ppm females. At 2 years, the incidence of cytoplasmic vacuolization was slightly increased in 1,000 and 2,500 ppm males and significantly increased in 1,000 and 2,500 ppm females. Also at 2 years, the incidence of fatty change was significantly increased in 2,500 ppm females. Cytoplasmic vacuolization was characterized by the presence of multiple, small vacuoles, whereas fatty change was indicated by the presence of single, large cytoplasmic vacuoles. In both instances, these changes are presumably the result of lipid accumulation.

At 15 months, the incidence of basophilic foci was significantly increased in 2,500 ppm males and these foci were present in all females; the incidences in exposed males and females at terminal sacrifice were similar to those in the controls. Incidences of mixed cell foci were significantly increased in 2,500 ppm males and females at 15 months and in 1,000 and 2,500 ppm males and females at the end of the study; at each time point, the incidence of mixed cell foci in 2,500 ppm females was twice that in 2,500 ppm males. Hepatocyte foci were characterized as basophilic, eosinophilic, clear, or mixed based on cytoplasmic staining properties. These differences in staining properties are generally attributed to variations in the amounts of rough or smooth endoplasmic reticulum, glycogen, or fat. Thus, basophilic foci consist predominantly of cells with greater amounts of rough endoplasmic reticulum, while eosinophilic foci consist of cells with more smooth endoplasmic reticulum. Clear cell foci consist of cells with vacuolated cytoplasm caused by the accumulation of lipid or with clear cytoplasm caused by the accumulation of glycogen. The mixed cell foci consist of cells with either basophilic or eosinophilic cytoplasm and cells with vacuolated or clear cytoplasm.

The incidences of hepatocellular adenoma or carcinoma (combined) in exposed male rats were not significantly greater than that in the control group (Tables 9 and A3).

TABLE 9
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Rats in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Dose (ppm)	0	500	1,000	2,500
Male				
15-Month Interim Evaluation				
Liver[a]	10	10	7	10
Kupffer Cell Hypertrophy[b]	0	0	0	10** (1.2)[c]
Cytoplasmic Vacuolization	1 (1.0)	10** (1.1)	7** (1.0)	10** (1.7)
Basophilic Focus	5	2	7	10*
Mixed Cell Focus	1	1	1	5
2-Year Study				
Liver	50	50	50	49
Kupffer Cell Hypertrophy	2 (1.5)	3 (1.0)	2 (1.0)	31** (2.1)
Cytoplasmic Vacuolization	13 (1.2)	11 (1.5)	19 (1.4)	18 (2.0)
Basophilic Focus	18	22	23	22
Mixed Cell Focus	6	14	18*	15*
Clear Cell Focus	2	0	1	1
Eosinophilic Focus	3	7	2	1
Hepatocellular Adenoma				
Overall rates[d]	1/50 (2%)	2/50 (4%)	3/50 (6%)	4/49 (8%)
Adjusted rates[e]	5.6%	7.1%	13.6%	17.0%
Terminal rates[f]	1/18 (6%)	2/28 (7%)	3/22 (14%)	2/18 (11%)
First incidence (days)	729 (T)	729 (T)	729 (T)	625
Logistic regression test[g]	P=0.091	P=0.653	P=0.377	P=0.177
Hepatocellular Carcinoma	0/50 (0%)	1/50 (2%)	0/50 (0%)	1/49 (2%)
Hepatocellular Adenoma or Carcinoma[h]				
Overall rates	1/50 (2%)	3/50 (6%)	3/50 (6%)	5/49 (10%)
Adjusted rates	5.6%	10.7%	13.6%	21.0%
Terminal rates	1/18 (6%)	3/28 (11%)	3/22 (14%)	2/18 (11%)
First incidence (days)	729 (T)	729 (T)	729 (T)	625
Logistic regression test	P=0.056	P=0.472	P=0.377	P=0.100

(continued)

TABLE 9
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Rats in the 2-Year Feed Study of 4,4'-Thiobis(6-*t*-Butyl-*m*-Cresol) (continued)

Dose (ppm)	0		500		1,000		2,500	
Female								
15-Month Interim Evaluation								
Liver	10		10		10		10	
Kupffer Cell Hypertrophy	1	(1.0)	0		5	(1.0)	10**	(2.7)
Cytoplasmic Vacuolization	0		1	(1.0)	1	(1.0)	8**	(1.0)
Basophilic Focus	10		10		10		10	
Eosinophilic Focus	0		0		1		0	
Mixed Cell Focus	0		1		0		10**	
2-Year study								
Liver	50		50		50		50	
Kupffer Cell Hypertrophy	11	(1.2)	10	(1.5)	9	(1.0)	42**	(2.7)
Cytoplasmic Vacuolization	12	(1.3)	10	(1.4)	20*	(1.3)	34**	(2.7)
Fatty Change	9	(1.4)	8	(1.5)	15	(1.3)	19*	(1.5)
Basophilic Focus	37		34		38		36	
Mixed Cell Focus	5		4		14*		34**	
Eosinophilic Focus	5		7		8		4	
Clear Cell Focus	0		1		1		1	
Adenoma	0		0		0		1	

* Significantly different (P≤0.05) by the Fisher exact test (15-month interim evaluation) or the logistic regression test (terminal sacrifice)
** (P≤0.01)
(T) Terminal sacrifice
[a] Number of animals with liver examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)
[d] Number of animals with neoplasm per number of animals with liver examined microscopically
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these neoplasms as nonfatal.
[h] Historical incidence for 2-year feed studies with untreated control groups (mean ± standard deviation): 41/1,251 (3.3% ± 3.6%); range 0%-10%

Kidney: Nephropathy is a common occurrence in aging F344/N rats and was observed in nearly all males and the majority of females in this study. In comparison to the control group, the severity of nephropathy was significantly increased in 2,500 ppm females both at 15 months and 2 years (Table 10). The number of females with a moderate severity of nephropathy was much higher in the 2,500 ppm group than in the control group, whereas the reverse was true for minimal nephropathy. The severity of nephropathy was similar among all groups of male rats.

TABLE 10
Incidences and Severity of Nephropathy in Female Rats in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Dose (ppm)	0	500	1,000	2,500
15-Month Interim Evaluation				
Kidney[a]	10	10	10	10
Nephropathy[b]	9	10	10	10
Absent (Grade 0)	1	0	0	0
Minimal (Grade 1)	6	8	9	0
Mild (Grade 2)	3	2	1	8
Moderate (Grade 3)	0	0	0	2
Marked (Grade 4)	0	0	0	0
Group average severity grade	1.2	1.2	1.1	2.2**
2-Year Study				
Kidney	50	50	50	50
Nephropathy	44	41	46	48
Absent (Grade 0)	6	9	4	2
Minimal (Grade 1)	17	14	19	1
Mild (Grade 2)	26	25	22	29
Moderate (Grade 3)	1	2	5	18
Marked (Grade 4)	0	0	0	0
Group average severity grade	1.4	1.4	1.6	2.3**

** Significantly different (P≤0.01) from the control group by the Mann-Whitney U test
[a] Number of animals with kidney examined microscopically
[b] Number of animals with lesion

Thyroid gland: The incidence of C-cell adenoma or carcinoma (combined) occurred with a significant positive trend in female rats and was slightly, but not significantly, increased in the 1,000 and 2,500 ppm groups at the end of the 2-year study (0 ppm, 3/49; 500 ppm, 4/49; 1,000 ppm, 8/50; 2,500 ppm, 9/50; Table B3). This positive trend was not considered chemical related because the incidence in 2,500 ppm females was only slightly above the historical average of 15% and well within the range of 6% to 31% for historical controls (Table B4b). Further, C-cell hyperplasia was decreased in females (28/49, 24/49, 27/50, 18/50; Table B5), although the decrease in 2,500 ppm females was not statistically significant by pairwise comparison.

Uterus: Stromal polyps occurred with a significant positive trend (0 ppm, 2/50; 500 ppm, 5/50; 1,000 ppm, 9/50; 2,500 ppm, 9/50; Table B3) in the uteri of female rats exposed to TBBC. Increased incidences of stromal polyps in females exposed to 1,000 or 2,500 ppm were significant; however, the incidences are only slightly above the historical control average of 16% and are well within the historical control range of 2% to 30% (Table B4c). The incidence in controls is unusually low compared to that in historical controls. Stromal sarcoma was also present in one 500 ppm and one 2,500 ppm female.

Mammary gland: The incidence of fibroadenoma occurred with a statistically significant negative trend in female rats (29/50, 24/50, 11/50, 16/50; Table B3), and the decreases were significant in the 1,000 and 2,500 ppm groups. There was also a significant negative trend in the incidence of mammary gland fibroadenoma, adenoma, or carcinoma (combined) in females (32/50, 24/50, 11/50, 16/50; Table B3).

MICE

15-DAY STUDY

All 10,000 and 25,000 ppm male and female mice and eight males and eight females receiving 5,000 ppm TBBC died (Table 11). The two surviving 5,000 ppm males had a mean body weight loss of 25% and a final mean body weight 35% lower than that of the controls; the final mean body weight of 2,500 ppm males was similar to that of the controls. The two surviving 5,000 ppm females had a mean body weight loss of 10% and a final mean body weight 27% lower than that of the controls; the final mean body weight of 2,500 ppm females was 13% lower than that of the controls. Male and female mice receiving 1,000 ppm TBBC had final mean body weights similar to those of the controls. Feed consumption by 5,000, 10,000, and 25,000 ppm males and females was markedly lower than that by controls. Mice exposed to 1,000, 2,500, or 5,000 ppm received approximate doses of 285, 585, or 475 mg TBBC per kilogram body weight per day (males) or 360, 950, or 1,030 mg/kg per day (females). Approximate doses for mice exposed to 10,000 or

TABLE 11
Survival, Body Weights, and Feed Consumption of Mice in the 15-Day Feed Study of 4,4'-Thiobis(6-t-Butyl-m-Cresol)

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 2
Male							
0	10/10	21.3 ± 0.4	24.2 ± 0.7	3.0 ± 0.6		6.7	9.1
1,000	10/10	21.6 ± 0.5	26.1 ± 0.5	4.5 ± 0.2	108	5.9	7.7
2,500	10/10	21.9 ± 0.2	23.8 ± 0.4	2.0 ± 0.5	98	4.0	6.7
5,000	2/10[d]	21.0 ± 0.6	15.9 ± 0.4**	−5.3 ± 0.3**	65	1.2	2.3
10,000	0/10[e]	21.7 ± 0.5	–	–	–	1.0	1.4
25,000	0/10[f]	22.0 ± 0.4	–	–	–	1.7	–[g]
Female							
0	10/10	15.7 ± 0.3	18.9 ± 0.4	3.1 ± 0.3	–	6.1	13.1
1,000	10/10	15.5 ± 0.3	19.3 ± 0.2	3.8 ± 0.4	103	4.8	7.8
2,500	10/10	16.2 ± 0.4	16.5 ± 0.5**	0.3 ± 0.4**	87	4.2	8.2
5,000	2/10[h]	15.3 ± 0.2	13.8 ± 0.1**	−1.2 ± 0.7**	73	2.2	3.8
10,000	0/10[i]	16.4 ± 0.3	–	–	–	1.3	–[g]
25,000	0/10[j]	16.8 ± 0.2*	–	–	–	0.9	–[g]

* Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test
** (P≤0.01)
a Number of animals surviving at 15 days/number initially in group
b Weights are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the studies. No final mean body weights or body weight changes were calculated for groups with 100% mortality.
c Feed consumption is expressed as grams per animal per day.
d Day of death: 10, 12, 12, 12, 13, 14, 15, 15
e Day of death: 8, 8, 9, 10, 10, 10, 11, 11, 12, 12
f Day of death: 4, 4, 4, 5, 5, 5, 6, 6, 6, 6
g All animals in these exposure groups died prior to the second week of the study
h Day of death: 9, 10, 10, 10, 11, 11, 11, 15
i Day of death: 6, 7, 7, 7, 7, 8, 8, 8, 8, 8
j Day of death: 4, 4, 4, 4, 5, 5, 5, 5, 5, 5

to 10,000 or 25,000 ppm cannot be calculated due to early deaths. Reduced feed consumption by exposed groups was seen as early as the first day of the study. The reduction in feed consumption was attributed to poor feed palatability.

Diarrhea was observed in 25,000 ppm mice beginning on either day 2 or day 3 of the study. Diarrhea was also present in most 10,000 ppm males (beginning on day 8) and females (beginning on day 2). Five 5,000 ppm males exhibited diarrhea (beginning on day 9), as did nine 5,000 ppm females (beginning on day 2).

Significantly different absolute or relative organ weights in exposed groups of mice were associated with lower mean body weights or were attributed to severe debilitation and stress (thymus, spleen) and were not considered to be the result of organ-specific toxicity (Table F4).

Because all 10,000 and 25,000 ppm male and female mice died and because of morbidity in surviving 5,000 ppm males, hematology parameters were measured only in males and females receiving 1,000 or 2,500 ppm and in 5,000 ppm females (Table G7). Segmented neutrophil counts were significantly higher in 2,500 and 5,000 ppm females. The increases were modest and were not accompanied by an increase in the number of immature cells, suggesting that these increases were not an inflammatory response. The increased numbers of circulating mature neutrophils may have been related to a shift in the total blood pool distribution without an absolute increase.

Significant increases in mean erythrocyte hemoglobin concentration values occurred in all surviving exposed male and female mice. Increased mean erythrocyte hemoglobin concentration is not a physiologic possibility and is usually an artifact caused by sample handling or analytical error. However, any condition that would cause increased erythrocyte fragility leading to increased post-sampling hemolysis could cause an increase in mean erythrocyte hemoglobin concentration values.

Microscopic examination was not performed on tissues from mice in the 10,000 or 25,000 ppm groups because they died before the end of the study. Kidneys were examined microscopically in the 2,500 and 5,000 ppm groups. The principal lesion caused by the ingestion of TBBC was minimal focal renal tubule necrosis in eight males and three females that received 5,000 ppm. Most of the affected mice also had a few protein casts within tubule lumens. Depletion of cells from the bone marrow and lymphoid organs was observed in many mice in the 5,000 ppm group. Bone marrow depletion was attributed to nutrient deficiency accompanying weight loss; depletion of lymphoid organs is commonly associated with low body weight, debilitation, and stress.

13-WEEK STUDY

All animals survived to the end of the study (Table 12). The final mean body weight of 2,500 ppm males was 15% lower than that of the controls. Female mice receiving 500, 1,000, or 2,500 ppm TBBC had final mean body weights 11%, 15%, and 22% lower than that of the controls, respectively. Final mean body weights of mice in other exposure groups were similar to those of the controls. Due to spillage and scattering, there were limitations in measuring feed consumption by mice and the data were difficult to interpret. Feed consumption by 2,500 ppm males averaged 24% less than that by the controls through week 3 of the study and was similar to that by the controls throughout the remainder of the study. No conclusions can be drawn from the slight variations in feed consumption observed in the male control group in the latter part of the study. Feed consumption by 2,500 ppm females averaged 27% less than that by the controls during most of the study. Mice exposed to 100, 250, 500, 1,000, or 2,500 ppm received approximate doses of 15, 30, 65, 145, or 345 mg TBBC per kilogram body weight per day (males) or 10, 35, 60, 165, or 340 mg/kg per day (females). Variations in feed consumption by males or females at other exposure levels did not appear to be chemical related. Since no clinical findings related to TBBC administration were observed in the present study, the reduction in feed consumption by 2,500 ppm females was probably due to poor feed palatability.

TABLE 12
Survival, Body Weights, and Feed Consumption of Mice in the 13-Week Feed Study of 4,4′-Thiobis(6-t-Butyl-m-Cresol)

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g) Initial	Final	Change	Final Weight Relative to Controls (%)	Feed Consumption[c] Week 1	Week 13
Male							
0	9/9	21.3 ± 0.4	30.8 ± 1.1	9.5 ± 0.8		3.3	2.8
100	10/10	21.5 ± 0.5	30.6 ± 1.0	9.0 ± 0.6	99	3.6	2.9
250	10/10	21.8 ± 0.4	31.7 ± 0.6	9.8 ± 0.6	103	3.1	3.5
500	10/10	21.6 ± 0.6	30.5 ± 0.9	8.9 ± 0.6	99	3.7	3.2
1,000	10/10	22.2 ± 0.4	30.8 ± 0.6	8.7 ± 0.6	100	–[d]	3.8
2,500	10/10	21.6 ± 0.4	26.3 ± 0.4**	4.7 ± 0.3**	85	2.6	4.0
Female							
0	10/10	17.7 ± 0.3	30.7 ± 0.8	13.0 ± 0.8		3.0	3.4
100	10/10	17.7 ± 0.3	28.1 ± 0.7	10.4 ± 0.6**	91	2.2	2.6
250	10/10	17.9 ± 0.3	29.2 ± 0.7	11.3 ± 0.6**	95	3.1	3.4
500	10/10	17.9 ± 0.4	27.3 ± 0.7**	9.4 ± 0.4**	89	2.8	3.4
1,000	10/10	17.7 ± 0.3	26.0 ± 0.4**	8.3 ± 0.3**	85	2.9	4.2
2,500	10/10	17.9 ± 0.3	23.8 ± 0.5**	5.9 ± 0.4**	78	2.0	3.7

** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test
[a] Number of animals surviving/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams per animal per day.
[d] Feed consumption values were invalid due to technical error.

Absolute and relative liver weights of 2,500 ppm males and females were slightly but significantly greater than those of the controls (Table F5). Males exposed to 500, 1,000, or 2,500 ppm and females exposed to 2,500 ppm had significantly increased absolute and relative spleen weights. Differences in the absolute or relative weights of other organs were related to reductions in mean body weights.

The erythrocyte counts, hematocrit and hemoglobin concentrations, and mean erythrocyte volume values of 2,500 ppm males and females were significantly less than those of the controls (Table G8). The hematocrit and erythrocyte counts of 1,000 ppm males and females were also significantly reduced. These differences were consistent with a developing mild microcytic, normochromic, nonresponsive anemia similar to differences observed in male rats in the 13-week study.

The principal lesions associated with the administration of TBBC to mice for 13 weeks occurred in the liver and were similar to those observed in rats (Table 13). The lesions were only observed in 2,500 ppm mice. The lesions in the liver consisted of individual or aggregates of enlarged Kupffer cells with abundant yellow-tan, pigmented cytoplasm (Kupffer cell hypertrophy), focal accumulations of similar macrophages in or adjacent to the portal areas, and a slight increase in small bile ductules in the portal areas (bile duct hyperplasia) (Plates 5 and 6). As in rats, the mesenteric lymph nodes of the 2,500 ppm mice contained increased numbers of enlarged macrophages.

Dose selection rationale: Because of the reduction in mean body weights, the increase in liver and spleen weights, and the accompanying histopathologic changes of the liver in 2,500 ppm males and females, the exposures selected for the 2-year study in mice were 250, 500, and 1,000 ppm.

TABLE 13
Incidences of Selected Nonneoplastic Lesions in Mice in the 13-Week Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Dose (ppm)	0	100	250	500	1,000	2,500
Male						
Liver[a]	9	–[c]	–	–	10	10
Bile Duct Hyperplasia[b]	0	–	–	–	0	10**(1.0)[d]
Kupffer Cell Hypertrophy	0	–	–	–	0	10**(4.0)
Lymph Node, Mesenteric	9	–	–	–	10	10
Macrophage, Hyperplasia	0	–	–	–	0	5* (1.0)
Female						
Liver	10	–	–	–	10	10
Bile Duct Hyperplasia	0	–	–	–	0	6**(1.0)
Kupffer Cell Hypertrophy	0	–	–	–	0	10**(3.4)
Lymph Node, Mesenteric	10	–	–	–	10	10
Macrophage, Hyperplasia	0	–	–	–	1 (1.0)	1 (2.0)

* Significantly different (P≤0.05) from the control group by Fisher's exact test
** P≤0.01
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Organ not examined microscopically
[d] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

2-YEAR STUDY

Survival

Estimates of survival probabilities for male and female mice administered TBBC in feed for 2 years are presented in Table 14 and in Kaplan-Meier survival curves (Figure 3). Survival rates of exposed males and females were similar to those of the controls.

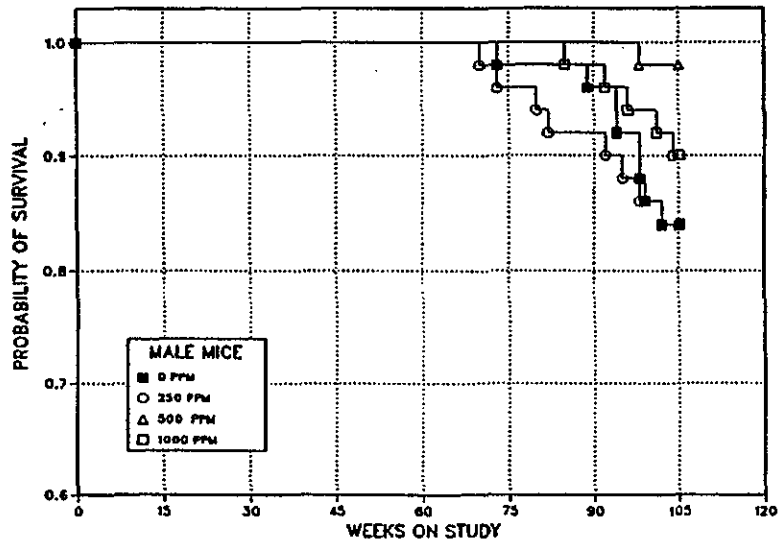
Body Weights, Feed Consumption, and Clinical Findings

The mean body weight of male mice receiving 1,000 ppm TBBC was approximately 10% lower than that of the controls from week 45 through the end of the study (Table 15). The mean body weight of males receiving 500 ppm TBBC was slightly lower than that of the controls throughout the study. The mean body weight of 250 ppm males was similar to that of the controls throughout the study. The mean body weight of 1,000 ppm females was 11% lower than that of the controls by week 45 and was 18% lower by the end of the study (Table 16 and Figure 4). Final mean body weights of 250 and 500 ppm females were approximately 9% lower than that of the controls. Exposure levels of 250, 500, or 1,000 ppm resulted in a daily ingestion of TBBC of 30, 60, or 145 mg/kg body weight for males or 45, 110, or 255 mg/kg for females. Feed consumption by exposed male mice was similar to that by the controls (Tables J3 and J4). No clinical findings were attributed to TBBC administration.

TABLE 14
Survival of Mice in the 2-Year Feed Study of 4,4'-Thiobis(6-t-Butyl-m-Cresol)

	0 ppm	250 ppm	500 ppm	1,000 ppm
Male				
Animals initially in study	60	60	60	60
15-month interim evaluation[a]	10	10	10	10
Natural deaths	6	6	1	4
Moribund	2	2	0	1
Animals surviving to study termination	42[e]	42[e]	49	45
Percent probability of survival at end of study[b]	84	84	98	90
Mean survival (days)[c]	673	667	683	678
Survival analysis[d]	P=0.242N	P=0.859	P=0.036N	P=0.536N
Female				
Animals initially in study	60	60	60	60
15-month interim evaluation[a]	9	9	10	10
Natural deaths	7	9	11	11
Moribund	4	3	3	4
Missing[a]		1		
Animals surviving to study termination	40[e]	38	36	35
Percent probability of survival at end of study	79	76	72	71
Mean survival (days)	658	660	654	644
Survival analysis	P=0.346	P=1.000	P=0.651	P=0.468

[a] Censored from survival analyses
[b] Kaplan-Meier determinations based on the number of animals alive on the first day of terminal sacrifice
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice)
[d] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed columns. A negative trend or lower mortality in an exposure group is indicated by N.
[e] Includes one animal that died the last week of the study



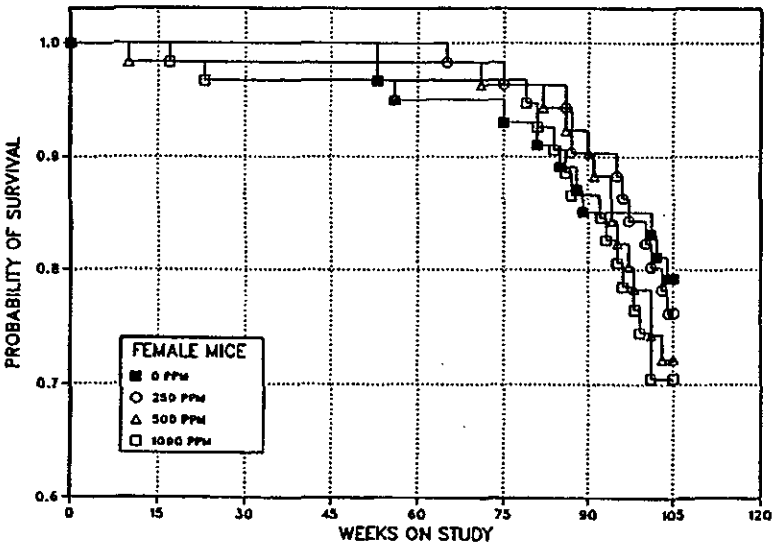


FIGURE 3
Kaplan-Meier Survival Curves for Male and Female Mice Administered 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) in Feed for 2 Years

TABLE 15
Mean Body Weights and Survival of Male Mice in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Weeks on Study	0 ppm		250 ppm			500 ppm			1,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	22.1	60	22.2	101	60	22.2	101	60	22.4	101	60
2	23.5	60	23.8	101	60	23.9	102	60	24.4	104	60
3	24.7	60	24.8	100	60	25.1	102	60	25.2	102	60
4	25.4	60	25.5	100	60	25.9	102	60	25.9	102	60
5	26.5	60	26.2	99	60	26.6	100	60	26.4	100	60
6	27.3	60	27.2	100	60	27.4	100	60	27.3	100	60
7	27.8	60	27.8	100	60	27.8	100	60	28.0	101	60
8	28.8	60	28.5	99	60	28.6	99	60	28.4	99	60
9	29.2	60	29.1	100	60	28.8	99	60	28.8	99	60
10	30.2	60	30.1	100	60	29.6	98	60	29.3	97	60
11	30.6	60	30.4	99	60	30.2	99	60	29.9	98	60
12	31.6	60	31.2	99	60	31.0	98	60	30.5	97	60
13	32.0	60	31.5	98	60	31.1	97	60	30.9	97	60
17	35.1	60	34.5	98	60	33.8	96	60	33.3	95	60
21	37.0	60	36.4	98	60	35.7	97	60	34.8	94	60
25	38.0	60	37.2	98	60	36.2	95	60	35.3	93	60
29	38.9	60	37.8	97	60	36.7	94	60	35.8	92	60
33	41.1	60	40.1	98	60	39.3	96	60	37.6	92	60
37	41.5	60	42.0	101	60	40.6	98	60	37.9	91	60
41	42.3	60	42.2	100	60	41.1	97	60	38.5	91	60
45	44.2	60	43.5	98	60	42.2	96	60	39.9	90	60
49	45.6	60	44.7	98	60	43.6	96	60	41.3	91	60
53	46.8	60	46.1	99	60	44.5	95	60	42.3	90	60
57	47.5	60	46.9	99	60	45.6	96	60	43.3	91	60
61	48.0	60	46.9	98	60	45.8	95	60	43.2	90	60
65[a]	48.3	60	47.5	98	60	45.9	95	60	44.1	91	60
69	47.7	50	47.1	99	50	46.0	96	50	43.7	92	50
73	47.8	50	47.5	99	49	46.0	96	50	43.4	91	50
77	48.8	49	49.0	100	48	47.5	97	50	44.9	92	50
81	48.3	49	48.8	101	47	47.5	98	50	43.9	91	50
85	47.5	49	48.5	102	46	45.8	96	50	42.8	90	50
89	46.9	49	47.2	101	46	45.3	97	50	42.8	91	49
93	46.4	48	47.4	102	45	44.5	96	50	42.3	91	48
97	46.5	46	49.2	106	44	45.2	97	50	42.6	92	47
101	46.0	43	48.3	105	43	45.0	98	49	42.8	93	46
104	47.0	42	49.5	105	42	46.2	98	49	43.2	92	45
Mean for weeks											
1-13	27.7		27.6	96		27.6	100		27.5	99	
14-52	40.4		39.8	99		38.8	96		36.2	90	
53-104	47.4		47.9	101		45.7	96		43.2	91	

[a] Interim evaluation occurred.

TABLE 16
Mean Body Weights and Survival of Female Mice in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Weeks on Study	0 ppm Av. Wt. (g)	0 ppm No. of Survivors	250 ppm Av. Wt. (g)	250 ppm Wt. (% of controls)	250 ppm No. of Survivors	500 ppm Av. Wt. (g)	500 ppm Wt. (% of controls)	500 ppm No. of Survivors	1,000 ppm Av. Wt. (g)	1,000 ppm Wt. (% of controls)	1,000 ppm No. of Survivors
1	18.2	60	18.2	100	60	18.5	102	60	18.6	102	60
2	20.2	60	20.5	102	60	20.5	102	60	20.7	103	60
3	21.3	60	21.7	102	60	21.8	102	60	21.9	103	60
4	22.6	60	22.6	100	60	22.5	100	60	22.7	100	60
5	23.6	60	23.6	100	60	23.5	100	60	23.6	100	60
6	24.6	60	24.5	100	60	24.3	99	60	24.5	100	60
7	25.2	60	25.3	100	60	25.1	100	60	25.3	100	60
8	26.1	60	25.8	99	60	25.8	99	60	25.8	99	60
9	27.0	60	26.6	99	60	26.5	98	60	26.4	98	60
10	28.2	60	27.8	99	60	27.2	97	60	27.2	97	60
11	28.6	60	28.3	99	60	27.8	97	59	27.8	97	60
12	29.4	60	29.0	99	60	28.4	97	59	28.4	97	60
13	30.3	60	29.8	98	60	28.7	95	59	28.9	95	60
17	33.3	60	32.8	99	60	32.1	96	59	31.3	94	59
21	35.8	60	34.9	98	60	34.2	96	59	33.5	94	59
25	36.2	60	35.3	98	59	34.4	95	59	33.6	93	58
29	37.8	60	35.9	95	59	35.2	93	59	34.3	91	58
33	40.6	60	39.1	96	59	38.5	95	59	36.8	91	58
37	41.1	60	40.6	99	59	40.0	97	59	37.3	91	58
41	41.9	60	40.8	97	59	40.0	96	59	38.0	91	58
45	43.9	60	42.7	97	59	41.7	95	59	39.2	89	58
49	45.1	60	44.1	98	59	43.0	95	59	40.3	89	58
53	46.8	60	45.8	98	59	44.6	95	59	42.1	90	58
57	49.1	57	47.0	96	59	45.8	93	59	42.7	87	58
61	49.8	57	47.5	95	59	46.8	94	59	43.0	86	58
65[a]	50.5	57	48.1	95	58	48.1	95	59	43.5	86	58
69	49.9	48	48.3	97	49	47.3	95	49	43.1	86	48
73	51.2	48	48.4	95	49	47.6	93	48	43.4	85	48
77	53.2	47	50.2	94	48	48.7	92	48	44.4	84	48
81	52.5	47	50.1	95	48	47.8	91	48	43.2	82	47
85	51.7	46	49.0	95	48	46.8	91	47	42.5	82	45
89	51.2	44	49.3	96	45	46.6	91	46	42.5	83	43
93	51.0	43	48.3	95	45	45.2	89	44	42.0	82	41
97	50.9	43	49.7	98	42	45.9	90	41	42.0	83	39
101	50.2	43	46.7	93	41	45.0	90	38	41.1	82	36
104	50.7	40	46.4	92	38	46.0	91	36	41.6	82	35
Mean for weeks											
1-13	25.0		24.9	100		24.7	99		24.8	99	
14-52	39.5		38.4	97		37.7	95		36.0	91	
53-104	50.6		48.2	95		46.6	92		42.7	84	

[a] Interim evaluation occurred.

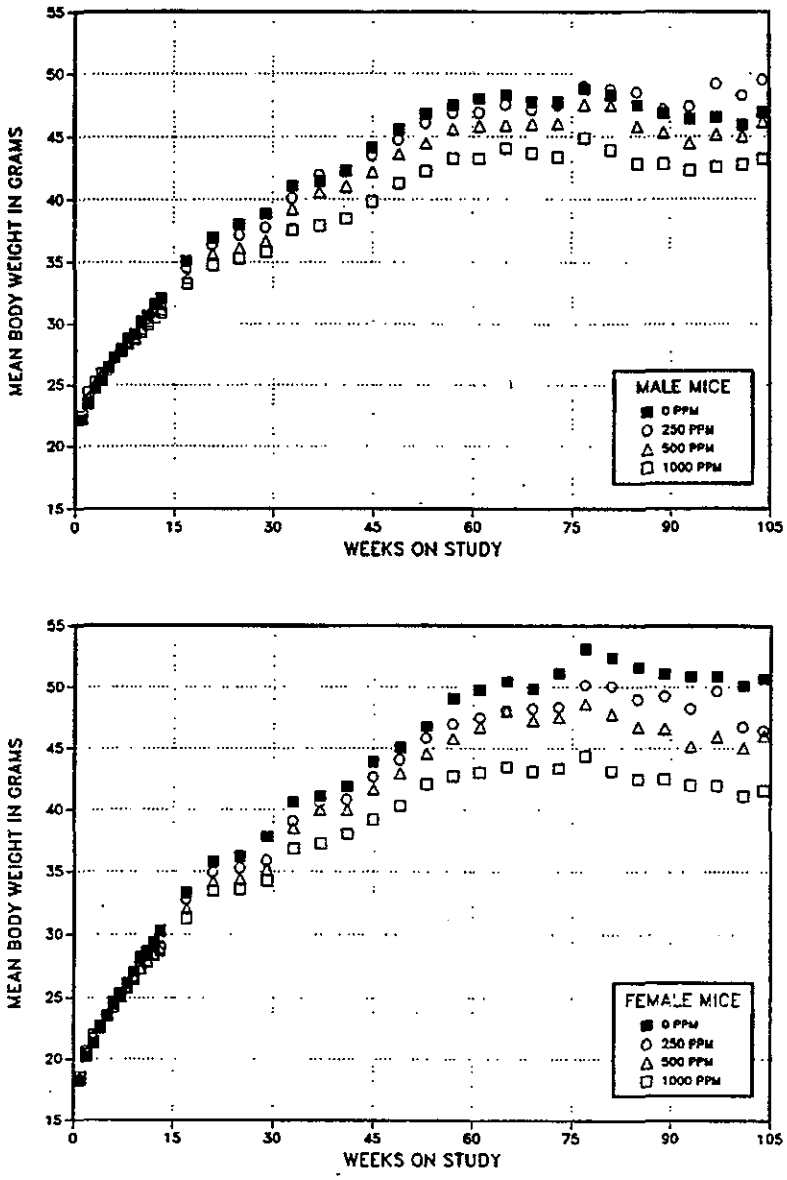


FIGURE 4
Growth Curves for Male and Female Mice Administered 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) in Feed for 2 Years

Hematology and Clinical Chemistry

Significantly lower hematocrit level, hemoglobin concentration, and erythrocyte count in 1,000 ppm males at 15 months were considered evidence of a mild normocytic normochromic nonresponsive anemia (Table G11). These decreases were similar to those that occurred in rats. Significantly decreased total leukocyte counts occurred in 500 and 1,000 ppm male mice at the 15-month interim evaluation.

Serum alkaline phosphatase (ALP) activities in 1,000 ppm males were slightly but significantly greater than those of the controls at 3 and 9 months (Tables G9 and G10). While ALP activity in 1,000 ppm males was numerically greater than that in controls at 15 months, the difference was not statistically significant. The ALP activity in 1,000 ppm females at 9 months was significantly greater than that in controls. Serum levels of total bilirubin in 250, 500, and 1,000 ppm males were significantly greater than those in controls at 9 and 15 months. At 9 months, the serum total bilirubin level in 250 ppm males was also significantly greater. These findings are consistent with hepatocellular damage.

Pathology and Statistical Evaluation

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and nonneoplastic lesions in the liver and bone marrow. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix C for male mice and Appendix D for female mice.

Liver: At the 15-month interim evaluation, the relative liver weight of 1,000 ppm females was greater than that of controls due to a decrease in mean body weight in this group (Table F6). Absolute and relative liver weights of all other exposed male and female mice were similar to those of the controls. The incidence and severity of cytoplasmic vacuolization occurred with a negative trend in male mice (lipid accumulation was characterized as cytoplasmic vacuolization at 15 months, and as fatty change at 2 years, based on criteria discussed previously on page 41 in the rat study) (Tables 17, C3, and C5). An eosinophilic focus was present in one 500 ppm male at 15 months. At the end of the study, the incidences of fatty change, clear cell and eosinophilic foci, and hepatocellular adenoma or carcinoma (combined) all occurred with negative trends in male mice. Most of the negative trends were statistically significant and most occurrences in 1,000 ppm males were significant by pairwise comparison. A basophilic focus was present in one 500 ppm male.

Bone marrow: Myelofibrosis was present in all groups of females with a significant positive trend (0 ppm, 21/51; 250 ppm, 18/50; 500 ppm, 23/50; 1,000 ppm, 34/50; Table D4) and the incidence in 1,000 ppm females was significant by pairwise comparison.

GENETIC TOXICOLOGY

TBBC (33 to 10,000 μg/plate) was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 when tested in a pre-incubation protocol with and without Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9 (Table E1; Zeiger *et al.*, 1987). A precipitate was observed on plates treated with 333 μg or greater TBBC. In cytogenetic tests with cultured Chinese hamster ovary cells, TBBC induced sister chromatid exchanges with and without S9, at doses which induced cell cycle delay (Table E2). No induction of chromosomal aberrations was observed in these cells, with or without S9 (Table E3). Because of TBBC-induced cell cycle delay, cultures analyzed for chromosomal aberrations were incubated for 20.5 hours, rather than the usual 12 hours, to allow sufficient cells to accumulate for harvest.

TABLE 17
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Male Mice in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Dose (ppm)	0	250	500	1,000
15-Month Interim Evaluation				
Liver[a]	10	10	10	10
Cytoplasmic Vacuolization[b]	6 (2.7)[c]	2 (2.0)	3 (2.3)	1* (1.0)
Eosinophilic Focus	0	0	1	0
Hepatocellular Adenoma	0	2	4	2
2-Year Study				
Liver	50	50	50	50
Fatty Change	19 (1.9)	17 (2.0)	5**(2.0)	6**(1.0)
Clear Cell Focus	6	5	2	0*
Eosinophilic Focus	2	3	2	0
Basophilic Focus	0	0	1	0
Focus, Any Type	8	8	5	0**
Hepatocellular Adenoma or Carcinoma[d]				
Overall rate[e]	25/50 (50%)	30/50 (60%)	27/50 (54%)	16/50 (32%)
Adjusted rate[f]	55.4%	62.4%	54.0%	34.7%
Terminal rate[g]	22/42 (52%)	24/42 (57%)	26/49 (53%)	15/45 (33%)
First incidence (days)	620	489	682	638
Logistic regression test[h]	P=0.018N	P=0.221	P=0.471	P=0.046N

* Significantly different (P≤0.05) from the control group by the Fisher exact test (15-month interim evaluation) or the logistic regression test (2-year study)
** P≤0.01
[a] Number of animals with liver examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)
[d] Historical incidence for 2-year feed studies with untreated control groups (mean ± standard deviation): 485/1,366 (35.5% ± 14.3%); range 10%-68%
[e] Number of animals with neoplasm per number of animals with liver examined microscopically
[f] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[g] Observed incidence at terminal kill
[h] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these neoplasms as nonfatal. A negative trend or lower incidence in an exposed group is indicated by N.

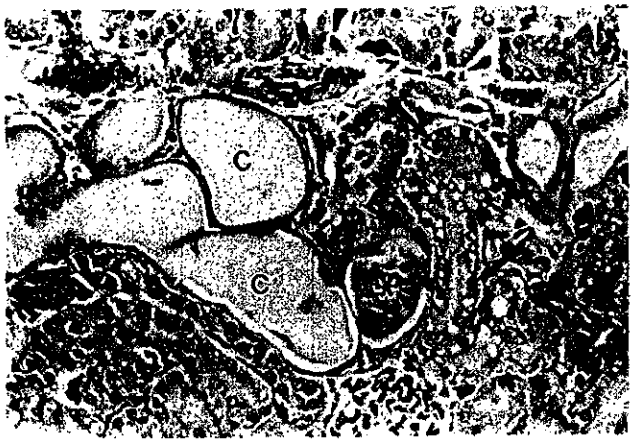


PLATE 1
Kidney of a female F344/N rat receiving 10,000 ppm 4,4′-thiobis(6-*t*-butyl-*m*-cresol) in the 15-day feed study. A segment of a proximal convoluted tubule with flattened epithelium is distended by a hyaline cast (C). Note the adjacent tubule filled with exfoliated necrotic cells (*) and other tubules with vacuolated epithelial cells and pyknotic nuclei (arrows). H&E, 80×

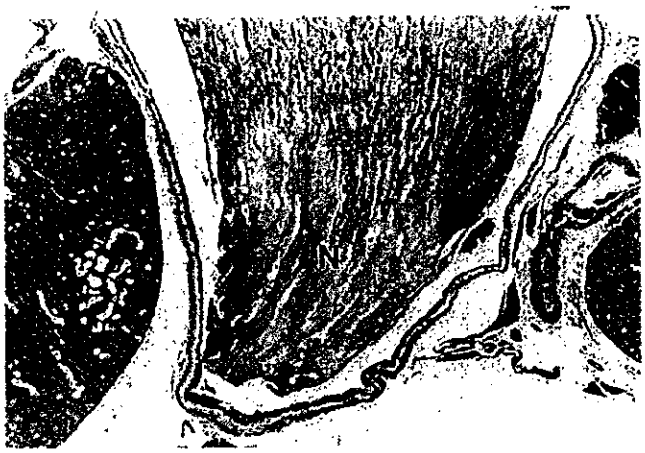


PLATE 2
Kidney of another female F344/N rat receiving 10,000 ppm 4,4′-thiobis(6-*t*-butyl-*m*-cresol) in the 15-day feed study. Note the coagulation necrosis of the renal papilla (N). H&E, 10×

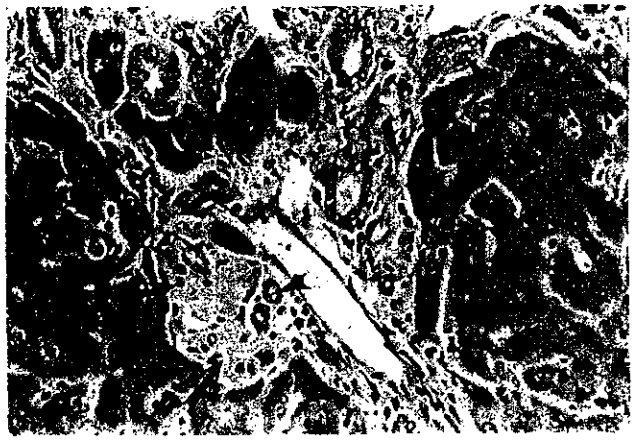


PLATE 3
Liver of a male F344/N rat receiving 5,000 ppm 4,4′-thiobis(6-*t*-butyl-*m*-cresol) in the 13-week feed study. Note the accumulation of enlarged Kupffer cells in the hepatic sinusoids and portal area (arrows) and proliferation of small bile ductules (arrow heads). H&E, 80×

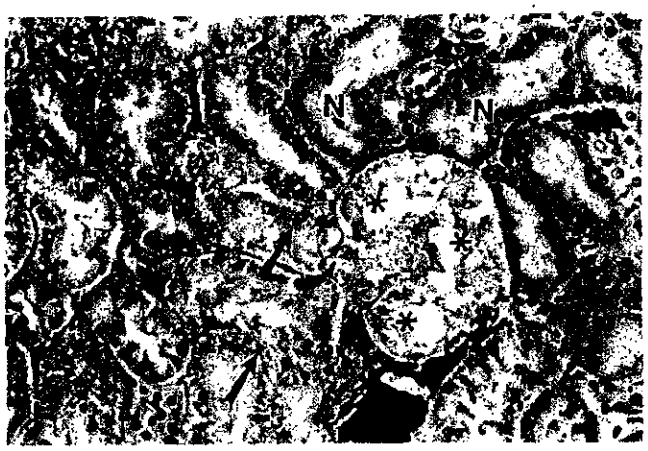


PLATE 4
Kidney of a male F344/N rat receiving 5,000 ppm 4,4′-thiobis(6-*t*-butyl-*m*-cresol) in the 13-week feed study. The segment of proximal convoluted tubule in the center of the field exhibits complete necrosis of the epithelium (*). Adjacent tubules exhibit necrosis of individual cells which have pyknotic nuclei (arrows). Compare with normal tubule epithelium (N). H&E, 80×

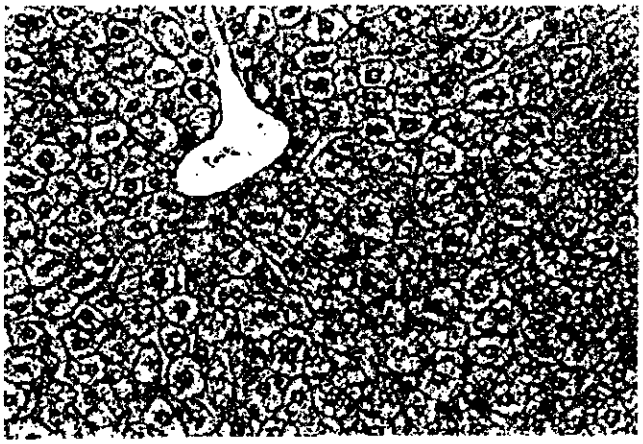


PLATE 5
Liver of a control male B6C3F$_1$ mouse in the 13-week feed study of 4,4′-thiobis(6-*t*-butyl-*m*-cresol). Compare with Plate 6. H&E, 80×

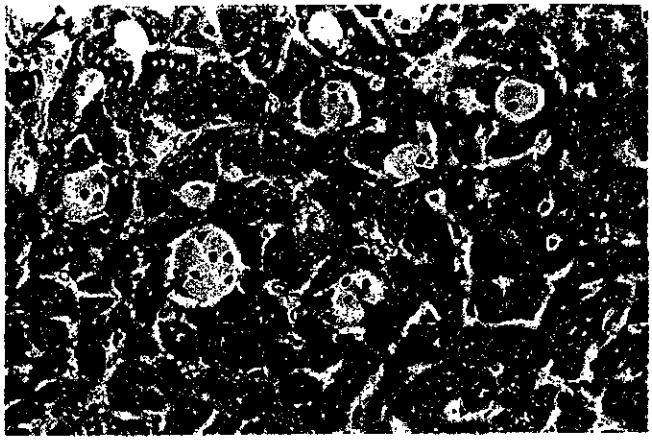


PLATE 6
Liver of a male B6C3F$_1$ mouse receiving 2,500 ppm 4,4′-thiobis(6-*t*-butyl-*m*-cresol) in the 13-week feed study. Note the scattered individual and small clusters of enlarged Kupffer cells (arrows) and the proliferation of small bile ductules (arrow heads). The hepatocyte nuclei are larger than normal and the cytoplasm contains an increased amount of basophilic material (rough endoplasmic reticulum). H&E, 80×

DISCUSSION AND CONCLUSIONS

4,4′-Thiobis(6-*t*-butyl-*m*-cresol) (TBBC) is used in the rubber and plastics industries as an antioxidant and as a stabilizer in polyethylene and polyolefin food packaging materials. Because of concern regarding the elevated cancer risk of workers in the rubber industry, the National Cancer Institute nominated TBBC for toxicology and carcinogenesis studies as a representative of the sulfur-containing class of antioxidants used in rubber processing. Because food packaging appeared to represent the most widespread potential for human exposure, the oral route of administration was chosen for the 15-day, 13-week, and 2-year studies in F344/N rats and B6C3F$_1$ mice.

The principal toxic effects associated with the administration of TBBC in the present studies occurred in the liver and kidney of rats and mice. With the exception of the renal lesions observed in the 15-day and 13-week studies, these findings are in agreement with the few studies reported in the literature. Birnbaum *et al.* (1983) reported that the liver was the major site of metabolism of TBBC in rats and that the compound was excreted primarily in the bile. In a 30-day feed study in rats, 2,500 ppm TBBC produced increased liver weight and growth retardation; rats fed diets containing 500 ppm for 90 days displayed only reduced feed consumption and slight growth retardation (Lefaux, 1968). A dose-related increase in liver weight accompanied by a slight increase in the number of Kupffer cells was reported in females exposed to 200 mg/kg in a study in which mice were administered 10, 100, or 200 mg/kg daily by gavage for 14 days (Munson *et al.*, 1988). In the NTP 15-day studies in rats or mice receiving TBBC in feed at doses ranging from 1,000 to 25,000 ppm, liver toxicity was not observed in surviving animals. However, in the NTP 13-week studies in rats, absolute and relative liver weights were significantly greater in females receiving 5,000 ppm than in controls. Males and females in the 2,500 and 5,000 ppm groups exhibited Kupffer cell hypertrophy, hepatocyte necrosis, and bile duct hyperplasia. In addition, males and females exposed to 5,000 ppm TBBC also exhibited centrilobular hepatocyte hypertrophy. Consistent with these histopathologic findings in the 13-week rat studies, there were significant elevations in serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Increased levels of ALT are usually associated with damage to hepatocytes; increases in ALP are usually associated with biliary disease. Male and female rats receiving 5,000 ppm in these studies exhibited a significant increase in size and number of macrophages in the mesenteric lymph nodes; a lesser, but similar response occurred in 2,500 ppm rats.

The 13-week NTP study in mice also elicited hepatotoxicity in 2,500 ppm males and females as exhibited by slight but significant increases in absolute and relative liver weights and the presence of Kupffer cell hypertrophy and bile duct hyperplasia. The response in rats at the same exposure level (2,500 ppm) was similar, except that liver weights in 2,500 ppm rats were unaffected and necrosis and centrilobular hypertrophy were observed in rats but not in mice. Based on average daily feed consumption, 2,500 ppm rats ingested roughly one-third as much TBBC on a body weight basis as mice. Thus, the liver of rats may be more sensitive than that of mice to the effects of this chemical. Additionally, there was a mild increase in size and number of macrophages in mesenteric lymph nodes of male and female mice administered 2,500 ppm; this response was similar to that observed in 2,500 ppm rats.

In the 2-year rat study, the highest exposure level (2,500 ppm TBBC) produced liver toxicity. At this exposure level, males and females exhibited increases in liver weights, Kupffer cell hypertrophy, cytoplasmic vacuolization, and basophilic and mixed cell foci at the 15-month interim evaluation and at the end of the 2-year study. In addition, marked significant increases in serum ALT and sorbitol dehydrogenase activities (SDH) occurred in males and females at the 15-month evaluation; these cytoplasmic enzymes are released into the blood following hepatocellular injury. The mild but significant increases in ALP which occurred in males in various exposure groups at the 3-, 9-, and 15-month evaluations are indicative of disturbances involving the hepatobiliary system. This increase did not occur in females. Although certain liver responses occurred in males and females, liver weight increase was more pronounced in females, there was a strong significant increase in the incidence of cytoplasmic vacuolization

in females but not in males, and mixed cell foci occurred in twice as many 2,500 ppm females as 2,500 ppm males. Thus, the preponderance of these responses occurred in females.

The incidence of hepatocellular adenoma or carcinoma (combined) was slightly increased in male rats administered 2,500 ppm TBBC (0 ppm, 1/50; 500 ppm, 3/50; 1,000 ppm, 3/50; 2,500 ppm, 5/49), but the increased incidence was not significant and did not exceed the range of 0% to 10% in historical control male rats. Furthermore, the incidences of these neoplasms were not increased in females, despite the fact that females demonstrated a greater number of different nonneoplastic responses. Therefore, the incidence of hepatocellular adenoma or carcinoma (combined) in male rats is not considered a carcinogenic response to TBBC.

In contrast to the findings in the 13-week study at 2,500 ppm, liver weights of mice were unaffected and there were no microscopic findings of hepatotoxicity in mice exposed to 1,000 ppm TBBC in feed for 2 years. Since 1,000 ppm male and female mice actually had a greater average daily ingestion of TBBC on a mg/kg body weight basis than did rats exposed to 2,500 ppm TBBC, the lack of microscopic findings in mice may indicate (as appeared to be the case in the 13-week studies) a higher degree of liver sensitivity in rats. This conclusion is strengthened by the marked significant increase in ALT and SDH found in rats but not mice. Total bilirubin in 1,000 ppm male mice was slightly but significantly greater than that in controls at 9 and 15 months. This response did not occur in female mice or in rats. In addition, the serum activity of ALP was significantly higher in male and female mice at various exposure levels and time points; these increases were milder in degree but similar to those that occurred in the rats. Increases in serum levels of total bilirubin would be consistent with either cholestasis or a liver function disorder in which circulating bilirubin could not be removed by the liver for conjugation and excretion. Increases in both ALP activity and total bilirubin concentration would be consistent with cholestasis. However, increases in total bilirubin concentration related to cholestasis are usually accompanied by increases in direct bilirubin, which did not occur in the present studies. In males, liver lesions which occurred with a significant negative trend included fatty change, clear cell foci, and hepatocellular adenoma or carcinoma (combined). The significant negative trends were considered to be related to the administration of TBBC. In 1,000 ppm male mice, the incidence of hepatocellular adenoma or carcinoma (combined) was significantly lower than that of controls by pairwise comparison. This result may be due to the reduction in mean body weight, since a significant positive association has been found between liver neoplasm prevalence and body weight in male B6C3F$_1$ mice (Rao *et al.*, 1990).

Evidence of kidney toxicity was present in rats and mice in the NTP 15-day studies and in rats in the 13-week study. In 10,000 ppm rats in the 15-day study, necrosis of the papilla was observed in one female and two males and focal necrosis of the tubules was observed in four males and seven females. Eight male mice and three female mice receiving 5,000 ppm in the 15-day study had tubule necrosis. Following 13 weeks of exposure, pigmentation and degeneration of the renal cortical tubule epithelial cells were present in male and female rats receiving 2,500 or 5,000 ppm; mild to moderate cortical tubule necrosis was also found in 5,000 ppm males and females. These lesions appear to be related to the administration of TBBC. Kidney lesions were not reported in the feed studies summarized by Lefaux (1968) in which rats were exposed to 500 or 2,500 ppm for 30 days and 50 or 500 ppm for 90 days. In the present NTP 2-year rat study, chronic nephropathy common in aging rats was found in nearly all animals. However, the severity of nephropathy in 2,500 ppm females was significantly greater than that in the control group, and the increase was attributed to the administration of TBBC. In remaining female exposure groups and in all exposed males, the severity of nephropathy was similar to that of the controls.

In the 13-week NTP studies, TBBC again affected hematology parameters in rats and mice. Significant decreases in hemoglobin and hematocrit values occurred in male rats and male and female mice; mean erythrocyte volume values were significantly lower in rats and mice; erythrocyte counts were significantly lower in mice but not in rats; and neutrophil counts were significantly higher in rats but not in mice.

In the 2-year study, results of hematocrit and hemoglobin analyses performed on two sets of male rats evaluated at 15 months were conflicting. However,

the results in each set of females indicated significant decreases; male mice also had a significant decrease in these parameters and in erythrocyte counts.

The significant increases in platelets which occurred mainly in 2,500 ppm male and female rats in the 2-year study are consistent with a reactive thrombocytosis. This condition has been observed with inflammations, trauma, surgery, hyposplenic or asplenic states, malignancies, acute blood loss, and hyperadrenocorticism.

The neurotoxicity evaluation in the 13-week study demonstrated statistically significant increases in grip strength in exposed rats, which did not occur in the 2-year study. While these evaluations were performed on animals of the same strain and age using the same methodology, they were conducted at two different laboratories. Therefore, the toxicologic significance of the positive findings in the 13-week study is uncertain. Further, no significant effects of TBBC were found on motor nerve excitability or conduction, neuromuscular transmission, muscle contractility, or neuropathology.

Although the rate of survival was less than 50% in 1,000 ppm male rats (42%) and 2,500 ppm male rats (36%), the survival rate for the control group was only 36% and reduced survival does not appear to be chemical related. Further, 50% of the 2,500 ppm males survived until week 97 and 50% of the 1,000 ppm male rats survived until week 101, allowing adequate time for the possible development of neoplasms. Some degree of chemical-related toxicity in 2,500 ppm rats was observed; mean body weights of rats in this group were slightly but consistently reduced, despite the fact that feed consumption by this group was similar to that by the controls. The final mean body weight of 2,500 ppm males was 5% less than that of the controls; the mean body weight of females exposed to 2,500 ppm TBBC dropped to 14% below that of the controls at week 65 and was 6% lower than that of the controls at the end of the study. There was also enough evidence of liver toxicity in the 2,500 ppm male and female rats in the 2-year study to indicate that a greater exposure level would have compromised the sensitivity of the study to detect neoplasia. In addition, exposure to 5,000 ppm TBBC in the 13-week study resulted in a significant increase in absolute and relative liver weight in females, marked reductions in final mean body weights and feed consumption in both males and females, and liver and kidney toxicity in males and females, as mentioned earlier. These observations indicate that rats could not have tolerated an exposure level much higher than 2,500 ppm.

Although no overt organ toxicity was observed in mice in the highest exposure group in the 2-year study (1,000 ppm), the reductions in final mean body weights were indicative of a toxic response to TBBC. The final mean body weights of 1,000 ppm male and female mice were 8% and 18% lower than that of the controls, respectively; feed consumption by the 1,000 ppm males was similar to that by the controls. In the 13-week study, 2,500 ppm males had a final mean body weight 15% lower than that of the controls and the final mean body weight of 2,500 ppm females was 22% lower than that of the controls. This exposure level also produced Kupffer cell hypertrophy and bile duct hyperplasia in males and females. At 15 months, males had a significant increase in total bilirubin at all exposure levels and 500 and 1,000 ppm females had a significant elevation in ALP. It is probable that an exposure level greater than 1,000 ppm for 2 years would have caused severe weight loss and liver toxicity in mice.

CONCLUSIONS

Under the conditions of these 2-year feed studies, there was *no evidence of carcinogenic activity** of 4,4'-thiobis(6-*t*-butyl-*m*-cresol) in male or female F344/N rats administered 500, 1,000, or 2,500 ppm or in male or female B6C3F$_1$ mice administered 250, 500, or 1,000 ppm.

Nonneoplastic lesions associated with exposure to TBBC included: Kupffer cell hypertrophy, cytoplasmic vacuolization, and mixed cell foci in the liver of male and female rats, fatty change in the liver of female rats, and an increase in the severity of nephropathy in the kidney of female rats. In addition, decreased incidences of fibroadenoma, adenoma, or carcinoma (combined) were observed in the mammary gland of female rats. Decreases also occurred in the incidences of fatty change, clear cell foci, and adenoma or carcinoma (combined) in the liver of male mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 11. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 13.

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(トリフルオロメチル)ベンゼンのラットを用いる反復経口投与毒性・生殖発生毒性併合試験

Combined Repeat Dose and Reproductive/Developmental Toxicity Screening Test of Trifluoromethylbenzene by Oral Administration in Rats

要　約

(トリフルオロメチル)ベンゼンは，化学産業の分野で染料あるいは高重合体の原料として使用されている化学物質である．本被験物質の経口投与によるLD_{50}値は，マウスで10000 mg/kg，ラットで15000 mg/kgであることが報告されているが[1]，反復投与毒性あるいは生殖発生毒性についての報告は見当たらない．今回，OECD既存化学物質安全性点検に係わる毒性試験の一環として，(トリフルオロメチル)ベンゼンの，20，100および500 mg/kgをCrj：CD(SD系)ラットの雌雄(各12匹/群)に交配前14日間，雄ではその後交配期間を含む35日間，雌では交配期間，妊娠期間および分娩後3日まで経口投与し，親動物に対する反復投与毒性および生殖能力ならびに次世代児の発生・発育に及ぼす影響について検討した．

1.反復投与毒性

一般状態，体重推移，摂餌量および血液学検査では，被験物質投与の影響はみられなかった．血液生化学検査では，500 mg/kg群に総蛋白質，アルブミン，総コレステロール，トリグリセライドおよびリン脂質の増加ならびにグルコースの減少が認められた．剖検では，500 mg/kg群の雄で腎臓の肥大および退色がみられ，器官重量では雄で100 mg/kg以上の群に肝臓および腎臓重量の増加，雌では500 mg/kg群に肝臓重量の増加が認められた．病理組織学検査では，100 mg/kg以上の群の雌雄で小葉中心性の肝細胞の肥大がみられ，雄で近位尿細管上皮の硝子滴の出現，壊死および好塩基性変化ならびに近位尿細管の拡張が認められた．

2.生殖発生毒性

親動物の生殖に関しては，性周期，雌雄の交尾率，授(受)胎率，黄体数，着床数，妊娠期間，出産率および分娩状態に被験物質投与の影響は認められなかった．死産率，出生児数，出生率および出生児の性比に被験物質投与の影響はみられず，外表異常の発生もなかった．出生児体重の増加抑制が20 mg/kg以上の投与群でみられたが，剖検では被験物質投与の影響は認められなかった．

以上のことから，本試験条件下における反復投与毒性に関する無影響量は雌雄とも20 mg/kg，生殖発生毒性に関する無影響量は親動物では500 mg/kg，出生児では20 mg/kg未満と推察された．

方　法

1.被験物質および投与液の調製

(トリフルオロメチル)ベンゼン(純度99.7%，Lot No.KCM2054，和光純薬工業㈱提供)は，エタノールおよびエーテルに易溶，水に不溶の無色透明の液体である．入手後の被験物質は室温で保管し，投与期間終了後に供給源にて分析を行って試験期間中安定であったことを確認した．媒体にはコーンオイル(キシダ化学㈱，Lot No L51257F)を使用し，これに被験物質を0.4，2および10 w/v%濃度になるように懸濁して投与液を調製した．調製した投与液は冷蔵保存した．なお，投与開始週に，投与液の濃度を測定し，設定値の±10%以内にあることを確認した．また，投与開始前に，本調製法による0.1，1および10 w/v%懸濁液が低温遮光下で調製後8日間安定であることを確認した．

2.使用動物および飼育条件

9週齢のSprague-Dawley系ラット(Crj：CD，日本チャールス・リバー㈱)を雌雄各55匹購入し，7日間の検疫馴化を行ったのち，雌雄各48匹を選んで10週齢で試験に使用した．投与開始時の体重は雄で319.7～391.2 g，雌で224.7～265.0 gであった．動物は温度24±2℃，湿度55±10%，照明12時間(午前7時～午後7時)および換気回数13回/時に設定したバリアーシステム飼育室でステンレススチール製ハンガーケージに，投与期間中は1匹(雌雄別)，交配期間中は2匹(雌雄各1匹)，妊娠および哺育期間中は床敷(ホワイトフレーク，日本チャールス・リバー㈱)を入れたポリカーボネイト製ケージに1匹ずつ(哺育期間中は哺育児を含む)収容し，飼育した．飼料は，固型飼料(MF，オリエンタル酵母工業㈱)を，飲水は次亜塩素酸ナトリウムを添加(約2 ppm)した水をそれぞれ自由に摂取させた．

3.投与量，投与方法，試験群構成および群分け

投与量は，予備試験の結果より設定した．すなわち，本被験物質の0，100，500および1000 mg/kgを2週間反復投与した結果，500 mg/kg以上の群でGPT，総コレステロールおよびリン脂質の増加または増加傾向ならびに肝臓重量の増加がみられ，100 mg/kg群にもその傾向が窺われた．したがって，本試験では高用量を500 mg/kgとし，以下100および20 mg/kgを設定した．

投与経路は経口とし，雄では交配前14日間およびその後交配期間を含む35日間の合計49日間，雌では交配前14日間，交配期間(最長14日間)，妊娠期間および哺育3日までの期間，1日1回，胃管を用いて投与した．投与容量は5 ml/kgとし，雄ならびに交配前および交配期間中の雌については最新の体重を基に，交尾成立後の雌については妊娠0日の体重を基にそれぞれ算出した．

試験群は，上記3用量にコーンオイルのみを投与する対照を加え計4群とした．1群当たりの動物数は雌雄各12匹とし，群分けは，投与開始前日の体重を基に層別連続無作為化法で行った。

4.反復投与毒性に関する観察・検査

1)一般状態

雌雄とも，全例について一般状態の観察および死亡の有無を毎日観察した．

2)体重および摂餌量

体重については，雄は投与期間を通じて週2回測定した．雌は，交配前の投与期間および交配期間中は週2回，妊娠期間中は妊娠0，4，7，10，14，17および21日，哺育期間中は哺育0(分娩日)および4日に測定した．摂餌量については，交配期間を除き体重測定日に測定したが，妊娠および哺育0日は測定せず，翌日測定した．

3)血液学検査

雄全例について，投与期間終了後に，18時間以上絶食させたのち，ペントバルビタール・ナトリウム麻酔下に開腹し，腹部大静脈から採血を行った．採取した血液は EDTA-2K処理(EDTA-2K加血液)して多項目自動血球計数装置(Sysmex CC-780，東亜医用電子㈱)を用いて，白血球数(電気抵抗検出方式)，赤血球数(電気抵抗検出方式)，ヘモグロビン量(オキシヘモグロビン法)，ヘマトクリット値(血球パルス波高値検出方式)および血小板数(電気抵抗検出方式)を測定し，これらを基に平均赤血球容積(MCV)，平均赤血球血色素量(MCH)および平均赤血球血色素濃度(MCHC)を算出した．

4)血液生化学検査

血液学検査に引き続き採取した血液を室温で約60分間放置後，3000回転/分で10分間遠心分離し，得られた血清を用いて自動分析装置(736-10，㈱日立製作所)により，総蛋白質(ビウレット法)，アルブミン(BCG法)，A/G比(総蛋白質およびアルブミンより算出)，総ビリルビン(アルカリアゾビリルビン法)，GOT(Karmen法)，GPT(Wróblewski-La Due法)，γ-グルタミルトランスペプチダーゼ(L-γ-グルタミル-DBHA基質法)，アルカリ性フォスファターゼ(*p*-ニトロフェニルリン酸基質法)，総コレステロール(COD-DAOS法)，トリグリセライド(GPO-DAOS法・グリセリン消去法)，リン脂質(酵素法・DAOS発色法)，グルコース(グルコキナーゼ・G-6-PDH法)，尿素窒素(ウレアーゼ-GlDH法)，クレアチニン(Jaffé法)，無機リン(モリブデン酸直接法)およびカルシウム(OCPC法)を測定した．また，電解質分析装置(PVA-αⅢ，㈱アナリティカル・インスツルメンツ)によりナトリウム(電極法)，カリウム(電極法)およびクロール(電量滴定法)を測定した．

5)病理学検査

雄では投与期間終了後の採血を行ったのちに，雌では哺育4日にエーテル麻酔下で外側腸骨動脈を切断して放血死させ，解剖して諸器官および組織の肉眼的観察を行い，雌について黄体数および着床痕数を調べた．剖検後，脳，心臓，肺(気管支を含む)，胸腺，肝臓，脾臓，腎臓，副腎，精巣，精巣上体および卵巣を摘出して器官重量(絶対重量)を測定するとともに，剖検日の体重を基に体重比器官重量(相対重量)を算出した．重量測定器官に加え，肉眼的異常部位を採取して10%中性緩衝ホルマリン溶液(精巣および精巣上体はブアン液で前固定)で固定した．対照群および500 mg/kg群の脳，心臓，肺(気管支を含む)，胸腺，肝臓，脾臓，腎臓，副腎，精巣，精巣上体および卵巣については，常法に従ってパラフィン切片を作製し，ヘマトキシリン・エオジン(HE)染色を施し，光学顕微鏡下で観察した．さらに，肝臓，胸腺および腎臓については，被験物質投与に関連したと考えられる変化がみられたため，100 mg/kg以下の投与群のこれらの器官についても同様の検査を行った．また，一部の動物で肝臓および腎臓の脂肪染色を実施した．なお，肉眼的異常部位，交尾が確認されなかった雄の精巣，精巣上体および雌の卵巣については，すべて病理組織学検査を行った．

5.生殖発生毒性に関する観察・検査

1)生殖機能

雌について交配開始日の2週間前(投与開始日)から交尾確認日まで，毎日午前の一定時間に膣垢を採取し，性周期検査を行った．

交配は雌雄(12週齢)1対1で一晩同居させる方法で行い，翌朝膣垢中の精子または膣栓が確認されたものを交尾成立とし，その日を妊娠0日とした．また，交配は同一群内で行い，交配期間は最長2週間とした．なお，交配相手が死亡した雌については，同群内の交尾が確認された雄と同居させた．交配期間終了後，交尾所要日数，交尾率［(交尾動物数/同居動物数)×100］および授(受)胎率［(受胎動物数/交尾動物数)×100］を算出した．

2)分娩および哺育状態ならびに新生児の観察

交尾が確認された雌は全例を自然分娩させ，分娩徴候を含め分娩状態および授乳，営巣などの哺育状態を観察するとともに，妊娠期間，出産率［(生児出産雌数/妊娠雌数)×100］を算出した．午後12時の時点で分娩が終了している動物を当該日分娩とし，その日を哺育0日とした。出産児については，分娩時に出産児数，出生児数，死産児数，出生児の性別および外表異常を検査した．出生児については，出生日および哺育4日に体重を個体

ごとに測定するとともに出生率[(出生児数/着床痕数)×100]および4日生存率 [(生後4日の生児数/出生児数)×100] を算出した．生後4日に出生児の全例をエーテル麻酔下で放血致死させ，器官・組織の肉眼的観察を行った．

6.統計処理

体重，摂餌量，血液学検査，血液生化学検査，交尾所要日数，性周期検査値(発情回数，発情周期)，器官重量，妊娠期間，黄体数，着床痕数，総出産児数および出生児数については各群ごとに平均値と標準偏差を求め，Bartlett法により分散の均一性を検定した．分散が均一な場合は一元配置型の分散分析を行い，ここで群間に有意差が認められ，かつ，各群の例数が同じ場合はDunnett法により，異なる場合はScheffé法により対照群と各群の一対比較検定を行った．分散が均一でない場合はKruskal-Wallisによって順位検定を行い，群間に有意差が認められ，かつ，各群の例数が同一の場合はDunnett型の，異なる場合はScheffé型の一対比較検定を行った．上記分散分析あるいはKruskal-Wallis法で群間に有意差を認めない場合は各群の多重比較は行わなかった．また交尾率，受(授)胎率，出産率および出生児の性比についてはχ^2検定により，死産率，出生率および4日生存率についてはWilcoxonの順位和検定により対照群と各投与群間の比較を行った．いずれの場合も有意水準を5%とした．なお，出生児に関する測定値については一腹単位で処理した．

結　果

1.反復投与毒性

1) 一般状態

各投与群の雌雄とも投与期間を通して被験物質投与による一般状態の変化は認められなかった．なお，投与過誤により500 mg/kg群の雌雄各1例が死亡した．

2) 体重(Fig.1) および摂餌量

各投与群の雌雄とも投与期間を通して体重および摂餌量に対照群との間の差は認められなかった．

3) 血液学検査(Table 1)

500 mg/kg群でMCHにごく軽度の減少がみられたが，他の赤血球系パラメータに変動はみられず，毒性学的意義はないものと考えられた．

4) 血液生化学検査(Table 2)

GOTの低下が100および500 mg/kg群で認められた．さらに，500 mg/kg群で総蛋白質，アルブミン，総コレステロール，トリグリセライド，リン脂質およびカルシウムの増加ならびにグルコースの減少が認められた．

5) 器官重量(Table 3)

雄では，500 mg/kg群で肝臓および腎臓の絶対および相対重量の増加がみられ，100 mg/kg群においても肝臓の絶対重量の増加傾向および相対重量の増加，腎臓の絶対および相対重量の増加傾向が認められた．

雌では，500 mg/kg群で肝臓の絶対および相対重量の増加が認められた．

6) 剖検所見

投与期間終了後の雄の剖検では，500 mg/kg群で腎臓の肥大が2例，退色が3例，小陥凹が1例に認められた．そのほか，対照群の1例に肺の灰白色化がみられた．

哺育4日の雌の剖検では，脾臓と周囲脂肪組織との癒着が対照群の1例，肝臓の横隔膜面結節が20 mg/kg群の1例に認められた．

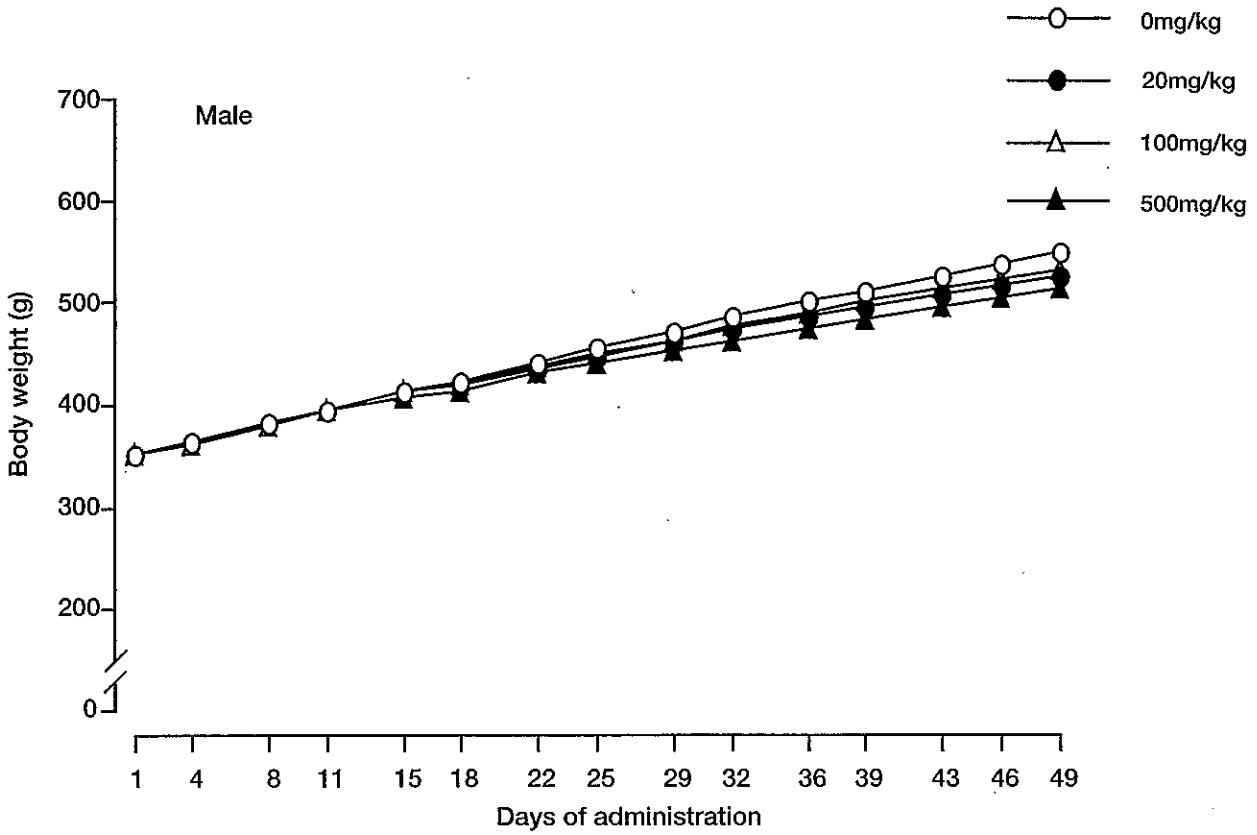
7) 病理組織学検査(Table 4)

雄では，小葉中心性の肝細胞の肥大が500 mg/kg群の11例，100 mg/kg群の10例に認められた．また，腎臓の近位尿細管上皮の硝子滴の出現および上皮の壊死が500 mg/kg群の11例，100 mg/kg群の12例，近位尿細管の拡張が500 mg/kg群の9例，100 mg/kg群の1例，近位尿細管上皮の好塩基性変化が500 mg/kg群の6例，100 mg/kg群の2例，腎臓の瘢痕が500 mg/kg群の1例に認められた．そのほか，偶発的変化として対照群では雄の1例に肺のマクロファージによる肺胞中隔あるいはリンパ球による血管周囲への細胞浸潤および動脈中膜の肥厚がみられた．

雌では，小葉中心性の肝細胞の肥大が500 mg/kg群の10例，100 mg/kg群の3例，胸腺皮質の萎縮が500 mg/kg群の2例に認められた．そのほか，偶発的変化として対照群および500 mg/kg群の各1例に脾臓の髄外造血の亢進，対照群の1例に脾臓と周囲脂肪組織との癒着が認められた．また，対照群の1例に肺のマクロファージによる肺胞中隔あるいはリンパ球による血管周囲への細胞浸潤および動脈中膜の肥厚がみられた．

500 mg/kg群の雌の死亡例では，生存例と同様の小葉中心性の肝細胞の肥大がみられたほか，腎臓の間質の毛細血管，小静脈および動脈ならびに糸球体毛細血管の血栓がみられ，さらに胸腺皮質のリンパ球の壊死および前胃の潰瘍が認められた．

100 mg/kg群の全児死亡例では，肝小葉辺縁部から中間帯にかけての脂肪化，近位尿細管上皮の脂肪化および壊死が認められた．



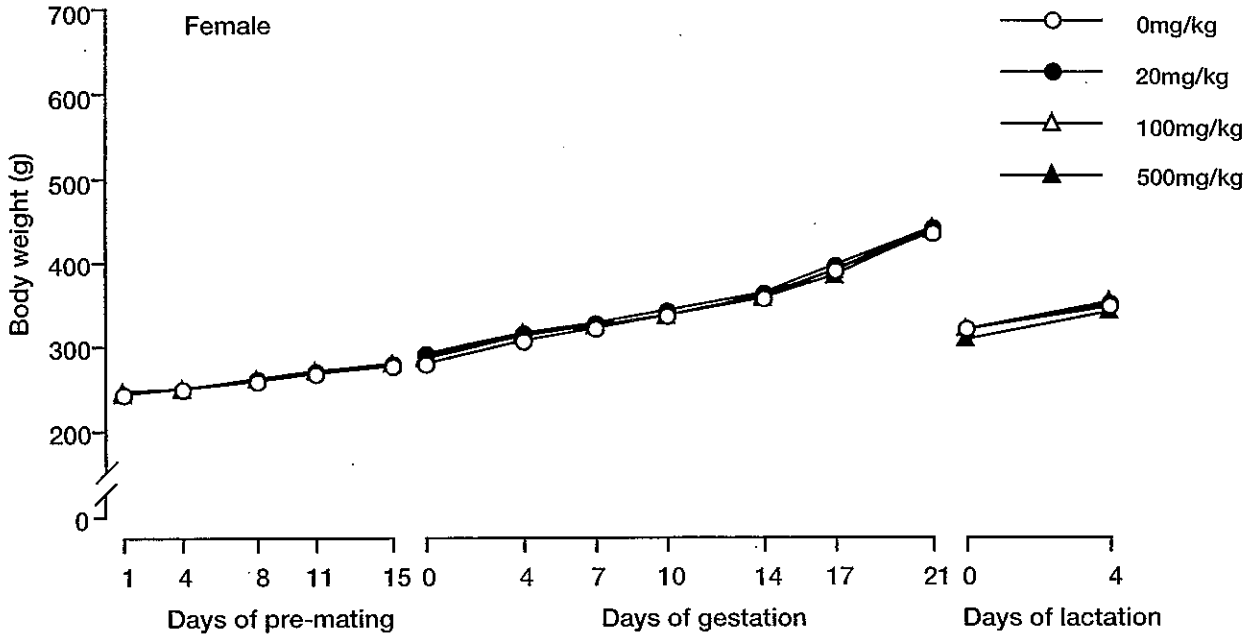


Fig. 1 Mean body weight changes of rats treated orally with trifluoromethylbenzene in the combined repeat dose and reproductive/developmental toxicity screening test

Table 1 Hematological findings of male rats treated orally with trifluoromethylbenzene in the combined repeat dose and reproductive/developmental toxicity screening test

Dose (mg/kg)	0	20	100	500
No. of animals	12	12	12	11
Leucocyte ($10^2/\mu l$)	79 ± 16	72 ± 18	86 ± 16	77 ± 19
Erythrocyte ($10^4/\mu l$)	853 ± 36	872 ± 35	868 ± 27	877 ± 30
Hemoglobin (g/dl)	14.8 ± 0.6	14.9 ± 0.6	14.6 ± 0.2	14.6 ± 0.5
Hematocrit (%)	47.3 ± 1.9	48.4 ± 1.8	47.4 ± 1.3	47.2 ± 2.0
Platelet ($10^4/\mu l$)	100.3 ± 12.3	92.0 ± 10.9	95.9 ± 8.2	102.5 ± 7.8
MCV (fl)	56 ± 1	56 ± 2	55 ± 2	54 ± 2
MCH (pg)	17.3 ± 0.6	17.1 ± 0.4	16.8 ± 0.5	16.6 ± 0.5*
MCHC (%)	31.2 ± 0.5	30.9 ± 0.5	30.8 ± 0.6	30.9 ± 0.7

* : $P<0.05$ (significantly different from control)

Values are mean ± S.D.

Table 2 Blood chemical findings of male rats treated orally with trifluoromethylbenzene in the combined repeat dose and reproductive/developmental toxicity screening test

Dose (mg/kg)	0	20	100	500
No. of animals	12	12	12	11
T.protein (g/dl)	5.3 ± 0.2	5.3 ± 0.3	5.5 ± 0.1	5.8 ± 0.2**
Albumin (g/dl)	3.7 ± 0.2	3.6 ± 0.1	3.8 ± 0.1	4.1 ± 0.2**
A/G ratio	2.23 ± 0.20	2.25 ± 0.29	2.25 ± 0.25	2.37 ± 0.19
GOT (IU/l)	98 ± 12	86 ± 12	82 ± 14*	77 ± 15**
GPT (IU/l)	24 ± 4	21 ± 2	23 ± 3	22 ± 4
γ-GTP (IU/l)	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.2
ALP (IU/l)	198 ± 23	203 ± 29	197 ± 27	184 ± 24
T.cholesterol (mg/dl)	50 ± 9	46 ± 9	56 ± 12	85 ± 10**
Triglycerides (mg/dl)	31 ± 10	28 ± 8	35 ± 12	66 ± 15**
Phospholipids (mg/dl)	80 ± 12	77 ± 12	93 ± 17	140 ± 14**
T.bilirubin (mg/dl)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Glucose (mg/dl)	138 ± 14	132 ± 12	123 ± 15	103 ± 13**
BUN (mg/dl)	14.0 ± 2.1	14.5 ± 2.2	14.9 ± 1.3	16.1 ± 1.9
Creatinine (mg/dl)	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
IP (mg/dl)	6.4 ± 0.6	6.4 ± 0.8	6.4 ± 0.6	6.1 ± 0.6
Ca (mg/dl)	9.5 ± 0.2	9.5 ± 0.2	9.6 ± 0.2	9.9 ± 0.2**
Na (mEq/l)	147.1 ± 1.0	147.5 ± 0.8	146.6 ± 0.8	147.1 ± 0.7
K (mEq/l)	4.38 ± 0.26	4.32 ± 0.20	4.36 ± 0.10	4.34 ± 0.17
Cl (mEq/l)	105.4 ± 1.4	106.2 ± 1.1	104.6 ± 0.8	104.2 ± 1.1

* : $P<0.05$, ** : $P<0.01$ (significantly different from control)

Values are mean ± S.D.

Table 3 Absolute and relative organ weights of rats treated orally with trifluoromethylbenzene in the combined repeat dose and reproductive/developmental toxicity screening test

Dose (mg/kg)	0	20	100	500
Male				
No. of animals	12	12	12	11
Absolute organ weight				
Final Body Weight (g)	516.2 ± 35.7	497.1 ± 40.6	501.1 ± 37.6	477.8 ± 41.6
Brain (g)	2.15 ± 0.09	2.15 ± 0.05	2.14 ± 0.09	2.13 ± 0.06
Heart (g)	1.59 ± 0.10	1.60 ± 0.08	1.52 ± 0.13	1.60 ± 0.20
Lungs (g)	1.60 ± 0.27	1.46 ± 0.13	1.51 ± 0.11	1.42 ± 0.12
Thymus (g)	0.39 ± 0.06	0.40 ± 0.11	0.36 ± 0.07	0.34 ± 0.08
Liver (g)	14.46 ± 1.75	13.35 ± 1.73	15.44 ± 1.20	18.86 ± 1.61**
Spleen (g)	0.78 ± 0.11	0.76 ± 0.13	0.75 ± 0.12	0.78 ± 0.12
Kidneys (g)	3.20 ± 0.28	3.27 ± 0.25	3.51 ± 0.23	4.33 ± 0.65**
Adrenals (mg)	64.5 ± 6.1	65.0 ± 7.4	61.3 ± 6.5	61.8 ± 6.9
Testes (g)	3.45 ± 0.24	3.34 ± 0.20	3.43 ± 0.21	3.47 ± 0.30
Epididymides (g)	1.32 ± 0.09	1.32 ± 0.09	1.31 ± 0.10	1.33 ± 0.10
Relative organ weight				
Brain (g/100 g B.W.)	0.42 ± 0.03	0.44 ± 0.03	0.43 ± 0.02	0.45 ± 0.04
Heart (g/100 g B.W.)	0.31 ± 0.03	0.32 ± 0.03	0.30 ± 0.03	0.33 ± 0.03
Lungs (g/100 g B.W.)	0.31 ± 0.07	0.29 ± 0.02	0.30 ± 0.02	0.30 ± 0.03
Thymus (g/100 g B.W.)	0.07 ± 0.01	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
Liver (g/100 g B.W.)	2.80 ± 0.21	2.68 ± 0.19	3.09 ± 0.25*	3.96 ± 0.24**
Spleen (g/100 g B.W.)	0.15 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.16 ± 0.03
Kidneys (g/100 g B.W.)	0.62 ± 0.04	0.66 ± 0.04	0.70 ± 0.06	0.91 ± 0.14**
Adrenals (mg/100 g B.W.)	12.5 ± 1.0	13.1 ± 1.3	12.3 ± 1.8	13.0 ± 1.5
Testes (g/100 g B.W.)	0.67 ± 0.06	0.68 ± 0.07	0.69 ± 0.06	0.73 ± 0.06
Epididymides (g/100 g B.W.)	0.26 ± 0.03	0.27 ± 0.03	0.26 ± 0.02	0.28 ± 0.02
Female				
No. of animals	12	12	10	10
Absolute organ weight				
Final Body Weight (g)	347.9 ± 14.2	348.9 ± 19.6	354.0 ± 21.6	342.2 ± 18.8
Brain (g)	2.03 ± 0.08	2.07 ± 0.09	2.07 ± 0.06	2.05 ± 0.05
Heart (g)	1.12 ± 0.12	1.11 ± 0.08	1.07 ± 0.10	1.08 ± 0.13
Lungs (g)	1.25 ± 0.20	1.26 ± 0.14	1.46 ± 0.79	1.19 ± 0.10
Thymus (g)	0.20 ± 0.03	0.20 ± 0.08	0.22 ± 0.07	0.17 ± 0.05
Liver (g)	14.62 ± 1.16	14.65 ± 1.36	15.21 ± 1.28	16.32 ± 1.59*
Spleen (g)	0.68 ± 0.12	0.65 ± 0.06	0.64 ± 0.09	0.62 ± 0.12
Kidneys (g)	2.07 ± 0.16	2.08 ± 0.14	2.10 ± 0.11	2.16 ± 0.07
Adrenals (mg)	76.1 ± 9.2	78.4 ± 8.3	78.6 ± 7.0	74.7 ± 9.6
Ovaries (mg)	101.0 ± 12.2	109.1 ± 14.4	110.9 ± 12.0	108.9 ± 7.3
Relative organ weight				
Brain (g/100 g B.W.)	0.58 ± 0.03	0.60 ± 0.05	0.59 ± 0.04	0.60 ± 0.02
Heart (g/100 g B.W.)	0.32 ± 0.03	0.32 ± 0.02	0.30 ± 0.04	0.32 ± 0.03
Lungs (g/100 g B.W.)	0.36 ± 0.06	0.36 ± 0.04	0.42 ± 0.27	0.35 ± 0.02
Thymus (g/100 g B.W.)	0.06 ± 0.01	0.06 ± 0.02	0.06 ± 0.02	0.05 ± 0.01
Liver (g/100 g B.W.)	4.20 ± 0.23	4.20 ± 0.29	4.30 ± 0.23	4.77 ± 0.44**
Spleen (g/100 g B.W.)	0.19 ± 0.03	0.19 ± 0.01	0.18 ± 0.03	0.18 ± 0.03
Kidneys (g/100 g B.W.)	0.60 ± 0.03	0.60 ± 0.04	0.59 ± 0.03	0.63 ± 0.04
Adrenals (mg/100 g B.W.)	21.9 ± 2.6	22.4 ± 2.0	22.2 ± 1.6	21.8 ± 2.3
Ovaries (mg/100 g B.W.)	29.0 ± 3.0	31.4 ± 4.4	31.4 ± 3.5	31.8 ± 2.1

*: $P<0.05$, **: $P<0.01$ (significantly different from control)

Values are mean ± S.D.

Table 4 Histopathological findings of rats treated orally with trifluoromethylbenzene in the combined repeat dose and reproductive/developmental toxicity screening test

Dose (mg/kg)	0				20				100											
	Terminal sacrifice				Terminal sacrifice				Terminal sacrifice				Imminent sacrifice/Dead				Total			
Organs and findings	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Male																				
Number of animals	12				12				12											
Digestive system																				
Liver	(12)				(12)				(12)											
Hypertrophy, centrilobular	12	0	0	0	12	0	0	0	2	9	1	0								
Respiratory system																				
Lung	(12)				(0)				(0)											
Cellular infiltration	11	1	0	0																
Thickening, tunica media, artery	11	1	0	0																
Urinary system																				
Kidney	(12)				(12)				(12)											
Hyaline droplets, proximal tubules	12	0	0	0	12	0	0	0	0	3	9	0								
Necrosis, proximal tubules	12	0	0	0	12	0	0	0	0	7	5	0								
Dilatation, proximal tubules	12	0	0	0	12	0	0	0	11	1	0	0								
Change, basophilic, proximal tubules	12	0	0	0	12	0	0	0	10	2	0	0								
Scar	12	0	0	0	12	0	0	0	12	0	0	0								
Female																				
Number of animals	12				12				11				1				12			
Digestive system																				
Liver	(12)				(12)				(11)				(1)				(12)			
Hypertrophy, centrilobular	12	0	0	0	12	0	0	0	8	3	0	0	1	0	0	0	9	3	0	0
Change, fatty, periportal and mid-zonal	12	0	0	0	12	0	0	0	11	0	0	0	0	0	1	0	11	0	1	0
Stomach	(0)				(0)				(0)				(0)				(0)			
Ulcer, forestomach																				
Ulcer, glandular stomach																				
Respiratory system																				
Lung	(12)				(0)				(1)				(1)				(2)			
Cellular infiltration	11	1	0	0					0	1	0	0	1	0	0	0	1	1	0	0
Thickening, tunica media, artery	11	1	0	0					0	1	0	0	1	0	0	0	1	1	0	0
Hematopoietic system																				
Thymus	(12)				(0)				(11)				(0)				(11)			
Atrophy	12	0	0	0					11	0	0	0					11	0	0	0
Necrosis, lymphocyte	12	0	0	0					11	0	0	0					11	0	0	0
Spleen	(12)				(0)				(0)				(1)				(1)			
Hematopoiesis, extramedullary, increased	11	1	0	0									1	0	0	0	1	0	0	0
Adhesion, adipose tissue	11	1	0	0									1	0	0	0	1	0	0	0
Proliferation, plasma cell, red pulp	12	0	0	0									1	0	0	0	1	0	0	0
Thoracic lymph node	(0)				(0)				(1)				(0)				(1)			
Proliferation, plasma cell, medulla									0	1	0	0					0	1	0	0
Urinary system																				
Kidney	(12)				(12)				(11)				(1)				(12)			
Change, fatty, proximal tubules	12	0	0	0	12	0	0	0	11	0	0	0	0	0	0	1	11	0	0	1
Thrombus	12	0	0	0	12	0	0	0	11	0	0	0	1	0	0	0	12	0	0	0
Necrosis, proximal tubules	12	0	0	0	12	0	0	0	11	0	0	0	0	0	1	0	11	0	1	0
Others																				
Thoracic cavity	(0)				(0)				(1)				(0)				(1)			
Abscess									0	1	0	0					0	1	0	0

Grade sign: -, none; +, mild; ++, moderate; +++, marked

Figure in parentheses represents the number of animals with tissues examined histopathologically.

Table 4 - continued Histopathological findings of rats treated orally with trifluoromethylbenzene in the combined repeat dose and reproductive/developmental toxicity screening test

Dose (mg/kg)	500											
	Terminal sacrifice				Imminent sacrifice/Dead				Total			
Organs and findings	-	+	++	+++	-	+	++	+++	-	+	++	+++
Male												
Number of animals	11				1				12			
Digestive system												
Liver	(11)				(1)				(12)			
Hypertrophy, centrilobular	0	0	11	0	0	0	1	0	0	0	12	0
Respiratory system												
Lung	(11)				(1)				(12)			
Cellular infiltration	11	0	0	0	1	0	0	0	12	0	0	0
Thickening, tunica media, artery	11	0	0	0	1	0	0	0	12	0	0	0
Urinary system												
Kidney	(11)				(1)				(12)			
Hyaline droplets, proximal tubules	0	0	11	0	0	0	1	0	0	0	12	0
Necrosis, proximal tubules	0	0	8	3	1	0	0	0	1	0	8	3
Dilatation, proximal tubules	2	6	1	2	1	0	0	0	3	6	1	2
Change, basophilic, proximal tubules	5	6	0	0	1	0	0	0	6	6	0	0
Scar	10	1	0	0	1	0	0	0	11	1	0	0
Female												
Number of animals	10				2				12			
Digestive system												
Liver	(10)				(2)				(12)			
Hypertrophy, centrilobular	0	0	10	0	1	1	0	0	1	1	10	0
Change, fatty, periportal and mid-zonal	10	0	0	0	2	0	0	0	12	0	0	0
Stomach	(0)				(2)				(2)			
Ulcer, forestomach					1	1	0	0	1	1	0	0
Ulcer, glandular stomach					1	1	0	0	1	1	0	0
Respiratory system												
Lung	(10)				(2)				(12)			
Cellular infiltration	10	0	0	0	2	0	0	0	12	0	0	0
Thickening, tunica media, artery	10	0	0	0	2	0	0	0	12	0	0	0
Hematopoietic system												
Thymus	(10)				(2)				(12)			
Atrophy	8	2	0	0	1	0	1	0	9	2	1	0
Necrosis, lymphocyte	10	0	0	0	1	0	1	0	11	0	1	0
Spleen	(10)				(2)				(12)			
Hematopoiesis, extramedullary, increased	9	1	0	0	2	0	0	0	11	1	0	0
Adhesion, adipose tissue	10	0	0	0	2	0	0	0	12	0	0	0
Proliferation, plasma cell, red pulp	10	0	0	0	1	0	1	0	11	0	1	0
Thoracic lymph node	(0)				(0)				(0)			
Proliferation, plasma cell, medulla												
Urinary system												
Kidney	(10)				(2)				(12)			
Change, fatty, proximal tubules	10	0	0	0	1	0	1	0	11	0	1	0
Thrombus	10	0	0	0	1	0	1	0	11	0	1	0
Necrosis, proximal tubules	10	0	0	0	2	0	0	0	10	0	0	0
Others												
Thoracic cavity	(0)				(1)				(1)			
Abscess					0	1	0	0	0	1	0	0

Grade sign: -, none; +, mild; ++, moderate; +++, marked

Figure in parentheses represents the number of animals with tissues examined histopathologically.

2.生殖発生毒性

1) 生殖機能(Table 5)

性周期検査では各投与群とも発情回数および発情周期に対照群との間の差は認められなかった.

生殖能力検査では100 mg/kg群の1組を除いてすべてに交尾がみられ,交尾確認例の全例に妊娠が認められた.したがって,交尾率は,対照群,20,100および500 mg/kg群でそれぞれ100,100,91.67および100%,受胎率はいずれも100%の成績を示し,交尾所要日数においても対照群と各投与群間の差は認められなかった.なお,100 mg/kg群の未交尾例では,雌雄とも生殖器の剖検および病理組織学検査に変化はみられず,授(受)胎能の欠如を示唆する所見は認められなかった.

2) 分娩および哺育ならびに新生児の観察(Table 6)

分娩時の検査では,500 mg/kg群で1母動物が妊娠23日の分娩後に死亡した.本例では,出産児(死産児4匹,生存児12匹)の発育には特に変化はみられなかったが,血液による下腹部の汚れが顕著にみられ,出産児の羊膜除去,胎盤処理ならびに児の回集などは不充分であった.剖検では,胸水貯留,肺の暗赤色化,気管に泡沫状水様液貯留および前胃粘膜の白色点点在が認められた.

20 mg/kg群の黄体数および100 mg/kg群の着床痕数にそれぞれ増加が認められたが,500 mg/kg群の黄体数および着床痕数に変化は認められなかった.

各投与群とも妊娠期間,総出産児数,出産率,死産率,出生児数,出生率および出生児の性比に対照群との間に差は認められなかった.また,外表異常児は,各投与群とも1例も認められなかった.

出生児の体重では,各投与群とも出生日および生後4日ともに減少が認められた.

哺育期の検査では,児の回集,授乳などの哺育行動の低下が100 mg/kg群の1母動物にみられ,分娩後2日までに出生児の全例が死亡した.

4日生存率では,500 mg/kg群に減少が認められた.なお,100 mg/kg群にも減少傾向が認められたが,この変化は当該群の1母動物に全児死亡がみられたことによるものであった.

出生児の剖検では,肝臓内側左葉の黄白色化が100 mg/kg群の1例に認められたのみであった.

考　察

1.反復投与毒性

肝臓および腎臓で被験物質投与に起因した変化が認められた.肝臓では,重量の増加が雄の100 mg/kg以上の投与群および雌の500 mg/kg群に認められ,小葉中心性の肝細胞の肥大が雌雄の100 mg/kg以上の投与群でみられた.また,腎臓では,重量の増加と近位尿細管上皮における硝子滴の出現,壊死,好塩基性変化および近位尿細管の拡張が雄の100 mg/kg以上の投与群に認められた.本被験物質とはハロゲン基が異なるトリクロロトルエンでも肝臓および腎臓に対する影響が報告されており[2],これらの類似化合物では肝臓および腎臓が標的器官になる可能性が示唆された.雄の血液生化学検査では,500 mg/kg群で総蛋白質,アルブミン,総コレステロール,トリグリセライドおよびリン脂質の増加ならびにグルコースの減少が認められたが,これらも本被験物質の肝臓に対する影響に関連した変化のように考えられた.また,当該群ではカルシウムの増加も認められたが,これはアルブミンの増加に伴った変化と思われた.しかし,被験物質投与に起因した症状の発現や,体重および摂餌量に対する影響はいずれの投与群にも認められず,全身状態の悪化を来すような毒性には至らなかったと考えられた.なお,腎臓では雄の500 mg/kg群の1例に肉眼的に小陥凹がみられ,組織学的には瘢痕が認められたが,本変化は正常ラットでも時折みられる変化であり,発生例数も少ないことから被験物質投与に起因したものとは考え難かった.また,胸腺では雌の500 mg/kg群の2例に組織学的に萎縮がみられたが,変化の程度は軽微で発生例数も少ないことおよび雄では胸腺をはじめリンパ系への作用を示唆する変化は認められなかったことから,被験物質の直接的な影響よりは分娩に伴ったストレス性の変化である可能性が高いと思われた.

以上のことから,本試験条件下における反復投与毒性に関する無影響量は雌雄とも20 mg/kgと推定された.

2.生殖発生毒性

親動物の生殖機能に関しては,性周期,雌雄の交尾率および授(受)胎率に被験物質投与の影響は認められなかった.

分娩時の観察では,500 mg/kg群の1母動物が妊娠23日の分娩の終了後に死亡した.本例では妊娠期間中の一般状態ならびに出産児の発育には特に変化はみられなかったが,分娩時に顕著な出血がみられ,出産児の羊膜除去,胎盤処理ならびに児の回集などの母性行動が正常に行われた形跡は認められなかったことから,その死因については分娩に伴う消耗性の変化と考えられた.しかしながら,この様な分娩異常は当該群のほかの母動物には観察されず,出現例数も少ないことから被験物質投与との直接的な関連性はないように思われた.当該群では妊娠期間,総出産児数,出産率,死産率,出生児数,出生率および出生児の性比に被験物質投与の影響は認められなかった.一方,20 mg/kg以上の群で出生日に体重減少が認められ,本被験物質の胎生期の発育に対する影響が示唆されたが,胚致死作用を窺わせる出生児数の減少はみられておらず,外表異常も観察されなかった.

哺育期の観察においても,出生児の継続した体重の増加抑制がみられ,500 mg/kg群に4日生存率の低下がごく軽度に認められた.体重の増加抑制は,胎生期の発育抑制を反映しているものと考えられ,その内,特に低い体重を示した児が生存できなかったものと考えられた.そのほか,哺育不良による全児死亡が100 mg/kg群の1母動物に認められたが,500 mg/kg群に全児死亡は観察されなかったことから特に問題視する変化とは考え難かった.

Table 5 Reproductive performance of rats treated orally with trifluoromethylbenzene in the combined repeat dose and reproductive/developmental toxicity screening test

Dose (mg/kg)	0	20	100	500
No. of females examined	12	12	12	12
Count of estrus[a]	3.42 ± 0.67	3.58 ± 0.51	3.50 ± 0.52	3.42 ± 0.51
Estrous cycle[b]	4.04 ± 0.14	4.08 ± 0.29	4.03 ± 0.10	4.08 ± 0.29
No. of mated				
Male	12	12	12	11
Female	12	12	12	12
No. of copulated[c]				
Male	12(100)	12(100)	11(91.67)	11(100)
Female	12(100)	12(100)	11(91.67)	12(100)
No. of impregnated[d]	12(100)	12(100)	11(100)	11(100)
No. of pregnant[d]	12(100)	12(100)	11(100)	12(100)
Duration of mating[b]	2.25 ± 1.29	2.58 ± 1.31	2.55 ± 2.02	1.67 ± 0.89

a) Values are mean ± S.D.

b) Values are mean ± S.D. (day)

c) Values in parentheses represent percentages to the number of mated.

d) Values in parentheses represent percentages to the number of copulated.

Table 6 Findings of delivery of F_0 dams treated orally with trifluoromethylbenzene and observations on their offspring in the combined repeat dose and reproductive/developmental toxicity screening test

Dose (mg/kg)	0	20	100	500
No. of dams	12	12	11	11
Gestational days[a]	22.33 ± 0.49	22.08 ± 0.29	22.00 ± 0.00	22.36 ± 0.50
No. of corpora lutea[b]	190(15.83 ± 1.53)	216(18.00 ± 2.13)*	192(17.45 ± 1.21)	192(17.45 ± 1.29)
No. of implantations[b]	179(14.92 ± 1.16)	197(16.42 ± 2.27)	187(17.00 ± 1.10)*	183(16.64 ± 1.21)
No. of litter[b]	168(14.00 ± 1.41)	191(15.92 ± 2.07)	171(15.55 ± 1.63)	173(15.73 ± 2.00)
Gestation index[c]	100	100	100	100
No. of stillborns[d]				
Male	0	6	2	5
Female	2	1	6	7
Total	2(1.19)	8(4.19)[i]	8(4.68)	12(6.94)
No. of live newborns[b]	166(13.83 ± 1.40)	183(15.25 ± 1.76)	163(14.82 ± 1.60)	161(14.64 ± 2.54)
Birth index[e]	92.74	92.89	87.17	87.98
Sex ratio of live newborns[f]	1.08(86/80)	1.01(92/91)	1.06(84/79)	0.99(80/81)
Body weight of live newborns (g)[g]				
Male On day 0	6.7 ± 0.5	6.1 ± 0.5*	6.2 ± 0.4	6.0 ± 0.4**
4	11.1 ± 1.0	9.9 ± 1.0*	9.8 ± 0.9**	9.4 ± 0.4**
Female On day 0	6.4 ± 0.5	5.7 ± 0.4**	5.8 ± 0.4**	5.7 ± 0.4**
4	10.5 ± 0.8	9.4 ± 0.9*	9.0 ± 0.7**	8.8 ± 0.5**
Viability index[h]	98.80	98.91	92.64	91.28*
No. of external anomalies	0	0	0	0

*: $P<0.05$, **: $P<0.01$ (significantly different from control)

a) Values are mean ± S.D. (day)

b) Values in parentheses represent mean ± S.D.

c) Gestation index=(Number of females with live newborns/Number of pregnant females)×100

d) Values in parentheses represent percentages to the number of litters.

e) Birth index=(Number of live newborns/Number of implantations)×100

f) Values in parentheses represent the number of male/female live newborns.

g) Values are mean ± S.D.

h) Viability index=(Number of live newborns on day 4 after birth/Number of live newborns)×100

i) Including 1 cannibalized

以上のことから，本試験条件下における生殖発生毒性に関する無影響量は雄および雌の生殖に対しては500 mg/kg，児の発生に対しては20 mg/kg未満と推察された．

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(トリフルオロメチル)ベンゼンの細菌を用いる復帰突然変異試験

Reverse Mutation Test of Trifluoromethylbenzene on Bacteria

要約

OECD既存化学物質安全性調査事業の一環として，(トリフルオロメチル)ベンゼンについて，細菌を用いる復帰突然変異試験をプレート法により実施し，陰性の結果を得た．検定菌として，*Salmonella typhimurium* TA100, TA1535, TA98, TA1537および*Escherichia coli* WP2 *uvrA*の5菌株を用い，S9 mix無添加および添加の条件でプレート法により，用量設定試験を50～5000 μg/プレートの用量で行ったところ，S9 mixの添加の有無にかかわらず抗菌性が認められた．したがって，本試験の最高用量はいずれも，当初 TA100, WP2 *uvrA*, TA98は2000 μg/プレート，TA1535および TA1537 は1000 μg/プレートとし，6用量を設定することとした．しかし，S9 mix 無添加試験では，本被験物質は弱い抗菌作用を幅広い用量にわたって示し，かつ試験ごとに抗菌性を示す用量に変動が認められたことから，WP2 *uvrA*とTA98の2回目の本試験，および TA100は2回とも S9 mix 無添加試験の最高用量 1000 μg/プレートに変更して6用量を設定し，本試験とした．

その結果，抗菌性は，S9 mix無添加試験では，500 μg/プレート(TA100, TA1535 およびTA1537)あるいは1000 μg/プレート(TA1535 およびWP2)用量で，S9 mix添加試験では，1000 μg/プレート(TA1535，TA98およびTA1537)あるいは2000 μg/プレート(TA100およびWP2)の用量で認められた．復帰変異コロニー数は，2回の本試験とも，用いた検定菌について，いずれの用量においても増加は認められなかったことから，(トリフルオロメチル)ベンゼンは，用いた試験系において変異原性を有しない(陰性)と判定された．

方法

〔検定菌〕

Salmonella typhimurium TA100
Salmonella typhimurium TA1535
Escherichia coli WP2 *uvrA*
Salmonella typhimurium TA98
Salmonella typhimurium TA1537

*S. typhimurium*の4菌株[1]は1975年10月31日にアメリカ合衆国，カリフォルニア大学のB. N. Ames博士から分与を受けた．

E. coli WP2 *uvrA* 株[2]は1979年5月9日に国立遺伝学研究所の賀田恒夫博士から分与を受けた．

検定菌は－80℃以下で凍結保存したものを用い，ニュートリエントブロスNo. 2(Oxoid)を入れたL字型試験管に解凍した種菌を一定量接種し，37℃で10時間往復振とう培養したものを検定菌液とした．

〔被験物質〕

(トリフルオロメチル)ベンゼン(CAS No. 98-08-8)は，分子量 146.11 の無色透明な液体である．試験には，和光純薬工業㈱製〔ロット番号：KCM2054，純度 98.0 %以上(不純物：水分0.2 %以下，その他不明)〕のものを購入し，使用時まで冷蔵保管して用いた．

(トリフルオロメチル)ベンゼンは，ジメチルスルホキシド(DMSO)に溶解性がよいことから，DMSOに 10～50 mg/mlの範囲で溶解した後，同溶媒で公比約3ないし2で希釈し，速やかに試験に用いた．

試験の開始に先立って，(トリフルオロメチル)ベンゼンの DMSO 溶液中での安定性試験および含量測定試験を実施した．安定性試験においては，低濃度(313 μg/ml)溶液は本試験Ⅱで調製したものについて，また高濃度(300 mg/ml)溶液は染色体異常試験における最高濃度として調製したものについて，室温遮光条件下で，安定性を調べた．その結果，調製4時間後における各濃度の平均含量は，それぞれ初期値(0時間)の平均値に対して，99.0および99.7 %であった．また，本試験Ⅱで調製した被験物質調製液について含量測定試験を行った結果，調製液の濃度は低濃度および高濃度(20.0 mg/ml)のいずれも103 %であった．

〔陽性対照物質〕

用いた陽性対照物質およびその溶媒は以下のとおりである．

AF2	：2-(2-フリル)-3-(5-ニトロ-2-フリル)アクリルアミド	(上野製薬㈱)
SA	：アジ化ナトリウム	(和光純薬工業㈱)
9AA	：9-アミノアクリジン	(Sigma Chem. Co.)
2AA	：2-アミノアントラセン	(和光純薬工業㈱)

AF2, 2AAはDMSO(和光純薬工業㈱)に溶解したものを－20℃で凍結保存し，用時解凍した．9AAはDMSOに，SAは純水に溶解し，速やかに試験に用いた．

〔培地および S9 mix の組成〕

1) トップアガー

下記の水溶液(A)および(B)を容量比10:1の割合で混合した．

(A) バクトアガー (Difco)	0.6 %
塩化ナトリウム	0.5 %
(B)* L-ヒスチジン	0.5 mM
D-ビオチン	0.5 mM

*:WP2 uvrA 用には，0.5 mM L-トリプトファン水溶液を用いた．

2) 合成培地

培地は，日清製粉㈱製の最少寒天培地を用いた．なお，培地1 lあたりの組成は下記のとおりである．

硫酸マグネシウム・7水和物	0.2 g
クエン酸・1水和物	2 g
リン酸水素二カリウム	10 g
リン酸一アンモニウム	1.92 g
水酸化ナトリウム	0.66 g
グルコース	20 g
バクトアガー(Difco)	15 g

径 90 mmのシャーレ1枚あたり30 mlを流して固めてある．

3) S9 mix

1 ml中下記の成分を含む

S9**	0.1 ml
塩化マグネシウム	8 μmol
塩化カリウム	33 μmol
グルコース-6-リン酸	5 μmol
NADH	4 μmol
NADPH	4 μmol
ナトリウム-リン酸緩衝液(pH 7.4)	100 μmol

**:7週齢の Sprague-Dawley 系雄ラットをフェノバルビタール(PB)および 5, 6-ベンゾフラボン(BF)の併用投与で酵素誘導して作製したS9 を用いた．

〔試験方法〕

プレート法により，S9 mix 無添加試験および S9 mix 添加試験を行った．

小試験管中にトップアガー2ml，被験物質調製液0.1 ml，リン酸緩衝液0.5 ml(S9 mix添加試験においては S9 mix 0.5 ml)，検定菌液 0.1 mlを混合したのち合成培地平板上に流して固めた．また，対照群として被験物質調製液の代わりに DMSO，または数種の陽性対照物質溶液を用いた．各検定菌ごとの陽性対照物質の名称および用量は各Table 中に示した．培養は37℃で48時間行い，生じた変異コロニー数を算定した．抗菌性の有無については，肉眼的あるいは実体顕微鏡下で，寒天表面の菌膜の状態から判断した．

用いた平板は用量設定試験においては，溶媒および陽性対照群では3枚ずつ，各用量については1枚ずつとした．また，本試験においては両対照群および各用量につき，3枚ずつを用い，それぞれの平均値と標準偏差を求めた．用量設定試験は1回，本試験は同一用量について2回実施し，結果の再現性の確認を行った．

〔判定基準〕

用いた5種の検定菌のうち，1種以上の検定菌のS9 mix無添加あるいはS9 mix添加条件において，被験物質を含有する平板上における変異コロニー数の平均値が，溶媒対照のそれに比べて2倍以上に増加し，かつ，その増加に再現性あるいは用量依存性が認められた場合に，当該被験物質は，本試験系において変異原性を有する(陽性)と判定することとした．

結果および考察

〔用量設定試験〕

50～5000 μg/プレートの範囲で公比を約3として，試験を実施したところ，S9 mix の添加の有無にかかわらず，TA100, WP2 *uvrA*, TA98は 1500 μg/プレート以上で，その他の検定菌では500 μg/プレート以上で抗菌性が認められた．

〔本試験〕

結果をそれぞれ Table 1，2に示した．(トリフルオロメチル)ベンゼンの用量は当初TA100 (S9 mix無添加)，TA1535, TA1537は31.3～1000 μg/プレート，TA100(S9 mix 添加)，WP2 *uvrA*, TA98は62.5～2000 μg/プレートの範囲で，公比を2として試験を行った．しかし，本被験物質は弱い抗菌作用が幅広い用量で認められ，特にS9 mix無添加試験の，WP2 *uvrA* およびTA98では試験ごとに抗菌性を示す用量に変動がみられ，抗菌性を示さない用量が4用量に満たない場合があったため，最高用量を1000 μg/プレートに変更して再試験を行った．その結果，本試験ⅠとⅡのいずれも，用いた5種類の検定菌のS9 mix無添加試験および添加試験において，溶媒対照値の2倍以上となる変異コロニー数の増加は認められなかった．

以上の結果に基づき，(トリフルオロメチル)ベンゼンは，用いた試験系において変異原性を有しないもの(陰性)と判定した．

Table 1. Mutagenicity of trifluoromethylbenzene** in reverse mutation test (I) on bacteria

With (+) or without (−) S9 mix	Test substance dose (μg/plate)	Number of revertants (number of colonies / plate, Mean ± S.D.)					
		Base - pair substitution type			Frameshift type		
		TA100	TA1535	WP2 *uvrA*	TA98	TA1537	
S9mix (−)	0	97 114 127 (113±15.0)	10 8 13 (10± 2.5)	18 22 20 (20± 2.0)	26 21 18 (22± 4.0)	11 8 5 (8± 3.0)	
	31.3	106 93 122 (107±14.5)	11 11 15 (12± 2.3)	ND	ND	10 8 6 (8± 2.0)	
	62.5	111 121 118 (117± 5.1)	13 14 8 (12± 3.2)	15 25 17 (19± 5.3)	23 25 26 (25± 1.5)	7 9 9 (8± 1.2)	
	125	102 141 123 (122±19.5)	10 11 6 (9± 2.6)	13 20 24 (19± 5.6)	30 13 26 (23± 8.9)	9 7 9 (8± 1.2)	
	250	111 103 102 (105± 4.9)	6 9 8 (8± 1.5)	18 17 21 (19± 2.1)	17 22 15 (18± 3.6)	8 5 10 (8± 2.5)	
	500	91 93 99 (94± 4.2)	7* 11* 6* (8± 2.6)	20 16 26 (21± 5.0)	14 13 18 (15± 2.6)	4 7 6 (6± 1.5)	
	1000	97* 100* 100* (99± 1.7)	10* 11* 11* (11± 0.6)	16 25 18 (20± 4.7)	15* 21* 22* (19± 3.8)	6* 4* 4* (5± 1.2)	
	2000			12* 24* 16* (17± 6.1)	25* 17* 14* (19± 5.7)		
S9mix (+)	0	122 108 105 (112± 9.1)	10 13 18 (14± 4.0)	23 22 20 (22± 1.5)	43 35 33 (37± 5.3)	9 7 17 (11± 5.3)	
	31.3	ND	14 16 8 (13± 4.2)	ND	ND	13 8 13 (11± 2.9)	
	62.5	89 123 102 (105±17.2)	8 10 7 (8± 1.5)	30 24 24 (26± 3.5)	29 31 39 (33± 5.3)	15 11 8 (11± 3.5)	
	125	91 83 105 (93±11.1)	14 15 18 (16± 2.1)	22 40 39 (34±10.1)	19 30 27 (25± 5.7)	14 13 11 (13± 1.5)	
	250	77 98 103 (93±13.8)	10 9 6 (8± 2.1)	24 28 23 (25± 2.6)	31 27 30 (29± 2.1)	14 9 8 (10± 3.2)	
	500	89 91 91 (90± 1.2)	8* 7* 15* (10± 4.4)	25 23 17 (22± 4.2)	33 36 23 (31± 6.8)	18 11 15 (15± 3.5)	
	1000	86 74 95 (85±10.5)	7* 15* 11* (11± 4.0)	33 18 23 (25± 7.6)	21 21 41 (28±11.5)	18* 14* 15* (16± 2.1)	
	2000	65* 72* 84* (74± 9.6)		16* 32* 31* (26± 9.0)	26* 27* 27* (27± 0.6)		
Positive control S9 mix (−)	Chemical	AF2	SA	AF2	AF2	9AA	
	Dose (μg/plate)	0.01	0.5	0.01	0.1	80	
	Number of colonies/plate	618 627 632 (626± 7.1)	268 257 282 (269±12.5)	146 138 104 (129±22.3)	727 733 772 (744±24.4)	628 786 686 (700±79.9)	
Positive control S9 mix (+)	Chemical	2AA	2AA	2AA	2AA	2AA	
	Dose (μg/plate)	1	2	10	0.5	2	
	Number of colonies/plate	970 981 1008 (986±19.6)	243 223 213 (226±15.3)	1423 1378 1280 (1360±73.1)	259 263 258 (260± 2.6)	184 179 194 (186± 7.6)	

AF2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, SA: Sodium azide, 9AA: 9-Aminoacridine, 2AA: 2-Aminoanthracene

*: Inhibition was observed against growth of the bacteria.

**: Purity was above 98.0% and water was contained below 0.2% as impurity.

ND: Not done

Table 2. Mutagenicity of trifluoromethylbenzene** in reverse mutation test (II) of on bacteria

With (+) or without (−) S9 mix	Test substance dose (μg/plate)	Number of revertants (number of colonies / plate, Mean ± S.D.)					
		Base - pair substitution type			Frameshift type		
		TA100	TA1535	WP2 *uvrA*	TA98	TA1537	
S9mix (−)	0	88 114 113 (105±14.7)	18 15 16 (16± 1.5)	20 15 19 (18± 2.6)	22 22 14 (19± 4.6)	11 14 9 (11± 2.5)	
	31.3	103 110 105 (106± 3.6)	14 11 14 (13± 1.7)	17 17 19 (18± 1.2)	30 22 20 (24± 5.3)	7 4 10 (7± 3.0)	
	62.5	95 100 100 (98± 2.9)	17 14 8 (13± 4.6)	20 21 34 (25± 7.8)	20 17 18 (18± 1.5)	5 11 10 (9± 3.2)	
	125	93 93 108 (98± 8.7)	14 13 13 (13± 0.6)	22 32 18 (24± 7.2)	21 14 20 (18± 3.8)	9 14 8 (10± 3.2)	
	250	106 82 114 (101±16.7)	13 7 14 (11± 3.8)	21 19 22 (21± 1.5)	17 22 21 (20± 2.6)	10 7 11 (9± 2.1)	
	500	80* 68* 78* (75± 6.4)	15* 5* 5* (8± 5.8)	22 14 16 (17± 4.2)	16 23 29 (23± 6.5)	8* 10* 7* (8± 1.5)	
	1000	3* 78* 79* (53±43.6)	4* 2* 4* (3± 1.2)	25* 28* 19* (24± 4.6)	18* 16* 19* (18± 1.5)	7* 7* 9* (8± 1.2)	
S9mix (+)	0	112 121 120 (118± 4.9)	8 12 12 (11± 2.3)	33 28 32 (31± 2.6)	44 36 29 (36± 7.5)	19 10 19 (16± 5.2)	
	31.3	ND	13 13 18 (15± 2.9)	ND	ND	10 17 12 (13± 3.6)	
	62.5	138 129 124 (130± 7.1)	17 14 6 (12± 5.7)	35 33 29 (32± 3.1)	45 32 42 (40± 6.8)	17 26 16 (20± 5.5)	
	125	133 103 118 (118±15.0)	17 18 17 (17± 0.6)	28 26 31 (28± 2.5)	27 38 44 (36± 8.6)	14 22 20 (19± 4.2)	
	250	124 95 104 (108±14.8)	9 14 13 (12± 2.6)	23 29 21 (24± 4.2)	43 38 25 (35± 9.3)	11 10 13 (11± 1.5)	
	500	81 96 107 (95±13.1)	12 12 11 (12± 0.6)	24 23 26 (24± 1.5)	36 24 33 (31± 6.2)	13* 11* 9* (11± 2.0)	
	1000	75 96 104 (92±15.0)	13* 6* 6* (8± 4.0)	24 23 19 (22± 2.6)	25* 27* 23* (25± 2.0)	7* 10* 11* (9± 2.1)	
	2000	59* 72* 84* (72±12.5)		18* 20* 22* (20± 2.0)	20* 23* 22* (22± 1.5)		
Positive control S9 mix (−)	Chemical	AF2	SA	AF2	AF2	9AA	
	Dose (μg/plate)	0.01	0.5	0.01	0.1	80	
	Number of colonies/plate	506 526 513 (515±10.1)	207 224 235 (222±14.1)	100 116 124 (113±12.2)	756 631 787 (725±82.6)	890 726 734 (783±92.5)	
Positive control S9 mix (+)	Chemical	2AA	2AA	2AA	2AA	2AA	
	Dose (μg/plate)	1	2	10	0.5	2	
	Number of colonies/plate	1164 1206 1145 (1172±31.2)	254 288 268 (270±17.1)	913 869 854 (879±30.7)	372 303 455 (377±76.1)	163 180 169 (171± 8.6)	

AF2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide , SA: Sodium azide, 9AA: 9-Aminoacridine, 2AA: 2-Aminoanthracene

*: Inhibition was observed against growth of the bacteria.

**: Purity was above 98.0% and water was contained below 0.2% as impurity.

ND : Not done

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トリフルオロメチルベンゼンの
チャイニーズ・ハムスター培養細胞を用いる染色体異常試験

In Vitro Chromosomal Aberration Test of Trifluoromethylbenzene on Cultured Chinese Hamster Cells

要約

OECD既存化学物質安全性点検に係る毒性調査事業の一環として，(トリフルオロメチル)ベンゼンの培養細胞に及ぼす細胞遺伝学的影響を評価するため，チャイニーズ・ハムスター培養細胞(CHL/IU)を用いて試験管内染色体異常試験を実施した．

連続処理(48時間)においては，50%を明らかに越える増殖抑制濃度，すなわち0.3 mg/mlの濃度を最高処理濃度とした．一方，短時間処理の(6時間)S9 mix存在下および非存在下では，50%を明らかに越える増殖抑制が認められなかったことから，1.5 mg/ml(10 mM)の濃度を最高処理濃度とした．最高処理濃度の1/2および1/4をそれぞれ中濃度，低濃度として設定した．連続処理では，S9 mix非存在下における24時間および48時間連続処理後，短時間処理ではS9 mix存在下および非存在下で6時間処理(18時間の回復時間)後，標本を作製し，検鏡することにより染色体異常誘発性を検討した．

CHL/IU細胞を24時間および48時間連続処理した高濃度群(0.3 mg/ml)においては，細胞毒性のため分析できなかったが，その他の処理群では，染色体の構造異常や倍数性細胞の誘発作用は認められなかった．また，短時間処理では，S9 mix存在下および非存在下で6時間処理したいずれの処理群においても，染色体の構造異常や倍数性細胞の誘発作用は認められなかった．

以上の結果より，(トリフルオロメチル)ベンゼンは，上記の試験条件下で染色体異常を誘発しないと結論した．

方法

1. 使用した細胞

リサーチ・リソースバンク(JCRB)から入手(1988年2月，入手時：継代4代，現在12代)したチャイニーズ・ハムスター由来のCHL/IU細胞を，解凍後継代10代以内で試験に用いた．

2. 培養液の調製

培養には，牛胎児血清(FCS：Biocell)を10%添加したイーグルMEM(日水製薬㈱)培養液を用いた．

3. 培養条件

(トリフルオロメチル)ベンゼンは揮発しやすいことまたプラスチック底面を溶解することから，培養にはガラス製フラスコ(25 cm^2，池本理化㈱)を用いた．2×10^4個のCHL/IU細胞を，培養液5 mlを入れたガラス製フラスコに播き，37℃のCO_2インキュベーター(5% CO_2)内で培養した．連続処理では，細胞播種3日目に被験物質を加え，24時間および48時間処理した．また，短時間処理では，細胞播種3日目にS9 mix存在下および非存在下で6時間処理し，処理終了後新鮮な培養液でさらに18時間培養した．

4. 被験物質

(トリフルオロメチル)ベンゼン(略号：TFMB，CAS No.：98-08-8，ロット番号：KCM2054，和光純薬工業㈱製造)は，無色透明の液体で，水に対しては不溶で，DMSOおよびアセトンには可溶であり，融点-29.02℃，沸点102.5℃，分子式$C_7H_5F_3$，分子量146.11，純度98%以上(不純物として水分2%以下)の物質である．

被験物質原体は，水分により分解して，刺激性の沸化水素と安息香酸になる．溶媒中(DMSO)では，313 μg/ml～300 mg/mlの濃度範囲で4時間安定であった．

5. 被験物質の調製

被験物質の調製は，使用のつど行った．溶媒はDMSO(和光純薬工業㈱)を用いた．原体を溶媒に溶解して原液を調製し，ついで原液を溶媒で順次希釈して所定の濃度の被験物質調製液を作製した．被験物質調製液は，すべての試験において培養液の0.5%(v/v)になるように加えた．染色体異常試験に用いた被験物質調製液の濃度は，許容範囲内(溶媒中での平均含量が添加量の90.0～110%)の値であった．なお濃度の記載について，純度換算は行わなかった．

6. 細胞増殖抑制試験による処理濃度の決定

染色体異常試験に用いる被験物質の処理濃度を決定するため，被験物質の細胞増殖に及ぼす影響を調べた．被験物質のCHL/IU細胞に対する増殖抑制作用は，フラスコあたりの観察細胞(400細胞)における分裂中期細胞の頻度(分裂指数：Mitotic index)を調べ，被験物質処理群の溶媒対照群に対する細胞増殖の比をもって指標とした．

その結果，連続処理における50%の増殖抑制濃度を明らかに越える濃度(約60%の増殖抑制濃度)を，60%増殖抑制濃度をはさむ2濃度より算出したところ，0.3 mg/mlであった．一方，短時間処理のS9 mix存在下および非存在下では，処理したすべての濃度範囲で50%

を明らかに越える増殖抑制は認められなかった(Fig. 1).

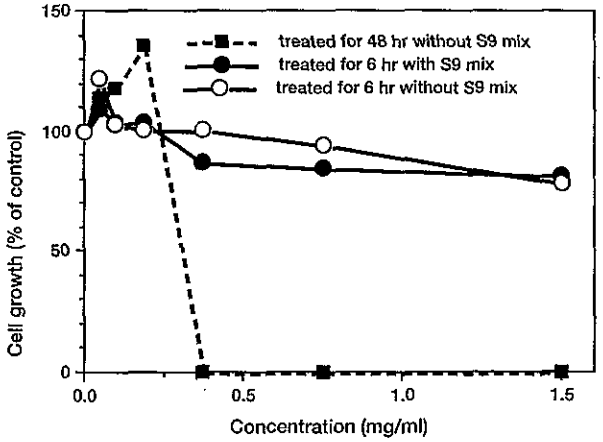

Fig. 1 Growth inhibition of CHL/IU cells treated with trifluoromethylbenzene

7. 実験群の設定

細胞増殖抑制試験の結果より，染色体異常試験で用いる被験物質の高濃度群を，連続処理では0.3 mg/ml，短時間処理では，それぞれ1.5 mg/ml(10 mM)とし，それぞれ高濃度群の1/2の濃度を中濃度，1/4の濃度を低濃度とした．陽性対照物質として用いたマイトマイシンC(MC，協和醗酵工業㈱)およびシクロホスファミド(CPA，Sigma Chemical Co.)は，注射用水(㈱大塚製薬工場)に溶解して調製した．それぞれ染色体異常を誘発することが知られている濃度を適用した．

8. 染色体標本作製法

培養終了の2時間前に，コルセミドを最終濃度が約0.1 μg/mlになるように培養液に加えた．染色体標本の作製は常法に従って行った．スライド標本は各フラスコにつき6枚作製した．作製した標本を3%ギムザ溶液で染色した．

9. 染色体分析

作製したスライド標本のうち，1つのフラスコから得られた異なるスライドを，4名の観察者がそれぞれ処理条件が分からないようにコード化した状態で分析した．染色体の分析は，日本環境変異原学会，哺乳動物試験(MMS)分科会[1]による分類法に基づいて行い，染色体型あるいは染色分体型のギャップ，切断，交換などの構造異常の有無と倍数性細胞(polyploid)の有無について観察した．また構造異常については1群200個，倍数性細胞については1群800個の分裂中期細胞を分析した．

10. 記録と判定

無処理対照，溶媒および陽性対照群と被験物質処理群についての分析結果は，観察した細胞数，構造異常の種類と数，倍数性細胞の数について集計し，各群の値を記録用紙に記入した．

染色体異常を有する細胞の出現頻度について，林[2]の方法を参考にして，溶媒の背景データと被験物質処理群間でフィッシャーの直接確率法[3](多重性を考慮してfamilywiseの有意水準を5%とした)により，有意差検定を実施した．また，フィッシャーの直接確率法で有意差が認められた場合には，用量依存性に関してコクラン・アーミテッジの傾向性検定[4]($p<0.05$)を行った．原則として以上2回の検定でともに有意差が認められた場合を陽性とした．傾向性検定で有意差が認められない場合には疑陽性とした．観察細胞数が，構造異常については100個未満，倍数性細胞については400個未満の場合を細胞毒性のため判定不能とした．

結果および考察

連続処理による染色体分析の結果をTable 1に示した．(トリフルオロメチル)ベンゼンを加えて24時間および48時間連続処理した高濃度群(0.3 mg/ml)では，細胞毒性により分析できなかったが，その他の処理群では，染色体の構造異常および倍数性細胞の誘発作用は認められなかった．

短時間処理による染色体分析の結果をTable 2に示した．(トリフルオロメチル)ベンゼンを加えてS9 mix存在下および非存在下で6時間処理したいずれの処理群においても，染色体の構造異常および倍数性細胞の誘発作用は認められなかった．

従って，(トリフルオロメチル)ベンゼンは，上記の試験条件下で，試験管内のCHL/IU細胞に染色体異常を誘発しないと結論した．

Table 1 Chromosome analysis of Chinese hamster cells (CHL/IU) continuously treated with trifluoromethylbenzene (TFMB)* without S9 mix

Group	Concentration (mg/ml)	Time of exposure (h)	No. of cells analysed	No. of structural aberrations							Others[3]	No. of cells with aberrations				Polyploid[4] (%)	Trend test[5]	
				gap	ctb	cte	csb	cse	mul[2]	total		TAG	(%)	TA	(%)		SA	NA
Control			200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
Solvent[1]	0	24	200	0	1	0	0	0	0	1	0	1	(0.5)	1	(0.5)	0.00		
TFMB	0.075	24	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
TFMB	0.15	24	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.25	NT	NT
TFMB	0.30	24	0[T]													T		
MC	0.00005	24	200	2	22	56	1	0	0	81	0	62	(31.0)	62	(31.0)	0.13		
Solvent[1]	0	48	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
TFMB	0.075	48	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
TFMB	0.15	48	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00	NT	NT
TFMB	0.30	48	0[T]													T		
MC	0.00005	48	200	3	28	60	2	6	20	119	13	68	(34.0)	67	(33.5)	0.50		

Abbreviations: gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring etc.), mul: multiple aberrations, TAG: total no. of cells with aberrations, TA: total no. of cells with aberrations except gap, SA: structural aberration, NA: numerical aberration, MC: mitomycin C, NT: not tested, T: Toxic; This group was excluded from judgement in case of less than one hundred cells for structural aberration analysed and less than four hundred cells for polyploid cells analysed. 1) Dimethyl sulfoxide was used as solvent. 2) More than ten aberrations in a cell were scored as 10. 3) Others, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations. 4) Eight hundred cells were analysed in each group. 5) Cochran · Armitage's trend test was done at $p<0.05$ when the incidence of TAG and polyploid in the treatment groups was significantly different from historical solvent control at $p<0.05$ by Fisher's exact test. *: Purity was more than 98%, and water was contained (less than 2%).

Table 2 Chromosome analysis of Chinese hamster cells (CHL/IU) treated with trifluoromethylbenzene (TFMB)* with and without S9 mix

Group	Concentration (mg/ml)	S9 mix	Time of exposure (h)	No. of cells analysed	No. of structural aberrations							Others[3]	No. of cells with aberrations				Polyploid[4] (%)	Trend test[5]	
					gap	ctb	cte	csb	cse	mul[2]	total		TAG	(%)	TA	(%)		SA	NA
Control				200	0	2	0	0	0	0	2	0	2	(1.0)	2	(1.0)	0.13		
Solvent[1]	0	-	6-(18)	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.13		
TFMB	0.38	-	6-(18)	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.38		
TFMB	0.75	-	6-(18)	200	1	1	0	1	0	0	3	0	2	(1.0)	1	(0.5)	0.13	NT	NT
TFMB	1.5	-	6-(18)	200	1	0	1	0	0	0	2	0	2	(1.0)	1	(0.5)	0.00		
CPA	0.005	-	6-(18)	200	0	1	0	0	2	0	3	0	3	(1.5)	3	(1.5)	0.13		
Solvent[1]	0	+	6-(18)	200	0	0	0	0	1	0	1	1	1	(0.5)	1	(0.5)	0.00		
TFMB	0.38	+	6-(18)	200	1	1	0	0	0	0	2	0	2	(1.0)	1	(0.5)	0.00		
TFMB	0.75	+	6-(18)	200	0	1	0	0	0	0	1	0	1	(0.5)	1	(0.5)	0.38	NT	NT
TFMB	1.5	+	6-(18)	200	0	0	2	0	0	0	2	4	1	(0.5)	1	(0.5)	0.13		
CPA	0.005	+	6-(18)	200	1	14	5	0	2	0	22	1	20	(10.0)	20	(10.0)	0.00		

Abbreviations: gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring etc.), mul: multiple aberrations, TAG: total no. of cells with aberrations, TA: total no. of cells with aberrations except gap, SA: structural aberration, NA: numerical aberration, CPA: cyclophosphamide, NT: not tested. 1) Dimethyl sulfoxide was used as solvent. 2) More than ten aberrations in a cell were scored as 10. 3) Others, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations. 4) Eight hundred cells were analysed in each group. 5) Cochran · Armitage's trend test was done at $p<005$ when the incidence of TAG and polyploid in the treatment groups was significantly different from historical solvent control at $p<0.05$ by Fisher's exact test. *: Purity was more than 98%, and water was contained (less than 2%).

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1,2,3-トリメチルベンゼンのラットを用いる28日間反復経口投与毒性試験

Twenty-eight-day Repeat Dose Oral Toxicity Test of 1,2,3-Trimethylbenzene in Rats

要約

トリメチルベンゼン(TMB)は，工業用に使用される多くの有機溶媒の主要な不純物であり，TMBはdimethyl benzoic acidおよびdimethyl hippuric acidに代謝される[1]．トリメチルベンゼンには，今回試験を実施した1,2,3-トリメチルベンゼンの他，異性体として1,2,4-および1,3,5-トリメチルベンゼンがあり，これら3種類の異性体のうち，1,2,4-トリメチルベンゼンが最も代謝および排泄が遅く，そのため毒性が強いといわれている[2]．また，毒性に関しては吸入時の毒性について，中枢神経抑制，粘膜刺激および呼吸器に対する障害等が明らかになっている[3]．今回，既存化学物質の安全点検に係わる毒性調査事業の一環として，1,2,3-トリメチルベンゼンのSD系ラットを用いる強制経口投与による28日間反復投与毒性試験を実施した．

ラットは1群雌雄各5匹で4試験群，対照群および高用量群には雌雄各5匹の回復群を設け，計60匹を使用した．

1,2,3-トリメチルベンゼンは，コーン油に溶解し，0，100，300および1000 mg/kgを毎日1回，4週間連続経口投与し，一般状態の観察，体重測定，摂餌量測定，血液学検査，血液凝固検査，血液生化学検査，尿検査，器官重量測定および病理学検査を行った．なお，回復期間は2週間とし，投与終了時と同様な検査を実施した．

その結果は，次のとおりである．

一般状態の観察では，雌雄の1000 mg/kg群で全例に流涎が認められたが，回復期間では発現がなかった．

体重は雌の1000 mg/kg群で増加が抑制され，雄の同群で増加抑制傾向がみられた．摂餌量は，雌雄とも群間で差が認められず，飼料効率は雄の300 mg/kg，雌雄の1000 mg/kg群で低値であった．

血液学検査では，被験物質投与の影響を示唆する変化は認められなかったが，血液凝固検査では，活性化部分トロンボプラスチン時間(APTT)が雌雄の被験物質投与群で延長または延長傾向を示し，さらに，プロトロンビン時間(PT)が雄のすべての被験物質投与群，雌の300 mg/kg群で延長または延長傾向を示し，フィブリノーゲン量が雄の1000 mg/kg群で高値を示した．回復試験では雄の1000 mg/kg群でAPTTのみ影響が継続して認められたが，その他の変化には回復が認められた．

血液生化学検査の結果，雌雄のすべての被験物質投与群でGOTの低値，雌のこれらの群および雄の1000 mg/kg群で塩素の低値，さらに雌雄の1000 mg/kg群で総コレステロールの高値，雌の1000 mg/kg群で総蛋白の高値が認められた．これらの変化のうち，雌の1000 mg/kg群で認められた総蛋白の高値は，回復終了時の検査でも僅かに高値であった．

尿検査の結果，雌雄の300および1000 mg/kg群で黄褐色尿が認められ，回復終了時の検査でも，雌雄の1000 mg/kg群で各1例ずつ認められた．

器官重量測定の結果，雄では300および1000 mg/kg群で腎臓，さらに1000 mg/kg群で肝臓の実重量および相対重量の高値が認められた．一方，雌では，300および1000 mg/kg群で胸腺の実重量の低値，さらに1000 mg/kg群で肝臓の実重量および相対重量の高値，脳の実重量の低値，相対重量の高値が認められた．回復終了時の測定では，これらの変化のうち，雄の1000 mg/kg群の腎臓相対重量の高値，雌の同群の胸腺実重量の低値が回復にまで至らなかった．

病理学検査の結果，被験物質の影響が示唆される病変として肉眼所見では投与終了時に腎臓の肥大および淡色化が雄の300および1000 mg/kg群に，肝臓の肥大が雌の1000 mg/kg群に観察された．このうち腎臓の淡色化は雄の回復試験終了時にも観察された．

組織所見では投与終了時に肝細胞腫脹が雌雄の1000 mg/kg群と雄の300 mg/kg群に，腎臓の硝子滴変性が雄の300および1000 mg/kg群に，石灰沈着などの変化が雄の1000 mg/kg群に観察された．腎臓の好酸性小体は雄で対照群から観察されたが，1000 mg/kg群で所見の程度の増強が観察されたほか，脾臓の鬱血が雌の1000 mg/kg群で観察された．

回復試験終了時には脾臓の色素沈着および赤血球系造血亢進が雌雄の1000 mg/kg群に観察された．

以上のことから，無影響量は雌雄とも100 mg/kg/day未満と判断され，低用量による追加試験を実施することとした．

材料および方法

1. 被験物質

1,2,3-トリメチルベンゼン(CAS No.526-73-8，東京化成工業㈱提供)は無色透明の液体で，非水溶性，分子式C_9H_{12}，分子量120.20の化合物である．本試験に用いたロットFJA01の純度は99.8％であった．

2. 供試動物

供試したラット[Crj:CD(SD)系，SPF]は日本チャー

ルス・リバー㈱(神奈川県)から4週齢で購入した．動物を検収後，試験環境に9日間馴化させた後，6週齢で投与を開始した．動物はあらかじめ体重によって層別化し，無作為抽出法により各試験群を構成するように群分けした．動物の識別は，個別飼育ケージに動物標識番号(Animal ID-No.)を付すことにより行った．投与開始時の体重は雄で131～146 g，雌で106～123 gであった．

3. 飼育条件

動物はバリアシステムの飼育室で飼育し，環境調節の目標値は温度23±2℃，相対湿度55±10%，換気回数20回／時，照明150～300 lux，12時間(午前7時点灯，午後7時消灯)とした．㈱東京技研サービスの水洗式飼育機を使用し，金属製前面・床網目飼育ケージに動物を1匹ずつ収容し，オリエンタル酵母工業㈱製造の放射線滅菌改良NIH公開ラット・マウス飼料および水道水を自由に摂取させた．飼育ケージは隔週1回，給餌器は週1回取り換えた．

なお，動物の馴化期間を含め，投与および回復期間中，データの信頼性に影響を及ぼしたと思われる環境要因の変化はなかった．

4. 試験群の構成

試験群は0，100，300および1000 mg/kgの4群とし，1群雌雄各5匹を用い，0および250 mg/kg群に雌雄各5匹の回復群を設け，計60匹を使用した．

〔用量設定理由〕

投与量設定のための2週間投与試験を0，100，300および1000 mg/kgの4用量で実施した．その結果，雌雄の1000 mg/kg群では，体重および摂餌量の低値傾向，肝臓の実重量および相対重量の高値が認められ，さらに雌の同群で流涎が認められた．また血液生化学検査では，雄の1000 mg/kg群，雌の300および1000 mg/kg群で被験物質投与によると考えられる軽微な変化が認められた．

従って，28日間反復投与毒性試験の用量は予備試験と同様の0，100，300および1000 mg/kgに設定した．

5. 投与方法

被験物質の投与経路は経口とした．被験物質はコーン油に溶解し，胃ゾンデを用いて経口投与した．投与容量は体重100 g当り0.5 mlとした．対照群には溶媒のみ投与した．

6. 投与液の調製，分析

被験物質は，各用量(100，300および1000 mg/kg)ごとに所定量を精秤し，コーン油(ナカライテスク㈱)に溶解した．投与液は調製後，冷蔵庫保存で1週間安定であることが確認されているので，本試験においては毎週1回調製を行い，1日分毎に小分けをし使用時まで冷蔵庫に保管した．投与液の濃度分析をすべての群に関し投与1および4週の調製液について実施した結果，設定濃度の98.5～106%の範囲であり，適切に調製されていた．

7. 投与期間

投与期間は28日間とし，投与終了後0および1000 mg/kg群について2週間の回復試験を実施した．

8. 観察，測定および検査

1) 一般状態の観察

全動物を毎日午前，午後の2回観察し，中毒症状の有無，行動異常，死期の迫った動物および死亡動物の有無等を記録した．

2) 体　重

投与開始から回復試験終了時まで，毎週1回測定した．

3) 摂餌量

毎週1回給餌した残量を測定し，飼料摂取量(g/week)を算出した．

4) 臨床検査

投与終了時および回復期間終了時の計2回実施した．採血するに当り，動物は約16時間絶食させた．動物をエーテルで麻酔後開腹し，腹部大動脈から採血した．

a. 血液学検査

EDTA-3Kを添加した初血を用い，白血球数(WBC：暗視野板法)，赤血球数(RBC：暗視野板法)，ヘモグロビン量(HGB：シアンメトヘモグロビン法)，ヘマトクリット値(HCT：RBC, MCVより算出)，平均赤血球容積(MCV：暗視野板法)，平均赤血球血色素量(MCH：HGB, RBCより算出)，平均赤血球血色素濃度(MCHC：HGB, HCTより算出)，血小板数(PLT：暗視野板法)および白血球百分率(フローサイトケミストリー法)を血液自動分析装置THMS H・1E(米国マイルス社)を用いて測定した．

網赤血球(RC)率算定用に，血液塗抹標本を作製しメイ・グリュンワルド・ギムザで染色後，鏡検した．

また，クエン酸ソーダ添加血液の血漿について，プロトロンビン時間(Quick 1段法)，活性化部分トロンボプラスチン時間(クロット法)およびフィブリノーゲン量(トロンビン時間法)を血液凝固自動測定装置KC-40(独国Amelung社)を用いて測定した．

b. 血液生化学検査

血清を用いて，総蛋白(ビューレット法)，アルブミン(B.C.G.法)，A/G比(計算値)，血糖(グルコースオキシダーゼ法)，中性脂肪(酵素法)，総コレステロール(酵素法)，尿素窒素(BUN：ウレアーゼアンモニア法)，総ビリルビン(ジアゾ色素法)，カルシウム(アルセナゾIII色素法)，無機リン(モリブデン酸ブルー法)，ナトリウム(電極法)，カリウム(電極法)および塩素(電極法)をEKTACHEM 700N(米国コダック社)で，クレアチニン(Jaffé法)，グルタミン酸オキザロ酢酸トランスアミナーゼ(GOT：IFCC法)，グルタミン酸ピルビン酸トラ

ンスアミナーゼ(GPT:IFCC法)，γ-グルタミルトランスペプチダーゼ(γ-GTP:Szasz改法)およびアルカリホスファターゼ(ALP:Bessey-Lowry-Brock改良法)をCentrifiChem ENCORE II(米国ベーカー社)で測定した．

c. 尿検査

血液学検査に先立ち，採尿器を用いて24時間(午前10時から翌日午前10時まで)尿を採取し，尿量，色調および濁度を検査後，尿比重計UR-S(㈱アタゴ)を用いて尿比重を測定した．また，尿を遠心分離後Sternheimer変法により沈渣を染色し，鏡検した．pH，潜血，ケトン体，糖，蛋白，ビリルビンおよびウロビリノーゲンについて，N-マルティスティックスSG試験紙(マイルス・三共㈱)およびCLINITEK 200(米国マイルス社)を用いて測定した．

5) 病理学検査

病理解剖は投与終了時および回復期間終了時に動物をエーテル麻酔し，放血致死させ実施した．肉眼的異常を病理解剖所見記録シートに記録した．また，脳，肝臓，腎臓，脾臓，副腎，精巣，卵巣および胸腺について重量を測定し，器官重量・体重比を算出した．上記重量測定器官と下垂体，眼球，甲状腺(上皮小体を含む)，心臓，肺，胃，膀胱，骨髄(大腿骨)および一般状態の観察で流涎が観察されたため唾液腺について10％中性緩衝ホルマリン液で固定した．

病理組織学検査は固定した器官・組織のうち，肝臓，脾臓および腎臓はすべての群について，心臓，副腎，骨髄(大腿骨)および唾液腺については対照群と高用量群について行った．常法に従って薄切標本を作製し，ヘマトキシリン・エオジン染色し鏡検した．

6) データの記録および統計分析

各試験群の体重，摂餌量，血液学検査値，血液生化学検査値，尿検査値(尿量および尿比重のみ)，器官重量および器官重量・体重比は，下記に示した自動判別方式に従い，最初にBartlettの等分散検定を実施した．等分散の場合は一元配置の分散分析を行い，分散が有意で各群の標本数が同数の場合はDunnettの多重比較検定，各群の標本数が異なる場合はDuncanの多重範囲検定で対照群と各投薬群間の有意差を検定した．Bartlettの等分散検定で不等分散の場合はKruskal-Wallisの順位検定を実施し，有意の場合はノンパラメトリックのDunnettの多重比較検定で対照群と各投群間の有意差を検定した．また，病理学検査結果についてはFisherの直接確率検定を実施した．なお，用量相関性については，Jonckheereの傾向検定を用いて有意差を検定した．

有意水準は5および1％の片側検定で実施した．

試験結果

1. 死亡率

投与期間中および回復期間中，雌雄ともいずれの群に

も死亡例は認められなかった．

2. 一般状態の観察

雌雄とも1000 mg/kg群で，投与2週から流涎を示す動物が認められ，雄では投与2週に1例，投与3週に2例，投与4週には全例に，雌では，投与2週に1例，投与3週に全例に観察された．流涎は下顎が濡れる程度であったが，いずれも投与2時間後には消失し，翌日また発現した．その他，雌雄とも異常は認められなかった．回復期間では，雌雄の1000 mg/kg群で，流涎は観察されなかった．

3. 体　　重(Figure 1)

雄では，投与期間および回復期間を通じて，対照群と被験物質投与群とで有意差が認められなかったが，1000 mg/kg群は僅かながら低値傾向にあった．雌では，対照群に比較して1000 mg/kg群で投与4週に低値が認められ，0～4週の体重増加量も低値であった．回復期間では，対照群と1000 mg/kg群とで差が認められなかった．

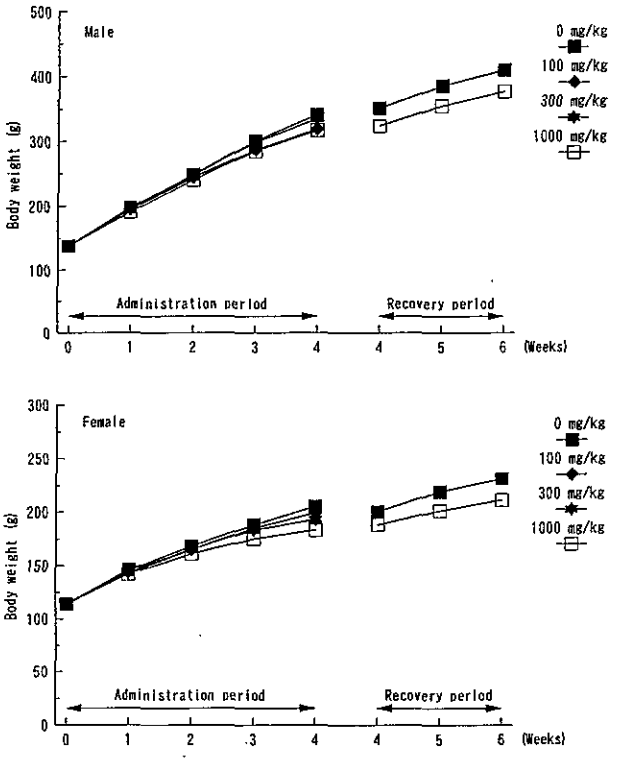

Fig. 1 Body weight changes of rats treated orally with 1,2,3-trimethylbenzene in the twenty-eight-day repeated dose toxicity test

4. 摂 餌 量

雌雄とも，投与期間および回復期間を通じて群間で差が認められなかった．

5. 血液学検査(Table 1)

〔投与終了時の検査結果〕

雄では，対照群に比較して100および300 mg/kg群で

Table 1 Hematology of rats treated orally with 1,2,3-trimethylbenzene in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)	
	0	100	300	1000	0	1000
Male						
No. of animals	5	5	5	5	5	5
HCT (%)	43.6 ± 0.9	43.4 ± 1.1	43.3 ± 1.8	42.2 ± 2.4	42.3 ± 0.9	43.4 ± 1.5
HGB (g/dl)	14.6 ± 0.3	14.4 ± 0.3	14.6 ± 0.5	14.1 ± 0.8	14.8 ± 0.5	14.9 ± 0.5
RBC ($\times 10^6$/mm^3)	7.39 ± 0.35	7.32 ± 0.17	7.30 ± 0.31	7.22 ± 0.48	7.79 ± 0.25	7.82 ± 0.44
MCV (μm^3)	59.0 ± 1.8N	59.4 ± 1.7	59.3 ± 0.2	58.5 ± 1.4	54.4 ± 0.8	55.6 ± 2.0
MCH (pg)	19.8 ± 0.6	19.6 ± 0.3	20.1 ± 0.6	19.5 ± 0.4	19.0 ± 0.4	19.1 ± 0.8
MCHC (%)	33.6 ± 0.5	33.1 ± 0.6	33.9 ± 0.8	33.3 ± 0.5	34.9 ± 0.5	34.3 ± 0.3
PLT ($\times 10^3$/mm^3)	1156 ± 87	1106 ± 124	1067 ± 99	1191 ± 155	1075 ± 74	1067 ± 102
WBC ($\times 10^3$/mm^3)	17.2 ± 3.5	10.9 ± 2.8*	11.0 ± 2.6*	14.0 ± 4.5	10.0 ± 1.9	9.5 ± 3.3
Differential leukocytecounts (%)						
NEUT	9 ± 4	11 ± 4	11 ± 3	14 ± 4	17 ± 4	18 ± 3
LYMPH	88 ± 5	86 ± 4	87 ± 3	83 ± 4	78 ± 5	77 ± 4
MONO	2 ± 1	1 ± 1	1 ± 0	1 ± 1	2 ± 1	2 ± 0
EOSN	1 ± 0	1 ± 1	1 ± 0	1 ± 0	2 ± 1	1 ± 1
BASO	1 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LUC	1 ± 1	0 ± 1	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Reticulocyte (‰)	25 ± 5	19 ± 5	24 ± 8	24 ± 4	20 ± 3	25 ± 4
PT (sec.)	13.7 ± 1.0N	19.4 ± 3.9	25.7 ± 5.3**	38.8 ± 17.9**	13.9 ± 0.6N	17.2 ± 3.0
APTT (sec.)	29.2 ± 2.0N	36.9 ± 4.4	43.1 ± 4.3**	57.3 ± 15.5**	26.2 ± 1.5	29.9 ± 2.4**
Fibrinogen (mg/dl)	250 ± 23	243 ± 17	255 ± 16	282 ± 25*	263 ± 21	265 ± 17
Female						
No. of animals	5	5	5	5	5	5
HCT (%)	42.0 ± 1.4	43.4 ± 1.3	42.7 ± 1.6	42.2 ± 1.2	41.8 ± 1.0	41.3 ± 2.1
HGB (g/dl)	14.3 ± 0.4	14.7 ± 0.6	14.8 ± 0.7	14.4 ± 0.5	14.7 ± 0.3	14.7 ± 0.5
RBC ($\times 10^6$/mm^3)	7.23 ± 0.12	7.45 ± 0.22	7.53 ± 0.25	7.50 ± 0.24	7.51 ± 0.11N	7.24 ± 0.50
MCV (μm^3)	58.0 ± 1.0	58.2 ± 1.0	56.7 ± 1.2	56.3 ± 0.6*	55.6 ± 0.6	57.2 ± 1.8
MCH (pg)	19.8 ± 0.3	19.8 ± 0.4	19.6 ± 0.4	19.3 ± 0.4	19.5 ± 0.2N	20.3 ± 0.9
MCHC (%)	34.1 ± 0.3	34.0 ± 0.5	34.7 ± 0.6	34.3 ± 0.6	35.2 ± 0.4N	35.6 ± 1.3
PLT ($\times 10^3$/mm^3)	1102 ± 89	1065 ± 122	1145 ± 91	1247 ± 94	1068 ± 52	1215 ± 145
WBC ($\times 10^3$/mm^3)	8.4 ± 1.9	10.8 ± 1.2*	5.9 ± 1.7*	8.7 ± 1.3	6.3 ± 3.3	5.4 ± 1.1
Differential leukocyte counts (%)						
NEUT	8 ± 2N	8 ± 2	12 ± 6	9 ± 1	17 ± 6	17 ± 5
LYMPH	89 ± 3	88 ± 3	84 ± 7	88 ± 2	79 ± 6	80 ± 6
MONO	2 ± 1	2 ± 1	2 ± 1	1 ± 1	2 ± 0	2 ± 1
EOSN	1 ± 0	1 ± 0	1 ± 0	1 ± 1	1 ± 0	1 ± 1
BASO	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LUC	1 ± 1	1 ± 0	1 ± 0	1 ± 1	1 ± 0	1 ± 0
Reticulocyte (‰)	14 ± 7	18 ± 7	15 ± 2	19 ± 4	22 ± 8	24 ± 5
PT (sec.)	13.9 ± 0.3	13.4 ± 0.6	16.6 ± 1.4**	14.1 ± 1.1	13.7 ± 0.4	14.1 ± 0.4
APTT (sec.)	23.0 ± 1.1	27.5 ± 3.0	33.7 ± 2.6**	34.0 ± 5.1**	21.2 ± 2.3	20.8 ± 1.8
Fibrinogen (mg/dl)	198 ± 27N	190 ± 6	171 ± 2	187 ± 14	210 ± 17	213 ± 25

NEUT: Neutrophil LYMPH: Lymphocyte MONO: Monocyte EOSN: Eosinophil BASO: Basophil LUC: Large unstained cells

Values are expressed as Mean ± S.D.

Significant difference from control group; *: P≦0.05 **: P≦0.01

N: Non parametric analysis

白血球数の低値が認められたが，対照群が高値傾向にあり，これらの群の値に問題はなかった(背景値　11.8±3.5×10^3/mm^3，n=80)．

雌では，対照群に比較して100 mg/kg群で白血球数の高値，300 mg/kg群で低値が認められたが用量相関性のない変化であった．

〔回復期間終了時の検査結果〕

雌雄とも検査したすべての項目について，対照群と1000 mg/kg群とで差が認められなかった．

6. 血液凝固検査(Table 1)

〔投与終了時の検査結果〕

雄では，対照群に比較して300および1000 mg/kg群でプロトロンビン時間(PT)および活性化部分トロンボプラスチン時間(APTT)が延長を示し，さらに1000 mg/kg群でフィブリノーゲン量が高値を示した．PTおよびAPTTに関しては，統計学的有意差は認められなかったものの，低用量の100 mg/kg群でも延長傾向にあった．雌では，対照群に比較して300および1000 mg/kg群でAPTTが延長を示し，300 mg/kg群でPTが延長を示した．

〔回復期間終了時の検査結果〕

雄の1000 mg/kg群でAPTTの延長が認められた．雌については，3検査項目とも対照群と1000 mg/kg群で差が認められなかった．

7. 血液生化学検査(Table 2)

〔投与終了時の検査結果〕

雄では，対照群に比較してすべての被験物質投与群でGOTが低値を示し，さらに1000 mg/kg群で総コレステロールが高値，塩素が低値を示した．

雌では，対照群に比較してすべての被験物質投与群でGOTおよび塩素が低値を示し，さらに1000 mg/kg群で総コレステロールおよび総蛋白が高値を示した．

〔回復期間終了時の検査結果〕

雄では，対照群に比較して1000 mg/kg群で尿素窒素およびGOTが低値を示し，雌では，対照群に比較して1000 mg/kg群でアルカリ性ホスファターゼが低値，総蛋白，アルブミンおよびカリウムが高値を示した．

8. 尿 検 査(Table 3)

〔投与終了時の検査結果〕

対照群に比較して，雌雄の300および1000 mg/kg群で尿色の変化が認められ，黄褐色尿動物の増加が認められた．

その他の検査項目は，雌雄とも対照群と被験物質投与群とで差が認められなかった．

〔回復期間終了時の検査結果〕

雌雄の1000 mg/kg群で黄褐色尿動物が各1例認められた以外，対照群と1000 mg/kg群で差は認められなかった．

9. 器官重量(Table 4)

〔投与終了時の検査結果〕

雄では，対照群に比較して300および1000 mg/kg群で腎臓重量が高値を示し，さらに1000 mg/kg群で肝臓重量が高値を示した．

雌では，対照群に比較して300および1000 mg/kg群で胸腺重量が低値を示し，さらに1000 mg/kg群で脳重量が低値，肝臓重量が高値を示した．その他，100 mg/kg群でも脳重量が低値を示したが，用量相関性のない変化であった．

〔回復期間終了時の検査結果〕

雄では，対照群に比較して1000 mg/kg群で脳および脾臓重量が低値を示した．

雌では，対照群に比較して1000 mg/kg群で胸腺重量が低値を示した．

10. 器官重量・体重比(相対重量)(Table 4)

〔投与終了時の検査結果〕

雄では，対照群に比較して300および1000 mg/kg群で腎臓相対重量が高値を示し，さらに1000 mg/kg群で肝臓相対重量が高値を示した．

雌では，対照群に比較して1000 mg/kg群で脳および肝臓相対重量が高値を示した．

〔回復期間終了時の検査結果〕

雄では，対照群に比較して1000 mg/kg群で腎臓相対重量が高値を示した．

雌では，対照群と1000 mg/kg群で差が認められなかった．

11. 病理学検査

a) 剖検所見(Table 5)

投与終了時において，対照群に比較して被験物質投与群で多く観察された所見として，腎臓の肥大が雄の300，1000 mg/kg群でそれぞれ1および4例，雌の1000 mg/kg群で1例に，腎臓の淡色化が雄の300，1000 mg/kg群でそれぞれ2および4例に観察された．また肝臓の肥大が雌の1000 mg/kg群で1例に観察された．その他観察された所見は，対照群，被験物質投与群で単発性の発生であった．

回復試験終了時において，対照群に比較して被験物質投与群で多く観察された所見として，腎臓の淡色化が雄の1000 mg/kg群で4例に観察された．その他観察された所見は，対照群，1000 mg/kg群でいずれも単発性の発生であった．

b) 組織所見 (Table 6)

投与終了時において，対照群に比較して被験物質投与群に多く観察された所見として，肝細胞腫脹が雌雄の1000 mg/kg群の全5例と，雄の300 mg/kg群の2例に，

Table 2 Blood chemistry of rats treated orally with 1,2,3-trimethylbenzene in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)	
	0	100	300	1000	0	1000
Male						
No. of animals	5	5	5	5	5	5
BUN (mg/dl)	12.1 ± 2.5	9.9 ± 1.5	9.9 ± 2.0	12.7 ± 4.1	13.2 ± 2.0	10.8 ± 0.7*
Creatinine (mg/dl)	0.62 ± 0.08	0.64 ± 0.05	0.60 ± 0.08	0.61 ± 0.12	0.56 ± 0.04	0.58 ± 0.05
T.cholesterol (mg/dl)	47 ± 15	33 ± 10	49 ± 12	69 ± 17*	52 ± 29	38 ± 10
T.protein (g/dl)	5.39 ± 0.21	5.43 ± 0.14	5.39 ± 0.10	5.48 ± 0.18	5.63 ± 0.42	5.77 ± 0.15
Albumin (g/dl)	3.11 ± 0.13	3.12 ± 0.08	3.13 ± 0.09	3.19 ± 0.12	3.17 ± 0.30	3.32 ± 0.10
A/G	1.37 ± 0.10	1.35 ± 0.11	1.39 ± 0.07	1.40 ± 0.08	1.30 ± 0.08	1.35 ± 0.06
Glucose (mg/dl)	134 ± 12	124 ± 17	124 ± 9	138 ± 19	132 ± 26	139 ± 15
Triglyceride (mg/dl)	50.5 ± 18.0	45.2 ± 16.1	60.9 ± 22.6	48.4 ± 15.7	55.2 ± 33.1	56.9 ± 10.7
GOT (U/l)	57 ± 19	40 ± 8*	34 ± 7**	36 ± 7*	53 ± 6	41 ± 5**
GPT (U/l)	11 ± 2	12 ± 2	12 ± 3	14 ± 3	12 ± 2	14 ± 3
ALP (U/l)	161 ± 25N	150 ± 7	167 ± 52	195 ± 34	143 ± 43	116 ± 19
γ-GTP (U/l)	0.7 ± 0.5	0.7 ± 0.4	0.6 ± 0.4	0.9 ± 0.2	0.8 ± 0.4	0.5 ± 0.3
T.bilirubin (mg/dl)	0.13 ± 0.05	0.12 ± 0.03	0.12 ± 0.04	0.10 ± 0.01	0.12 ± 0.05N	0.11 ± 0.02
Sodium (mmol/l)	142.8 ± 1.6	143.9 ± 1.5	143.2 ± 1.2	141.7 ± 1.5	142.9 ± 1.0	143.6 ± 0.8
Potassium (mmol/l)	4.87 ± 0.42	4.38 ± 0.13	4.38 ± 0.27	4.66 ± 0.42	4.49 ± 0.26	4.70 ± 0.31
Chloride (mmol/l)	109.3 ± 1.8	108.3 ± 1.0	107.7 ± 1.4	106.1 ± 1.5**	107.9 ± 1.4	109.3 ± 1.0
Calcium (mg/dl)	9.86 ± 0.14	9.81 ± 0.19	10.02 ± 0.44	10.08 ± 0.41	9.76 ± 0.39	9.88 ± 0.23
I.phosphate (mg/dl)	8.37 ± 0.58	7.67 ± 0.81	7.67 ± 0.76	7.78 ± 0.52	6.90 ± 0.60	6.99 ± 0.26
Female						
No. of animals	5	5	5	5	5	5
BUN (mg/dl)	14.5 ± 4.7N	13.1 ± 1.4	14.2 ± 0.8	11.7 ± 1.4	13.1 ± 1.1	14.9 ± 2.5
Creatinine (mg/dl)	0.65 ± 0.10	0.58 ± 0.05	0.63 ± 0.04	0.58 ± 0.09	0.56 ± 0.06	0.58 ± 0.10
T.cholesterol (mg/dl)	43 ± 14	52 ± 18	46 ± 11	74 ± 12**	50 ± 19	61 ± 12
T.protein (g/dl)	5.34 ± 0.11	5.59 ± 0.19	5.67 ± 0.28	5.99 ± 0.35**	5.79 ± 0.20	6.07 ± 0.10*
Albumin (g/dl)	3.21 ± 0.04N	3.41 ± 0.13	3.45 ± 0.22	3.62 ± 0.28	3.41 ± 0.17	3.60 ± 0.06*
A/G	1.51 ± 0.04N	1.56 ± 0.02	1.55 ± 0.10	1.53 ± 0.11	1.44 ± 0.07	1.46 ± 0.05
Glucose (mg/dl)	103 ± 9	107 ± 10	102 ± 12	115 ± 15	116 ± 14	112 ± 7
Triglyceride (mg/dl)	28.6 ± 6.8N	40.5 ± 12.2	28.9 ± 1.3	34.2 ± 2.2	49.1 ± 25.8	36.9 ± 10.3
GOT (U/l)	62 ± 15	44 ± 4**	44 ± 7**	42 ± 8**	54 ± 7	56 ± 3
GPT (U/l)	12 ± 1	11 ± 2	12 ± 3	14 ± 2	12 ± 1	13 ± 2
ALP (U/l)	95 ± 22	75 ± 28	85 ± 12	79 ± 30	90 ± 22	61 ± 12*
γ-GTP (U/l)	0.6 ± 0.4	0.6 ± 0.5	0.6 ± 0.4	0.6 ± 0.4	0.8 ± 0.2	1.3 ± 0.5
T.bilirubin (mg/dl)	0.12 ± 0.02	0.15 ± 0.05	0.13 ± 0.03	0.16 ± 0.05	0.16 ± 0.01N	0.18 ± 0.02
Sodium (mmol/l)	142.8 ± 0.5	142.5 ± 0.6	142.6 ± 0.8	141.9 ± 0.6	142.8 ± 1.5	143.0 ± 0.9
Potassium (mmol/l)	4.60 ± 0.30	4.59 ± 0.31	4.61 ± 0.33	4.25 ± 0.14	4.18 ± 0.12	4.50 ± 0.22*
Chloride (mmol/l)	113.3 ± 1.2	110.9 ± 1.3*	110.9 ± 1.8*	108.8 ± 1.1**	110.8 ± 1.7	109.6 ± 0.7
Calcium (mg/dl)	9.74 ± 0.13	9.99 ± 0.12	9.84 ± 0.29	9.96 ± 0.08	9.62 ± 0.12	9.78 ± 0.17
I.phosphate (mg/dl)	6.83 ± 0.51N	7.08 ± 0.17	6.99 ± 0.56	7.23 ± 0.14	6.34 ± 0.62	6.95 ± 1.37

Values are expressed as Mean ± S.D.

Significant difference from control group; *: P≦0.05 **: P≦0.01

N: Non parametric analysis

Table 3 Urinalysis of rats treated orally with 1,2,3-trimethylbenzene in the twenty-eight-day repeated dose toxicity test

Item		28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)	
		0	100	300	1000	0	1000
Male							
No. of animals		5	5	5	5	5	5
Volume (ml)		22 ± 15	11 ± 4	15 ± 6	23 ± 7	21 ± 5	20 ± 10
Specific gravity		1.039 ± 0.021	1.052 ± 0.016	1.052 ± 0.015	1.044 ± 0.018	1.032 ± 0.015	1.033 ± 0.016
Color	Slignt yellow	5	5	2	1	5	4
	Yellow-brown	0	0	3	4	0	1
Turbidity	Clear muddy	5	5	5	5	5	5
pH	5	0	0	1	0	0	0
	6	0	1	1	2	0	0
	6.5	0	1	1	2	0	0
	7	0	1	1	0	0	0
	7.5	1	0	1	1	0	0
	8	0	1	0	0	3	0
	8.5	2	0	0	0	0	1
	≧9	2	1	0	0	2	4
Occult blood	-	4	5	5	5	5	5
	+/-	1	0	0	0	0	0
Ketones	-	0	1	0	2	1	1
	+/-	3	2	1	2	3	2
	1+	2	2	4	1	1	2
Glucose (g/dl)	-	5	5	5	5	5	5
Protein	-	1	1	0	0	0	0
(mg/dl)	30	3	0	1	2	2	2
	100	1	3	1	2	3	2
	≧300	0	1	3	1	0	1
Bilirubin	-	5	4	3	4	5	4
	1+	0	1	2	1	0	1
Urobilinogen	0.1	2	1	1	4	3	2
(E.U./dl)	1.0	3	4	4	1	2	3
Erythrocytes	-	5	5	5	5	5	5
Leukocytes	-	5	5	5	5	5	5
Epith. cells	-	5	5	5	5	5	5
Casts	-	5	5	5	5	5	5
Fat glob.	-	5	5	5	5	5	5
M. threads	-	5	5	5	5	4	5
	+	0	0	0	0	1	0
others	-	2	0	0	1	2	2
	+	3	5	5	4	3	3

Fat glob.: Fat globule, M. threads: Mucous threads, others: Crystals

Values of volume and specific gravity are expressed as Mean±S.D., other values are expressed as No. of animals

Table 3 (continued)

Item		28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)	
		0	100	300	1000	0	1000
Female							
No. of animals		5	5	5	5	5	5
Volume (ml)		13 ± 2N	12 ± 4	11 ± 5	20 ± 10	10 ± 3	11 ± 4
Specific gravity		1.046 ± 0.006	1.050 ± 0.025	1.057 ± 0.031	1.040 ± 0.019	1.054 ± 0.015	1.057 ± 0.027
Color	Slight yellow	5	5	3	2	5	4
	Yellow-brown	0	0	2	3	0	1
Turbidity	Clear muddy	5	5	5	5	5	5
pH	5	0	0	2	1	0	0
	5.5	1	1	0	1	0	0
	6	1	0	1	1	1	0
	6.5	1	1	1	2	1	1
	7	1	2	0	0	1	1
	7.5	1	1	1	0	1	1
	8	0	0	0	0	1	1
	8.5	0	0	0	0	0	1
Occult blood	−	5	5	5	5	5	5
Ketones	−	1	1	1	2	0	0
	+/−	0	3	1	2	4	3
	1+	4	1	3	1	1	2
Glucose (g/dl)	−	5	5	5	5	5	5
Protein	−	0	1	1	2	0	0
(mg/dl)	+/−	1	2	0	1	0	0
	30	0	1	1	1	2	2
	100	2	0	1	1	2	1
	≧300	2	1	2	0	1	2
Bilirubin	−	3	5	4	5	3	2
	1+	2	0	1	0	2	3
Urobilinogen	0.1	1	1	2	3	0	0
(E.U./dl)	1.0	4	4	3	2	5	5
Erythrocytes	−	5	5	5	5	5	5
Leukocytes	−	5	5	5	5	5	5
Epith. cells	−	5	5	5	5	5	5
Casts	−	5	5	5	5	5	5
Fat glob.	−	5	5	5	5	5	5
M. threads	−	5	5	5	5	5	5
others	−	1	0	2	2	1	2
	+	4	5	3	3	4	3

Fat glob.: Fat globule , M. threads: Mucous threads, others: Crystals

Values of volume and specific gravity are expressed as Mean ± S.D., other values are expressed as No. of animals

N: Non parametric analysis

Table 4 Absolute and relative organ weights of rats treated orally with 1,2,3-trimethylbenzene in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)	
	0	100	300	1000	0	1000
Male						
No. of animals	5	5	5	5	5	5
Body weight (g)	332 ± 34	319 ± 20	335 ± 30	311 ± 16	410 ± 30	377 ± 18
Absolute organ weight						
Brain (g)	2.05 ± 0.03	2.01 ± 0.10	2.03 ± 0.11	1.99 ± 0.07	2.15 ± 0.04	2.03 ± 0.10*
Liver (g)	9.97 ± 1.50	9.78 ± 1.01	10.78 ± 1.56	12.35 ± 1.64*	11.99 ± 2.02	11.57 ± 1.27
Kidneys (g)	2.50 ± 0.23	2.58 ± 0.17	2.95 ± 0.33*	3.19 ± 0.38**	2.87 ± 0.21	3.08 ± 0.34
Spleen (g)	0.64 ± 0.07	0.55 ± 0.07	0.58 ± 0.07	0.55 ± 0.03	0.70 ± 0.02	0.63 ± 0.05**
Adrenals (mg)	47 ± 4	46 ± 5	51 ± 3	49 ± 6	52 ± 4	54 ± 8
Testes (g)	2.86 ± 0.15	2.85 ± 0.10	2.93 ± 0.12	2.62 ± 0.27	3.23 ± 0.20	3.06 ± 0.16
Thymus (mg)	717 ± 133	639 ± 118	593 ± 56	592 ± 104	471 ± 79	508 ± 40
Relative organ weight						
Brain (%)	0.622 ± 0.055	0.629 ± 0.028	0.608 ± 0.025	0.644 ± 0.058	0.527 ± 0.036	0.539 ± 0.027
Liver (%)	2.992 ± 0.162N	3.057 ± 0.157	3.199 ± 0.190	4.010 ± 0.788**	2.911 ± 0.310	3.065 ± 0.232
Kidneys (%)	0.754 ± 0.053	0.810 ± 0.063	0.879 ± 0.045**	1.025 ± 0.080**	0.701 ± 0.061	0.814 ± 0.059**
Spleen (%)	0.195 ± 0.036	0.173 ± 0.029	0.174 ± 0.027	0.177 ± 0.020	0.171 ± 0.012	0.166 ± 0.016
Adrenals (%)	0.014 ± 0.001	0.015 ± 0.002	0.015 ± 0.001	0.016 ± 0.002	0.013 ± 0.001	0.014 ± 0.002
Testes (%)	0.864 ± 0.043	0.897 ± 0.058	0.877 ± 0.065	0.843 ± 0.057	0.789 ± 0.035	0.813 ± 0.068
Thymus (%)	0.221 ± 0.060	0.199 ± 0.028	0.179 ± 0.033	0.192 ± 0.039	0.115 ± 0.021	0.135 ± 0.014
Female						
No. of animals	5	5	5	5	5	5
Body weight (g)	209 ± 7	199 ± 16	193 ± 20	178 ± 6**	231 ± 25	211 ± 14
Absolute organ weight						
Brain (g)	1.97 ± 0.05	1.83 ± 0.06**	1.89 ± 0.05	1.85 ± 0.09**	1.88 ± 0.05	1.89 ± 0.09
Liver (g)	6.03 ± 0.42	5.91 ± 0.59	5.86 ± 0.73	7.03 ± 0.52*	6.28 ± 0.82	6.11 ± 0.72
Kidneys (g)	1.66 ± 0.26	1.62 ± 0.23	1.65 ± 0.15	1.68 ± 0.22	1.74 ± 0.16	1.66 ± 0.14
Spleen (g)	0.41 ± 0.086	0.35 ± 0.05	0.33 ± 0.06	0.37 ± 0.03	0.40 ± 0.02	0.42 ± 0.04
Adrenals (mg)	64 ± 5	59 ± 8	59 ± 7	55 ± 10	60 ± 8	64 ± 14
Ovaries (mg)	86 ± 11	87 ± 17	82 ± 18	78 ± 10	84 ± 7	86 ± 9
Thymus (mg)	485 ± 46	411 ± 24	365 ± 57**	401 ± 87*	468 ± 99	336 ± 78*
Relative organ weight						
Brain (%)	0.945 ± 0.035N	0.925 ± 0.066	0.987 ± 0.108	1.034 ± 0.015*	0.819 ± 0.082	0.894 ± 0.042
Liver (%)	2.885 ± 0.146	2.961 ± 0.029	3.032 ± 0.093	3.941 ± 0.233**	2.716 ± 0.190	2.885 ± 0.227
Kidneys (%)	0.796 ± 0.112	0.810 ± 0.080	0.860 ± 0.063	0.942 ± 0.093	0.760 ± 0.090	0.787 ± 0.046
Spleen (%)	0.197 ± 0.024	0.177 ± 0.017	0.174 ± 0.034	0.207 ± 0.015	0.173 ± 0.021	0.201 ± 0.018
Adrenals (%)	0.030 ± 0.001	0.030 ± 0.004	0.031 ± 0.003	0.031 ± 0.005	0.026 ± 0.005	0.030 ± 0.007
Ovaries (%)	0.041 ± 0.005	0.043 ± 0.007	0.042 ± 0.005	0.044 ± 0.004	0.036 ± 0.002	0.041 ± 0.005
Thymus (%)	0.232 ± 0.019	0.207 ± 0.020	0.190 ± 0.031	0.244 ± 0.045	0.201 ± 0.024	0.159 ± 0.035

Values are expressed as Mean ± S.D.
Significant difference from control group; *: P≦0.05 **: P≦0.01
N: Non parametric analysis

Table 5 Summary of gross findings in rats treated orally with 1,2,3-trimethylbenzene in the twenty-eight-day repeated dose toxicity test

Item		28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)	
Organ	Findings	0	100	300	1000	0	1000
Male							
No. of animals necropsied		5	5	5	5	5	5
HEMATOPOIETIC SYSTEM							
thymus	red patch/zone	1	0	1	0	0	0
RESPIRATORY SYSTEM							
lung	colored patch/zone	0	0	0	0	0	1
	red patch/zone	0	0	0	0	0	1
URINARY SYSTEM							
kidney	enlarged	0	0	1	4*	0	0
	pale	0	0	2	4*	0	4*
Female							
No. of animals necropsied		5	5	5	5	5	5
HEMATOPOIETIC SYSTEM							
lymph node	enlarged	0	0	0	1	0	0
RESPIRATORY SYSTEM							
lung	colored patch/zone	0	1	0	0	0	0
DIGESTIVE SYSTEM							
small intestine							
	deformed	0	0	1	0	0	0
liver	enlarged	0	0	0	1	0	0
URINARY SYSTEM							
kidney	enlarged	0	0	0	1	0	0
REPRODUCTIVE SYSTEM							
ovary	cyst	0	0	0	0	1	0
uterus	dilated lumen	1	0	1	0	0	0
broad ligament of uterus							
	cyst	0	0	0	1	0	0

Significant difference from control group; *: $P \leqq 0.05$

腎臓の硝子滴変性が雄の300, 1000 mg/kg群でそれぞれ4および5例に観察された．雄において対照群から観察されたものの，腎臓の好酸性小体が対照群，100，300および1000 mg/kgの各群でそれぞれ1，1，2（軽度：1，中度：1）および3（軽度：1，重度：2）例と，300および1000 mg/kg群で程度の増強を示した．また腎臓の好酸性小体の程度の増強に伴って，腎臓の石灰沈着，蛋白円柱および管腔拡張が雄の1000 mg/kg群でそれぞれ4，3および2例に観察された．また脾臓の鬱血が雌の1000 mg/kg群で全5例に観察された．発生数は1例のみであったが，雄の1000 mg/kg群に脾臓の赤血球系造血亢進および肝臓の糖質蓄積が観察された．その他，被験物質投与群で減少した所見として雄の1000 mg/kg群および雌の全被験物質投与群で肝臓の脂肪化の発生がなかった．なお，被験物質投与群において一般状態で流涎が観察されたため唾液腺の組織学的検査を行ったが，異常は認められなかった．また，対照群を含め試験群で肝臓の肉芽巣，腎臓の細胞浸潤および好塩基化，副腎の空胞化が観察されたが，群間で発生数に差は認められなかった．

その他，観察された所見は単発性の発生にとどまった．

回復試験終了時において，対照群に比較して被験物質投与群で多く観察された所見として，脾臓の色素沈着が雌雄の1000 mg/kg群で全5例に，赤血球系造血亢進が雌雄の1000 mg/kg群にそれぞれ雄全5例，雌2例に観察

Table 6 Summary of histopathological findings in rats treated orally with 1,2,3-trihydroxybenzene in the 28-day repeated dose toxicity test

Item		28 days dosing groups (mg/kg)												14 days recovery groups (mg/kg)					
		0			100			300			1000			0			1000		
Organ	Findings	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Male																			
No. of animals necropsied			5			5			5			5			5			5	
HEMATOPOIETIC SYSTEM																			
spleen		(5)			(5)			(5)			(5)			(5)			(5)		
	deposit of pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0**
	erythropoiesis, increased	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	5	0	0**
DIGESTIVE SYSTEM																			
liver		(5)			(5)			(5)			(5)			(5)			(5)		
	fatty change	3	0	0	3	0	0	1	0	0	0	0	0	3	0	0	0	0	0
	glycogen storage	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	swelling of liver cells	0	0	0	0	0	0	2	0	0	5	0	0**	0	0	0	0	0	0
	granulation	3	0	0	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0
URINARY SYSTEM																			
kidney		(5)			(5)			(5)			(5)			(5)			(5)		
	basophilic change	3	0	0	3	0	0	2	0	0	4	1	0	5	0	0	3	1	0
	deposit of calcium	0	0	0	0	0	0	0	0	0	3	1	0*	1	0	0	1	0	0
	eosinophilic body	1	0	0	0	1	0	1	1	0	1	0	2	0	0	0	2	0	1
	hyaline droplet	0	0	0	0	0	0	4	0	0*	5	0	0**	0	0	0	0	0	0
	protein cast	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	1	0	0
	tubular dilatation	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	1	0	0
	infiltration/cellular	1	0	0	1	0	0	1	0	0	2	0	0	0	0	0	3	0	0
	lymphocytic infiltration	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ENDOCRINE SYSTEM																			
adrenal gland		(5)			(0)			(0)			(5)			(0)			(0)		
	vacuolic change	5	0	0	-	-	-	-	-	-	4	0	0	-	-	-	-	-	-
Female																			
No. of animals necropsied			5			5			5			5			5			5	
HEMATOPOIETIC SYSTEM																			
spleen		(5)			(5)			(5)			(5)			(5)			(5)		
	congestion	0	0	0	0	0	0	0	0	0	5	0	0**	0	0	0	0	0	0
	deposit of pigment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0**
	erythropoiesis, increased	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
DIGESTIVE SYSTEM																			
liver		(5)			(5)			(5)			(5)			(5)			(5)		
	fatty change	4	0	0	0	0	0*	0	0	0*	0	0	0*	0	0	0	0	0	0
	swelling of liver cells	0	0	0	0	0	0	0	0	0	5	0	0**	0	0	0	0	0	0
	granulation	3	0	0	4	0	0	3	0	0	1	0	0	2	0	0	1	0	0
URINARY SYSTEM																			
kidney		(5)			(5)			(5)			(5)			(5)			(5)		
	basophilic change	2	0	0	1	0	0	1	0	0	0	0	0	2	0	0	3	0	0
	deposit of calcium	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	lymphocytic infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

1:slight 2:moderate 3:marked
Numbers in parenthesis indicate No. of animals examined microscopically at this site.
Significant difference from control group; *:P≦0.05 **:P≦0.01

された．また，腎臓の好酸性小体が雄の1000 mg/kg群で3例に観察されたが，そのうちの1例には投与終了時と同様の重度の好酸性小体と蛋白円柱および管腔拡張も観察された．その他，肝臓の肉芽巣および脂肪化，腎臓の好塩基化および細胞浸潤が観察された．

その他，観察された所見は単発性の発生にとどまった．

考察および結論

一般状態の観察では，雌雄とも1000 mg/kg群で流涎が認められたが，投与2時間後には消失した．回復期間に入ると，雌雄の1000 mg/kg群で認められた流涎は1例も観察されず，この症状が被験物質投与に起因することを裏付けた．

雌雄とも摂餌量の増減は認められなかったが，体重が雌の1000 mg/kg群で低値，雄の同用量群で低値傾向を示したため，これらの群では飼料効率が低値を示した．

血液学検査に関しては，病理組織学的検査で脾臓に赤血球系造血亢進が認められたが，被験物質投与の影響を示唆する変化は認められなかった．

血液凝固検査において，雌雄ともAPTTに低用量の100 mg/kgから延長傾向または延長が認められ，雄ではPTも同様な傾向を示した．雄の1000 mg/kg群では，フィブリノーゲン量も高値を示しており，抗凝固薬や抗血小板薬で認められるものと同様な変化であったが，これらの薬剤で認められる血小板数の増加は本被験物質では認められていない．

血液生化学検査の結果，雌雄のすべての被験物質投与群でGOTの低値が認められた．

GOTはGPT同様逸脱酵素であり高値の場合のみ意義のある変化といわれている．GPTに関しては，セファロスポリン系抗生物質の投与で直接活性阻害を引き起こすことが知られているが[4]，GOTに関しての報告は見あたらず，本試験における他の諸検査の結果からもGOT低値の原因は明らかではない．その他雌雄で認められた総コレステロールおよび塩素，雌で認められた総蛋白についても変化の程度は僅かであり，特定器官の障害を明確に示唆するものではないと考えられた．

尿検査の結果，尿色の黄褐色化が認められたが，潜血やビリルビンなどの増加は認められなかった．被験物質の代謝物による色調変化が示唆されるが，回復期間終了時の検査でも雌雄各1例に認められていることから，原因は明らかではない．

器官重量測定の結果，雌雄で肝臓重量の高値，さらに雄で腎臓重量の高値，雌で胸腺重量の低値が認められ，胸腺の変化を除き，病理学検査で重量増加の要因となる対応所見が認められた．

病理学検査の結果，被験物質投与の影響と考えられる変化として，肉眼所見では投与終了時の雄に腎臓の淡色化と肥大，雌に肝臓の肥大が観察され，回復試験終了時の雄にも腎臓の淡色化が観察された．その他の所見は自然発生性の所見と考えられた．組織所見では被験物質の影響と考えられる変化として，投与終了時の雌雄に中心性肝細胞腫脹，雄に腎臓の硝子滴変性が観察された．また被験物質投与によって，対照群にも観察された腎臓の好酸性小体の程度が増強し，それに伴って対照群には見られなかった腎臓の石灰沈着，蛋白円柱および管腔拡張が観察された．その他，各1例のみであったが，1000 mg/kg群の雄に肝臓の糖質蓄積および脾臓の赤血球系造血亢進，雌に脾臓の鬱血が観察された．回復試験終了時の雌雄に脾臓の赤血球系造血亢進および色素沈着が観察された．逆に投与終了時の雌雄に肝臓の脂肪化の発生が減少した．また，投与終了時に雌の腎臓の肉眼的な肥大が観察されたが，組織学的には異常は認められなかった．その他の所見は，自然発生性の変化と考えられた．

投与終了時に観察された雌の肝臓の肉眼的な肥大，雌雄の中心性の肝細胞腫脹は肝機能に関する臨床検査値の明らかな上昇もなく，毒性変化というより生体の適応反応と考えられた．すなわち被験物質投与によって，薬物代謝誘導などにより，肝細胞が肥大したものと考えられた[5]．この変化は回復試験終了時には観察されず，可逆性の変化と考えられた．

投与終了時に観察された雄の腎臓の硝子滴変性および投与終了時と回復試験終了時に観察された雄の腎臓の好酸性小体は，近位尿細管に観察された．揮発性炭化水素類の薬物投与により，肝臓で合成されたα2u-グロブリンが揮発性炭化水素と結合し，蛋白代謝の低下した近位尿細管に蓄積するとされ，蓄積が強くなると尿細管上皮細胞の変性，壊死，再生変化，蛋白円柱が観察される[6]．本試験の1,2,3-トリメチルベンゼンもベンゼン環をもつ揮発性炭化水素であり，同様の所見が観察されたと考えられた．

回復試験終了時の雄の1000 mg/kg群の1例にも同様の変化が認められたものの，発生数の減少より回復傾向はあるものと考えられた．

また投与終了時に観察された雌の脾臓の鬱血および雄の脾臓の赤血球系造血亢進，回復試験終了時に観察された雌雄の脾臓の赤血球系造血亢進と色素沈着は，投与期間および回復期間中の赤血球系の臨床検査値の異常はみられず，骨髄の異常も観察されなかったことから原因は不明であった．しかし血液凝固系の延長が起こっており，また造血障害を示唆する文献もあることから[3]，投与期間中に何らかの赤血球への障害があったことが示唆され，回復期での造血亢進は障害に対する回復反応とも考えられた．

以上のことから，本被験物質は腎臓，血液凝固系を主な標的とし，無影響量は雌雄とも100 mg/kg/day未満と判断された．従って無影響量が求まらなかったことから，さらに低用量による追加試験を実施した．

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1,2,3-トリメチルベンゼンのラットを用いる28日間反復経口投与毒性試験(追加試験)

Twenty-eight-day Repeat Dose Oral Toxicity Test of 1,2,3-Trimethylbenzene in Rats (Additional test)

要約

先に実施した1,2,3-トリメチルベンゼンのSD系ラットを用いる強制経口投与による28日間反復投与毒性試験において最低用量の100 mg/kg群でも雌雄で活性化部分トロンボプラスチン時間(APTT), さらに雄の100 mg/kg群でプロトロンビン時間(PT)の延長傾向が認められたため, 無影響量を見い出すための追加試験を実施した.

ラットは1群雌雄各5匹で4試験群, 計40匹を使用した.

1,2,3-トリメチルベンゼンは, コーン油に溶解し, 0, 3, 10および30 mg/kgを毎日1回, 4週間連続経口投与し, 一般状態の観察, 体重測定, 摂餌量測定, 血液凝固能検査, 血液生化学検査(GOTおよび塩素のみ)および病理学的検査(剖検)を行った.

その結果は, 次のとおりであった.

一般状態の観察では, 雌雄とも投与期間を通じて異常動物は認められなかった.

体重, 摂餌量および飼料効率は, 雌雄とも群間で差が認められなかった.

血液凝固能検査では, 活性化部分トロンボプラスチン時間(APTT), プロトロンビン時間(PT)およびフィブリノーゲン量のいずれも, 雌雄ともに被験物質投与群と対照群とで差が認められなかった.

血液生化学検査の結果, 雌雄のすべての被験物質投与群ともGOTおよび塩素の2項目について, 対照群と差が認められなかった.

病理学的検査の結果, 被験物質の影響が示唆される肉眼所見は雌雄いずれの群にも認められなかった.

以上のことから, 無影響量は雌雄とも30 mg/kg/dayと判断された.

材料および方法

1. 被験物質

1,2,3-トリメチルベンゼン(CAS No.526-73-8, 東京化成工業㈱提供)は無色透明の液体で, 非水溶性, 分子式C_9H_{12}, 分子量120.20の化合物である. 本試験に用いたロットFJA01の純度は99.8%であった.

2. 供試動物

供試したラット [Crj:CD(SD)系, SPF] は日本チャールス・リバー㈱(神奈川県)から4週齢で購入した. 動物を検収後, 試験環境に8日間馴化させた後, 6週齢で投与を開始した. 動物はあらかじめ体重によって層別化し, 無作為抽出法により各試験群を構成するように群分けした. 動物の識別は, 個別飼育ケージに動物標識番号(Animal ID-No.)を付すことにより行った. 投与開始時の体重は雄で130～144 g, 雌で104～121 gであった.

3. 飼育条件

動物はバリアシステムの飼育室で飼育し, 環境調節の目標値は温度23±2℃, 相対湿度55±10%, 換気回数20回/時, 照明150～300 lux, 12時間(午前7時点灯, 午後7時消灯)とした. ㈱東京技研サービスの水洗式飼育機を使用し, 金属製前面・床網目飼育ケージに動物を1匹ずつ収容し, オリエンタル酵母工業㈱製造の放射線滅菌改良ＮＩＨ公開ラット・マウス飼料および水道水を自由に摂取させた. 飼育ケージは隔週1回, 給餌器は週1回取り換えた.

なお, 動物の馴化期間を含め, 投与および回復期間中, データの信頼性に影響を及ぼしたと思われる環境要因の変化はなかった.

4. 試験群の構成

試験群は0, 3, 10および30 mg/kgの4群とし, 1群雌雄各5匹, 計40匹を使用した.

〔用量設定理由〕

28日間反復投与毒性試験を0, 100, 300および1000 mg/kgで実施した結果, 雌雄とも100 mg/kgにおいてもPT, APTTの延長のみが影響として認められた. 無影響量を把握するため, さらに公比約3で30, 10および3 mg/kgを設定した.

5. 投与方法

被験物質の投与経路は経口とした. 被験物質はコーン油に溶解し, 胃ゾンデを用いて経口投与した. 投与容量は体重100 g当り0.5 mlとした. 対照群には溶媒のみ投与した.

6. 投与液の調製, 分析

被験物質は, 各用量(3, 10および30 mg/kg)ごとに所定量を精秤し, コーン油(ナカライテスク㈱)に溶解した. 投与液は調製後, 冷蔵庫保存で1週間安定であることが確認されているので, 本試験においては毎週1回調製を行い, 1日分毎に小分けをし使用時まで冷蔵庫に保

官した．投与液の濃度分析をすべての群に関し投与1および4週の調製液について実施した結果，設定濃度の89.5～99.3％の範囲であり，適切に調製されていた．

7. 投与期間

投与期間は28日間とした．

8. 観察，測定および検査

1) 一般状態の観察

全動物を毎日午前，午後の2回観察し，中毒症状の有無，行動異常，死期の迫った動物および死亡動物の有無等を記録した．

2) 体　重

投与開始から投与終了時まで，毎週1回測定した．

3) 摂餌量

毎週1回給餌した残量を測定し，飼料摂取量(g/week)を算出した．

4) 臨床検査

投与終了時に実施した．

採血するに当り，動物は約16時間絶食させた．動物をエーテルで麻酔後開腹し，腹部大動脈から採血した．

a. 血液凝固能検査

クエン酸ソーダ添加血液の血漿について，プロトロンビン時間(Quick 1段法)，活性化部分トロンボプラスチン時間(クロット法)およびフィブリノーゲン量(トロンビン時間法)を血液凝固自動測定装置KC-40(独国Amelung社)を用いて測定した．

b. 血液生化学検査

血清を用いて，塩素(電極法)をEKTACHEM 700N(米国コダック社)で，グルタミン酸オキザロ酢酸トランスアミナーゼ(GOT：Karmen改良法)をCentrifiChem ENCORE Ⅱ(米国ベーカー社)で測定した．

5) 病理学検査

病理解剖は投与終了時に動物をエーテル麻酔し，放血致死させ実施した．肉眼的異常を病理解剖所見記録シートに記録した．また，肝臓，腎臓，脾臓および骨髄(大腿骨)について10％中性緩衝ホルマリン液で固定し保存した．

6) データの記録および統計分析

各試験群の体重，摂餌量，血液凝固能検査値および血液生化学検査値は，下記に示した自動判別方式に従い，最初にBartlettの等分散検定を実施した．等分散の場合は一元配置の分散分析を行い，分散が有意で各群の標本数が同数の場合はDunnettの多重比較検定，各群の標本数が異なる場合はDuncanの多重範囲検定で対照群と各投薬群間の有意差を検定した．Bartlettの等分散検定で不等分散の場合はKruskal-Wallisの順位検定を実施し，有意の場合はノンパラメトリックのDunnettの多重比較検定で対照群と各投薬群間の有意差を検定した．また，病理学的検査結果についてはFisherの直接確率検定を実施した．

有意水準は5および1％の片側検定で実施した．

試験結果

1. 死 亡 率

投与期間中，雌雄ともいずれの群にも死亡例は認められなかった．

2. 一般状態の観察

雌雄いずれの群にも異常動物は認められなかった．

3. 体　重

雌雄とも全投与期間を通じて，対照群と被験物質投与群とで有意差が認められなかった．

4. 摂 餌 量

雌雄とも，投与期間を通じて群間で差が認められなかった．

5. 血液凝固能検査(Table 1)

雌雄ともPT，APTTおよびフィブリノーゲン量の3検査項目について群間で差は認められなかった．

6. 血液生化学検査(Table 2)

雌雄ともGOTおよび塩素は被験物質投与群と対照群とで差が認められなかった．

7. 病理学検査

a) 剖検所見

被験物質投与群で多く観察された所見はなく，観察された所見は，いずれも単発性の発生であった．

考察および結論

雌雄とも投与期間を通じて死亡例はなく，一般状態に異常のある動物は観察されなかった．

体重，摂餌量および飼料効率は，雌雄とも被験物質投与群で差が認められなかった．

血液凝固能検査については，前試験(投与量0，100，300および1000 mg/kg)で認められたPTおよびAPTTの延長傾向が，雌雄の30 mg/kg群では認められなかった．

血液生化学的検査の結果，GOTおよび塩素は，雌雄とも対照群と被験物質投与群とで差が認められなかった．

剖検所見にも被験物質投与と関連づけられる異常は認められなかった．

以上のことから，無影響量は雌雄とも30 mg/kg/dayと判断された．

Table 1 Coagulation of rats treated orally with 1,2,3-trimethylbenzene in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups(mg/kg)			
	0	3	10	30
Male				
No. of animals	5	5	5	5
PT (sec.)	13.6 ± 0.3	13.5 ± 0.3	13.5 ± 0.6	13.8 ± 0.4
APTT (sec.)	23.0 ± 0.6	23.5 ± 0.7	22.8 ± 1.2	23.2 ± 1.1
Fibrinogen (mg/dl)	284 ± 19	273 ± 17	283 ± 17	266 ± 9
Female				
No. of animals	5	5	5	5
PT (sec.)	13.8 ± 0.2	14.1 ± 0.5	14.0 ± 0.4	13.8 ± 0.4
APTT (sec.)	20.5 ± 1.7	20.7 ± 1.4	20.1 ± 0.9	20.8 ± 1.3
Fibrinogen (mg/dl)	231 ± 22	221 ± 31	229 ± 11	219 ± 16

Values are expressed as Mean ± S.D.

Table 2 Blood chemistry of rats treated orally with 1,2r3-trimethylbenzene in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)			
	0	3	10	30
Male				
No. of animals	5	5	5	5
GOT (U/l)	48 ± 10	46 ± 9	38 ± 3	49 ± 5
Chloride (mmol/l)	110.6 ± 0.9	110.3 ± 0.4	109.6 ± 1.0	110.9 ± 0.9
Female				
No. of animals	5	5	5	5
GOT (U/l)	52 ± 11	59 ± 7	51 ± 8	57 ± 10
Chloride (mmol/l)	112.3 ± 1.3	112.1 ± 1.2	111.7 ± 1.0	113.8 ± 0.8

Values are expressed as Mean±S.D.

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1,2,3-トリメチルベンゼンの細菌を用いる復帰突然変異試験

Reverse Mutation Test of 1,2,3-Trimethylbenzene on Bacteria

要約

既存化学物質安全性点検作業の一環として，1,2,3-トリメチルベンゼンの変異原性について遺伝子突然変異誘発性を検討するため，ネズミチフス菌(*Salmonella typhimurium*)TA100，TA98，TA1535およびTA1537株ならびに大腸菌(*Escherichia coli*)WP2*uvrA*株を用いる復帰突然変異試験を行った．予備的な試験の結果を基に，試験用量を設定した．すなわち，直接法(-S9 mix)の各菌株ならびに代謝活性化法(+S9 mix)のTA100，TA1535およびTA1537でそれぞれ，1.42～45.4 μg/プレート，代謝活性化法のWP2*uvrA*およびTA98で5.68～182 μg/プレートの6用量を設定し試験した．その結果，直接法および代謝活性化法のいずれにおいても，ラット肝ミクロソーム(S9)添加の有無にかかわらず，溶媒対照に比べ復帰突然変異コロニー数の明確な増加は認められず，再現性も確認された．一方，各系での陽性対照物質は，それぞれの試験菌株に対し明確な突然変異誘発作用を示した．従って，本試験条件下において，1,2,3-トリメチルベンゼンは微生物に対し遺伝子突然変異を誘起しないものと判断した．

材料および方法

1. 試験菌株

細菌を用いる復帰突然変異試験に広く使用されていることから，試験菌株としてヒスチジン要求性の*Salmonella typhimurium* TA100，TA98，TA1535およびTA1537[1]ならびにトリプトファン要求性の*Escherichia coli* WP2*uvrA*[2]の5種類の菌株を選択した．

ネズミチフス菌は昭和58年9月9日にカリフォルニア大学のB. N. Ames教授から，また，大腸菌については昭和58年3月16日に国立衛生試験所から分与を受けた．平成6年9月5日に菌株の特性検査を実施し，本試験に用いた菌株が規定の特性を保持していることを確認した．各菌株の菌懸濁液はジメチルスルホキシド(DMSO: MERCK社)を添加した後，凍結保存用チューブに0.2 mlずつ分注した．これを液体窒素を用いて凍結し，超低温フリーザーに-80℃で保存した．

2. 培地の調製

1) 最少グルコース寒天平板培地(プレート)

日清製粉㈱製のテスメディアAN培地を購入し，試験に用いた．本プレートは，Vogel-Bonnerの最少培地Eを含む水溶液(0.02%硫酸マグネシウム・7水塩，0.2%クエン酸・1水塩，1%リン酸二カリウム・無水塩，0.192%リン酸一アンモニウム，0.066%水酸化ナトリウム[いずれも最終濃度])に2%のグルコース(和光純薬工業㈱)と1.5%の寒天(OXOID社:No.1)を加え，30 mlをシャーレに分注したものである．

2) トップアガー(軟寒天)

Bacto-agar(DIFCO社)0.6%を含む0.5%塩化ナトリウム水溶液10容量に対し，ネズミチフス菌を用いる試験の場合，0.5 mM L-ヒスチジン(関東化学㈱)-0.5 mM D-ビオチン(関東化学㈱)水溶液を1容量加え，大腸菌を用いる試験の場合，0.5 mM L-トリプトファン(関東化学㈱)水溶液を同じく1容量加え用いた．

3. 前培養条件

内容量200 mlの円筒容器(ストレージボトル：Corning Costar社)に2.5%ニュートリエントブロス(OXOID社)溶液を25 ml分注し，これに融解した菌懸濁液を50 μl接種した．ウォーターバスシェーカー(MM-10:タイテック㈱)を用い，37℃で8時間振盪(往復振盪:120回/分)培養し，試験に使用した．

4. S9 mix

製造後6ヵ月以内のキッコーマン㈱製S9 mixを試験に使用した．S9 mix中のS9は誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawley系雄ラットの肝臓から調製されたものである．S9 mixの組成を以下に示す．

成　分	S9 mix 1ml中の量
S9	0.1 ml
$MgCl_2$	8 μmol
KCl	33 μmol
G-6-P	5 μmol
NADPH	4 μmol
NADH	4 μmol
リン酸緩衝Na-液(pH 7.4)	100 μmol

5. 被験物質

被験物質の1,2,3-トリメチルベンゼン(ロット番号:FJA01，CAS No.:526-73-8)は分子式C_9H_{12}，分子量120.20，純度90.8%の液体である．東京化成工業㈱から提供された被験物質を使用した．試験終了後，当センターにおいて残余被験物質を分析した結果，安定性に問題

はなかった.

6. 被験物質溶液の調製

DMSOに被験物質を溶解して調製原液とした. 調製原液を使用溶媒を用いて順次所定濃度に希釈した後, 直ちに処理を行った(用時調製).

7. 試験用量の設定

7.26, 36.3, 182, 908および4540 μg/プレート(50 mg/ml溶液を本被験物質の純度90.8%で補正した)の用量を用いて予備的な試験を実施した. その結果, 直接法の全菌株ならびに代謝活性化法のTA100, TA1535およびTA1537で36.3 μg/プレート以上, 代謝活性化法のWP2*uvrA*およびTA98で182 μg/プレート以上において試験菌株に対する生育阻害作用が観察された. 従って, 本試験においては直接法の各菌株ならびに代謝活性化法のTA100, TA1535およびTA1537で45.4 μg/プレート, 代謝活性化法のWP2*uvrA*およびTA98で182 μg/プレートを最高用量とし, それぞれ6用量(公比2)を設定した.

8. 陽性対照物質

陽性対照物質として下記に示した物質を使用した. これらの陽性対照物質は, DMSOを用いて溶解し, 少量ずつ分注した後凍結保存(-20℃)した.

2-(2-フリル)-3-(5-ニトロ-2-フリル)アクリルアミド(AF-2:和光純薬工業㈱)
アジ化ナトリウム(NaN_3:和光純薬工業㈱)
9-アミノアクリジン(ACR:ALDRICH社)
2-アミノアントラセン(2-AA:和光純薬工業㈱)

9. 試験方法

Amesらの原法の改良法であるプレインキュベーション法[1]に準じて, 直接法および代謝活性化法それぞれについて試験を実施した. 試験管に, 使用溶媒, 被験物質溶液あるいは陽性対照物質溶液を100 μl, 次いで直接法の場合, 0.1Mナトリウム・リン酸緩衝液(pH 7.4)を500 μl, 代謝活性化法の場合, S9 mixを500 μlおよび試験菌液100 μlを加え, 37℃で20分間振盪培養(プレインキュベーション)した. 培養終了後, トップアガーを2 ml添加し, 混合液をプレート上に重層した. 37℃の条件で48時間各プレートを培養した後, 被験物質の試験菌株に対する生育阻害作用を確認するため, 実体顕微鏡(×60)を用いてプレート上の試験菌株の生育状態を観察した. 次いで, 復帰突然変異により生じたコロニーを計数した. 計測に際してはコロニーアナライザー(CA-11:システムサイエンス㈱)を用いた. 独立して試験を2回実施した.

10. 結果の解析

復帰突然変異コロニー数が溶媒対照のほぼ2倍以上に増加し, かつ, 再現性あるいは被験物質の用量に依存性が認められた場合に, 陽性と判定した.

なお, 統計学的手法を用いた検定は実施しなかった.

結果および考察

試験結果をTable 1～4に示した. 直接法(-S9 mix)ならびに代謝活性化法(+S9 mix)のいずれとも高用量群において, 1,2,3-トリメチルベンゼン処理による生育阻害作用が観察された. また, 復帰突然変異コロニー数については, 直接法, 代謝活性化法とも溶媒対照と同等の値であり, 明確な増加傾向は認められなかった. 一方, 陽性対照物質はそれぞれの菌株において, 溶媒対照群の2倍以上の復帰突然変異コロニーを誘発した. なお, S9 mix添加時182 μg/プレートの用量において, 試験管内の反応液が僅かに白濁したが, コロニー計数時には析出等の特筆すべき変化は観察されなかった. 以上の試験結果から, 本試験条件下において1,2,3-トリメチルベンゼンの微生物に対する遺伝子突然変異に関し, 陰性と判定した.

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[direct method : -S9]

Compound	Dose	Revertant colonies per plate [Mean±S.D.]				
	(μg/plate)	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO#	0	97 104 103 [101 ± 4]	10 12 14 [12 ± 2]	15 21 18 [18 ± 3]	18 24 25 [22 ± 4]	4 6 10 [7 ± 3]
Test sub.	1.42	87 101 85 [91 ± 9]	7 14 16 [12 ± 5]	22 23 13 [19 ± 6]	18 19 29 [22 ± 6]	8 9 8 [8 ± 1]
	2.84	97 91 82 [90 ± 8]	12 8 9 [10 ± 2]	18 19 24 [20 ± 3]	22 19 26 [22 ± 4]	7 9 10 [9 ± 2]
	5.68	74 94 94 [87 ± 12]	4 11 5 [7 ± 4]	13 19 22 [18 ± 5]	20 22 27 [23 ± 4]	5 9 4 [6 ± 3]
	11.4	80 83 69 [77 ± 7]	5 13 12 [10 ± 4]	17 21 16 [18 ± 3]	25 21 22 [23 ± 2]	7 10 8 [8 ± 2]
	22.7	83 88 95 [89 ± 6]	7 8 7 [7 ± 1]	31 18 21 [23 ± 7]	26 20 15 [20 ± 6]	6 7 7 [7 ± 1]
	45.4	77* 66* 88* [77 ± 11]	8* 6* 11* [8 ± 3]	25* 14* 26* [22 ± 7]	19* 26* 22* [22 ± 4]	5* 9* 5* [6 ± 2]
Positive control		558 579 517[a] [551 ± 32]	402 345 345[b] [364 ± 33]	107 140 106[a] [118 ± 19]	528 588 565[c] [560 ± 30]	620 580 585[d] [595 ± 22]

#: Solvent control *: The background lawn was thin
a): AF-2; 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, 0.01 μg/plate b): NaN_3; Sodium azide, 0.5 μg/plate c): AF-2, 0.1 μg/plate
d): ACR; 9-Aminoacridine, 80 μg/plate

Table 2. Results of the bacterial reversion test of 1,2,3-trimethylbenzene (1st trial)
[activation method : +S9]

Compound	Dose	Revertant colonies per plate [Mean±S.D.]				
	(μg/plate)	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO#	0	109 100 97 [102 ± 6]	8 11 14 [11 ± 3]	26 23 27 [25 ± 2]	28 25 33 [29 ± 4]	14 11 15 [13 ± 2]
Test sub.	1.42	97 94 96 [96 ± 2]	9 7 15 [10 ± 4]	-	-	23 23 16 [21 ± 4]
	2.84	94 102 90 [95 ± 6]	13 15 12 [13 ± 2]	-	-	17 20 23 [20 ± 3]
	5.68	89 110 107 [102 ± 11]	13 20 11 [15 ± 5]	21 26 21 [23 ± 3]	32 30 40 [34 ± 5]	20 17 22 [20 ± 3]
	11.4	114 99 106 [106 ± 8]	18 9 9 [12 ± 5]	29 26 17 [24 ± 6]	40 37 41 [39 ± 2]	24 23 27 [25 ± 2]
	22.7	108 121 105 [111 ± 9]	6 10 14 [10 ± 4]	24 26 24 [25 ± 1]	33 37 43 [38 ± 5]	25 17 22 [21 ± 4]
	45.4	110* 121* 118* [116 ± 6]	8* 10* 11* [10 ± 2]	25 24 30 [26 ± 3]	33 37 24 [31 ± 7]	14* 24* 15* [18 ± 6]
	90.8	-	-	14 25 22 [20 ± 6]	38 33 30 [34 ± 4]	-
	182	-	-	28* 28* 26* [27 ± 1]	26* 21* 24* [24 ± 3]	-
Positive control		620 580 583[a] [594 ± 22]	272 367 321[b] [320 ± 48]	577 554 646[c] [592 ± 48]	314 287 288[d] [296 ± 15]	130 120 97[b] [116 ± 17]

#: Solvent control *: The background lawn was thin -: Not tested
a): 2-AA; 2-Aminoanthracene, 1 μg/plate b): 2-AA, 2 μg/plate c): 2-AA, 10 μg/plate d): 2-AA, 0.5 μg/plate

Table 3. Results of the bacterial reversion test of 1,2,3-trimethylbenzene (2nd trial) [direct method:−S9]

Compound	Dose	Revertant colonies per plate [Mean±S.D.]				
	(μg/plate)	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO#	0	94 103 106 [101 ± 6]	16 15 14 [15 ± 1]	20 21 27 [23 ± 4]	21 20 24 [22 ± 2]	7 8 7 [7 ± 1]
Test sub.	1.42	101 98 100 [100 ± 2]	14 16 15 [15 ± 1]	27 21 22 [23 ± 3]	21 20 22 [21 ± 1]	5 5 8 [6 ± 2]
	2.84	94 105 99 [99 ± 6]	16 14 13 [14 ± 2]	23 24 25 [24 ± 1]	24 20 22 [22 ± 2]	8 8 7 [8 ± 1]
	5.68	97 98 92 [96 ± 3]	15 17 17 [16 ± 1]	22 25 25 [24 ± 2]	22 23 24 [23 ± 1]	8 6 7 [7 ± 1]
	11.4	102 103 104 [103 ± 1]	16 11 12 [13 ± 3]	26 21 20 [22 ± 3]	26 26 25 [26 ± 1]	6 8 4 [6 ± 2]
	22.7	103 93 100 [99 ± 5]	15 15 15 [15 ± 0]	24 26 23 [24 ± 2]	27 23 24 [25 ± 2]	7 6 10 [8 ± 2]
	45.4	107* 94* 97* [99 ± 7]	10* 10* 9* [10 ± 1]	22* 22* 20* [21 ± 1]	23* 20* 21* [21 ± 2]	9* 6* 9* [8 ± 2]
Positive control		428 394 437[a] [420 ± 23]	375 442 502[b] [440 ± 64]	109 107 110[a] [109 ± 2]	631 645 659[c] [645 ± 14]	586 597 450[d] [544 ± 82]

#: Solvent control *: The background lawn was thin
a): AF-2; 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, 0.01 μg/plate b): NaN_3; Sodium azide, 0.5 μg/plate c): AF-2, 0.1 μg/plate
d): ACR; 9-Aminoacridine, 80 μg/plate

Table 4. Results of the bacterial reversion test of 1,2,3-trimethylbenzene (2nd trial) [activation method:+S9]

Compound	Dose	Revertant colonies per plate [Mean±S.D.]				
	(μg/plate)	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO#	0	101 99 102 [101 ± 2]	10 16 15 [14 ± 3]	27 23 28 [26 ± 3]	28 32 34 [31 ± 3]	14 14 13 [14 ± 1]
Test sub.	1.42	96 109 91 [99 ± 9]	13 16 10 [13 ± 3]	-	-	14 13 12 [13 ± 1]
	2.84	98 94 102 [98 ± 4]	12 18 14 [15 ± 3]	-	-	9 9 14 [11 ± 3]
	5.68	99 96 105 [100 ± 5]	13 17 16 [15 ± 2]	26 21 27 [25 ± 3]	35 36 34 [35 ± 1]	14 10 15 [13 ± 3]
	11.4	102 107 106 [105 ± 3]	15 15 13 [14 ± 1]	27 27 21 [25 ± 3]	34 39 32 [35 ± 4]	14 14 14 [14 ± 0]
	22.7	90 105 103 [99 ± 8]	15 18 13 [15 ± 3]	27 26 27 [27 ± 1]	33 37 38 [36 ± 3]	10 13 14 [12 ± 2]
	45.4	106* 98* 90* [98 ± 8]	9* 12* 12* [11 ± 2]	24 25 21 [23 ± 2]	34 31 39 [35 ± 4]	15* 15* 13* [14 ± 1]
	90.8	-	-	21* 23* 23* [22 ± 1]	35* 30* 32* [32 ± 3]	-
	182	-	-	24* 20* 27* [24 ± 4]	33* 30* 25* [29 ± 4]	-
Positive control		658 716 764[a] [713 ± 53]	299 321 294[b] [305 ± 14]	528 504 607[c] [546 ± 54]	406 324 322[d] [351 ± 48]	104 105 111[b] [107 ± 4]

#: Solvent control *: The background lawn was thin -: Not tested
a): 2-AA; 2-Aminoanthracene, 1 μg/plate b): 2-AA, 2 μg/plate c): 2-AA, 10 μg/plate d): 2-AA, 0.5 μg/plate

1,2,3-トリメチルベンゼンのチャイニーズ・ハムスター培養細胞を用いる染色体異常試験

In Vitro Chromosomal Aberration Test of 1,2,3-Trimethylbenzene on Cultured Chinese Hamster Cells

要約

既存化学物質安全性点検作業の一環として，1,2,3-トリメチルベンゼンの変異原性について染色体異常誘発性の有無を検討するため，チャイニーズ・ハムスター肺線維芽細胞株(CHL)を用いる*in vitro*染色体異常試験を行った．細胞増殖抑制試験結果を基に，細胞毒性が観察される濃度を最高用量として設定した．すなわち，連続24時間処理法で90.0, 180および360 μg/ml，同48時間処理法で60.0, 120および240 μg/ml，短時間+S9 mix処理法で120, 240, 480および960 μg/ml，同-S9 mix処理法で120, 240および480 μg/mlの3～4用量(公比2)について染色体標本を作製した後，顕微鏡観察を実施した．短時間+S9 mix処理法の240および480 μg/mlにおいて，僅かではあるが染色体構造異常の誘発傾向が観察された．同処理法において350, 500, 650および800 μg/mlの4用量を用いた確認試験を実施した結果，800 μg/mlにおいて5.5%の構造異常細胞が出現した．一方，連続処理法の陽性対照物質マイトマイシンC(MMC)および短時間+S9 mix処理の陽性対照物質シクロホスファミド(CP)は，いずれも染色体構造異常を高頻度に誘発した．従って，本試験条件下の*in vitro*試験系において，1,2,3-トリメチルベンゼンの染色体異常誘起性について疑陽性と判断した．

材料および方法

1. 試験細胞株

哺乳類培養細胞を用いる染色体異常試験に広く使用されていることから，試験細胞株としてチャイニーズ・ハムスターの肺由来の線維芽細胞株(CHL)を選択した．昭和59年11月15日に国立衛生試験所から分与を受け，一部はジメチルスルホキシド(DMSO:MERCK社)を10%添加した後，液体窒素中に保存し，残りは3～5日ごとに継代した．なお，本染色体異常試験では解凍後継代数7の細胞を，確認試験においては同14の細胞を用いた．

2. 培養液の調製

Eagle-MEM培地(LIFE TECHNOLOGIES社)を1000mlの精製水で溶解した後，2.2 gの炭酸水素ナトリウム(関東化学㈱)を加えた．1N塩酸を用いてpHを7.2に調整した後，メンブランフィルター(0.2 μm:Gelman Sciences社)を用いて加圧濾過除菌した．非働化(56℃，30分)済み仔牛血清(LIFE TECHNOLOGIES社)を最終濃度で10%になるよう加えた後，試験に使用した．

3. 培養条件

CO_2インキュベーター(FORMA社あるいは三洋電機特機㈱)を用い，CO_2濃度5%，37℃の条件で細胞を培養した．

4. S9 mix

製造後6ヵ月以内のキッコーマン㈱製S9 mixを試験に使用した．S9 mix中のS9は誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawley系雄ラットの肝臓から調製されたものである．S9 mixの組成は松岡らの方法に従った[1]．

5. 被験物質

被験物質の1,2,3-トリメチルベンゼン(ロット番号:FJA01，CAS No.:526-73-8)は分子式C_9H_{12}，分子量120.20，純度90.8%の液体である．東京化成工業㈱から提供された被験物質を使用した．試験終了後，当センターにおいて残余被験物質を分析した結果，安定性に問題はなかった．

6. 被験物質溶液の調製

DMSOに被験物質を溶解して調製原液とした．調製原液を使用溶媒を用いて順次所定濃度に希釈した後，直ちに処理を行った(用時調製)．

なお，本被験物質の純度は95%未満であるため，秤量に際して換算した．

7. 予備試験(細胞増殖抑制試験)

細胞培養用マルチプレートに細胞を播種し，培養3日後に被験物質溶液を処理した．連続処理法の場合，24あるいは48時間連続して処理を実施し，短時間処理法ではS9 mix存在下(+S9 mix)あるいは非存在下(-S9 mix)で6時間処理した後，新鮮な培養液に交換してさらに18時間培養を続けた．

細胞を10%中性緩衝ホルマリン液(和光純薬工業㈱)で固定した後，0.1%クリスタル・バイオレット(関東化学㈱)水溶液で10分間染色した．色素溶出液(30%エタノール，1%酢酸水溶液)を適量加え，5分間程度放置して色素を溶出した後，580 nmでの吸光度を測定した．各用量群について溶媒対照群での吸光度に対する比，すなわち細胞生存率を算出した．

その結果，いずれの処理法においても顕著な細胞増殖抑制が観察された(Fig. 1)．プロビット法を用いて算出した50%細胞増殖抑制濃度は連続24時間処理で180 μg/ml，同48時間処理で130 μg/ml，短時間+S9 mix処理で218 μg/ml，同-S9 mix処理で213 μg/mlであった．

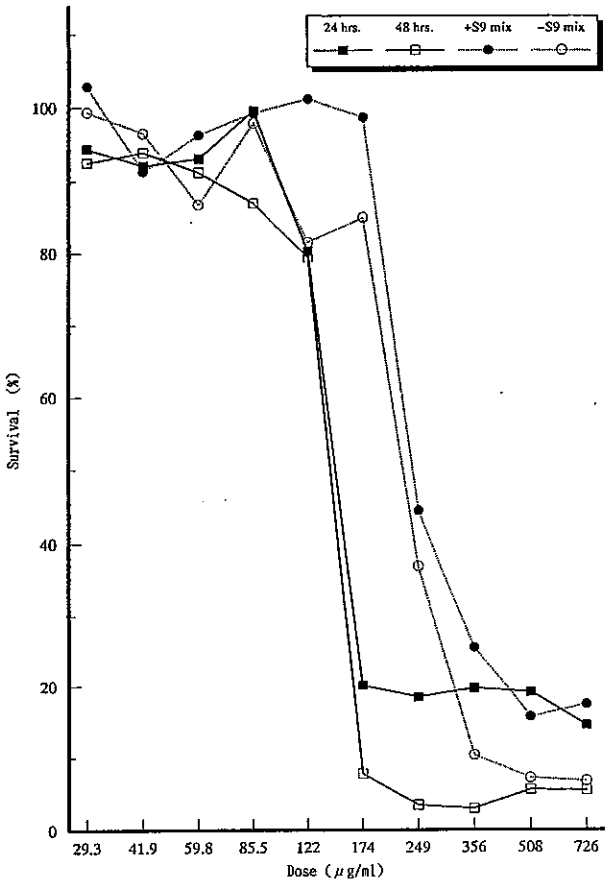

Fig. 1 Dose-survival curves of 1,2,3-trimethylbenzene

8. 試験用量および試験群の設定

細胞増殖抑制試験結果を基に，染色体異常試験では連続24時間処理で360 μg/ml，同48時間処理で240 μg/ml短時間+S9 mix処理で960 μg/ml，同-S9 mix処理で480 μg/mlを最高用量とし，以下公比2で減じた計3～4用量ならびに溶媒対照群を設定した．

陽性対照として，連続処理法の場合，マイトマイシンC(MMC:協和醗酵工業㈱)を，24時間処理で0.05 μg/ml，48時間処理で0.025 μg/mlの用量で，短時間処理法の場合，シクロホスファミド(CP:塩野義製薬㈱)を，12.5 μg/mlの用量で試験した．

また，確認試験においては350, 500, 650および800 μg/mlの4用量(等差数列)を設定した．

9. 染色体標本の作製

直径60 mmのプレートを用い，予備試験と同様に被験物質等の処理を行った．培養終了2時間前に，最終濃度で0.2 μg/mlとなるようコルセミド(LIFE TECHNOLOGIES社)を添加した．トリプシン処理で細胞を剥離させ，遠心分離により細胞を回収した．75 mM塩化カリウム水溶液で低張処理を行った後，固定液(メタノール3容:酢酸1容)で細胞を固定した．空気乾燥法で染色体標本を作製した後，1.2%ギムザ染色液で12分間染色した．

10. 染色体の観察

各プレートあたり100個，すなわち用量当たり200個の分裂中期像を顕微鏡下で観察し，染色体の形態的変化としてギャップ(gap)，染色分体切断(ctb)，染色体切断(csb)，染色分体交換(cte)，染色体交換(cse)およびその他(oth)の構造異常に分類した．同時に，倍数性細胞の出現率を記録した．染色体の分析は日本環境変異原学会・哺乳動物試験分科会[2]による分類法に従って実施した．

すべての標本をコード化した後，観察した．

11. 結果の解析

ギャップのみ保有する細胞を含めた場合(+gap)と，含めない場合(-gap)とに区別して染色体構造異常の出現頻度を表示した．

各試験群の構造異常を有する細胞あるいは倍数性細胞の出現頻度を，石館ら[3]の基準に従って判定した．染色体異常を有する細胞の出現頻度が5%未満を陰性(-)，5%以上10%未満を疑陽性(±)，10%以上を陽性(+)とした．最終的には再現性あるいは用量に依存性が認められた場合に陽性と判定した．

なお，統計学的手法を用いた検定は実施しなかった．

結果および考察

連続処理群での試験結果をTable 1に示した．1,2,3-トリメチルベンゼン処理群の場合，24時間ならびに48時間処理のいずれの用量においても染色体の構造異常および倍数性細胞の誘発傾向は観察されなかった．一方，陽性対照物質のMMCで処理した細胞では染色体の構造異常の顕著な誘発が認められた．短時間処理群での試験結果をTable 2に示した．被験物質処理群の場合，+S9 mix処理において240および480 μg/mlにおいて染色体の構造異常の出現頻度が5%を超え，疑陽性と判定された．再現性を調査するため確認試験を実施した結果，被験物質処理群において僅かではあるが染色体の構造異常の増加傾向が観察された(Table 3)．また，陽性対照物質のCPで処理した細胞ではS9 mix存在下でのみ染色体の構造異常の顕著な誘発が認められた．以上の試験結果から，本試験条件下において1,2,3-トリメチルベンゼンの哺乳類培養細胞に対する染色体異常誘発性に関し，疑陽性と判定した．

Table 1. Chromosomal aberration test on CHL cells treated with 1,2,3-trimethylbenzene [long-term treatment]

Compound	Dose (μg/ml)	Time of exposure (hr)	Number of cells analyzed	Number of cells with structural aberrations						Total [+gap] (%)	Total [-gap] (%)	Polyploid cells (%)	Final judgement
				gap	ctb	csb	cte	cse	oth				
DMSO*	0	24	200	2	1	0	1	0	0	1.5	1.0	0.5	-
Test Sub.	90.0	24	200	3	4	0	2	0	0	4.5	3.0	0.5	-
	180	24	200	0	0	0	1	0	0	0.5	0.5	0.0	-
	360	24	200	0	1	0	1	0	0	1.0	1.0	0.0	-
MMC**	0.05	24	200	19	63	0	100	0	1	64.5	63.5	0.0	+
DMSO*	0	48	200	1	2	0	1	0	0	2.0	1.5	0.0	-
Test Sub.	60.0	48	200	1	2	0	4	0	0	3.5	3.0	0.5	-
	120	48	200	1	2	0	1	0	0	2.0	1.5	0.0	-
	240	48	200	0	2	0	3	0	0	2.5	2.5	2.0	-
MMC**	0.025	48	200	14	38	0	76	2	0	46.5	45.0	0.0	+

*: Solvent control **: Positive control (mitomycin C)
ctb: chromatid break csb: chromosome break cte: chromatid exchange cse: chromosome exchange oth: others

Table 2. Chromosomal aberration test on CHL cells treated with 1,2,3-trimethylbenzene [short-term treatment]

Compound	Dose (μg/ml)	S 9 mix	Time of exposure (hr)	Number of cells analyzed	Number of cells with structural aberrations						Total [+gap] (%)	Total [-gap] (%)	Polyploid cells (%)	Final judgement
					gap	ctb	csb	cte	cse	oth				
DMSO*	0	+	6	200	0	1	0	2	0	0	1.5	1.5	0.0	-
Test Sub.	120	+	6	200	0	5	0	4	0	0	3.5	3.5	2.0	-
	240	+	6	200	3	7	0	12	0	0	6.5	6.0	1.0	±
	480	+	6	200	2	4	0	6	1	0	5.5	4.5	3.0	±
	960	+	6	Toxic										
CP**	12.5	+	6	200	5	40	0	89	1	0	51.5	51.5	0.0	+
DMSO*	0	-	6	200	1	2	0	1	1	0	2.5	2.0	1.0	-
Test Sub.	120	-	6	200	1	4	0	1	0	0	3.0	2.5	1.0	-
	240	-	6	200	1	2	0	0	0	0	1.5	1.0	0.0	-
	480	-	6	200	2	2	0	0	0	0	1.5	1.0	1.0	-
CP**	12.5	-	6	200	1	0	0	2	0	0	1.5	1.0	1.0	-

*: Solvent control **: Positive control (cyclophosphamide)
ctb: chromatid break csb: chromosome break cte: chromatid exchange cse: chromosome exchange oth: others

Table 3. Results of the confirmative examination using CHL cells of 1,2,3-trimethylbenzene [short-term treatment]

Compound	Dose (μg/ml)	S9 mix	Time of exposure (hr)	Number of cells analyzed	Number of cells with structural aberrations gap	ctb	csb	cte	cse	oth	Total [+gap] (%)	Total [-gap] (%)	Polyploid cells (%)	Final judgement
DMSO*	0	+	6	200	1	0	1	3	0	1	3.0	2.5	0.0	-
Test Sub.	350	+	6	200	1	1	0	3	0	0	1.5	1.5	1.0	-
	500	+	6	200	0	1	0	6	1	0	3.5	3.5	0.5	-
	650	+	6	200	2	2	0	5	1	0	4.5	3.5	0.5	-
	800	+	6	200	2	7	0	7	0	0	5.5	5.0	1.5	±
CP**	12.5	+	6	200	10	34	0	105	0	0	59.0	58.5	0.0	+

*:Solvent control **:Positive control (cyclophosphamide)
ctb:chromatid break csb:chromosome break cte:chromatid exchange cse:chromosome exchange oth:others

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2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンのラットを用いる28日間反復経口投与毒性試験

Twenty-eight-day Repeat Dose Oral Toxicity Test of 2-Hydroxy-4-(octyloxy)benzophenone in Rats

要約

2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンは，プラスチックスや合成繊維の耐候性改良，食品や医療品などの容器・包装材に使用して内容物の紫外線からの保護，日焼け防止，シャンプーの分離防止，UVカットフィルムに用いる紫外線吸収材の目的で利用されているベンゾフェノン系紫外線吸収材である．毒性情報として，経口投与のLD_{50}値はマウスで10985 mg/kg以上と報告されている[1]．

今回，2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの20，140および1000 mg/kg/dayをSD系ラットの雌雄に28日間反復投与し，その毒性について検討した．対照および1000 mg/kg/day群については14日間回復群を設けた．

全試験期間を通して死亡はみられず，一般状態，体重，摂餌量に変化はなく，血液学検査，血液生化学検査，尿検査および病理検査結果に，被験物質投与に起因した毒性変化は認められなかった．

以上の結果より，本試験条件下における2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの無影響量は，雌雄ともに1000 mg/kg/dayと考えられる．

方法

1. 被験物質

2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノン(住友化学工業㈱，ロット番号：40650，純度：99%以上)は，融点45～50℃，水，熱，光等に安定，*n*-ヘキサンおよびベンゼンに可溶で，水には不溶の淡黄(白)色粉末である．本ロットについては試験期間中安定であることが確認された．投与液は被験物質を0.1% Tween80添加0.5%カルボキシメチルセルロース・ナトリウム水溶液に懸濁させ調製し，冷蔵保存した．投与液中の被験物質は冷蔵保存条件下で少なくとも8日間安定であり，また使用した投与液にはほぼ所定量の被験物質が均一に含有されていることを確認した．

2. 試験動物および飼育条件

日本チャールス・リバー㈱より入手したSD系ラット(Crj:CD, SPF)の雌雄を8日間検疫・馴化し，試験に使用した．投与開始前に動物を体重別層化無作為抽出法により群分けした．1群の動物数は雌雄各6匹とし，対照および高用量群についてはこの他に雌雄各6匹の14日間回復群を設けた．投与開始時の週齢は雌雄とも5週齢，体重範囲は雄が165～190 g，雌が129～153 gであった．

検疫・馴化期間を含めた全飼育期間中，温度20～25℃，湿度40～70%，換気約12回/時，照明12時間(7:00～19:00)に自動調節された飼育室を使用した．動物は，実験動物用床敷(ベータチップ：日本チャールス・リバー㈱)を敷いたポリカーボネート製ケージに1ケージ当り2匹で収容し飼育した．

動物には，実験動物用固型飼料(MF：オリエンタル酵母工業㈱)および5 μmのフィルター濾過後，紫外線照射した水道水を，それぞれ自由摂取させた．

3. 投与量および投与方法

被験物質を500および1000 mg/kgの各用量でSD系ラットに7日間反復経口投与した結果，1000 mg/kg群でも毒性変化は認められなかった．従って，本試験では高用量をガイドラインの上限である1000 mg/kgとし，以下公比約7で中用量を140 mg/kg，低用量を20 mg/kgとした．

被験物質は28日間毎日1回，午前中に胃ゾンデを用いて強制経口投与した．投与液量は10 ml/kgとし，至近測定日の体重を基に算出した．対照群には同様に溶媒を投与した．

4. 観察および検査方法

1) 一般状態，体重および摂餌量

全例について一般状態を毎日観察した．体重は投与開始日およびその後週1回測定した．また，摂餌量については，投与開始日およびその後週1回測定し，各期間毎の1匹当りの1日の平均摂餌量を算出した．

2) 血液学検査

各計画剖検時の全動物について，チオペンタールナトリウムの腹腔内投与による麻酔下で後大静脈より採血し，赤血球数(シースフローDCインピーダンス検出法)，白血球数(RF/DCインピーダンス検出法)，血小板数(シースフローDCインピーダンス検出法)，ヘモグロビン濃度(SLSヘモグロビン法)，ヘマトクリット値(赤血球パルス波高値検出法)を多項目自動血球分析装置(NE-4500：東亞医用電子㈱)，白血球百分率(Wright染色塗抹標本)を血液細胞自動分析装置(MICROX HEG-70A：㈱立石電機)，網状赤血球数(アルゴンレーザーを用いたフローサイトメトリー法)を自動網赤血球測定装置(R-2000：東亞医用電子㈱)，プロトロンビン時間(PT：

Quick一段法），活性化部分トロンボプラスチン時間（APTT：活性化セファロプラスチン法）を血液凝固自動測定装置（KC 10A：アメルング社）により測定した．また，検査の結果から平均赤血球容積（MCV），平均赤血球血色素量（MCH），平均赤血球血色素濃度（MCHC）を算出した．凝固阻止剤として，プロトロンビン時間および活性化部分トロンボプラスチン時間測定には3.13%クエン酸ナトリウム水溶液を，それ以外の項目の測定にはEDTA-2Kを用いた．

3） 血液生化学検査

採取した血液を室温で約30分間放置した後，3000 r.p.m.，10分間遠心分離し，得られた血清を用いて総蛋白（Biuret法），アルブミン（BCG法），A/G比（総蛋白およびアルブミンから算出），グルコース（GK-G6PDH法），トリグリセライド（LPL-GK-G3PO-POD法），総コレステロール（CES-CO-POD法），尿素窒素（Urease-GLDH法），クレアチニン（Jaffé法），カルシウム（O-CPC法），無機リン（UV法），GOT（SSCC改良法），GPT（SSCC改良法），γ-GTP（SSCC改良法），ALP（GSCC改良法），ナトリウム，カリウム，クロール（イオン選択電極法）を自動分析装置（日立736-10形：㈱日立製作所）により測定した．

4） 尿検査

投与終了時の解剖の2日前に全生存動物の新鮮尿を採取し，pH，潜血，蛋白，糖，ケトン体，ビリルビン，ウロビリノーゲン（試験紙法，マルティスティックス：マイルス・三共㈱）を尿分析器（クリニテック100：マイルス・三共㈱）で検査した．その結果，1000 mg/kg群の雌で変化がみられたので，雌のみ更に21時間蓄積尿を採取し，尿量をメスシリンダーで，比重（屈折法）を尿比重計（ユリコン-S：㈱アタゴ）で，ナトリウムおよびカリウム（炎光光度法）を全自動炎光光度計（FLAME-30C/AD-3：日本分光メディカル㈱）で，クロール（電量滴定法）をクロライドメーター（Model 925：コーニングメディカル㈱）により測定した．

投与期間に変化のみられた雌については，回復期間終了時の解剖の2日前にも同様の検査を実施した．

5） 病理検査

各計画殺時，全動物について採血後に腹大動脈を切断して放血致死させ剖検し，脳，肝臓，腎臓，副腎，胸腺，脾臓，精巣および卵巣の重量を測定した．また，これらの器官に加え，下垂体，眼球（付属腺を含む），肺，胃，甲状腺（上皮小体を含む），心臓，膀胱，骨髄（大腿骨）を採取し，10%中性リン酸緩衝ホルマリン液（眼球およびハーダー腺はDavidson液）にて固定後保存した．

投与終了時解剖動物の対照および1000 mg/kg群の雌雄の心臓，肝臓，脾臓，腎臓，副腎を対象に，常法に従いヘマトキシリン・エオジン染色標本を作製し，鏡検した．また，肉眼的に変化のみられた投与期間終了時の20 mg/kg群の雄1例および140 mg/kg群の雄2例の腎臓，1000 mg/kg群の雄1例の精巣と回復期間終了時の対照群の雄1例の肺，1000 mg/kg群の雄1例の胸腺についても同様に検査した．

6） 統計解析

計量データについては，Bartlett法による等分散の検定を行い，分散が一様の場合は一元配置分散分析を行った後，Dunnett法またはScheffé法により平均値の比較検定を行った．分散が一様でない場合にはKruskal-Wallisの検定を行い，Dunnett型またはScheffé型の順位和検定を行った．尿検査で得られた計数データについては，Armitageのχ^2検定を用いた．有意水準は5%以下とした．

結果

1. 一般状態，体重および摂餌量

全試験期間を通して，死亡および異常所見は認められなかった．体重および摂餌量は全ての被験物質投与群で対照群と同様な推移を示した．

2. 血液学検査（Table 1）

投与期間終了時の検査において，20 mg/kg群の雄でMCHCの低値がみられたが，140および1000 mg/kg群では認められないことから，被験物質投与とは関連のない変化と判断した．また，回復期間終了時の検査において，1000 mg/kg群の雌で単球比の高値がみられたが，投与期間終了時にはみられなかったことから，被験物質投与とは関連のない変化と判断した．

3. 血液生化学検査（Table 2）

投与期間終了時の検査において，変化の認められた項目はなかった．なお，回復期間終了時の検査において，1000 mg/kg群の雌で尿素窒素の低値がみられたが，投与期間終了時にはみられなかったことから，被験物質投与とは関連のない変化と判断した．

4. 尿検査（Table 3）

投与期間の検査において，1000 mg/kg群の雌でpHのアルカリ側への変動がみられた．なお，20 mg/kg群の雄でビリルビンの上昇がみられたが，140および1000 mg/kg群では認められなかったことから，被験物質投与とは関連のない変化と判断した．

雌のみについて実施した回復期間の検査においては，pHの変化は認められなかった．なお，1000 mg/kg群でカリウムの低値が認められたが，投与期間にはみられなかったことから，被験物質投与とは関連のない変化と判断した．

5. 器官重量（Table 4）

投与期間終了時の検査において，140 mg/kg群の雌で副腎の相対重量の低値がみられたが，1000 mg/kg群では認められなかったことから，被験物質投与とは関連の

Table 1 Hematology of rats treated orally with 2-hydroxy-4-(octyloxy)benzophenone in 28-day repeat dose toxicity test

Sex / Dose level	28 Days				Recovery	
	0mg/kg	20mg/kg	140mg/kg	1000mg/kg	0mg/kg	1000mg/kg
Male						
Number of animals	6	6	6	6	6	6
RBC ($\times10^4$/mm^3)	743 ± 21.9	720 ± 40.9	753 ± 19.0	739 ± 23.2	779 ± 23.1	770 ± 41.1
Hematocrit (%)	43.6 ± 0.64	43.1 ± 1.74	44.6 ± 0.83	44.3 ± 1.67	43.4 ± 1.33	43.9 ± 1.25
Hemoglobin (g/dl)	15.0 ± 0.21	14.6 ± 0.57	15.2 ± 0.25	15.1 ± 0.50	14.9 ± 0.41	15.1 ± 0.60
Reticulocyte (‰)	36 ± 3.6	33 ± 3.2	31 ± 2.3	32 ± 2.9	29 ± 2.9	29 ± 1.6
MCV (μm^3)	58.8 ± 1.61	60.0 ± 1.87	59.2 ± 1.08	60.0 ± 2.09	55.7 ± 2.22	57.1 ± 2.22
MCH (pg)	20.2 ± 0.51	20.3 ± 0.67	20.2 ± 0.34	20.5 ± 0.73	19.1 ± 0.73	19.7 ± 0.81
MCHC (%)	34.3 ± 0.18	33.9 ± 0.14*	34.1 ± 0.29	34.2 ± 0.27	34.3 ± 0.14	34.5 ± 0.50
Platelet ($\times10^4$/mm^3)	114.2 ± 13.61	106.9 ± 8.42	103.8 ± 6.81	117.6 ± 15.41	103.9 ± 14.43	108.2 ± 13.03
PT (sec)	13.6 ± 0.21	13.5 ± 0.37	13.5 ± 0.36	13.5 ± 0.35	13.2 ± 0.28	13.2 ± 0.27
APTT (sec)	17.7 ± 1.14	17.4 ± 0.95	16.6 ± 1.30	18.2 ± 0.75	18.3 ± 1.16	17.0 ± 1.54
WBC ($\times10^2$/mm^3)	121 ± 24.4	120 ± 33.0	140 ± 26.9	143 ± 35.9	102 ± 23.6	105 ± 14.9
Differential leukocyte counts (%)						
Lymphocytes	88 ± 5.7	84 ± 3.0	91 ± 6.2	88 ± 4.9	86 ± 4.9	85 ± 4.1
Neutrophils						
segmented	8 ± 3.4	11 ± 3.5	5 ± 4.0	7 ± 4.0	9 ± 3.7	8 ± 2.2
band	1 ± 0.5	1 ± 0.5	0 ± 0.8	0 ± 0.4	0 ± 0.5	0 ± 0.5
Eosinophils	1 ± 0.5	1 ± 0.5	0 ± 0.8	1 ± 0.8	1 ± 0.8	1 ± 0.5
Basophils	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Monocytes	4 ± 2.7	5 ± 3.7	4 ± 2.6	5 ± 1.0	4 ± 1.4	6 ± 3.5
Female						
Number of animals	6	6	6	6	6	6
RBC ($\times10^4$/mm^3)	703 ± 26.1	727 ± 29.2	727 ± 29.3	737 ± 24.6	724 ± 34.3	740 ± 9.1
Hematocrit (%)	41.7 ± 1.20	42.1 ± 1.05	43.0 ± 1.45	42.5 ± 0.92	40.8 ± 1.19	41.1 ± 1.08
Hemoglobin (g/dl)	14.5 ± 0.45	14.5 ± 0.32	14.8 ± 0.34	14.7 ± 0.24	14.1 ± 0.55	14.2 ± 0.31
Reticulocyte (‰)	27 ± 4.1	28 ± 1.5	22 ± 3.9	23 ± 4.0	26 ± 5.2	25 ± 4.7
MCV (μm^3)	59.3 ± 1.10	57.9 ± 1.41	59.2 ± 1.35	57.7 ± 1.87	56.4 ± 1.81	55.5 ± 1.28
MCH (pg)	20.6 ± 0.32	20.0 ± 0.54	20.4 ± 0.48	20.0 ± 0.55	19.5 ± 0.68	19.1 ± 0.29
MCHC (%)	34.7 ± 0.18	34.5 ± 0.28	34.4 ± 0.58	34.6 ± 0.27	34.6 ± 0.34	34.5 ± 0.42
Platelet ($\times10^4$/mm^3)	99.8 ± 9.57	92.1 ± 10.67	99.5 ± 9.38	102.0 ± 6.27	95.1 ± 8.26	101.1 ± 8.16
PT (sec)	13.8 ± 0.45	13.6 ± 0.51	13.4 ± 0.78	14.1 ± 0.45	14.1 ± 0.70	14.0 ± 0.71
APTT (sec)	14.6 ± 1.20	15.3 ± 0.74	15.2 ± 1.36	15.5 ± 1.70	15.2 ± 0.98	14.5 ± 0.68
WBC ($\times10^2$/mm^3)	102 ± 24.0	89 ± 36.1	99 ± 19.2	97 ± 22.9	72 ± 23.3	80 ± 23.1
Differential leukocyte counts (%)						
Lymphocytes	90 ± 4.1	88 ± 3.9	90 ± 3.8	89 ± 3.9	85 ± 5.6	84 ± 5.2
Neutrophils						
segmented	6 ± 2.3	6 ± 2.4	6 ± 4.1	6 ± 2.5	12 ± 5.2	9 ± 3.2
band	0 ± 0.5	0 ± 0.5	1 ± 0.5	0 ± 0.4	1 ± 0.5	0 ± 0.4
Eosinophils	1 ± 0.8	2 ± 1.5	1 ± 1.0	0 ± 0.5	1 ± 0.8	1 ± 1.1
Basophils	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Monocytes	4 ± 2.4	4 ± 2.6	3 ± 1.9	5 ± 2.5	2 ± 1.0	6 ± 6.2*

Values are expressed as Mean±S.D.

Significantly different from control group; *, $P<0.05$.

Table 2 Blood chemical examination of rats treated orally with 2-hyroxy-4-(octyloxy)benzophenone in 28-day repeat dose toxicity test

		28 Days				Recovery	
Sex	Dose leve	0mg/kg	20mg/kg	140mg/kg	1000mg/kg	0mg/kg	1000mg/kg
Male							
Number of animals		6	6	6	6	6	6
GOT (IU/l)		87 ± 17.3	85 ± 14.6	95 ± 26.4	82 ± 14.6	95 ± 17.3	89 ± 22.3
GPT (IU/l)		27 ± 4.2	31 ± 5.7	28 ± 4.2	29 ± 4.8	25 ± 4.2	23 ± 3.4
γ-GTP (IU/l)		0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
ALP (IU/l)		566 ± 189.7	577 ± 90.0	642 ± 97.8	533 ± 97.8	556 ± 94.8	520 ± 71.7
Urea nitrogen (mg/dl)		15.2 ± 3.33	16.5 ± 1.49	16.4 ± 2.16	16.8 ± 2.16	20.8 ± 2.82	20.2 ± 1.46
Creatinine (mg/dl)		0.5 ± 0.05	0.5 ± 0.04	0.5 ± 0.05	0.5 ± 0.00	0.5 ± 0.04	0.5 ± 0.05
Glucose (mg/dl)		147 ± 9.4	149 ± 12.9	151 ± 4.6	143 ± 9.5	160 ± 10.0	153 ± 7.5
Total chol. (mg/dl)		69 ± 8.5	68 ± 9.3	64 ± 5.6	74 ± 9.6	67 ± 12.5	73 ± 7.4
Triglyceride (mg/dl)		210 ± 55.0	172 ± 64.0	187 ± 72.6	173 ± 45.0	195 ± 54.2	181 ± 71.1
Total protein (g/dl)		6.83 ± 0.360	6.59 ± 0.231	6.70 ± 0.150	6.78 ± 0.271	6.79 ± 0.258	6.81 ± 0.364
Albumin (g/dl)		3.75 ± 0.072	3.65 ± 0.063	3.80 ± 0.037	3.78 ± 0.091	3.63 ± 0.118	3.63 ± 0.093
A/G ratio		1.23 ± 0.099	1.25 ± 0.083	1.31 ± 0.055	1.27 ± 0.076	1.15 ± 0.046	1.15 ± 0.077
Calcium (mg/dl)		9.7 ± 0.26	9.6 ± 0.16	9.8 ± 0.12	9.8 ± 0.23	9.4 ± 0.13	9.4 ± 0.23
Inorganic phos. (mg/dl)		9.5 ± 0.28	9.9 ± 0.15	9.3 ± 0.55	9.4 ± 0.54	7.4 ± 0.38	7.3 ± 0.59
Na (mEq/l)		142 ± 0.8	143 ± 0.9	143 ± 1.5	143 ± 1.5	143 ± 0.9	143 ± 1.2
K (mEq/l)		4.9 ± 0.18	4.7 ± 0.13	4.7 ± 0.20	4.6 ± 0.18	4.4 ± 0.15	4.6 ± 0.10
Cl (mEq/l)		98 ± 1.4	99 ± 2.1	99 ± 1.2	99 ± 1.0	100 ± 1.2	101 ± 1.4
Female							
Number of animals		6	6	6	6	6	6
GOT (IU/l)		78 ± 7.1	90 ± 21.6	82 ± 16.7	83 ± 9.9	89 ± 9.3	86 ± 15.8
GPT (IU/l)		24 ± 2.3	24 ± 3.6	20 ± 1.2	27 ± 8.0	26 ± 5.2	23 ± 3.1
γ-GTP (IU/l)		0 ± 0.0	0 ± 0.4	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
ALP (IU/l)		322 ± 65.3	296 ± 41.2	280 ± 71.9	399 ± 88.9	362 ± 68.6	296 ± 58.4
Urea nitrogen (mg/dl)		15.2 ± 3.29	14.1 ± 1.96	14.1 ± 1.85	16.8 ± 2.51	21.2 ± 2.38	15.8 ± 1.54**
Creatinine (mg/dl)		0.5 ± 0.05	0.6 ± 0.05	0.6 ± 0.05	0.6 ± 0.08	0.6 ± 0.04	0.6 ± 0.00
Glucose (mg/dl)		144 ± 7.0	146 ± 5.3	143 ± 7.6	139 ± 11.1	161 ± 15.8	158 ± 7.3
Total chol. (mg/dl)		69 ± 5.0	77 ± 13.7	78 ± 8.2	70 ± 10.7	71 ± 10.3	76 ± 15.4
Triglyceride (mg/dl)		69 ± 30.9	61 ± 14.6	60 ± 15.3	87 ± 61.6	77 ± 34.1	75 ± 10.5
Total protein (g/dl)		6.77 ± 0.250	6.80 ± 0.333	6.75 ± 0.221	6.84 ± 0.354	6.89 ± 0.181	7.11 ± 0.262
Albumin (g/dl)		3.96 ± 0.113	3.94 ± 0.186	3.95 ± 0.081	3.99 ± 0.142	3.86 ± 0.056	3.85 ± 0.142
A/G ratio		1.41 ± 0.066	1.38 ± 0.055	1.42 ± 0.109	1.41 ± 0.065	1.28 ± 0.086	1.19 ± 0.112
Calcium (mg/dl)		9.4 ± 0.17	9.2 ± 0.20	9.4 ± 0.15	9.5 ± 0.20	9.1 ± 0.20	9.2 ± 0.20
Inorganic phos. (mg/dl)		8.6 ± 0.37	7.9 ± 0.39	8.5 ± 0.48	8.7 ± 0.73	6.3 ± 0.98	6.6 ± 0.58
Na (mEq/l)		143 ± 1.0	143 ± 0.5	142 ± 0.8	143 ± 0.4	142 ± 0.8	142 ± 0.9
K (mEq/l)		4.2 ± 0.19	4.2 ± 0.08	4.4 ± 0.18	4.2 ± 0.12	3.9 ± 0.28	3.9 ± 0.12
Cl (mEq/l)		101 ± 1.4	102 ± 1.0	101 ± 1.4	101 ± 1.2	102 ± 1.8	102 ± 0.9

Values are expressed as Mean±S.D.

Significantly different from control group; **, $P<0.01$.

Table 5 Urinary pH of female rats treated orally with 2-hydroxy-4-(octyloxy)benzophenone in 28-day repeat dose toxicity test

Sex	Dose level	28 Days 0mg/kg	28 Days 20mg/kg	28 Days 100mg/kg	28 Days 500mg/kg	Recovery 0mg/kg	Recovery 500mg/kg
Female							
Number of animals		12	6	6	12 *	6	6
pH	6.5	2	0	1	0	0	0
	7.0	1	0	0	0	1	0
	7.5	0	1	1	0	2	2
	8.0	3	0	0	4	1	3
	8.5	6	4	3	5	2	1
	>9.0	0	1	1	3	0	0

Values are expressed as number of animals.
Significantly different from control group; *, $P<0.05$.

ない変化と判断した．なお，回復期間終了時の検査において，1000 mg/kg群の雌で卵巣の相対重量の低値がみられたが，投与期間終了時にはみられなかったことから，被験物質投与とは関連のない変化と判断した．

6. 剖検所見

被験物質投与に起因する変化は認められなかった．偶発性変化として腎臓の軽度の褪色，腎臓ののう胞，副腎の腫大，精巣の小型化，肺の出血斑，胸腺の出血が認められた．

7. 組織所見

被験物質投与に起因する変化は認められなかった．偶発性変化として肝臓の微小肉芽腫，腎臓の尿細管上皮の好塩基性変化，腎臓の尿細管ののう胞状拡張，腎臓の単核細胞浸潤，精細管の低形成，肺の出血，胸腺の出血が認められた．

考察

2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの20，140および1000 mg/kg/dayをSD系ラットの雌雄に28日間反復経口投与し，その毒性について検討した．

その結果，1000 mg/kg/dayを投与した群でも，死亡は認められず，一般状態，体重および摂餌量に変化はなく，血液学検査，血液生化学検査および病理検査においても被験物質投与に起因した毒性変化は認められなかった．

尿検査において，投与期間中に1000 mg/kg/day群の雌でpHのアルカリ側への変動がみられた．アルカリ尿は一般的には代謝性および呼吸性アルカローシス，腎不全や腎盂腎炎による腎臓のH^+排泄障害でみられることが知られている[2]．しかし，今回の変動は，当研究所の背景データの範囲内にあり，かつ，腎障害を示唆する病理変化は認められていないことから，偶発的な偏りによるものと判断した．

以上の結果から，本試験条件下における2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの無影響量は雌雄ともに1000 mg/kg/dayと考えられる．

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Table 4 Absolute and relative organ weight of rats treated orally with 2-hydroxy-4-(octyloxy)benzophenone in 28-day repeat dose toxicity test

Sex	Dose leve	28 Days				Recovery	
		0mg/kg	20mg/kg	140mg/kg	1000mg/kg	0mg/kg	1000mg/kg
Male							
Number of animals		6	6	6	6	6	6
Body weight (g)		418 ± 44.1	398 ± 21.4	412 ± 33.5	398 ± 20.8	479 ± 21.3	475 ± 25.0
Absolute organ weight							
Brain (g)		2.08 ± 0.072	2.09 ± 0.056	2.09 ± 0.096	2.01 ± 0.059	2.13 ± 0.123	2.17 ± 0.031
Thymus (mg)		712 ± 108.0	618 ± 91.4	691 ± 73.4	698 ± 99.9	577 ± 152.6	642 ± 125.3
Liver (g)		16.71 ± 2.869	15.10 ± 1.309	16.15 ± 1.756	15.92 ± 1.752	19.33 ± 0.823	18.03 ± 2.196
Kidneys (g)		2.84 ± 0.267	2.71 ± 0.185	2.94 ± 0.196	2.81 ± 0.150	3.09 ± 0.243	3.05 ± 0.315
Adrenals (mg)		62.1 ± 14.38	55.8 ± 12.32	54.4 ± 4.52	53.0 ± 7.27	56.7 ± 5.82	57.7 ± 6.70
Spleen (g)		0.87 ± 0.079	0.80 ± 0.086	0.82 ± 0.086	0.80 ± 0.138	0.91 ± 0.122	0.90 ± 0.039
Testes (g)		3.18 ± 0.185	3.02 ± 0.229	3.19 ± 0.357	2.73 ± 0.911	3.32 ± 0.336	3.26 ± 0.224
Relative organ weight							
Brain (g%)		0.50 ± 0.049	0.53 ± 0.037	0.51 ± 0.046	0.51 ± 0.024	0.45 ± 0.024	0.46 ± 0.026
Thymus (mg%)		171 ± 27.5	155 ± 17.9	169 ± 25.0	176 ± 24.2	120 ± 26.4	136 ± 28.5
Liver (g%)		3.98 ± 0.279	3.80 ± 0.231	3.92 ± 0.266	3.99 ± 0.260	4.04 ± 0.128	3.78 ± 0.276
Kidneys (g%)		0.68 ± 0.011	0.68 ± 0.037	0.72 ± 0.024	0.71 ± 0.056	0.65 ± 0.039	0.64 ± 0.033
Adrenals (mg%)		14.8 ± 2.47	14.0 ± 2.78	13.2 ± 1.23	13.4 ± 2.33	11.9 ± 1.30	12.2 ± 1.45
Spleen (g%)		0.21 ± 0.019	0.20 ± 0.016	0.20 ± 0.032	0.20 ± 0.033	0.19 ± 0.021	0.19 ± 0.013
Testes (g%)		0.77 ± 0.076	0.76 ± 0.072	0.78 ± 0.121	0.69 ± 0.233	0.69 ± 0.066	0.69 ± 0.066
Female							
Number of animals		6	6	6	6	6	6
Body weight (g)		241 ± 14.8	234 ± 13.9	243 ± 10.5	246 ± 18.8	258 ± 10.6	258 ± 14.6
Absolute organ weight							
Brain (g)		1.89 ± 0.049	1.92 ± 0.050	1.91 ± 0.086	1.84 ± 0.087	1.95 ± 0.045	1.94 ± 0.043
Thymus (mg)		494 ± 97.5	546 ± 147.1	557 ± 91.6	589 ± 131.9	488 ± 89.8	437 ± 94.9
Liver (g)		8.70 ± 0.626	8.39 ± 0.720	8.34 ± 0.353	8.59 ± 0.787	9.07 ± 0.620	9.12 ± 0.560
Kidneys (g)		1.62 ± 0.063	1.65 ± 0.137	1.61 ± 0.149	1.70 ± 0.088	1.68 ± 0.089	1.62 ± 0.111
Adrenals (mg)		76.3 ± 2.58	72.5 ± 14.17	63.3 ± 5.59	71.6 ± 9.33	64.4 ± 2.59	62.4 ± 5.18
Spleen (g)		0.56 ± 0.070	0.57 ± 0.099	0.53 ± 0.071	0.52 ± 0.048	0.54 ± 0.034	0.59 ± 0.090
Ovaries (mg)		97.9 ± 14.11	102.9 ± 11.55	100.2 ± 19.82	90.6 ± 11.17	100.9 ± 10.08	91.9 ± 6.06
Relative organ weight							
Brain (g%)		0.79 ± 0.059	0.82 ± 0.042	0.79 ± 0.035	0.75 ± 0.042	0.76 ± 0.046	0.76 ± 0.039
Thymus (mg%)		205 ± 42.2	232 ± 53.7	231 ± 43.5	240 ± 52.9	189 ± 29.5	170 ± 34.9
Liver (g%)		3.61 ± 0.125	3.58 ± 0.128	3.44 ± 0.086	3.48 ± 0.151	3.52 ± 0.193	3.54 ± 0.065
Kidneys (g%)		0.67 ± 0.036	0.71 ± 0.030	0.67 ± 0.085	0.69 ± 0.030	0.65 ± 0.048	0.63 ± 0.023
Adrenals (mg%)		31.8 ± 1.40	30.9 ± 4.75	26.2 ± 2.99*	29.0 ± 2.43	25.0 ± 1.37	24.4 ± 3.14
Spleen (g%)		0.23 ± 0.025	0.24 ± 0.036	0.22 ± 0.028	0.21 ± 0.019	0.21 ± 0.008	0.23 ± 0.041
Ovaries (mg%)		40.6 ± 5.05	43.9 ± 3.17	41.6 ± 9.71	37.0 ± 6.03	39.1 ± 3.21	35.7 ± 1.85*

Values are expressed as Mean±S.D.

Significantly different from control group; *, $P<0.05$.

2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの細菌を用いる復帰突然変異試験

Reverse Mutation Test of 2-Hydroxy-4-(octyloxy)benzophenone on Bacteria

要約

OECD既存化学物質安全性点検に係わる毒性調査事業の一環として，2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンについて*Salmonella typhimurium* TA100, TA1535, TA98, TA1537および*Escherichia coli* WP2 *uvrA*を用いる復帰突然変異試験をプレインキュベーション法により実施した．

予備試験の結果，いずれの菌株にも抗菌性が認められなかったことから，本試験ではS9 mix非共存下および共存下のいずれも，5000～313 μg/プレートの公比2で5濃度を設定した．

本試験を2回実施した結果，被験物質の各濃度において誘発された復帰変異コロニー数は，いずれの菌株においても陰性対照値の2倍以上を示さなかった．従って，2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの変異原性は，陰性と結論した．

方法

〔使用菌株〕

カリフォルニア大学B. N. Ames教授より1983年5月27日に入手した*Salmonella typhimurium* TA98, TA100, TA1535, TA1537および東京大学医科学研究所 松島教授より1985年10月14日に入手した*Escherichia coli* WP2 *uvrA*の5菌株を用いた．各使用菌株は超低温槽で-80℃以下に凍結保存した．

試験に際して，各凍結菌株を融解後，その20 μlをニュートリエントブロス(Oxoid Nutrient Broth No.2，ロット番号：067 54134, Unipath社) 25 gを1 lの精製水に溶解して作成した液体完全培地10 mlに接種し，37℃で8時間振盪培養した．培養終了後の菌懸濁液は菌濃度を測定した後，試験に使用した．

〔被験物質〕

2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノン(CAS No.：1843-05-6，ロット番号：40650，純度：99%以上；住友化学工業㈱製造，日本化学工業協会提供)は分子量326.44，融点45～50℃の淡黄(白)色粉末であり，水，熱，光等に安定である．また，*n*-ヘキサンおよびベンゼンに可溶で，水には不溶である．なお，本ロットについては試験期間中安定であることを確認した．

2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンはジメチルスルホキシド(DMSO，ロット番号：603E2089，純度：99.7%，関東化学㈱)を用いて最高濃度(50 mg/ml)の溶液を調製した後，同溶媒で公比2で希釈したものを用いた．なお，本試験の1回目に調製した最高および最低濃度の溶液について濃度分析を実施し，いずれも所定濃度の100±5%以内であることを確認した．

〔陽性対照物質〕

陽性対照物質として下記のものを用いた．

AF-2 ：2-(2-フリル)-3-(5-ニトロ-2-フリル)アクリルアミド(純度：98.0%，和光純薬工業㈱)

NaN_3 ：アジ化ナトリウム(純度：96.5%，和光純薬工業㈱)

ENNG：*N*-エチル-*N'*-ニトロ-*N*-ニトロソグアニジン(純度：99.0%，Sigma Chemical Co.)

9-AA ：9-アミノアクリジン(純度：99%，Sigma Chemical Co.)

2-AA ：2-アミノアントラセン(純度：95.0%，和光純薬工業㈱)

NaN_3は注射用水(㈱大塚製薬工場)に，その他はDMSOに溶解したものを使用した．

〔培地およびS9 mixの組成〕

1) トップアガー

アミノ酸水溶液として，精製水を用いてD-ビオチン，L-ヒスチジンおよびL-トリプトファンの0.5 mM混合水溶液を調製し，これをろ過滅菌後，冷蔵庫に保管した．精製水100 mlに対して，粉末寒天(Bacto-Agar；Difco社)0.6 g，塩化ナトリウム0.5 gの割合で加え，オートクレーブで滅菌し完全に溶解させた後，上記のアミノ酸水溶液を1/10量加えて混和し，約45℃に保温した．

2) 最少グルコース寒天平板培地

クリメディアAM-N培地(日清製粉㈱)を購入し，使用した．なお，培地1 lあたりの組成は下記のとおりである．

硫酸マグネシウム七水塩	0.2 g
クエン酸一水塩	2 g
リン酸水素二カリウム無水塩	10 g
リン酸一アンモニウム	1.92 g
水酸化ナトリウム	0.66 g
ブドウ糖	20 g
寒天(OXOID Agar No.1)	15 g

径90 mmのシャーレ1枚あたり30 mlを流して固めてある．

3) S9 mix

S9 mix 1 mlあたり以下の組成で調製し，使用時まで氷中に保存した．

S9*	0.1 ml
塩化マグネシウム六水塩	8 μmol
塩化カリウム	33 μmol
D-グルコース6-リン酸	5 μmol
β-NADPH	4 μmol
β-NADH	4 μmol
ナトリウム-リン酸緩衝液(pH 7.4)	100 μmol
滅菌精製水	残量

*：購入したS9(キッコーマン㈱)を使用した．このS9は，7週齢の雄のSD系ラットにフェノバルビタールと5,6-ベンゾフラボンを併用投与して作製した肝ホモジネートの9000×g遠心上清分画である．

〔試験方法〕

試験はプレインキュベーション法で実施した．

試験管に被験物質溶液0.1 mlを分注し，S9 mix 0.5 mlと菌懸濁液0.1 mlを加え，37℃で20分間振盪し，プレインキュベーションを行った．S9 mixを共存させない場合には，S9 mixの代わりに0.1 Mナトリウム-リン酸緩衝液(pH 7.4) 0.5 mlを加えた．プレインキュベーション後，トップアガー2 mlを上記の試験管に加えて混和し，最少グルコース寒天平板培地に重層した．重層したトップアガーが凝固した後，37℃で48時間培養し，復帰変異コロニー数を数えた．同時に実体顕微鏡を用いてバックグランドの菌の生育を観察し，被験物質による抗菌性の有無を調べた．予備試験は各濃度あたり1枚のプレートを使用した．本試験は各濃度あたり3枚のプレートを用い，2回実施した．また，被験物質溶液の代わりに陰性対照物質(溶媒)および各菌株毎の陽性対照物質を用いて，被験物質群と同様の操作を行う対照群を設けた．

〔試験結果の判定基準〕

被験物質処理プレートにおける復帰変異コロニー数(平均値)が陰性対照値の2倍以上を示し，明確な用量相関性および再現性が認められる場合に陽性と判定した．

結果および考察

〔予備試験〕

5000, 2500, 1250, 625, 313, 156, 78.1, 39.1 μg/プレートの濃度で実施したところ，S9 mix非共存下および共存下のいずれについても，すべての菌株で抗菌性が認められなかった．従って，S9 mix非共存下および共存下のいずれについても本試験では5000, 2500, 1250, 625, 313 μg/プレートの5濃度を設定した．

〔本試験〕

結果をTable 1, 2に示した．上記の濃度範囲で試験を実施したところ，2回の本試験とも各テスト菌株の復帰変異コロニー数は，S9 mix 非共存下および共存下のいずれにおいても，陰性(溶媒)対照値の2倍以上を示さなかった．また，抗菌性はS9 mixの有無によらずいずれの菌株も5000 μg/プレートまで認められなかった．S9 mix 非共存下および共存下の625 μg/プレート以上の濃度で，沈殿物が認められた．

以上の結果から，2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの変異原性は陰性と結論した．

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Table 1 Results of reverse mutation test(I) of 2-hydroxy-4-(octyloxy) benzophenone on bacteria

With(+) or Without(-) S9 mix	Test Substance Concentration (μg/plate)	Number of revertants (number of colonies / plate)				
		Base-pair change type			Frameshift type	
		TA100	TA1535	WP2 *uvrA*	TA98	TA1537
S9 mix (-)	0	85 81 (83) 82 (± 2)	9 11 (11) 12 (± 2)	32 17 (27) 32 (± 9)	16 20 (18) 17 (± 2)	4 7 (5) 4 (± 2)
	313	90 80 (80) 70 (± 10)	9 9 (9) 8 (± 1)	19 23 (23) 27 (± 4)	19 22 (17) 10 (± 6)	2 4 (5) 8 (± 3)
	625 C	67 88 (74) 67 (± 12)	8 8 (8) 7 (± 1)	28 31 (29) 29 (± 2)	17 17 (18) 19 (± 1)	10 7 (7) 5 (± 3)
	1250 C	87 82 (83) 79 (± 4)	9 12 (10) 10 (± 2)	18 31 (24) 23 (± 7)	24 17 (20) 18 (± 4)	4 5 (4) 4 (± 1)
	2500 C	82 81 (81) 80 (± 1)	10 12 (11) 10 (± 1)	34 24 (24) 14 (± 10)	19 18 (19) 20 (± 1)	5 4 (4) 3 (± 1)
	5000 C	91 72 (83) 87 (± 10)	5 4 (5) 5 (± 1)	22 32 (24) 19 (± 7)	14 14 (13) 12 (± 1)	4 2 (3) 4 (± 1)
S9 mix (+)	0	73 89 (86) 95 (± 11)	6 3 (6) 8 (± 3)	24 19 (23) 27 (± 4)	29 29 (29) 30 (± 1)	7 14 (10) 10 (± 4)
	313	85 87 (88) 92 (± 4)	6 9 (9) 11 (± 3)	26 29 (25) 20 (± 5)	23 30 (31) 39 (± 8)	7 9 (11) 18 (± 6)
	625 C	83 66 (68) 54 (± 15)	8 13 (10) 8 (± 3)	30 18 (22) 19 (± 7)	27 30 (29) 29 (± 2)	7 8 (8) 8 (± 1)
	1250 C	100 122 (99) 75 (± 24)	8 11 (10) 12 (± 2)	36 25 (29) 27 (± 6)	27 22 (27) 31 (± 5)	6 8 (8) 11 (± 3)
	2500 C	90 82 (84) 80 (± 5)	4 7 (7) 9 (± 3)	28 31 (29) 29 (± 2)	26 26 (27) 29 (± 2)	8 12 (10) 11 (± 2)
	5000 C	103 88 (86) 68 (± 18)	12 10 (11) 10 (± 1)	35 32 (30) 24 (± 6)	43 24 (32) 30 (± 10)	12 13 (13) 14 (± 1)
Positive control	Name	AF-2	NaN_3	ENNG	AF-2	9-AA
	Concentration (μg/plate)	0.01	0.5	2	0.1	80
S9 mix (-)	Number of revertants	385 412 (410) 433 (± 24)	283 331 (314) 327 (± 27)	301 308 (335) 397 (± 54)	406 486 (454) 470 (± 42)	876 836 (851) 842 (± 22)
Positive control	Name	2-AA	2-AA	2-AA	2-AA	2-AA
	Concentration (μg/plate)	1	2	10	0.5	2
S9 mix (+)	Number of evertants	559 625 (572) 532 (± 48)	356 284 (303) 269 (± 47)	897 897 (888) 869 (± 16)	312 355 (316) 280 (± 38)	159 152 (163) 177 (± 13)

AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, NaN_3: sodium azide
ENNG: N-ethyl-N'-nitro-N-nitrosoguanidine, 9-AA: 9-aminoacridine, 2-AA: 2-aminoanthracene

(Mean)*
(± S.D.)

C: Precipitates were observed on the surface of agar plates.

Table 2 Results of reverse mutation test (II) of 2-hydroxy-4-(octyloxy)benzophenone on bacteria

With (+) or Without (−) S9 mix	Test Substance Concentration (μg/plate)	Number of revertants (number of colonies / plate)				
		Base-pair change type			Frameshift type	
		TA100	TA1535	WP2 *uvrA*	TA98	TA1537
S9 mix (−)	0	80 91 (83) 79 (± 7)	6 6 (7) 8 (± 1)	18 24 (21) 21 (± 3)	12 18 (14) 13 (± 3)	9 9 (7) 4 (± 3)
	313	88 83 (81) 71 (± 9)	11 5 (9) 12 (± 4)	32 27 (29) 28 (± 3)	13 15 (14) 14 (± 1)	7 5 (5) 2 (± 3)
	625 C	76 81 (78) 76 (± 3)	4 7 (8) 12 (± 4)	26 20 (19) 12 (± 7)	10 15 (15) 20 (± 5)	3 6 (5) 6 (± 2)
	1250 C	94 97 (99) 107 (± 7)	9 11 (9) 6 (± 3)	24 25 (24) 22 (± 2)	19 18 (20) 22 (± 2)	8 7 (7) 6 (± 1)
	2500 C	98 93 (93) 87 (± 6)	10 11 (9) 6 (± 3)	33 35 (34) 34 (± 1)	21 21 (21) 20 (± 1)	6 7 (8) 10 (± 2)
	5000 C	87 80 (84) 84 (± 4)	12 5 (8) 7 (± 4)	26 23 (26) 29 (± 3)	21 20 (18) 13 (± 4)	7 7 (5) 2 (± 3)
S9 mix (+)	0	97 80 (90) 92 (± 9)	13 7 (10) 10 (± 3)	22 31 (25) 22 (± 5)	34 27 (30) 30 (± 4)	15 14 (12) 8 (± 4)
	313	88 94 (92) 95 (± 4)	7 11 (8) 5 (± 3)	30 30 (27) 22 (± 5)	29 28 (30) 32 (± 2)	14 14 (14) 13 (± 1)
	625 C	83 84 (78) 66 (± 10)	5 6 (6) 6 (± 1)	31 37 (32) 27 (± 5)	27 26 (29) 33 (± 4)	10 11 (13) 19 (± 5)
	1250 C	123 85 (103) 101 (± 19)	13 6 (10) 11 (± 4)	43 31 (35) 31 (± 7)	35 32 (32) 30 (± 3)	13 13 (12) 9 (± 2)
	2500 C	98 89 (99) 110 (± 11)	12 5 (9) 9 (± 4)	36 25 (30) 28 (± 6)	37 28 (31) 29 (± 5)	5 11 (9) 11 (± 3)
	5000 C	98 107 (100) 96 (± 6)	9 9 (9) 8 (± 1)	32 30 (29) 25 (± 4)	30 21 (31) 41 (± 10)	12 9 (10) 9 (± 2)
Positive control	Name	AF-2	NaN_3	ENNG	AF-2	9-AA
	Concentration (μg/plate)	0.01	0.5	2	0.1	80
S9 mix (−)	Number of revertants	472 434 (443) 424 (± 25)	280 315 (308) 329 (± 25)	592 674 (651) 688 (± 52)	410 420 (428) 453 (± 23)	714 779 (751) 760 (± 33)
Positive control	Name	2-AA	2-AA	2-AA	2-AA	2-AA
	Concentration (μg/plate)	1	2	10	0.5	2
S9 mix (+)	Number of revertants	560 715 (606) 542 (± 95)	304 337 (318) 312 (± 17)	696 722 (692) 658 (± 32)	217 234 (224) 221 (± 9)	111 131 (131) 150 (± 20)

AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, NaN_3: sodium azide
ENNG: N-ethyl-N'-nitro-N-nitrosoguanidine, 9-AA: 9-aminoacridine, 2-AA: 2-aminoanthracene

(Mean)
(±S.D.)

C: Precipitates were observed on the surface of agar plates.

2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンのチャイニーズ・ハムスター培養細胞を用いる染色体異常試験

In Vitro Chromosomal Aberration Test of 2-Hydroxy-4-(octyloxy)benzophenone on Cultured Chinese Hamster Cells

要約

OECD既存化学物質安全性点検に係わる毒性調査事業の一環として，2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの培養細胞に及ぼす細胞遺伝学的影響を評価するため，チャイニーズ・ハムスター培養細胞(CHL/IU，以下CHLと略す)を用いて試験管内染色体異常試験を実施した．

染色体異常試験に用いる濃度を決定するため，細胞増殖抑制試験を行ったところ，連続処理法の24時間処理および短時間処理法のいずれの処理濃度群(0.156～5.00 mg/ml)においても50%を超える増殖抑制作用は認められなかった．連続処理法の48時間処理では0.156～1.25 mg/mlの細胞増殖率はいずれも50%程度であり顕著な差が認められなかった．従って染色体異常試験において，連続処理法および短時間処理法では5.00 mg/mlを高濃度とし，それぞれその1/2の濃度を中濃度，1/4の濃度を低濃度に設定した．48時間処理では，さらに最低の細胞生存率を示した0.625 mg/ml(高濃度の1/8の濃度)を加えた4濃度を設定した．

CHL細胞を被験物質で24時間および48時間連続処理した結果，すべての処理群において，染色体の構造異常や倍数性細胞の出現頻度は5%未満であった．また，短時間処理法のS9 mix存在下および非存在下においても，すべての処理群において，染色体の構造異常や倍数性細胞の出現頻度は5%未満であった．

以上の結果より2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンは，上記の試験条件下で，試験管内のCHL細胞に染色体異常を誘発しないと結論した．

材料および方法

1. 使用した細胞

大日本製薬㈱から入手(1994年8月，入手時：継代14代)したチャイニーズ・ハムスター由来のCHL細胞を，解凍後継代5代以内で試験に用いた．

2. 培養液の調製

培養には，仔牛血清(CS:GIBCO LABORATORIES，ロット番号：43N1140)を10%添加したイーグルMEM培養液を用いた．

3. 培養条件

2×10^4個のCHL細胞を，培養液5mlを入れたディシュ(径6cm，Becton Dickinson and Company)に播き，37℃のCO_2インキュベーター(5%CO_2)内で培養した．

連続処理法では，細胞播種3日目に被験物質を加え，24時間および48時間処理した．また，短時間処理法では，細胞播種3日目にS9 mixの存在下および非存在下で6時間処理し，処理終了後新鮮な培養液でさらに18時間培養した．

4. 被験物質

2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノン(CAS No.：1843-05-6，ロット番号：40650，純度：99%以上，住友化学工業㈱製造，日本化学工業協会提供)は，分子量326.44，融点45～50℃の淡黄(白)色粉末であり，水，熱，光に安定である．また，*n*-ヘキサンおよびベンゼンに可溶で，水には不溶である．なお，本ロットについては試験期間中安定であることを確認した．

5. 被験物質溶液の調製

被験物質調製液は，用時調製した．溶媒はアセトン(和光純薬工業㈱，ロット番号：KCF1401)を用いた．原体を溶媒に溶解して原液を調製し，ついで原液を溶媒で順次希釈して所定の濃度の被験物質調製液を作製した．被験物質調製液は，すべての試験において培養液の1.0(v/v)%になるように加えた．染色体異常試験に用いた最高および最低濃度の被験物質調製液について濃度分析を実施し，いずれも所定濃度の100±5%以内であることを確認した．

6. 細胞増殖抑制試験による処理濃度の決定

染色体異常試験に用いる被験物質の処理濃度を決定するため，被験物質の細胞増殖に及ぼす影響を調べた．被験物質のCHL細胞に対する増殖抑制作用は，血球計算盤を用いて各群の生存細胞を数え，陰性対照群に対す細胞増殖の比をもって指標とした．

その結果，2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンの約50%の増殖抑制を示す濃度を，50%をはさむ2濃度の値より算出したところ，連続処理法の48時間処理では0.404 mg/mlであったが，この値の前後の濃度の細胞生存率は50%前後で差が認められなかった．一方，連続処理法の24時間処理と短時間処理法ではいずれの処理濃度群(0.156～5.00 mg/ml)においても50%を超える増殖抑制は認められなかった(Fig. 1)．

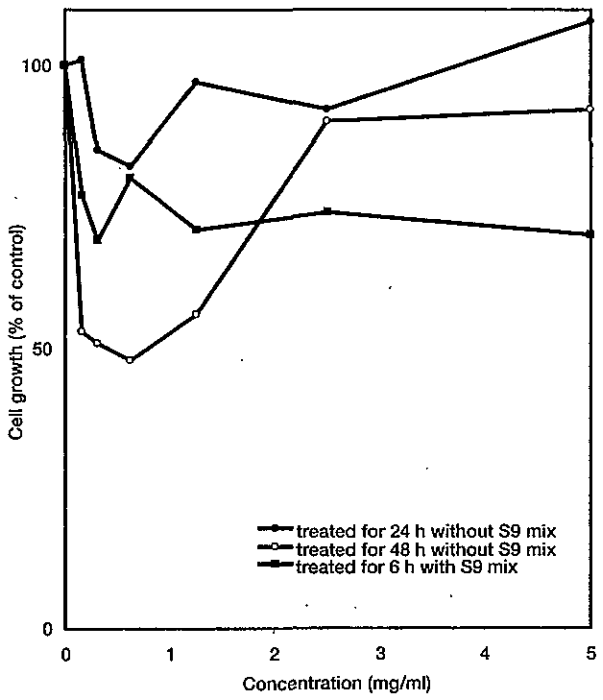

Fig. 1 Inhibition of cell growth treated with 2-hydroxy-4-(octyloxy)benzophenone

7. 実験群の設定

細胞増殖抑制試験の結果より，染色体異常試験で用いる被験物質の高濃度群を連続処理法および短時間処理法では5.00 mg/mlを高濃度とし，それぞれその1/2の濃度を中濃度，1/4の濃度を低濃度とした．48時間処理では，さらに最低の細胞生存率を示した0.625 mg/ml(高濃度の1/8の濃度)を加えた4濃度を設定した．

8. 染色体標本作製法

培養終了の2時間前に，コルセミドを最終濃度が約0.1 μg/mlになるように培養液に加えた．染色体標本の作製は常法に従って行った．スライド標本は各シャーレにつき2枚作製した．作製した標本を，3%ギムザ溶液で20分間染色した．

9. 染色体分析

作製したスライド標本のうち，1枚のシャーレから得られたスライドを処理条件が分からないようにコード化した状態で分析した．染色体の分析は，日本環境変異原学会・哺乳動物試験分科会(MMS)[1]による分類法に基づいて行い，染色体型あるいは染色分体型のギャップ，切断，交換などの構造異常の有無と倍数性細胞(polyploid)の有無について観察した．また，構造異常および倍数性細胞については1群200個の分裂中期細胞を分析した．

10. 記録と測定

溶媒および陽性対照群と被験物質処理群についての分析結果は，観察した細胞数，構造異常の種類と数，倍数性細胞の数について集計し，各群の値を記録用紙に記入した．被験物質の染色体異常誘発性についての判定は，石館ら[2]の判定基準に従い，染色体異常を有する細胞の頻度が5%未満を陰性，5%以上10%未満を疑陽性，10%以上を陽性とした．

結果および考察

連続処理法による染色体分析の結果をTable 1に示した．2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンを加えて24時間および48時間処理した各濃度群で，いずれも染色体の構造異常および倍数性細胞の出現頻度は5%未満であった．

短時間処理法による染色体分析の結果をTable 2に示した．2-ヒドロキシ-4-(オクチルオキシ)ベンゾフェノンを加えてS9 mix存在下および非存在下で6時間処理した各濃度群で，いずれも染色体の構造異常および倍数性細胞の出現頻度は5%未満であった．

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Table 1 Chromosomal analysis of Chinese hamster cells (CHL) continuously treated with 2-hydroxy-4-(octyloxy)benzophenone without S9 mix

Group	Concentration (mg/ml)	Time of exposure (h)	No. of cells analysed	No. of structural aberrations							No. of cells with aberrations		Polyploid[2] (%)	Judgement[3]	
				gap	ctb	cte	csb	cse	f	total	-g (%)	+g (%)		SA	NA
Solvent[1]	0	24	200	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.0		
HOBP	1.25	24	200	0	0	0	1	0	0	1	1 (0.5)	1 (0.5)	0.0	-	-
	2.50	24	200	0	1	0	0	1	0	2	2 (1.0)	2 (1.0)	0.0	-	-
	5.00	24	200	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.0	-	-
MC	0.00003	24	200	2	35	17	1	0	0	55	48 (24.0)	49 (24.5)	0.0	+	-
Solvent	0	48	200	1	0	0	1	0	0	2	1 (0.5)	2 (1.0)	0.5		
HOBP	0.625	48	200	0	0	0	1	0	0	1	1 (0.5)	1 (0.5)	0.0	-	-
	1.25	48	200	0	0	0	1	0	0	1	1 (0.5)	1 (0.5)	0.0	-	-
	2.50	48	200	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.0	-	-
	5.00	48	200	0	0	0	1	0	0	1	1 (0.5)	1 (0.5)	0.5	-	-
MC	0.00003	48	200	2	34	23	7	1	0	67	57 (28.5)	59 (29.5)	0.0	+	-

Abbreviations : gap : chromatid gap and chromosome gap, ctb : chromatid break, cte : chromatid exchange, csb : chromosome break, cse : chromosome exchange (dicentric and ring etc.), f : acentric fragment (chromatid type), -g : total no. cells with aberrations except gap, +g : total no. of cells with aberrations, SA : structural aberration, NA : numerical aberration, HOBP : 2-hydroxy-4-(octyloxy)benzophenone, MC : mitomycin C

1) Acetone was used as solvent. 2) Two hundred cells were analysed in each group. 3) Judgement was done on the basis of the criteria of Ishidate et al. (1987).

Table 2 Chromosomal analysis of Chinese hamster cells (CHL) treated with 2-hydroxy-4-(octyloxy)benzophenone with and without S9 mix

Group	Concentration (mg/ml)	S9 mix	Time of exposure (h)	No. of cells analysed	No. ofstructural aberrations							No. of cells with aberrations		Polyploid[2] (%)	Judgement[3]	
					gap	ctb	cte	csb	cse	f	total	-g (%)	+g (%)		SA	NA
Solvent[1]	0	-	6-(18)	200	0	1	0	0	0	0	1	1 (0.5)	1 (0.5)	0.0		
HOBP	1.25	-	6-(18)	200	0	0	0	2	0	0	2	2 (1.0)	2 (1.0)	0.0	-	-
	2.50	-	6-(18)	200	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.0	-	-
	5.00	-	6-(18)	200	0	1	0	0	1	0	2	2 (1.0)	2 (1.0)	0.0	-	-
BP	0.020	-	6-(18)	200	0	1	0	0	0	0	1	1 (0.5)	1 (0.5)	0.0	-	-
Solvent	0	+	6-(18)	200	0	0	1	0	1	0	2	2 (1.0)	2 (1.0)	0.0		
HOBP	1.25	+	6-(18)	200	0	1	0	0	0	0	1	1 (0.5)	1 (0.5)	0.0	-	-
	2.50	+	6-(18)	200	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.0	-	-
	5.00	+	6-(18)	200	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.0	-	-
BP	0.020	+	6-(18)	200	2	25	140	3	0	0	170	142 (71.0)	144 (72.0)	0.0	+	-

Abbreviations : gap : chromatid gap and chromosome gap, ctb : chromatid break, cte : chromatid exchange, csb : chromosome break, cse : chromosome exchange (dicentric and ring etc.), f : acentric fragment (chromatid type), -g : total no. cells with aberrations except gap, +g : total no. of cells with aberrations, SA : structural aberration, NA : numerical aberration, HOBP : 2-hydroxy-4-(octyloxy)benzophenone, BP : benzo[a]pyrene

1) Acetone was used as solvent. 2) Two hundred cells were analysed in each group. 3) Judgement was done on the basis of the criteria of Ishidate et al. (1987).

ノニルフェノールのラットを用いる28日間反復経口投与毒性試験

Twenty-eight-day Repeat Dose Oral Toxicity Test of Nonylphenol in Rats

要約

既存化学物質の毒性評価の一環として，ノニルフェノールの0(オリーブ油)，4，15，60および250 mg/kgをSDラットに28日間強制経口投与し，その毒性を検討した．0，60および250 mg/kgについては，別に14日間の回復群を設けた．

流涎および体重増加抑制が250 mg/kg群の雌雄にみられた．

尿検査では，尿量の増加と比重の低下が250 mg/kg群の雌雄に，摂水量の増加，沈渣中への扁平上皮細胞の増加と小円形上皮細胞の出現が250 mg/kg群の雌に，血液学検査では，ヘモグロビンとヘマトクリット値の減少が250 mg/kg群の雌に，血液生化学検査では，尿素窒素および無機リンの増加と塩素の減少が250 mg/kg群の雄に，総蛋白およびトリグリセライドの増加が250 mg/kg群の雌にみられた．

病理学検査では，肝臓，腎臓，膀胱および盲腸に変化がみられた．肝臓では，重量増加が60 mg/kg群の雄と250 mg/kg群の雌雄にみられ，組織学的には小葉中心帯肝細胞の肥大が250 mg/kg群の雌雄にみられた．腎臓では，250 mg/kg群で重量増加が雄に，肉眼的に白色点散在，腫大および腎盂拡張が雌にみられ，組織学的には，皮髄境界部において近位尿細管の好塩基性化が雌雄に，同部近位尿細管の単細胞性壊死，間質の細胞浸潤および円柱が雌に，集合管の好塩基性化と拡張が雌雄に，腎盂粘膜の単純性過形成と腎盂拡張が雌にみられた．膀胱では，移行上皮の単純性過形成が250 mg/kg群の雌雄にみられた．盲腸では，肉眼的な拡張が250 mg/kg群の雌雄にみられたが，組織学的変化は認められなかった．

回復群においては，腎臓および膀胱を除く変化は消失した．腎臓および膀胱の変化は投与終了時と比べ軽減していたことから，いずれの変化も可逆性のものと考えられた．

以上の結果から，本試験条件下におけるノニルフェノールの無影響量は雄で15 mg/kg/day，雌で60 mg/kg/dayと考えられた．

方法

1. 被験物質および被験液の調製

被験物質ノニルフェノールは，分子量220.36，融点2℃，沸点295℃，比重0.95，刺激臭のある無色～黄色の粘稠な液体で水に溶けにくい．本試験にはロット番号F1132(三井東圧㈱製)，純度99.0%のものを用いた．なお，投与終了後の残余被験物質について分析を行った結果，使用期間中は安定であったことが確認された．

投与容量が2.5 ml/kg体重となるよう，オリーブ油(日本薬局方)に溶解して最高用量群の投与液(10%(w/v))を調製した．高，中および低用量群の投与液は，10%液をオリーブ油で段階的に希釈してそれぞれ2.4，0.6および0.16%(w/v)液とした．0.05～10%(w/v)液は，室温で1日間および冷蔵(約4℃)・暗所(褐色ガラス瓶)・窒素置換で8日間まで安定であったことから，被験液は最大1週間分を一括して調製し，1日分ずつ褐色ガラス瓶に分注し，窒素置換した上で，冷蔵庫(約4℃)に保存した．また，投与開始前および投与終了週の2回，投与に使用する各濃度液について当施設で濃度を測定した結果，いずれも適正であった．

2. 使用動物および飼育条件

5週齢のCrj:CD(SD)系SPF雌雄ラットを日本チャールス・リバー㈱から購入し，当所で約1週間検疫・馴化飼育した後，体重増加が順調で一般状態に異常を認めなかった雌雄各48匹を選び，6週齢で試験に供した．投与開始日の体重範囲は，雄で199～234 g(平均値：217.0 g)，雌で146～175 g(平均値：161.9 g)であった．

動物は，群分け当日の体重に基づいて層別化し、各群の平均体重がほぼ均等となるよう，コンピュータを用いて各群に割り付けた．

動物は，温度23±3℃，相対湿度50±20%，換気回数1時間当たり11～13回，照明1日12時間の飼育室で，金属製網ケージに1匹ずつ収容し，固型飼料(放射線滅菌CRF-1，オリエンタル酵母工業㈱)および飲料水(水道水)を自由に摂取させ飼育した．

3. 投与量および投与方法

2週間投与による予備試験(投与量：0，5，60，250および1000 mg/kg)の結果，1000 mg/kg群で全例が死亡し，250 mg/kg群では尿素窒素および総コレステロールの増加，盲腸の拡張などがみられた．60 mg/kg以下の投与群では肝臓あるいは副腎重量の増加がみられた．これらの成績から，本試験では250，60，15および4 mg/kgの4用量を設定し，これに対照群を加えて計5群を使用した．さらに，対照群，60および250 mg/kg群では14日間の回復群を設けた．動物数はいずれの群も雌雄各6匹とした．

被験液の投与容量は2.5 ml/kg体重とし，金属製胃ゾンデを用いて1日1回28日間強制経口投与した．対照群には溶媒(オリーブ油)を同様に投与した．

4. 検査項目

1) 一般状態の観察

投与期間中は毎日2回以上，回復期間中は毎日1回観察した．

2) 体重

投与期間および回復期間を通じ，週2回の頻度で体重を測定した．

3) 摂餌量測定

投与期間および回復期間を通じ，週2回の頻度で摂餌量を測定した．

4) 血液学検査

投与期間および回復期間終了の翌日の剖検時に検査を行った．前日から一夜(約16時間)絶食させた動物をエーテル麻酔下で開腹し，腹大動脈から抗凝固剤(EDTA-2K)を加えた採血ビンに血液を採取し，赤血球数(電気抵抗変化検出法)，ヘモグロビン量(シアンメトヘモグロビン法)，ヘマトクリット値(平均赤血球容積および赤血球数から算出)，平均赤血球容積(電気抵抗変化検出法)，平均赤血球血色素量(ヘモグロビン量および赤血球数から算出)，平均赤血球血色素濃度(ヘモグロビン量およびヘマトクリット値から算出)，血小板数(電気抵抗変化検出法)，白血球数(電気抵抗変化検出法)(以上コールター全自動8項目血球アナライザーT890，㈱日科機)，網赤血球率(Brecher法)および白血球百分率(May-Giemsa鏡検法)を測定した．また，3.8%クエン酸ナトリウムを加えた容器に採取した血液を遠心分離(3000 rpm，10分間)し，得られた血漿を用いてプロトロンビン時間および活性化部分トロンボプラスチン時間(以上クロット法，血液凝固自動測定装置，ACL-100，Instrumentation Laboratory)を測定した．

5) 血液生化学検査

血液学検査のための採血と同時に腹大動脈から採血し，遠心分離(3000 rpm，10分間)により得られた血清を用いてAlP(Bessey-Lowry法)，総コレステロール(CEH-COD-POD法)，トリグリセライド(GK-GPO-POD法)，リン脂質(PLD-ChOD-POD法)，総ビリルビン(アゾビリルビン法)，血糖(Hexokinase-G6PD法)，尿素窒素(Urease-GLDH法)，クレアチニン(Jaffé法)，ナトリウム，カリウムおよび塩素(イオン選択電極法)，カルシウム(OCPC法)，無機リン(モリブデン酸法)，総蛋白質(Biuret法)，アルブミン(BCG法)およびA/G比(総蛋白質およびアルブミンから算出)を測定した．また，ヘパリンを加えた容器に採血し，遠心分離(3000 rpm，10分間)により得られた血漿を用いてGOT，GPT，LDH(UV-rate法)，γ-GTP(γ-グルタミル-3-カルボキシ-4-ニトロアニリド法)およびCHE(BTND法)(以上いずれも自動分析装置Monarch，Instrumentation Laboratory)を測定した．

6) 尿検査

投与終了時剖検動物は投与第4週(検査当日の投与後)に，回復群の動物は回復第2週に検査を行った．検査動物を代謝ケージに個別に収容し，絶食・自由摂水下で4時間尿を，次いで自由摂食・自由摂水下でその後の20時間尿を採取した．採取した最初の4時間尿を用いてpH，蛋白質，ケトン体，ブドウ糖，潜血，ビリルビン，ウロビリノーゲン(以上URIFLET7A試験紙，㈱京都第一科学)，色調(肉眼観察)および沈渣(鏡検)を検査した．また，その後に得られた20時間尿を用いて比重(屈折法，アタゴ屈折計，㈱アタゴ)を測定し，4時間尿量および20時間尿量から1日の尿量を算出した．さらに，代謝ケージに収容した状態で，前日からの1日の摂水量を給水瓶を用いて測定した．

7) 剖検および器官重量

上記血液学検査および血液生化学検査のための採血後に放血致死させ，外表異常の有無を観察した後，頭部，胸部および腹部を含む全身の器官・組織について肉眼的に異常の有無を観察した．続いて，以下に示す器官を摘出後，器官重量(絶対重量)を測定した．また，絶食後の体重および絶対重量から体重100 g当たりの相対重量を算出した．

脳，胸腺，心臓，肺(気管支を含む)，肝臓，脾臓，腎臓，副腎，精巣，卵巣

8) 病理組織学検査

全動物について以下に示す全器官・組織を採取し，リン酸緩衝10%ホルマリン液(但し，眼球およびハーダー腺は3%グルタルアルデヒド・2.5%ホルマリン液)で固定した．さらに*印を施した器官・組織についてパラフィンに包埋した．投与終了時剖検動物では，このうち対照群と最高用量群は包埋した全ての器官・組織について，また，高，中および低用量群は被験物質投与によると考えられる変化のみられた肝臓，腎臓および膀胱についてそれぞれ切片とし，ヘマトキシリン・エオジン(H.E.)染色を施して鏡検した．回復群では，被験物質投与による変化が疑われた上記の肝臓，腎臓および膀胱のうち腎臓と膀胱は全動物について，肝臓については対照群と最高用量群の動物を検査した．また，肉眼的異常部位については用量に関係なく鏡検した．

脳*，脊髄*，坐骨神経*，胸大動脈，心臓*，気管*，肺(気管支を含む)*，舌，食道，胃*，十二指腸*，空腸*，回腸*，盲腸*，結腸*，直腸*，唾液腺(顎下腺・舌下腺)，肝臓*，膵臓*，下垂体*，甲状腺(上皮小体を含む)*，副腎*，胸腺*，脾臓*，腸間膜リンパ節*，頸部リンパ節*，腎臓*，膀胱*，精巣*，精巣上体*，精嚢，前立腺*，卵巣*，子宮*，膣*，乳腺，皮膚，眼球*，ハーダー腺，骨及び骨髄(胸骨・大腿骨)*，大腿筋，肉眼

的異常部位*

5. 統計解析

各検査項目のうち，数値化した成績についてまずBartlett法により各群の分散の均一性の検定を行った．その結果，分散が均一の場合には一元配置法による分散分析を行い，群間に有意差が認められたならば，Dunnett法(各群の例数が等しいとき)またはScheffé法(各群の例数が異なるとき)を用いて対照群と各投与群との平均値の差の検定を行った．分散が均一でない場合には，Kruskal-Wallisの順位検定を行い，有意であればDunnett型(各群の例数が等しいとき)またはScheffé型(各群の例数が異なるとき)を用いて対照群と各投与群との平均順位の差の検定を行った．検定はいずれも両側で，有意水準は5および1%とした[7]．

試験結果

1. 一般状態

雄では，流涎が投与13日から投与期間終了まで250 mg/kg群の1～4例で投与直後または投与2時間後にみられた．

雌では，流涎が投与14日から21日まで250 mg/kg群の1～2例で投与直後にみられた．回復期間中はいずれの動物にも異常はみられなかった．

2. 体重(Fig.1)

1) 投与期間

雄では，250 mg/kg群の体重は投与15日頃から対照群をやや下回って推移し，投与期間中の体重増加量は有意に低かった．雌では，各投与群の体重は対照群と同様に推移した．

2) 回復期間

雄では，250 mg/kg群の体重は回復10日まで対照群を有意に下回って推移したが，回復期間中の体重増加量は対照群とほぼ同等であった．雌では，各投与群の体重は対照群と同様に推移した．なお，60 mg/kg群の回復期間中の体重増加量は有意な低値を示したが，用量に関連した変化ではなかった．

3. 摂餌量

1) 投与期間

雌雄ともに，各投与群の摂餌量は対照群と同様に推移した．なお，60 mg/kg群の雌で投与1日(投与開始前日から投与開始直前までの値)に有意な低値を示したが，投与前であり偶発的な変動であった．

2) 回復期間

雄では，60 mg/kg群の摂餌量は回復期間を通じて対照群を有意に上回ったが，用量に関連した変化ではなかった．雌では，各投与群の摂餌量は対照群と同様に推移した．

4. 血液学検査(Table 1)

1) 投与終了時

雄では，被験物質投与による変化はみられなかった．雌では，250 mg/kg群でヘモグロビン量およびヘマトクリット値の有意な減少がみられた．

2) 回復終了時

雄では，桿状核好中球比率の有意な増加が60 mg/kg群にみられたが，用量に関連した変化ではなかった．雌では，各投与群ともに対照群との間に有意差はみられなかった．

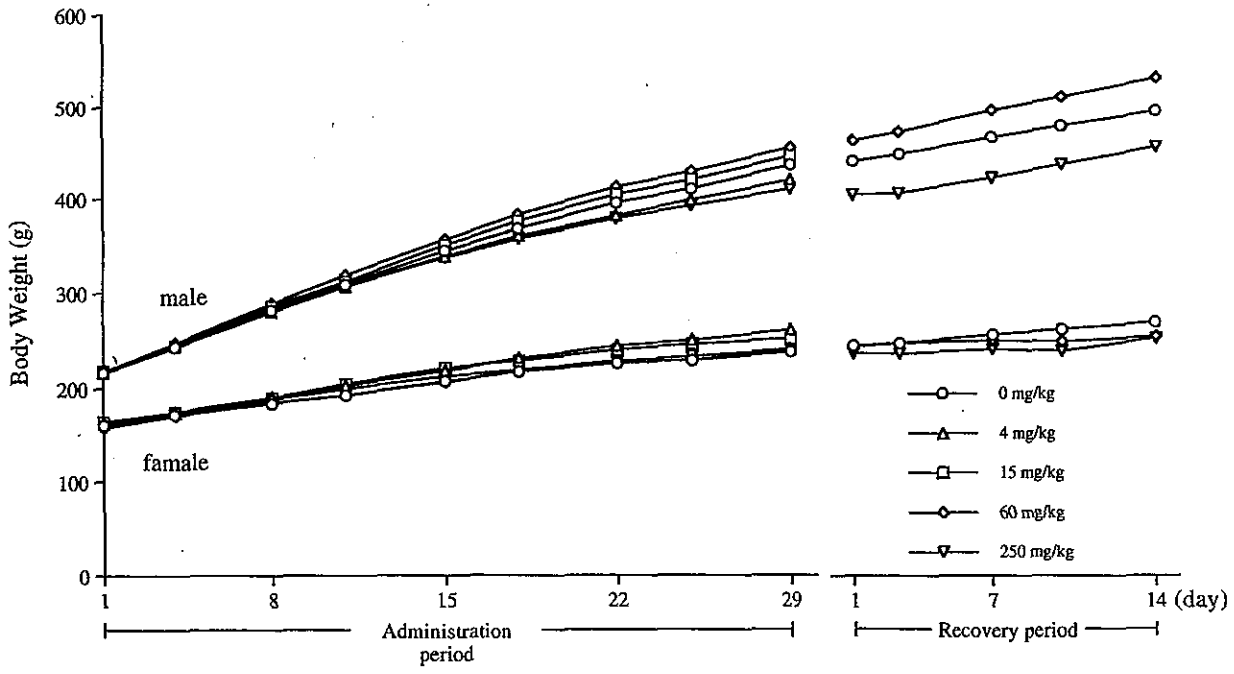

Fig. 1 Body weight changes of rats treated orally with nonylphenol in the twenty-eight-day repeated dose toxicity test

Table 1 Hematology of rats treated orally with nonylphenol in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)					14 days recovery groups (mg/kg)		
	0	4	15	60	250	0	60	250
Male								
No. of animals	6	6	6	6	6	6	5[a]	6
RBC ($\times 10^4$/mm^3)	766 ± 24	784 ± 32	753 ± 13	740 ± 25	746 ± 24	780 ± 33	795 ± 35	793 ± 32
Hb (g/l)	15.5 ± 0.5	15.6 ± 0.3	15.6 ± 0.4	14.8 ± 0.6	15.1 ± 0.7	15.3 ± 0.5	15.5 ± 0.3	15.2 ± 0.6
Ht (%)	46 ± 2	46 ± 1	46 ± 2	44 ± 2	44 ± 1	45 ± 2	46 ± 2	45 ± 2
MCV (μ^3)	59.6 ± 0.7	59.1 ± 1.8	61.4 ± 1.8	59.0 ± 1.6	58.9 ± 1.0	57.6 ± 1.8	57.2 ± 0.8	56.5 ± 1.0
MCH (pg)	20.2 ± 0.3	19.9 ± 0.9	20.7 ± 0.3	20.0 ± 0.6	20.2 ± 0.5	19.6 ± 0.8	19.4 ± 0.6	19.1 ± 0.5
MCHC (%)	33.9 ± 0.3	33.7 ± 0.8	33.8 ± 0.6	33.9 ± 0.4	34.3 ± 0.7	34.1 ± 0.8	33.9 ± 0.8	33.9 ± 0.6
Reticulocyte (‰)	19 ± 1	18 ± 3	20 ± 2	21 ± 6	19 ± 3	18 ± 2	20 ± 3	19 ± 3
Platelet ($\times 10^4$/mm^3)	122.6 ± 8.9	107.5 ± 8.1	111.5 ± 7.8	115.5 ± 16.1	117.9 ± 11.3	95.0 ± 11.7	103.7 ± 10.7	109.3 ± 11.7
WBC ($\times 10^2$/mm^3)	97 ± 22	104 ± 50	89 ± 25	112 ± 37	104 ± 17	104 ± 36	135 ± 33	114 ± 37
Differential leukocyte counts (%)								
Lymph	89.1 ± 5.7	90.0 ± 6.3	89.8 ± 3.8	89.9 ± 6.2	90.0 ± 5.4	92.0 ± 2.5	93.1 ± 1.5	89.8 ± 5.9
Stab	0.3 ± 0.4	0.0 ± 0.0	0.2 ± 0.3	0.3 ± 0.3	0.3 ± 0.4	0.0 ± 0.0	0.3 ± 0.3*	0.0 ± 0.0
Seg	10.5 ± 5.5	9.8 ± 6.3	9.3 ± 3.1	9.3 ± 5.7	9.3 ± 5.0	7.5 ± 2.6	6.2 ± 1.6	9.8 ± 6.0
Eosino	0.2 ± 0.3	0.3 ± 0.3	0.5 ± 0.6	0.3 ± 0.5	0.4 ± 0.4	0.4 ± 0.2	0.3 ± 0.7	0.4 ± 0.4
Baso	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Mono	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.3	0.3 ± 0.3	0.1 ± 0.2	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.0
Others	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PT (Sec.)	11.6 ± 0.6	12.0 ± 0.9	12.2 ± 0.5	11.7 ± 0.7	11.4 ± 0.5	11.7 ± 1.0	11.7 ± 0.6	11.1 ± 0.4
APTT (Sec.)	16.9 ± 2.5	17.5 ± 1.5	17.0 ± 1.5	16.6 ± 1.6	16.8 ± 1.5	16.6 ± 3.3	17.4 ± 1.2	16.2 ± 1.0
Female								
No. of animals	6	6	6	6	6	6	6	6
RBC ($\times 10^4$/mm^3)	808 ± 17	786 ± 33	796 ± 29	787 ± 45	756 ± 36	818 ± 36	807 ± 29	809 ± 25
Hb (g/l)	15.8 ± 0.5	15.7 ± 0.3	16.0 ± 0.6	15.8 ± 0.5	14.8 ± 0.7*	15.6 ± 0.3	15.4 ± 0.4	15.1 ± 0.4
Ht (%)	47 ± 2	46 ± 1	47 ± 1	47 ± 2	44 ± 2*	47 ± 1	46 ± 1	46 ± 1
MCV (μ^3)	58.2 ± 1.1	58.7 ± 2.0	59.6 ± 1.7	59.2 ± 3.0	58.5 ± 2.0	57.3 ± 1.5	56.8 ± 1.8	56.5 ± 1.9
MCH (pg)	19.6 ± 0.4	19.9 ± 0.6	20.1 ± 0.6	20.0 ± 0.9	19.6 ± 0.9	19.1 ± 0.8	19.1 ± 1.0	18.7 ± 0.3
MCHC (%)	33.6 ± 0.7	33.9 ± 0.9	33.8 ± 0.4	33.9 ± 0.4	33.4 ± 0.5	33.3 ± 0.5	33.6 ± 1.1	33.1 ± 0.6
Reticulocyte (‰)	17 ± 3	19 ± 3	17 ± 2	17 ± 3	17 ± 2	17 ± 2	19 ± 3	18 ± 3
Platelet ($\times 10^4$/mm^3)	113.5 ± 13.3	116.1 ± 14.2	116.5 ± 14.8	111.2 ± 5.7	118.1 ± 9.5	104.0 ± 5.3	100.0 ± 10.7	109.0 ± 4.6
WBC ($\times 10^2$/mm^3)	74 ± 18	84 ± 22	98 ± 26	101 ± 27	109 ± 33	67 ± 14	77 ± 18	74 ± 22
Differential leukocyte counts (%)								
Lymph	90.3 ± 5.2	87.5 ± 3.3	92.0 ± 5.3	90.3 ± 4.4	92.3 ± 2.4	88.2 ± 3.5	90.2 ± 5.1	85.4 ± 6.0
Stab	0.3 ± 0.6	0.0 ± 0.0	0.4 ± 0.6	0.2 ± 0.3	0.0 ± 0.0	0.3 ± 0.4	0.2 ± 0.3	0.3 ± 0.3
Seg	8.6 ± 5.2	11.4 ± 3.0	7.4 ± 5.4	9.3 ± 4.2	7.0 ± 2.4	10.9 ± 2.8	8.8 ± 4.9	13.3 ± 5.7
Eosino	0.7 ± 0.8	1.1 ± 0.7	0.2 ± 0.3	0.2 ± 0.3	0.6 ± 0.4	0.6 ± 0.6	0.8 ± 0.9	0.8 ± 0.5
Baso	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Mono	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.0	0.1 ± 0.2
Others	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PT (Sec.)	11.0 ± 0.2	11.0 ± 0.4	10.9 ± 0.2	10.8 ± 0.7	10.8 ± 0.7	10.8 ± 0.3	10.9 ± 0.3	10.9 ± 0.7
APTT (Sec.)	14.1 ± 1.3	14.2 ± 2.5	14.1 ± 0.7	14.4 ± 0.5	13.1 ± 1.8	14.7 ± 0.9	14.4 ± 0.8	14.5 ± 1.3

Values are expressed as Mean ± S.D.
Significant difference from control group; *: $P<0.05$
a) : One sample was not available due to technical error at the time of blood collection.

5. 血液生化学検査(Table 2)

1) 投与終了時

雄では，250 mg/kg群で尿素窒素および無機リンの有意な増加と塩素の有意な減少がみられた．雌では，250 mg/kg群で総蛋白およびトリグリセライドの有意な増加がみられた．

2) 回復終了時

雄では，250 mg/kg群で血糖の有意な減少がみられたが，その値は生理的範囲内のものであった．雌では，各投与群とも対照群との間に有意差はなかった．

6. 尿検査(Table 3)

1) 投与第4週

雄では，250 mg/kg群で尿量の有意な増加および尿比重の有意な低下がみられた．雌では，250 mg/kg群で尿沈渣中への扁平上皮細胞の増加傾向がみられたほか，小円形上皮細胞が2例にみられ，さらに，摂水量と尿量の有意な増加および尿比重の有意な低下がみられた．

2) 回復第2週

雄では，各投与群で摂水量の有意な増加がみられたが，生理的範囲内の変動であった．雌では，各投与群ともに対照群との間に差はみられなかった．

7. 器官重量(Table 4)

1) 投与終了時剖検例

肝臓で，相対重量の有意な増加が60 mg/kg群の雄に，絶対および相対重量の有意な増加が250 mg/kg群の雌雄にみられた．

腎臓で，絶対および相対重量の有意な増加が250 mg/kg群の雄にみられた．

他には被験物質投与によると考えられる変化は認められなかった．

2) 回復終了時剖検例

腎臓において，相対重量の有意な増加が250 mg/kg群の雌雄にみられた．

他には被験物質投与によると考えられる変化は認められなかった．

8. 剖検所見(Table 5)

1) 投与終了時剖検例

250 mg/kg群において，盲腸の拡張が雄全例と雌5例にみられたほか，腎臓で雌1例に白色点散在(両側性)が，さらに，他の1例に白色点散在を伴う腫大(両側性)と腎盂拡張(片側性)が重複してみられた．

他には被験物質投与によると考えられる変化はみられなかった．

2) 回復終了時剖検例

被験物質投与によると考えられる変化はみられなかった．

9. 病理組織学検査(Table 6)

1) 投与終了時剖検例

被験物質投与によると考えられる変化が肝臓，腎臓および膀胱にみられた．

肝臓：小葉中心帯肝細胞のごく軽度な肥大が250 mg/kg群の雄6例全例と雌5例にみられた．他に，微小肉芽腫，クッパー細胞の増殖と変異細胞巣がみられたが，出現状況とその病理学的性状から偶発所見と判断した．

腎臓：主な変化が皮髄境界部の近位尿細管，集合管および腎盂粘膜にみられた．皮髄境界部の近位尿細管では，ごく軽度から軽度な好塩基性化が250 mg/kg群の雄4例と雌2例にみられ，さらに，250 mg/kg群の雌2例ではごく軽度ながら単細胞性壊死もみられた．また，上記の好塩基性化あるいは壊死を示した動物のうち，雌の2例では間質にごく軽度から軽度な細胞浸潤が，雌1例では軽度な円柱もみられた．集合管では，ごく軽度から中等度の好塩基性化が250 mg/kg群の雌雄各6例全例にみられ，うち，雄2例と雌4例の集合管はごく軽度から軽度に拡張していた．腎盂粘膜では，ごく軽度な単純性過形成が250 mg/kg群の雌2例にみられた．また，肉眼的に腎盂拡張を示した250 mg/kg群の雌1例では，組織学的にも中等度な腎盂拡張が認められた．なお，肉眼的に白色点散在を示した250 mg/kg群の雌2例の腎実質における病変の程度は，他の個体と比べやや強かった．

膀胱：移行上皮のごく軽度から軽度な単純性過形成が250 mg/kg群の雄2例と雌6例全例にみられた．

上記以外の所見は出現状況とその病理学的性状からいずれも偶発所見と判断した．

2) 回復終了時剖検例

肝臓：肝細胞の肥大は認められなかった．なお，微小肉芽腫が250 mg/kg群の雌1例にみられた．

腎臓：皮髄境界部における近位尿細管のごく軽度から軽度な好塩基性化と円柱が，250 mg/kg群の雄でそれぞれ5および4例に，集合管のごく軽度な拡張が250 mg/kg群の雌1例にみられた．

膀胱：移行上皮のごく軽度から軽度な単純性過形成が250 mg/kg群の雌6例全例にみられた．

他には被験物質投与によると考えられる変化は認められなかった．

考察

一般状態の観察では，250 mg/kg群で投与直後あるいは2時間後に流涎がみられた．

体重では，250 mg/kg群の雄に増加抑制がみられた．一方，摂餌量には対照群と差がなかったことから，食餌効率の低下が示唆された．

尿検査では，250 mg/kg群で摂水量と尿量の増加，尿比重の低下がみられ，さらに雌では沈渣中への扁平上皮細胞の増加と小円形上皮細胞の出現がみられた．腎臓では，250 mg/kg群の雄で重量が増加し，組織学的には250 mg/kg群で皮髄境界部における近位尿細管の好塩基

Table 2 Blood chemistry of rats treated orally with nonylphenol in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)					14 days recovery groups (mg/kg)		
	0	4	15	60	250	0	60	250
Male								
No. of animals	6	6	6	6	6	6	5[a)]	6
GOT (IU/l)	55 ± 6	52 ± 10	61 ± 5	65 ± 19	62 ± 13	57 ± 11	65 ± 8	65 ± 11
GPT (IU/l)	36 ± 2	35 ± 3	34 ± 3	36 ± 6	38 ± 6	38 ± 4	42 ± 5	38 ± 3
LDH (IU/l)	24 ± 5	24 ± 8	27 ± 4	39 ± 21	28 ± 10	30 ± 12	35 ± 5	29 ± 4
AlP (IU/l)	327 ± 62	349 ± 80	294 ± 41	301 ± 26	375 ± 154	253 ± 43	282 ± 76	214 ± 28
γ-GTP (IU/l)	1.5 ± 0.2	1.5 ± 0.3	1.7 ± 0.3	1.7 ± 0.4	1.8 ± 0.7	1.8 ± 0.3	1.8 ± 0.4	1.8 ± 0.4
ChE (IU/l)	745 ± 92	697 ± 54	782 ± 121	755 ± 98	742 ± 58	642 ± 60	732 ± 168	732 ± 111
TP (g/dl)	6.0 ± 0.2	6.0 ± 0.2	6.0 ± 0.2	58 ± 0.4	6.2 ± 0.4	6.2 ± 0.1	6.4 ± 0.2	6.2 ± 0.1
Albumin (g/dl)	3.3 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.3 ± 0.2	3.5 ± 0.1	3.4 ± 0.2	3.5 ± 0.1	3.3 ± 0.1
A/G (%)	1.24 ± 0.11	1.40 ± 0.08	1.37 ± 0.07	1.36 ± 0.13	1.31 ± 0.08	1.21 ± 0.12	1.18 ± 0.10	1.13 ± 0.06
T. cho (mg/dl)	73 ± 12	64 ± 15	64 ± 8	59 ± 13	80 ± 37	66 ± 9	67 ± 11	73 ± 12
TG (mg/dl)	111 ± 43	95 ± 34	109 ± 34	104 ± 7	106 ± 36	104 ± 38	107 ± 31	74 ± 14
PL (mg/dl)	132 ± 19	122 ± 25	122 ± 11	116 ± 14	150 ± 48	126 ± 12	124 ± 13	129 ± 9
T. bilirubin (mg/dl)	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02
Glucose (mg/dl)	139 ± 18	133 ± 19	144 ± 24	142 ± 24	129 ± 11	168 ± 19	166 ± 22	137 ± 18*
BUN (mg/dl)	11 ± 1	12 ± 1	12 ± 1	12 ± 1	14 ± 2**	14 ± 1	13 ± 2	15 ± 1
Creatinine (mg/dl)	0.59 ± 0.04	0.59 ± 0.05	0.63 ± 0.06	0.62 ± 0.05	0.64 ± 0.05	0.67 ± 0.07	0.67 ± 0.06	0.64 ± 0.03
Na (mEq/l)	144 ± 1	143 ± 1	143 ± 1	143 ± 1	143 ± 1	143 ± 1	142 ± 2	143 ± 1
K (mEq/l)	4.4 ± 0.3	4.3 ± 0.3	4.2 ± 0.2	4.2 ± 0.3	4.4 ± 0.2	4.4 ± 0.3	4.5 ± 0.3	4.8 ± 0.2
Cl (mEq/l)	110 ± 2	108 ± 2	109 ± 1	108 ± 1	106 ± 2**	109 ± 2	107 ± 2	109 ± 3
Ca (mg/dl)	9.1 ± 0.2	9.3 ± 0.3	9.3 ± 0.6	9.2 ± 0.2	9.5 ± 0.4	8.9 ± 0.3	8.9 ± 0.2	8.7 ± 0.3
P (mg/dl)	8.9 ± 0.4	9.0 ± 0.7	9.2 ± 0.6	8.6 ± 0.7	10.0 ± 0.7*	7.8 ± 0.4	8.1 ± 0.4	7.7 ± 0.4
Female								
No. of animals	6	6	6	6	6	6	6	6
GOT (IU/l)	67 ± 14	63 ± 3	62 ± 9	66 ± 12	58 ± 8	65 ± 7	66 ± 10	75 ± 10
GPT (IU/l)	32 ± 4	32 ± 5	29 ± 2	34 ± 9	34 ± 4	35 ± 11	34 ± 5	38 ± 10
LDH (IU/l)	18 ± 1	20 ± 3	21 ± 5	26 ± 6	19 ± 9	17 ± 3	16 ± 4	19 ± 7
AlP (IU/l)	167 ± 22	187 ± 43	176 ± 38	204 ± 42	221 ± 146	138 ± 26	157 ± 43	141 ± 27
γ-GTP (IU/l)	2.3 ± 0.7	2.3 ± 0.5	2.2 ± 0.6	2.3 ± 0.6	3.2 ± 1.0	1.8 ± 0.5	1.8 ± 0.4	1.4 ± 0.5
ChE (IU/l)	2002 ± 259	2158 ± 873	2148 ± 880	2230 ± 339	1513 ± 499	2705 ± 830	2598 ± 1470	1960 ± 451
TP (g/dl)	6.2 ± 0.2	6.3 ± 0.2	6.3 ± 0.3	6.2 ± 0.3	6.8 ± 0.5*	6.8 ± 0.4	6.5 ± 0.3	6.7 ± 0.5
Albumin (g/dl)	3.5 ± 0.1	3.5 ± 0.1	3.6 ± 0.2	3.5 ± 0.1	3.7 ± 0.3	3.7 ± 0.2	3.5 ± 0.2	3.7 ± 0.3
A/G (%)	1.30 ± 0.10	1.27 ± 0.12	1.29 ± 0.05	1.28 ± 0.10	1.18 ± 0.10	1.20 ± 0.07	1.19 ± 0.05	1.25 ± 0.09
T. cho (mg/dl)	73 ± 12	79 ± 22	63 ± 6	65 ± 11	71 ± 24	76 ± 15	76 ± 14	82 ± 19
TG (mg/dl)	32 ± 5	41 ± 17	37 ± 5	33 ± 7	47 ± 12*	38 ± 7	36 ± 7	35 ± 7
PL (mg/dl)	143 ± 16	152 ± 28	135 ± 10	132 ± 16	160 ± 31	159 ± 20	148 ± 20	164 ± 40
T. bilirubin (mg/dl)	0.11 ± 0.01	0.11 ± 0.02	0.10 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.11 ± 0.02	0.11 ± 0.03	0.10 ± 0.01
Glucose (mg/dl)	103 ± 5	108 ± 7	107 ± 7	105 ± 7	109 ± 5	129 ± 18	111 ± 15	114 ± 19
BUN (mg/dl)	15 ± 1	16 ± 2	15 ± 1	14 ± 2	20 ± 7	15 ± 1	15 ± 1	16 ± 2
Creatinine (mg/dl)	0.55 ± 0.06	0.58 ± 0.05	0.60 ± 0.07	0.59 ± 0.08	0.61 ± 0.12	0.66 ± 0.09	0.66 ± 0.07	0.65 ± 0.07
Na (mEq/l)	141 ± 1	141 ± 1	142 ± 0	141 ± 1	142 ± 1	142 ± 2	142 ± 1	142 ± 1
K (mEq/l)	4.5 ± 0.3	4.4 ± 0.6	4.4 ± 0.4	4.4 ± 0.4	4.4 ± 0.2	4.7 ± 0.3	4.6 ± 0.3	4.6 ± 0.4
Cl (mEq/l)	112 ± 1	111 ± 1	111 ± 1	111 ± 2	110 ± 1	112 ± 1	113 ± 1	113 ± 1
Ca (mg/dl)	9.3 ± 0.3	9.4 ± 0.3	9.4 ± 0.3	9.2 ± 0.2	9.6 ± 0.2	9.2 ± 0.3	9.1 ± 0.4	9.2 ± 0.4
P (mg/dl)	8.7 ± 0.7	9.1 ± 0.5	9.1 ± 0.5	8.8 ± 0.9	8.8 ± 0.7	7.9 ± 0.8	8.0 ± 0.8	7.9 ± 1.1

Values are expressed as Mean ± S.D.

Significant difference from control group; *:P<0.05 **:P<0.01

Table 3 Urinalysis of rats treated orally with nonylphenol in the twenty-eight-day repeated dose toxicity test

Item		28 days dosing groups (mg/kg)					14 days recovery groups (mg/kg)		
		0	4	15	60	250	0	60	250
Male									
No. of animals		6	6	6	6	6	6	6	6
Volume (ml)		8.0 ± 3.8	9.0 ± 3.4	10.3 ± 5.2	9.9 ± 2.7	15.4 ± 3.9**	12.2 ± 4.8	14.3 ± 7.1	14.4 ± 2.1
Specific gravity		1.079 ± 0.018	1.074 ± 0.010	1.064 ± 0.010	1.078 ± 0.007	1.043 ± 0.011**	1.075 ± 0.015	1.071 ± 0.015	1.057 ± 0.005
Water intake (ml)		45 ± 7	41 ± 5	47 ± 11	42 ± 3	54 ± 7	38 ± 6	46 ± 7*	51 ± 4**
pH	7	0	1	0	0	0	0	0	0
	7.5	0	0	0	1	0	0	0	0
	8	1	0	0	0	4	1	0	0
	8.5	3	2	3	1	1	0	2	3
	9	2	3	3	4	1	5	4	3
Protein	-	1	0	1	0	0	0	0	0
	-/+	3	2	3	2	0	1	1	0
	1+	1	3	2	4	6	4	3	5
	2+	1	1	0	0	0	1	2	1
Ketons	-	6	6	6	5	6	6	6	6
	-/+	0	0	0	1	0	0	0	0
Glucose	-	6	6	6	6	6	6	6	6
Occult blood	-	4	5	5	6	5	6	4	5
	-/+	1	1	0	0	0	0	1	0
	1+	0	0	0	0	0	0	1	0
	2+	1	0	1	0	0	0	0	1
	3+	0	0	0	0	1	0	0	0
Bilirubin	-	6	6	6	6	6	6	6	6
Urobilinogen	-/+	6	6	6	6	6	6	6	6
Color	Yellow	6	6	6	6	6	6	6	6
	Dark yellow	0	0	0	0	0	0	0	0
RBC	-	6	6	6	6	6	6	6	6
WBC	-	6	6	6	6	6	6	6	6
SEC	-	0	0	0	0	0	0	0	0
	-/+	6	6	6	6	5	6	6	6
	1+	0	0	0	0	1	0	0	0
	3+	0	0	0	0	0	0	0	0
SREC	-	6	6	6	6	6	6	6	6
Cast	-	6	6	6	6	6	6	6	6
PS	-	5	6	4	3	5	5	2	5
	-/+	1	0	2	1	1	1	3	1
	1+	0	0	0	2	0	0	1	0
Co	-	6	6	6	6	6	6	6	6

Values of volume, specific gravity and water intake are expressed as Mean±S.D., other values are expressed as No. of animals
Significant difference from control group; *: $P<0.05$ **: $P<0.01$

Table 3 (Continued)

Item		28 days dosing groups (mg/kg)					14 days recovery groups (mg/kg)		
		0	4	15	60	250	0	60	250
Female									
No. of animals		6	6	6	6	6	6	6	6
Volume (ml)		4.2 ± 3.0	6.4 ± 4.3	5.5 ± 1.3	5.0 ± 2.2	28.4 ± 6.7**	7.3 ± 3.5	4.2 ± 1.4	6.6 ± 2.4
Specific gravity		1.081 ± 0.029	1.077 ± 0.019	1.070 ± 0.011	1.074 ± 0.016	1.021 ± 0.004**	1.060 ± 0.013	1.071 ± 0.017	1.062 ± 0.012
Water intake (ml)		28 ± 5	31 ± 6	31 ± 6	30 ± 8	64 ± 13**	35 ± 3	30 ± 4	30 ± 7
pH	6	0	0	0	1	0	0	0	0
	6.5	1	0	1	0	0	0	3	0
	7	0	1	2	0	2	2	1	1
	7.5	0	0	0	1	1	1	0	0
	8	3	1	0	0	1	0	1	0
	8.5	2	4	3	3	2	2	0	2
	9	0	0	0	1	0	1	1	3
Protein	-	0	0	4	0	3	2	1	1
	-/+	1	3	0	3	3	2	3	4
	1+	5	3	2	2	0	2	2	1
	2+	0	0	0	1	0	0	0	0
Ketons	-	5	4	6	6	6	6	6	6
	-/+	0	2	0	0	0	0	0	0
	1+	1	0	0	0	0	0	0	0
Glucose	-	6	6	6	6	4	6	6	6
	-/+	0	0	0	0	2	0	0	0
Occult blood	-	4	4	5	6	4	6	6	6
	-/+	1	1	1	0	0	0	0	0
	1+	1	1	0	0	2	0	0	0
Bilirubin	-	6	6	6	6	6	6	6	6
Urobilinogen	-/+	6	6	6	6	6	6	6	6
Color	Yellow	6	6	6	6	6	6	6	6
RBC	-	6	6	6	6	6	6	6	6
WBC	-	6	6	6	6	6	6	6	6
SEC	-	0	0	0	0	0	0	0	0
	-/+	6	5	6	6	2	6	6	6
	1+	0	1	0	0	4	0	0	0
SREC	-	6	6	6	6	4	6	6	6
	-/+	0	0	0	0	2	0	0	0
Cast	-	6	6	6	6	6	6	6	6
PS	-	4	5	5	5	6	4	6	3
	-/+	1	1	1	1	0	1	0	2
	1+	1	0	0	0	0	1	0	1
Co	-	6	6	6	6	6	6	6	6

Values of volume, specific gravity and water intake are expressed as Mean ± S.D., other values are expressed as No. of animals
Significant difference from control group; *: $P<0.01$

Table 4 Absolute and relative organ weights of rats treated orally with nonylphenol in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)					14 days recovery groups (mg/kg)		
	0	4	15	60	250	0	60	250
Male								
No. of animals	6	6	6	6	6	6	6	6
Body weight (g)	400 ± 17	393 ± 17	417 ± 29	413 ± 23	382 ± 37	472 ± 30	509 ± 21	433 ± 31
Absolute organ weight								
Brain (g)	2.03 ± 0.10	2.05 ± 0.08	2.05 ± 0.06	2.06 ± 0.04	2.06 ± 0.07	2.13 ± 0.06	2.10 ± 0.05	2.08 ± 0.11
Thymus (mg)	555 ± 85	591 ± 133	605 ± 207	687 ± 308	576 ± 150	464 ± 92	606 ± 161	439 ± 114
Heart (g)	1.25 ± 0.04	1.35 ± 0.06	1.29 ± 0.08	1.27 ± 0.10	1.17 ± 0.11	1.54 ± 0.21	1.56 ± 0.20	1.34 ± 0.13
Lung (g)	1.33 ± 0.10	1.32 ± 0.04	1.39 ± 0.09	1.35 ± 0.07	1.35 ± 0.15	1.50 ± 0.10	1.55 ± 0.12	1.40 ± 0.10
Liver (g)	12.85 ± 0.80	12.39 ± 1.01	14.13 ± 2.42	14.75 ± 1.20	15.68 ± 2.37*	14.74 ± 1.92	16.54 ± 1.24	13.37 ± 1.42
Spleen (g)	0.67 ± 0.05	0.67 ± 0.09	0.68 ± 0.08	0.91 ± 0.55	0.68 ± 0.07	0.82 ± 0.07	1.86 ± 0.13	0.84 ± 0.21
Kidneys (g)	3.06 ± 0.16	2.99 ± 0.26	2.95 ± 0.17	3.22 ± 0.13	3.57 ± 0.27**	3.07 ± 0.23	3.47 ± 0.25	3.47 ± 0.44
Adrenals (mg)	66 ± 12	66 ± 6	68 ± 8	67 ± 9	69 ± 11	69 ± 9	67 ± 6	67 ± 6
Testes (g)	3.15 ± 0.20	3.25 ± 0.14	3.12 ± 0.17	3.32 ± 0.26	3.21 ± 0.30	3.17 ± 0.23	2.89 ± 0.92	3.22 ± 0.22
Rerative organ weight								
Brain (%)	0.51 ± 0.03	0.53 ± 0.02	0.50 ± 0.04	0.50 ± 0.03	0.55 ± 0.04	0.45 ± 0.02	0.41 ± 0.02*	0.48 ± 0.02
Thymus (%)	139 ± 19	150 ± 34	146 ± 52	167 ± 75	151 ± 35	98 ± 16	119 ± 32	102 ± 28
Heart (%)	0.31 ± 0.02	0.35 ± 0.02*	0.31 ± 0.01	0.31 ± 0.01	0.31 ± 0.02	0.32 ± 0.03	0.31 ± 0.04	0.31 ± 0.02
Lung (%)	0.33 ± 0.02	0.34 ± 0.01	0.34 ± 0.03	0.33 ± 0.02	0.35 ± 0.01	0.32 ± 0.02	0.31 ± 0.02	0.32 ± 0.02
Liver (%)	3.21 ± 0.16	3.16 ± 0.20	3.38 ± 0.35	3.58 ± 0.19*	4.09 ± 0.25**	3.12 ± 0.22	3.25 ± 0.22	3.09 ± 0.17
Spleen (%)	0.17 ± 0.01	0.17 ± 0.03	0.16 ± 0.02	0.22 ± 0.15	0.18 ± 0.02	0.17 ± 0.01	0.17 ± 0.03	0.19 ± 0.04
Kidneys (%)	0.77 ± 0.04	0.76 ± 0.05	0.71 ± 0.03	0.79 ± 0.04	0.94 ± 0.05**	0.65 ± 0.03	0.68 ± 0.05	0.80 ± 0.09**
Adrenals (%)	17 ± 2	17 ± 2	17 ± 2	17 ± 3	18 ± 2	15 ± 3	13 ± 1	16 ± 2
Testes (%)	0.79 ± 0.06	0.83 ± 0.04	0.76 ± 0.09	0.81 ± 0.09	0.85 ± 0.09	0.67 ± 0.04	0.57 ± 0.18	0.75 ± 0.05
Female								
No. of animals	6	6	6	6	6	6	6	6
Body weight (g)	222 ± 8	245 ± 21	239 ± 10	222 ± 22	221 ± 11	256 ± 21	249 ± 20	240 ± 20
Absolute organ weight								
Brain (g)	1.87 ± 0.07	1.87 ± 0.08	1.91 ± 0.10	1.82 ± 0.05	1.83 ± 0.07	1.94 ± 0.03	1.91 ± 0.07	1.91 ± 0.11
Thymus (mg)	379 ± 75	449 ± 113	453 ± 114	383 ± 66	351 ± 44	338 ± 66	338 ± 99	324 ± 36
Heart (g)	0.80 ± 0.04	0.88 ± 0.09	0.83 ± 0.06	0.80 ± 0.09	0.78 ± 0.07	0.89 ± 0.09	0.88 ± 0.05	0.87 ± 0.10
Lung (g)	1.01 ± 0.01	1.05 ± 0.08	1.06 ± 0.06	1.06 ± 0.06	1.01 ± 0.07	1.07 ± 0.09	1.07 ± 0.07	1.06 ± 0.08
Liver (g)	6.44 ± 0.25	7.11 ± 0.95	6.80 ± 0.35	6.79 ± 0.75	8.25 ± 0.59**	7.09 ± 0.85	6.91 ± 0.68	7.19 ± 1.03
Spleen (g)	0.49 ± 0.06	0.51 ± 0.10	0.50 ± 0.07	0.52 ± 0.12	0.49 ± 0.04	0.50 ± 0.08	0.53 ± 0.05	0.49 ± 0.05
Kidneys (g)	1.69 ± 0.08	1.78 ± 0.19	1.81 ± 0.13	1.78 ± 0.08	2.11 ± 0.36	1.74 ± 0.13	1.74 ± 0.18	1.83 ± 0.17
Adrenals (mg)	66 ± 8	75 ± 11	72 ± 3	78 ± 4*	60 ± 3	75 ± 11	79 ± 10	72 ± 3
Ovaries (mg)	89.1 ± 11.8	87.6 ± 13.8	89.3 ± 7.3	96.9 ± 17.1	80.5 ± 12.8	102.0 ± 22.0	102.7 ± 12.9	92.4 ± 12.1
Rerative organ weight								
Brain (%)	0.84 ± 0.04	0.77 ± 0.04	0.80 ± 0.08	0.83 ± 0.10	0.83 ± 0.03	0.76 ± 0.07	0.77 ± 0.05	0.80 ± 0.07
Thymus (%)	170 ± 32	182 ± 35	190 ± 51	172 ± 23	159 ± 18	132 ± 28	137 ± 42	137 ± 21
Heart (%)	0.36 ± 0.02	0.36 ± 0.02	0.35 ± 0.03	0.36 ± 0.03	0.35 ± 0.02	0.35 ± 0.02	0.36 ± 0.02	0.36 ± 0.03
Lung (%)	0.45 ± 0.02	0.43 ± 0.01	0.44 ± 0.03	0.48 ± 0.06	0.46 ± 0.02	0.42 ± 0.03	0.43 ± 0.02	0.44 ± 0.03
Liver (%)	2.90 ± 0.15	2.89 ± 0.17	2.84 ± 0.10	3.06 ± 0.21	3.74 ± 0.21**	2.76 ± 0.13	2.78 ± 0.14	2.99 ± 0.23
Spleen (%)	0.22 ± 0.03	0.21 ± 0.03	0.21 ± 0.03	0.23 ± 0.04	0.22 ± 0.01	0.20 ± 0.04	0.21 ± 0.03	0.21 ± 0.03
Kidneys (%)	0.76 ± 0.05	0.73 ± 0.04	0.76 ± 0.07	0.81 ± 0.05	0.95 ± 0.14	0.68 ± 0.03	0.70 ± 0.03	0.77 ± 0.07*
Adrenals (%)	30 ± 3	31 ± 3	30 ± 2	36 ± 5**	27 ± 2	29 ± 3	32 ± 2	30 ± 3
Ovaries (%)	40.2 ± 5.5	35.6 ± 2.8	37.5 ± 4.3	43.5 ± 5.7	36.4 ± 4.9	39.8 ± 7.2	41.4 ± 5.6	38.9 ± 6.5

Values are expressed as Mean ± S.D.
Significant difference from control group; *:P<0.05 **:P<0.01

Table 4. Summary of gross findings in rats treated orally with nonylphenol in the twenty eight day repeated dose toxicity test

Item / Organ / Findings	28 days dosing groups (mg/kg)					14 days recovery groups (mg/kg)		
	0	4	15	60	250	0	60	250
Male								
No. of animals necropsied	6	6	6	6	6	6	6	6
Spleen								
Enlargement with disseminated white spots	0	0	0	1	0	0	0	0
White spot	0	0	0	0	0	0	0	1
Stomach								
Dark red spot in gl. stomach	0	0	0	0	0	1	0	0
Large intestine								
Dilatation of cecum	0	0	0	0	6	0	0	0
Testis								
Small in size (bilateral)	0	0	0	0	0	0	1	0
Epididymis								
Small in size (bilateral)	0	0	0	0	0	0	1	0
Female								
No. of animals necropsied	6	6	6	6	6	6	6	6
Kidney								
Disseminated white spots (bilateral)	0	0	0	0	1	0	0	0
Enlargement with white spots (bilateral) and dilatation of pelvis (unilateral)	0	0	0	0	1	0	0	0
Stomach								
Dark red spot in gl. stomach	1	0	0	0	0	0	0	1
Large intestine								
Dilatation of cecum	0	0	0	0	5	0	0	0

性化，集合管の好塩基性化と拡張がみられたほか，雌では皮髄境界部の近位尿細管に単細胞性壊死，間質の細胞浸潤，円柱，腎盂拡張もみられ，雌の少数例では肉眼的にも白色点などが認められた．従って，上述の検査所見は，腎実質(特に皮髄境界部の近位尿細管と集合管)に対する障害と関連した変化と考えられる．また，250 mg/kg群の膀胱では移行上皮の単純性過形成がみられ，さらに，雌では腎盂粘膜にも過形成がみられ，腎実質のみならず移行上皮から成る尿路系も本被験物質の標的器官と考えられた．

血液学検査では，250 mg/kg群の雌にヘモグロビンとヘマトクリット値の低下が認められ，赤血球に対する影響が示唆された．

血液生化学検査では，250 mg/kg群の雌に総蛋白質およびトリグリセライドの増加がみられたほか，雄では腎障害の反映と考えられる尿素窒素と無機リンの増加および塩素の減少がみられた．

病理学検査では，既に述べた腎臓と膀胱のほかに，肝臓と盲腸に変化がみられた．肝臓では，重量増加が60 mg/kg群の雄と250 mg/kg群の雌雄にみられ，組織学的には，小葉中心帯肝細胞の肥大が250 mg/kg群にみられた．血液生化学検査では，GOTやGPTなど肝機能障害を示す所見はみられなかったことから，肝臓の所見は薬物代謝酵素の誘導を示唆するもの[2]と推察される．また，盲腸では肉眼的な拡張が250 mg/kg群にみられたが，組織学的変化は認められなかった．

回復群においては，被験物質投与に関連すると考えられる変化のうち，250 mg/kg群において，腎臓で重量の増加，皮髄境界部における近位尿細管の好塩基性化，円柱および集合管の拡張，また，膀胱で移行上皮の単純性過形成がみられたが，その他の変化は認められず，被験物質投与によって惹起された変化は概ね可逆性のものと考えられた．また，回復終了時の腎臓でみられた近位尿細管の好塩基性化は，障害を受けた尿細管の再生像と考えられることから，腎臓の変化も本質的には可逆性のものと推定された．

以上の如く，ノニルフェノールをラットに28日間反復投与した結果，主な変化が250 mg/kg群の腎臓，膀胱および肝臓にみられ，本被験物質の標的器官は腎臓，膀胱および肝臓と考えられた．また，60 mg/kg群では，肝臓相対重量の増加がみられたが，15 mg/kg以下の投与群では変化は認められなかった．これらの結果から，

Table 6 Summary of histopathological findings in rats treated orally with nonylphenol in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)					14 days recovery groups (mg/kg)		
	0	4	15	60	250	0	60	250
	1 2 3 P	1 2 3 P	1 2 3 P	1 2 3 P	1 2 3 P	1 2 3 P	1 2 3 P	1 2 3 P
Male								
No. of animals necropsied	6	6	6	6	6	6	6	6
Heart	(6)	(0)	(0)	(0)	(6)	(0)	(0)	(0)
myocarditis/focal	0 0 0 0	- - - -	- - - -	- - - -	1 0 0 0	- - - -	- - - -	- - - -
Trachea	(6)	(0)	(0)	(0)	(6)	(0)	(0)	(0)
cellular infiltratin/mucosa	0 0 0 0	- - - -	- - - -	- - - -	1 0 0 0	- - - -	- - - -	- - - -
Cecum	(6)	(0)	(0)	(0)	(6)	(0)	(0)	(0)
cellular infiltration/mucosa	0 1 0 0	- - - -	- - - -	- - - -	1 1 0 0	- - - -	- - - -	- - - -
Rectum	(6)	(0)	(0)	(0)	(6)	(0)	(0)	(0)
cellular infiltration/mucosa	1 0 0 0	- - - -	- - - -	- - - -	1 0 0 0	- - - -	- - - -	- - - -
Liver	(6)	(6)	(6)	(6)	(6)	(6)	(0)	(6)
hypertrophy/hepatocyte/ centrilobular	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	6 0 0 0	0 0 0 0	- - - -	0 0 0 0
microgranuloma	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0	0 0 0 0	- - - -	0 0 0 0
proliferation/kupffer cell	0 0 0 0	0 0 0 0	0 0 0 0	0 1 0 0	0 0 0 0	0 0 0 0	- - - -	0 0 0 0
Spleen	(6)	(0)	(0)	(1)	(6)	(0)	(0)	(0)
granloma	0 0 0 0	- - - -	- - - -	0 1 0 0	0 0 0 0	- - - -	- - - -	- - - -
hematopoiesis/ extramedullary/ increased	0 0 0 0	- - - -	- - - -	0 1 0 0	0 0 0 0	- - - -	- - - -	- - - -
Kidney	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
basophilic change/ proximal tubule/ cortico-medullary junction	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	3 1 0 0	0 0 0 0	0 0 0 0	2 3 0 0
basophilic change/ proximal tubule/ focal	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0	0 0 0 0
basophilic change/ collecting tubule	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	6 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
dilatation/ collecting tubule	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	2 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
cast	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	3 1 0 0
Urinary bladder	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
hyperplasia/ transitional cell/ simple	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	2 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
Prostate	(6)	(0)	(0)	(0)	(6)	(0)	(0)	(0)
inflammation	0 0 0 0	- - - -	- - - -	- - - -	1 0 0 0	- - - -	- - - -	- - - -
Female								
No. of animals necropsied	6	6	6	6	6	6	6	6
Cecum	(6)	(0)	(0)	(0)	(6)	(0)	(0)	(0)
cellular infiltration/ mucosa	0 1 0 0	- - - -	- - - -	- - - -	0 2 0 0	- - - -	- - - -	- - - -
Liver	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
hypertrophy/ hepatocyte/ centrilobular	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	5 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
microgranuloma	1 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0
altered hepatocellular foci	0 0 0 0	0 0 0 0	0 0 0 1	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
Kidney	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
basophilic change/ proximal tubule/ cortico-medullary junction	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 2 0 0	0 0 0 0	0 0 0 0	0 0 0 0
basophilic change/ collecting tubule	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	2 2 2 0	0 0 0 0	0 0 0 0	0 0 0 0
dilatation/ collecting tubule	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	1 3 0 0	0 0 0 0	0 0 0 0	1 0 0 0
necrosis/ single cell/ proximal tubule/ cortico-medullary junction	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	2 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
cast	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 1 0 0	0 0 0 0	0 0 0 0	0 0 0 0
hyperplasia/ pelvic mucosa/ simple	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	2 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
cellular infiltration/ interstitium	0 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0	1 1 0 0	0 0 0 0	0 0 0 0	0 0 0 0
dilatation/ pelvis	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 1 0	0 0 0 0	0 0 0 0	0 0 0 0
basophilic change/ proximal tubule/ focal	0 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
Urinary bladder	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
hyperplasia/ transitional cell/ simple	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	4 2 0 0	0 0 0 0	0 0 0 0	5 1 0 0

1:Slight 2:Milde 3:Moderate P:Present (grading of severity was not done, such as case in the neoplastic lesion)
Numbers in parenthesis indecate No. of animals examined microscopically at this site.

本試験条件下におけるクールアミノールの無影響は雄では15 mg/kg/day，雌では60 mg/kg/dayと考えられた．

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ノニルフェノールの細菌を用いる復帰突然変異試験

Reverse Mutation Test of Nonylphenol on Bacteria

要約

既存化学物質安全性点検作業の一環として，ノニルフェノールの変異原性について遺伝子突然変異誘発性を検討するため，ネズミチフス菌(*Salmonella typhimurium*) TA100，TA98，TA1535およびTA1537株ならびに大腸菌(*Escherichia coli*) WP2*uvrA*株を用いる復帰突然変異試験を行った．予備的な試験の結果を基に，試験用量を設定した．すなわち，直接法(-S9 mix)ならびに代謝活性化法(+S9 mix)の各菌株についてそれぞれ，0.195-200 μg/プレートの6用量を設定し試験した．その結果，直接法および代謝活性化法のいずれにおいても，ラット肝ミクロソーム(S9)添加の有無にかかわらず，溶媒対照に比べ復帰突然変異コロニー数の明確な増加は認められず，再現性も確認された．一方，各系での陽性対照物質は，それぞれの試験菌株に対し明確な突然変異誘発作用を示した．従って，本試験条件下において，ノニルフェノールは微生物に対し遺伝子突然変異を誘起しないものと判断した．

材料および方法

1. 試験菌株

細菌を用いる復帰突然変異試験に広く使用されていることから，試験菌株としてヒスチジン要求性の*Salmonella typhimurium* TA100，TA98，TA1535および TA1537[1]ならびにトリプトファン要求性の*Escherichia coli* WP2*uvrA*[2]の5種類の菌株を選択した．

ネズミチフス菌は昭和58年9月9日にカリフォルニア大学のB. N. Ames教授から，また，大腸菌については昭和58年3月16日に国立衛生試験所から分与を受けた．平成6年11月25日に菌株の特性検査を実施し，本試験に用いた菌株が規定の特性を保持していることを確認した．各菌株の菌懸濁液はジメチルスルホキシド(DMSO:MERCK社)を添加した後，凍結保存用チューブに0.2 mlずつ分注した．これを液体窒素を用いて凍結し，超低温フリーザーに-80℃で保存した．

2. 培地の調製

1) 最少グルコース寒天平板培地(プレート)

日清製粉㈱製のテスメディアAN培地を購入し，試験に用いた．本プレートは，Vogel-Bonnerの最少培地Eを含む水溶液(0.02%硫酸マグネシウム・7水塩，0.2%クエン酸・1水塩，1%リン酸カルシウム・無水塩，0.192%リン酸一アンモニウム，0.066%水酸化ナトリウム[いずれも最終濃度])に2%のグルコース(和光純薬工業㈱)と1.5%の寒天(OXOID社：No.1)を加え，30 mlをシャーレに分注したものである．

2) トップアガー(軟寒天)

Bacto-agar(DIFCO社)0.6%を含む0.5%塩化ナトリウム水溶液10容量に対し，ネズミチフス菌を用いる試験の場合，0.5 mM L-ヒスチジン(関東化学㈱)-0.5 mM D-ビオチン(関東化学㈱)水溶液を1容量加え，大腸菌を用いる試験の場合，0.5 mM L-トリプトファン(関東化学㈱)水溶液を同じく1容量加え用いた．

3. 前培養条件

内容量200 mlの円筒容器(ストレージボトル：Corning Costar社)に2.5%ニュートリエントブロス(OXOID社)溶液を25 ml分注し，これに融解した菌懸濁液を50 μl接種した．ウォーターバスシェーカー(MM-10：タイテック㈱)を用い，37℃で8時間振盪(往復振盪：120回/分)培養し，試験に使用した．

4. S9 mix

製造後6ヵ月以内のキッコーマン㈱製S9 mixを試験に使用した．S9 mix中のS9は誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawley系雄ラットの肝臓から調製されたものである．S9 mixの組成を以下に示す．

成　分	S9 mix 1ml中の量
S9	0.1 ml
$MgCl_2$	8 μmol
KCl	33 μmol
G-6-P	5 μmol
NADPH	4 μmol
NADH	4 μmol
リン酸緩衝Na-液(pH 7.4)	100 μmol

5. 被験物質

被験物質のノニルフェノール(ロット番号：F1132，CAS No.：25154-52-3)は分子式$C_{15}H_{21}O$，分子量220.36，純度99.0%以上の無色～黄色の粘調液体である．三井東圧化学㈱から提供された被験物質を使用した．試験終了後，被験物質提供元において残余被験物質を分析した結果，安定性に問題はなかった．

6. 被験物質溶液の調製

DMSOに被験物質を溶解して調製原液とした．調製原液を使用溶媒を用いて順次所定濃度に希釈した後，直ちに処理を行った(用時調製)．

7. 試験用量の設定

8.00，40.0，200，1000および5000 μg/プレートの用量を用いて予備的な試験を実施した．その結果，直接法でネズミチフス菌の8.00 μg/プレート以上およびWP2*uvrA*の40.0 μg/プレート以上の用量において試験菌株に対する生育阻害作用が観察された．また，代謝活性化法ではTA100，TA1535およびTA1537の40.0 μg/プレート以上，WP2*uvrA*およびTA98の200 μg/プレート以上の用量において同作用が観察された．従って，本試験においては直接法のネズミチフス菌で12.5 μg/プレートおよびWP2*uvrA*で50.0 μg/プレートを，代謝活性化法ではTA100，TA1535およびTA1537で50.0 μg/プレート，WP2*uvrA*およびTA98で200 μg/プレートを最高用量とし，それぞれ6用量(公比2)を設定した．

8. 陽性対照物質

陽性対照物質として下記に示した物質を使用した．これらの陽性対照物質は，DMSOを用いて溶解し，少量ずつ分注した後凍結保存(-20℃)した．

2-(2-フリル)-3-(5-ニトロ-2-フリル)アクリルアミド(AF-2:和光純薬工業㈱)

アジ化ナトリウム(NaN_3:和光純薬工業㈱)

9-アミノアクリジン(ACR:ALDRICH社)

2-アミノアントラセン(2-AA:和光純薬工業㈱)

9. 試験方法

Amesらの原法の改良法であるプレインキュベーション法[1]に準じて，直接法および代謝活性化法それぞれについて試験を実施した．試験管に，使用溶媒，被験物質溶液あるいは陽性対照物質溶液を100 μl，次いで直接法の場合，0.1M ナトリウム・リン酸緩衝液(pH 7.4)を500 μl，代謝活性化法の場合，S9 mixを500 μlおよび試験菌液100 μlを加え，37℃で20分間振盪培養(プレインキュベーション)した．培養終了後，トップアガーを2 ml添加し，混合液をプレート上に重層した．37℃の条件で48時間各プレートを培養した後，被験物質の試験菌株に対する生育阻害作用を確認するため，実体顕微鏡(×60)を用いてプレート上の試験菌株の生育状態を観察した．次いで，復帰突然変異により生じたコロニーを計数した．計測に際してはコロニーアナライザー(CA-11:システムサイエンス㈱)を用いた．独立して試験を2回実施した．

10. 結果の解析

復帰突然変異コロニー数が溶媒対照のほぼ2倍以上に増加し，かつ，再現性あるいは被験物質の用量に依存性が認められた場合に，陽性と判定した．

なお，統計学的手法を用いた検定は実施しなかった．

結果および考察

試験結果をTable 1-4に示した．直接法(-S9 mix)ならびに代謝活性化法(+S9 mix)のいずれとも高用量群において，ノニルフェノール処理による生育阻害作用が観察された．また，復帰突然変異コロニー数については，直接法，代謝活性化法とも溶媒対照と同等の値であり，増加傾向は認められなかった．一方，陽性対照物質はそれぞれの菌株において，溶媒対照群の2倍以上の復帰突然変異コロニーを誘発した．なお，S9 mix添加時試験管内で反応液が僅かに白濁したが，コロニー計数時においては特筆すべき変化は観察されなかった．以上の試験結果から，本試験条件下においてノニルフェノールの微生物に対する遺伝子突然変異に関し，陰性と判定した．

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Table 1. Results of the bacterial reversion test of nonylphenol (1st trial) [direct method: −S9]

Compound	Dose	Revertant colonies per plate [Mean±S.D.]				
	(μg/plate)	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO#	0	85 95 97 [92 ± 6]	13 17 19 [16 ± 3]	17 17 23 [19 ± 3]	24 31 33 [29 ± 5]	10 10 6 [9 ±2]
Test sub.	0.195	111 84 91 [95 ± 14]	17 14 11 [14 ± 3]	−	26 27 28 [27 ± 1]	13 8 8 [10 ± 3]
	0.391	92 102 89 [94 ± 7]	13 23 17 [18 ± 5]	−	28 22 26 [25 ± 3]	7 9 7 [8 ± 1]
	0.781	95 109 101 [102 ± 7]	10 17 12 [13 ± 4]	−	31 16 27 [25 ± 8]	9 8 5 [7 ± 2]
	1.56	86 95 84 [88 ± 6]	21 14 16 [17 ± 4]	24 15 17 [19 ± 5]	27 16 28 [24 ± 7]	8 6 6 [7 ± 1]
	3.13	92 92 107 [97 ± 9]	13 11 9 [11 ± 2]	18 23 17 [19 ± 3]	30* 13* 32* [25 ± 10]	11* 10* 12* [11 ± 1]
	6.25	84* 84* 82* [83 ± 1]	10* 10* 11* [10 ± 1]	17 22 17 [19 ± 3]	19* 15* 24* [19 ± 5]	7* 6* 4* [6 ± 2]
	12.5	83* 61* 75* [73 ± 11]	13* 9* 10* [11 ± 2]	16 27 16 [20 ± 6]	17* 16* 25* [19 ± 5]	2* 1* 4* [± 2]
	25.0	−	−	20* 14* 16* [17 ± 3]	−	−
	50.0	−	−	14* 22* 14* [17 ± 5]	−	−
Positive control		466 378 397[a] [414 ± 46]	517 517 487[b] [507 ± 17]	112 117 116[a] [115 ± 3]	575 543 551[c] [556 ± 17]	465 551 438[d] [485 ± 59]

#: Solvent control *: The background lawn was thin −: Not tested
a): AF-2; 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, 0.01 μg/plate b): NaN_3; Sodium azide, 0.5 μg/plate c): AF-2, 0.1 μg/plate d): ACR; 9-Aminoacridine, 80 μg/plate

Table 2. Results of the bacterial reversion test of nonylphenol (1st trial) [activation method: +S9]

Compound	Dose	Revertant colonies per plate [Mean±S.D.]				
	(μg/plate)	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO#	0	87 94 97 [93 ± 5]	9 15 12 [12 ± 3]	32 36 24 [31 ± 6]	37 34 36 [36 ± 2]	15 15 19 [16 ± 2]
Test sub.	1.56	105 94 92 [97 ± 7]	11 21 14 [15 ± 5]	−	−	19 13 12 [15 ± 4]
	3.13	112 116 114 [114 ± 2]	15 18 23 [19 ± 4]	− [19 ± 3]	−	17 18 22
	6.25	126 95 91 [104 ± 19]	13 16 9 [13 ± 4]	25 21 31 [26 ± 5]	38 36 32 [35 ± 3]	17 12 9 [13 ± 4]
	12.5	124 105 100 [110 ± 13]	16 13 16 [15 ± 2]	17 20 33 [23 ± 9]	35 34 36 [35 ± 1]	17 18 7 [14 ± 6]
	25.0	100 114 94 [103 ± 10]	16 8 12 [12 ± 4]	25 17 30 [24 ± 7]	54 50 57 [54 ± 4]	9 12 17 [13 ± 4]
	50.0	87* 97* 95* [93 ± 5]	8* 10* 16* [11 ± 4]	21 28 29 [26 ± 4]	46 42 38 [42 ± 4]	9* 17* 14* [13 ± 4]
	100	−	−	30* 24* 31* [28 ± 4]	34* 32* 31* [32 ± 2]	−
	200	−	−	16* 15* 18* [16 ± 2]	25* 25* 29* [26 ± 2]	−
Positive control		866 793 819[a] [826 ± 37]	483 592 538[b] [538 ± 55]	811 845 824[c] [827 ± 17]	374 306 338[d] [339 ± 34]	186 149 170[b] [168 ± 19]

#: Solvent control *: The background lawn was thin −: Not tested
a): 2-AA; 2-Aminoanthracene, 1 μg/plate b): 2-AA, 2 μg/plate c): 2-AA, 10 μg/plate d): 2-AA, 0.5 μg/plate

Table 3. Results of the bacterial reversion test of nonylphenol (2nd trial) [direct method: −S9]

Compound	Dose	Revertant colonies per plate [Mean±S.D.]				
	(μg/plate)	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO#	0	106 93 98 [99 ± 7]	11 10 12 [11 ± 1]	27 19 24 [23 ± 4]	18 26 24 [23 ± 4]	10 7 9 [9 ± 2]
Test sub.	0.195	100 108 103 [104 ± 4]	14 14 13 [14 ± 1]	−	24 29 31 [28 ± 4]	10 12 8 [10 ± 2]
	0.391	105 105 109 [106 ± 2]	16 15 13 [15 ± 2]	−	25 29 32 [29 ± 4]	10 12 9 [10 ± 2]
	0.781	103 106 102 [104 ± 2]	15 11 15 [14 ± 2]	−	27 28 32 [29 ± 3]	9 7 7 [8 ± 1]
	1.56	94 107 99 [100 ± 7]	16 14 16 [15 ± 1]	15 14 16 [15 ± 1]	30 23 25 [26 ± 4]	10 5 6 [7 ± 3]
	3.13	101* 104* 101* [102 ± 2]	13 13 14 [13 ± 1]	23 19 21 [21 ± 2]	24 25 33 [27 ± 5]	7* 9* 13* [10 ± 3]
	6.25	79* 83* 85* [82 ± 3]	11* 14* 14* [13 ± 2]	20 24 19 [21 ± 3]	20* 14* 20* [18 ± 3]	6* 5* 4* [5 ± 1]
	12.5	82* 76* 85* [81 ± 5]	5* 8* 10* [8 ± 3]	19 21 23 [21 ± 2]	16* 16* 22* [18 ± 3]	3* 6* 3* [4 ± 2]
	25.0	−	−	16* 18* 15* [16 ± 2]	−	−
	50.0	−	−	12* 13* 13* [13 ± 1]	−	−
Positive control		432 403 397[a] [411 ± 19]	460 557 453[b] [490 ± 58]	127 134 100[a] [120 ± 18]	526 527 473[c] [509 ± 31]	613 591 463[d] [556 ± 81]

#: Solvent control *: The background lawn was thin −: Not tested
a): AF-2; 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, 0.01 μg/plate b): NaN_3; Sodium azide, 0.5 μg/plate c): AF-2, 0.1 μg/plate d): ACR; 9-Aminoacridine, 80 μg/plate

Table 4. Results of the bacterial reversion test of nonylphenol (2nd trial) [activation method: +S9]

Compound	Dose	Revertant colonies per plate [Mean±S.D.]				
	(μg/plate)	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
DMSO#	0	97 91 102 [97 ± 6]	16 18 15 [16 ± 2]	29 32 22 [28 ± 5]	40 39 29 [36 ± 6]	17 16 16 [16 ± 1]
Test sub.	1.56	102 99 105 [102 ± 3]	16 14 16 [15 ± 1]	−	−	17 14 17 [16 ± 2]
	3.13	97 100 110 [102 ± 7]	14 15 14 [14 ± 1]	−	−	14 19 14 [16 ± 3]
	6.25	99 106 100 [102 ± 4]	14 17 14 [15 ± 2]	32 25 34 [30 ± 5]	40 31 41 [37 ± 6]	17 14 17 [16 ± 2]
	12.5	114 102 96 [104 ± 9]	15 17 16 [16 ± 1]	21 33 29 [28 ± 6]	37 33 31 [34 ± 3]	13 16 15 [15 ± 2]
	25.0	105* 101* 107* [104 ± 3]	15 14 7 [12 ± 4]	36 30 33 [33 ± 3]	40 44 34 [39 ± 5]	13 13 12 [13 ± 1]
	50.0	102* 101* 94* [99 ± 4]	8* 11* 11* [10 ± 2]	31 31 32 [31 ± 1]	30 29 35 [31 ± 3]	14* 8* 9* [10 ± 3]
	100	−	−	24* 21* 23* [23 ± 2]	28* 22* 37* [29 ± 8]	−
	200	−	−	24* 23* 21* [23 ± 2]	33* 22* 24* [26 ± 6]	−
Positive control		646 660 634[a] [647 ± 13]	360 414 432[b] [402 ± 37]	611 786 719[c] [705 ± 88]	316 276 335[d] [309 ± 30]	140 127 127[b] [131 ± 8]

#: Solvent control *: The background lawn was thin −: Not tested
a): 2-AA; 2-Aminoanthracene, 1 μg/plate b): 2-AA, 2 μg/plate c): 2-AA, 10 μg/plate d): 2-AA, 0.5 μg/plate

ノニルフェノールのチャイニーズ・ハムスター培養細胞を用いる染色体異常試験

In Vitro Chromosomal Aberration Test of Nonylphenol on Cultured Chinese Hamster Cells

要約

既存化学物質安全性点検作業の一環として，ノニルフェノールの変異原性について染色体異常誘発性の有無を検討するため，チャイニーズ・ハムスター肺線維芽細胞株(CHL)を用いる*in vitro*染色体異常試験を行った．細胞増殖抑制試験結果を基に，細胞毒性が観察される濃度を最高用量として設定した．すなわち，連続24時間処理法で6.25, 12.5, 25.0および50.0 μg/ml，同48時間処理法で3.13, 6.25, 12.5および25.0 μg/ml，短時間処理法(6時間処理の+S9 mixおよび-S9 mix)で7.50, 15.0, 30.0および60.0 μg/mlの4用量(公比2)について染色体標本を作製した後，顕微鏡観察を実施した．細胞の増殖が強く抑制される用量まで検討した結果，連続処理法ならびに短時間処理法のいずれとも染色体異常，すなわち構造異常あるいは倍数性細胞の誘発は認められなかった．一方，連続処理法の陽性対照物質マイトマイシンC(MMC)および短時間処理+S9 mixの陽性対照物質シクロホスファミド(CP)は，いずれも染色体構造異常を高頻度に誘発した．従って，本試験条件下の*in vitro*試験系において，ノニルフェノールには染色体異常を誘起する可能性がないものと判断した．

材料および方法

1. 試験細胞株

哺乳類培養細胞を用いる染色体異常試験に広く使用されていることから，試験細胞株としてチャイニーズ・ハムスターの肺由来の線維芽細胞株(CHL)を選択した．昭和59年11月15日に国立衛生試験所から分与を受け，一部はジメチルスルホキシド(DMSO:MERCK社)を10%添加した後，液体窒素中に保存し，残りは3～5日ごとに継代した．なお，本染色体異常試験では解凍後継代数32の細胞を用いた．

2. 培養液の調製

Eagle-MEM培地(LIFE TECHNOLOGIES社)を1000 mlの精製水で溶解した後，2.2 gの炭酸水素ナトリウム(関東化学㈱)を加えた．1N塩酸を用いてpHを7.2に調整した後，メンブランフィルター(0.2 μm:Gelman Sciences社)を用いて加圧濾過除菌した．非働化(56℃，30分)済み仔牛血清(LIFE TECHNOLOGIES社)を最終濃度で10%になるよう加えた後，試験に使用した．

3. 培養条件

CO_2インキュベーター(FORMA社あるいは三洋電機特機㈱)を用い，CO_2濃度5%，37℃の条件で細胞を培養した．

4. S9 mix

製造後6ヵ月以内のキッコーマン㈱製S9 mixを試験に使用した．S9 mix中のS9は誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawley系雄ラットの肝臓から調製されたものである．S9 mixの組成は松岡らの方法に従った[1]．

5. 被験物質

被験物質のノニルフェノール(ロット番号:F1132, CAS No.:25154-52-3)は分子式$C_{15}H_{24}O$，分子量220.36，純度99.0%以上の無色～黄色の粘調液体である．三井東圧化学㈱から提供された被験物質を使用した．試験終了後，被験物質提供元において残余被験物質を分析した結果，安定性に問題はなかった．

6. 被験物質溶液の調製

DMSOに被験物質を溶解して調製原液とした．調製原液を使用溶媒を用いて順次所定濃度に希釈した後，直ちに処理を行った(用時調製)．

7. 予備試験(細胞増殖抑制試験)

細胞培養用マルチプレートに細胞を播種し，培養3日後に被験物質溶液を処理した．連続処理法の場合，24あるいは48時間連続して処理を実施し，短時間処理法ではS9 mix存在下(+S9 mix)あるいは非存在下(-S9 mix)で6時間処理した後，新鮮な培養液に交換してさらに18時間培養を続けた．

細胞を10%中性緩衝ホルマリン液(和光純薬工業㈱)で固定した後，0.1%クリスタル・バイオレット(関東化学㈱)水溶液で10分間染色した．色素溶出液(30%エタノール，1%酢酸水溶液)を適量加え，5分間程度放置して色素を溶出した後，580 nmでの吸光度を測定した．各用量群について溶媒対照群での吸光度に対する比，すなわち細胞生存率を算出した．

その結果，いずれの処理法においても顕著な細胞増殖抑制が観察された(Fig. 1)．プロビット法あるいは対数確立紙を用いて算出した50%細胞増殖抑制濃度は連続24時間処理で23.2 μg/ml，同48時間処理で25.9 μg/ml，短時間+S9 mix処理で31.8 μg/ml，同-S9 mix処理で29.3 μg/mlであった．

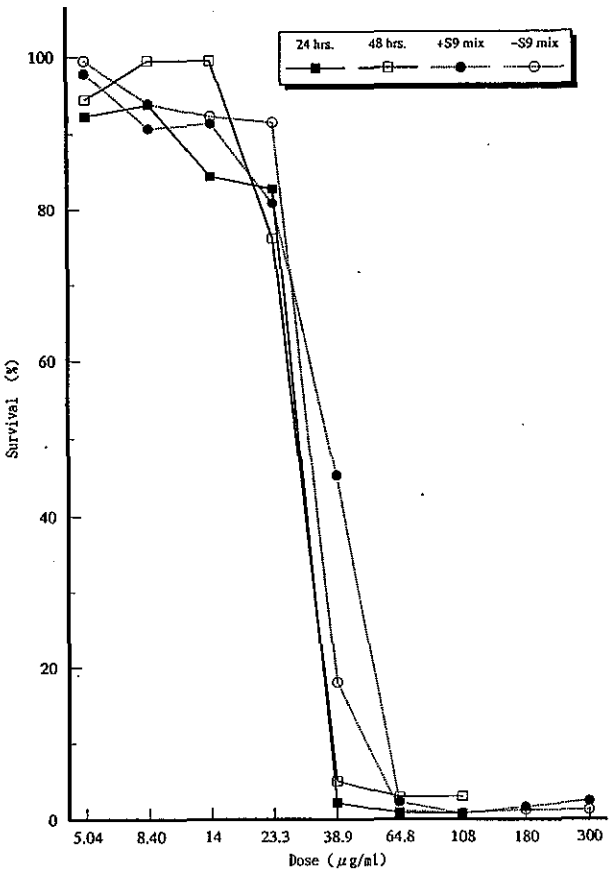

Fig. 1 Dose-survival curves of nonylphenol

8. 試験用量および試験群の設定

細胞増殖抑制試験結果を基に，染色体異常試験では連続24時間処理で50.0 μg/ml，同48時間処理で25.0 μg/ml，短時間処理法で60.0 μg/mlを最高用量とし，以下公比2で減じた計4用量ならびに溶媒対照群を設定した．

陽性対照として，連続処理法の場合，マイトマイシンC(MMC:協和醗酵工業㈱)を，24時間処理で0.05 μg/ml，48時間処理で0.025 μg/mlの用量で，短時間処理法の場合，シクロホスファミド(CP:塩野義製薬㈱)を，12.5 μg/mlの用量で試験した．

9. 染色体標本の作製

直径60 mmのプレートを用い，予備試験と同様に被験物質等の処理を行った．培養終了2時間前に，最終濃度で0.2 μg/mlとなるようコルセミド(LIFE TECHNOLOGIES社)を添加した．トリプシン処理で細胞を剥離させ，遠心分離により細胞を回収した．75 mM塩化カリウム水溶液で低張処理を行った後，固定液(メタノール3容:酢酸1容)で細胞を固定した．空気乾燥法で染色体標本を作製した後，1.2%ギムザ染色液で12分間染色した．

10. 染色体の観察

各プレートあたり100個，すなわち用量当たり200個の分裂中期像を顕微鏡下で観察し，染色体の形態的変化としてギャップ(gap)，染色分体切断(ctb)，染色体切断(csb)，染色分体交換(cte)，染色体交換(cse)およびその他(oth)の構造異常に分類した．同時に，倍数性細胞の出現率を記録した．染色体の分析は日本環境変異原学会・哺乳動物試験分科会[2]による分類法に従って実施した．

すべての標本をコード化した後，観察した．

11. 結果の解析

ギャップのみ保有する細胞を含めた場合(+gap)と，含めない場合(-gap)とに区別して染色体構造異常の出現頻度を表示した．

各試験群の構造異常を有する細胞あるいは倍数性細胞の出現頻度を，石館ら[3]の基準に従って判定した．染色体異常を有する細胞の出現頻度が5%未満を陰性(-)，5%以上10%未満を疑陽性(±)，10%以上を陽性(+)とした．最終的には再現性あるいは用量に依存性が認められた場合に陽性と判定した．

なお，統計学的手法を用いた検定は実施しなかった．

結果および考察

連続処理群での試験結果をTable 1に示した．ノニルフェノール処理群の場合，24時間ならびに48時間処理のいずれにおいても最高用量で強い細胞毒性作用が観察された．しかしながら，染色体の構造異常および倍数性細胞の誘発傾向は観察されなかった．一方，陽性対照物質のMMCで処理した細胞では染色体の構造異常の顕著な誘発が認められた．短時間処理群での試験結果をTable 2に示した．被験物質処理群の場合，+S9 mixならびに-S9 mixの最高用量で細胞毒性作用が認められたものの，いずれの用量においても染色体構造異常および倍数性細胞の誘発傾向は観察されなかった．また，陽性対照物質のCPで処理した細胞ではS9 mix存在下でのみ染色体の構造異常の顕著な誘発が認められた．以上の試験結果から，本試験条件下においてノニルフェノールの哺乳類培養細胞に対する染色体異常誘発性に関し，陰性と判定した．

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Table 1. Chromosomal aberration test on CHL cells treated with nonylphenol [long-term treatment]

Compound	Dose (μg/ml)	Time of exposure (hr)	Number of cells analyzed	Number of cells with structural aberrations						Total [+gap] (%)	Total [-gap] (%)	Polyploid cells (%)	Final judgement
				gap	ctb	csb	cte	cse	oth				
DMSO*	0	24	200	0	0	0	1	0	0	0.5	0.5	0.0	-
Test Sub.	6.25	24	200	1	0	0	1	0	0	1.0	0.5	0.0	-
	12.5	24	200	0	0	0	0	0	0	0.0	0.0	0.0	-
	25.0	24	200	1	1	0	1	2	0	2.5	2.0	0.5	-
	50.0	24	Toxic										
MMC**	0.05	24	200	19	49	0	95	1	0	65.5	61.5	0.5	+
DMSO*	0	48	200	0	0	0	0	0	0	0.0	0.0	0.0	-
Test Sub.	3.13	48	200	1	0	0	0	0	0	0.5	0.0	1.0	-
	6.25	48	200	0	0	1	5	0	0	2.5	2.5	0.0	-
	12.5	48	200	0	1	0	0	0	0	0.5	0.5	0.0	-
	25.0	48	Toxic										
MMC**	0.025	48	200	14	47	0	91	6	0	60.0	59.0	1.0	+

*:Solvent control **:Positive control (mitomycin C)
ctb:chromatid break csb:chromosome break cte:chromatid exchange cse:chromosome exchange oth:others

Table 2. Chromosomal aberration test on CHL cells treated with nonylphenol [short-term treatment]

Compound	Dose (μg/ml)	S 9 mix	Time of exposure (hr)	Number of cells analyzed	Number of cells with structural aberrations						Total [+gap] (%)	Total [-gap] (%)	Polyploid cells (%)	Final judgement
					gap	ctb	csb	cte	cse	oth				
DMSO*	0	+	6	200	0	0	0	0	0	0	0.0	0.0	0.5	-
Test Sub.	7.50	+	6	200	0	0	0	0	0	0	0.0	0.0	0.0	-
	15.0	+	6	200	0	1	0	0	0	0	0.5	0.5	0.0	-
	30.0	+	6	200	0	0	0	2	1	0	1.5	1.5	3.0	-
	60.0	+	6	200	2	1	0	6	0	0	4.0	3.5	0.0	-
CP**	12.5	+	6	200	7	17	0	66	1	0	39.5	38.0	0.0	+
DMSO*	0	-	6	200	4	0	1	1	1	0	3.5	1.5	1.0	-
Test Sub.	7.50	-	6	200	1	0	0	2	1	0	2.0	1.5	1.0	-
	15.0	-	6	200	0	0	0	1	0	0	0.5	0.5	0.0	-
	30.0	-	6	200	1	0	0	1	0	0	1.0	0.5	0.5	-
	60.0	-	6	Toxic										
CP**	12.5	-	6	200	0	1	0	0	0	0	0.5	0.5	0.0	-

*:Solvent control **: Positive control (cyclophosphamide)
ctb:chromatid break csb:chromosome break cte:chromatid exchange cse:chromosome exchange oth:others

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4.1.2.5.2 Respiratory tract

No data are available, although it can be predicted from its low chemical reactivity that nonylphenol is unlikely to be a respiratory allergen.

4.1.2.5.3 Summary of sensitisation

No human data are available. The results of several guinea pig maximisation tests suggest that nonylphenol does not have significant skin sensitising potential. No information on respiratory tract sensitisation is available, although it can be predicted from its low chemical reactivity that nonylphenol is unlikely to be a respiratory allergen.

4.1.2.6 Repeated dose toxicity

4.1.2.6.1 Animal data

There are no data for the inhalation or dermal routes. Two high-quality oral repeated dose studies in rats, of 28 and 90 days duration, have been conducted. The studies followed OECD guidelines and were in compliance with GLP. Additionally, the influence of nonylphenol on growth and cell proliferation and of the mammary gland has been investigated in the rat in a non-standard study involving subcutaneous administration.

In the 28-day study, groups of five male and five female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at nominal dose levels of 0, 25, 100 or 400 mg/kg/day (Hüls 1989). Clinical signs of toxicity, bodyweights and food consumption were recorded and towards the end of the study routine haematology, blood clinical chemistry and urinalysis examinations were made. A full necropsy was performed on all animals at termination. Adrenals, liver, kidneys and testes with epididymides were weighed and a limited range of major organs was examined microscopically.

There were no mortalities or treatment related clinical signs of toxicity. At 400 mg/kg/day, bodyweight gain was significantly reduced throughout the study, and by week four mean bodyweights were 26% and 13% less than the controls for males and females, respectively. The amount of food consumed and food utilisation was also reduced at 400 mg/kg/day for both sexes. For males only at 400 mg/kg/day there were slight differences in comparison with the controls for certain clinical chemistry parameters; urea and cholesterol levels were increased and glucose levels were reduced. Also, there were increases in the group mean relative kidney, liver and testes weights (all by about 20% compared with controls). Histopathological examination revealed hyaline droplet accumulation in the renal proximal tubules (an effect considered to be of no relevance to human health) and a minor vacuolation in the periportal hepatocytes for males at 400 mg/kg/day. Among the females at this level, there were no treatment-related changes in the organs.

For males and females at 25 and 100 mg/kg/day, there were no differences in any of the parameters examined that could be conclusively related to treatment. It should be noted that minor increases in comparison with the concurrent control group were reported for kidney, adrenal and liver weights and for the incidence of minimal hyaline droplet formation in the kidney among males at 25 and 100 mg/kg/day. However, all values were within the laboratory's historical control range (personal communication with study sponsor) and confirmatory changes were not seen for adrenal and liver weight or hyaline droplet formation in the 90-day study (see

below). Consequently these marginal changes could not be reliably attributed to nonylphenol treatment. Overall, this study identifies a NOAEL of 100 mg/kg/day for 28-day exposure.

In the 90-day study, groups of fifteen male and fifteen female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at concentrations of 0 (control), 200, 650 or 2000 ppm (Chemical Manufacturers Association 1997a, Cunny et al., 1997). Calculated nonylphenol intakes were about 0, 15, 50 and 140 mg/kg/day, respectively. Additionally, control and high dose satellite groups of ten animals of each sex were included; these were given a 28 day recovery period at the end of the 90-day exposure. Clinical signs of toxicity, bodyweights and food consumption were recorded and towards the end of the study routine haematology, blood clinical chemistry and ophthalmoscopy examinations were made. A full necropsy was performed on all animals at termination. A number of organs were weighed and histopathological examinations were conducted on a comprehensive range of organs and tissues. Also, oestrous cycles were monitored during week 8 and sperm motility, sperm number (in epididymis) and sperm morphology were evaluated at necropsy.

There were no treatment-related mortalities or clinical signs of toxicity. At 140 mg/kg/day only, there were adverse effects on bodyweight gain, the amount of food consumed and food utilisation throughout the dosing period for both males and females. At 90 days, the mean bodyweights for both sexes at this exposure level were about 7% less than the controls. In the satellite group, some recovery of bodyweight and food consumption values was seen after exposure was discontinued. Haematology and ophthalmoscopy findings and oestrous cycle patterns were not affected by treatment. There was no evidence of effects on spermatogenesis. However, one interesting clinical chemistry change was seen among females from the 140 mg/kg/day group. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were markedly elevated in two females, which correlated with some histopathological changes reported in the liver (see below).

At necropsy, no treatment-related macroscopic findings were reported. Among the males killed at 90 days, there was a dose-related increase in group mean absolute (by 6, 9 and 13%, relative to controls, at 15, 50 and 140 mg/kg/day respectively) and bodyweight-related (by 8, 11 and 24%, respectively) kidney weight. In the recovery group, the bodyweight-related kidney weight among males was also increased, although the effect was less marked. However, this organ weight increase could not be correlated with any clinical chemistry or histopathological change and consequently this finding was considered unlikely to be of toxicological significance, particularly at 15 and 50 mg/kg/day where magnitude of the change was small. Also, ovary weight was slightly decreased in females from the 140 mg/kg/day group, in comparison with the controls, at 90 days. In contrast, the weight of this organ was slightly increased in the recovery group. Again, this difference could not be correlated with any histopathological change which, together with the inconsistency between the findings for the main and satellite groups, makes the interpretation of this finding uncertain. Bodyweight-related liver weight was increased at 90 days only in males at 50 and 140 mg/kg/day and females at 140 mg/kg/day, by about 10% compared with controls. This was considered likely to be an adaptive rather than toxicological response.

The only noteworthy microscopic changes were seen in the kidneys and liver. Among males at 140 mg/kg/day in both the main and satellite groups there was a decrease in the occurrence of renal tubular hyaline droplets/globules in comparison with the control group. The biological significance of this change is uncertain. Also, a lack of correlation with the findings of the 28-day repeated dose study, in which an actual increase in the incidence of renal hyaline droplets occurred, casts doubt on whether these changes should be considered to be related to treatment. Slight or moderate individual hepatic cell necrosis was seen in three females at 140 mg/kg/day; two of the affected females also had raised serum ALT and AST. This provides evidence that the liver may be a target organ for nonylphenol toxicity, although this evidence is weak in view of the mild nature of response and small number of animals affected.

The renal histopathological findings have been reviewed by a pathologist not involved in the original investigation (Hard 1998), because of a lack of coherence between the results of this study and a multigeneration study summarised below (NTP 1997). An increased incidence of deposits of intratubular mineralisation in the P3 (straight) segment of the proximal tubule at the outer stripe of the outer medulla/inner stripe of outer medulla (OSOM/ISOM) junction was seen in males at 140 mg/kg/day; 11 out of 25 from this group were affected, compared with 1 out of 25 control males.

Overall, a NOAEL of about 50 mg/kg/day can be derived from this study. At 140 mg/kg day there were reductions in bodyweight gain, food consumption and food utilisation together with evidence of morphological changes in the liver and possibly kidneys.

Further information on repeated dose toxicity can be derived from a good-quality multigeneration study (NTP 1997, see section 4.1.2.9.2 for full details of this study, including information on any findings in reproductive organs). Groups of thirty male and thirty female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at concentrations of 0 (control) 200, 650 or 2000 ppm over three generations. Calculated nonylphenol intakes were, respectively, about 0, 15, 50 and 160 mg/kg/day during non-reproductive phases. The F_0 generation were exposed for 15 weeks, the F_1 and F_2 generations from soon after birth to about 20 weeks of age and the F3 generation from birth to about 8 weeks of age.

Evidence of general toxicity was seen in adults of all generations, although there were no treatment-related clinical signs, mortalities or adverse effects on food consumption. At 160 mg/kg/day, bodyweight gain was reduced in comparison with controls in adults across all generations, with the terminal bodyweights being about 10% lower than the controls. Similar reductions in bodyweight gain were also seen at 50 mg/kg/day in F_1 females, F_2 males and F_3 females. Relative kidney weights were increased at 50 and/or 160 mg/kg/day in adult males of the F_0, F_1 and F_2 generations and also at 160 mg/kg/day in F_1 adult females. Histopathological examination revealed an increase, although often without a convincing dose-response relationship, in the incidence of renal tubular degeneration and/or dilatation in adult males from all generations and all nonylphenol treated groups; similar findings were reported for adult females at 160 mg/kg/day in the F_1, F_2 and F_3 generations and at 15 and 50 mg/kg/day in the F_3 generation. These data are given in **Table 4.13.**

Table 4.13 Number of animals with histopathological abnormalities in the kidney (n=10)

Males

Generation	Finding	Dose level (mg/kg/day)			
		0	15	50	160
F_0	Renal tubule degeneration	1	3	5	5
	Renal tubule dilatation	0	1	0	0
F_1	Renal tubule degeneration	1	2	7	8
	Renal tubule dilatation	1	1	0	2
F_2	Renal tubule degeneration	3	6	6	6
	Renal tubule dilatation	1	2	0	4
F_3	Renal tubule degeneration	0	7	10	2
	Renal tubule dilatation	0	0	3	3

Females

Generation	Finding	Dose level (mg/kg/day)			
		0	15	50	160
F_0	Renal tubule degeneration	3	3	0	0
	Renal tubule dilatation	0	0	1	0
F_1	Renal tubule degeneration	0	1	1	6
	Renal tubule dilatation	0	0	0	3
F_2	Renal tubule degeneration	1	2	0	4
	Renal tubule dilatation	0	0	0	1
F_3	Renal tubule degeneration	0	8	9	7
	Renal tubule dilatation	0	0	1	1

It is difficult to decide for certain whether or not this increased incidence of renal tubular degeneration and/or dilatation is related to treatment because these changes were not seen to the same extent in the 90-day study, which was conducted using the same strain of rats, and because a dose-dependent trend was not apparent in all generations/sexes. The lack of concordance between the studies cannot be explained on the basis of a slightly longer exposure period in the multigeneration study because kidney effects were seen in the F_3 generation which was exposed for only 8 weeks, nor on the basis of *in utero* and neonatal exposure because the effect also occurred in the F_0 generation. Giving special emphasis to the fact that the increased incidence occurred consistently across all four generations in the multigeneration study, it is considered that this cannot be dismissed as background variation. Consequently, a conclusion has been drawn from this study that there is a LOAEL for repeated exposure of 15 mg/kg/day, based on histopathological changes in the kidneys; this value will be taken forward to the risk characterisation.

The renal histopathological findings have been reviewed by a pathologist not involved in the original investigation (Hard, 1998). The presence of renal lesions in all nonylphenol exposed groups was confirmed, as was the lack of a consistent dose-dependent trend in all generations. The

predominant renal lesions were described as tubular mineralisation at the OSOM/ISOM junction, cystic tubules surrounded by fibrosis, or granular cast formation at the OSOM/ISOM junction.

A briefly reported oral (gavage) study investigating the testicular toxicity of nonylphenol (de Jager et al., 1999a) is summarised in the toxicity to reproduction section. In this study, mortality was observed at 100 (the lowest dose level tested), 250 and 400 mg/kg/day; 3, 15 and 18, respectively, out of 20 animals in each group died during a 10-week dosing period. No further information on these mortalities is available. The presence of mortality at such dose levels contrasts with the findings of the dietary administration studies (Hüls, 1989; Chemical Manufacturers Association, 1997a; NTP, 1997). The differences can probably be accounted for by the method of administration; gavage dosing is likely to produce higher peak concentrations of nonylphenol in the blood than dietary administration.

The influence of nonylphenol on growth and cell proliferation and of the mammary gland has been investigated in rats of the Nobel strain in two studies using non-standard methods. The Nobel strain of rat is particularly sensitive to oestrogenic activity. In the original study, groups of six female juvenile rats were exposed to nonylphenol by the subcutaneous route for 11 days, administered via osmotic minipumps implanted in the dorsal cervical region (Colerangle and Roy, 1996). The dose levels were 0 (DMSO vehicle control), 0.01 and 7.12 mg/day (0.05 and 35.6 mg/kg/day, assuming a bodyweight of 200 g). An additional group received diethylstilbestrol (DES) at 0.01 mg/day (0.05 mg/kg/day) for 11 days by an unspecified route. At the end of the exposure period the rats were killed and the abdominal mammary glands removed for evaluation. Mammary gland growth was assessed by counting the number of mammary structures (terminal ducts, terminal end buds or lobules) and cells in 16 mm^2 areas of the mammary gland. Cell proliferation and cell-cycle kinetics were evaluated using immunohistochemical techniques (reaction with antiproliferating cell nuclear antigen (PCNA)) which allowed cells in S, G1 and G0 phases to be identified. The labelling index (LI, proportion of cells in S phase) growth fraction (GF, proportion of cells in the G1 or S phase) were calculated.

In the group receiving the highest dose of nonylphenol there was a 1.5-fold increase in the number of mammary structures and a 4-fold increase in the number cells/16 mm^2 area, compared with the vehicle control group. At the lowest level the number of structures was similar to the controls, but there was a 2-fold increase in the number of cells. DES caused a 6-fold increase in the number of cells. The LI was increased by 1.3 and 1.8 fold and GF by 1.2 and 2 fold in the nonylphenol low and high dose groups, respectively, in comparison with the vehicle control. DES had a much greater influence on the indices, with increases of 4 and 5 fold for the LI and GF. Cell cycle time was unchanged in the low dose group, slightly decreased (by about 10%) in the high dose group and markedly deceased (by more than half) in the DES group. This study shows that nonylphenol at dose levels of 0.05 and 35.6 mg/kg/day increases growth and proliferation activity in a dose-related manner in the mammary gland of the Nobel rat, although the effects at 0.05 mg/kg/day are marginal. The significance for human health of such a finding is unknown. Furthermore, the use of the subcutaneous route of administration and selection of the oestrogen-sensitive Nobel rat as the model casts doubts about the relevance of these findings to humans. Ashby and Odum (1998) draw attention to the fact that same positive control (DES) data reported for this study also appear in two other reports by Colerangle and Roy (1995 and 1997), and that the vehicle control data of the nonylphenol study is duplicated in the 1997 study. This raises some uncertainties as whether the control data were generated concurrently with the nonylphenol data and questions the validity of this study.

The Colerangle and Roy (1996) study was duplicated by Odum et al. (1999). Groups of ten female OVR^+ Noble rats were exposed to nonylphenol at dose levels of 0 (DMSO vehicle control), 0.073 or 53.2 mg/kg/day or DES at 0.076 mg/kg/day by the subcutaneous route for 11 days, administered via osmotic minipumps implanted in the dorsal cervical region. Mammary gland differentiation and mammary gland cell proliferation were assessed following similar methodology to Colerangle and Roy (1996), except that BRDU as well as PNCA staining was used (BRDU incorporation was considered to be a more sensitive and robust technique) and a more objective method was used to quantitate mammary gland changes. The quantitative determination of the numbers and areas of mammary gland structures showed no differences between the vehicle control and nonylphenol exposed groups, in contrast to the findings of the original study. DES, however, had a marked influence of the differentiation of mammary structures. Terminal ducts were completely absent and terminal end buds were present only in peripheral regions. Also, the numbers and areas of lobules were markedly increased in peripheral and central areas. The mammary gland cell proliferation assessment revealed, in comparison with the vehicle control group, no changes in the nonylphenol exposed groups, and a marked increase (about 4 fold in the lobules) in the DES group. This study shows the DES can induce growth and proliferation activity in the mammary gland of the Nobel rat, but failed to confirm the observation in the Colerangle and Roy (1996) study of such activity following nonylphenol exposure at similar dose levels.

4.1.2.6.2 Human data

The effects of nonylphenol exposure have not been evaluated in humans. There are two isolated case reports of leucoderma on the hands and forearms, with subsequent spreading to other areas, among Japanese workers exposed to alkaline detergents containing polyethylene alkylphenylether (Ikeda et al., 1970). The authors speculated that this might be caused by free octylphenol or nonylphenol, which were found to be present in the detergents. However, in the absence of corroborative reports from elsewhere, no firm conclusions regarding causality can be made.

4.1.2.6.3 Summary of repeated dose toxicity

No useful human data are available. In a multigeneration study in the rat involving oral exposure via the diet for up to 20 weeks, a LOAEL for repeated dose toxicity of 15 mg/kg/day was identified, based on histopathological changes in the kidneys (tubular degeneration or dilatation), although such changes were not apparent at this dose level in a 90-day dietary exposure rat study. At higher dose levels the liver may also be a target organ; minor histopathological changes in the liver (vacuolation in the periportal hepatocytes or occasional individual cell necrosis) were seen at doses of 140 mg/kg/day and above in some dietary studies. The oral toxicity of nonylphenol appears to be enhanced when dosed by gavage, with mortalities being reported at dose levels of 100 mg/kg/day and above. No studies involving dermal or inhalation exposure have been conducted. Nonylphenol has been reported to induce cell proliferation in the mammary gland of the Nobel rat following subcutaneous exposure at levels down to 0.05 mg/kg/day, but this finding could not be reproduced in a duplicate study; furthermore, there are doubts about the relevance of this finding to humans and regarding the validity of the original study.

4.1.2.7 Mutagenicity

Only data from *in vitro* test systems and animals are available.

4.1.2.7.1 Studies in vitro

Two pre-incubation bacterial mutagenicity (Ames) tests have been conducted. Both were negative. The first cannot be fully appraised because only a summary report is available (Hüls 1984). *Salmonella typhimurium* strains TA1537, TA 1538, TA 98 and TA 100 were exposed to nonylphenol at concentrations to 5000 µg/plate, both in the presence and absence of metabolic activation (Aroclor induced rat liver S9). The same *Salmonella* strains were used in the second study, together with *Escherichia coli* strain WP2urvA (Shimizu et al., 1985). Concentrations up to 100 µg/plate were tested, both in the presence and absence of metabolic activation (polychlorinated biphenyl induced rat liver S9), and toxicity was reported at the highest concentrations tested. A limitation of both studies is that the results of neither appeared to have been confirmed by a second independent experiment.

In a well-conducted *in vitro* mammalian cell gene mutation test, following OECD guideline 476 and in compliance with GLP, the potential for nonylphenol to induce mutations at the HPRT-locus was investigated in Chinese hamster V79 cells (Hüls, 1990). The exposure period was 5 hours, and a range of concentrations up to 2.5 µg/ml (without metabolic activation) or 1.25 µg/ml (with metabolic activation) were tested. At higher concentrations there was no cell survival. The results were confirmed by independent experiment. The test was negative.

4.1.2.7.2 Studies in vivo

Two micronucleus studies are available.

In the most recent study, conducted according to OECD guideline 474, groups of 5 male and 5 female NMRI strain mice received a single intraperitoneal dose of 50, 150 or 300 mg/kg (Hüls, 1999b). Appropriate positive (cyclophosphamide) and negative (vehicle) control groups were included. The highest treatment level was chosen as the maximum tolerated dose, based on the results of a preliminary study. Bone marrow was sampled 24 hours after treatment. There was a second sampling time of 48 hours for additional groups receiving either nonylphenol at 300 mg/kg or only the vehicle. Toxicity was elicited at 150 and 300 mg/kg, seen as clinical signs such as sedation, squatting posture, abnormal gait and piloerection. There was no consistent effect on the polychromatic to normochromatic erythrocyte (PCE/NCE) ratio. No increases in the frequency of micronucleated PCEs were seen in the nonylphenol exposed groups; thus the tests is considered to be negative. The anticipated response was seen in the positive control group. Although the PCE/NCE ratio was not affected, the fact that the study was conducted at the maximum tolerated dose and using the intraperitoneal route of administration, it can be presumed that exposure of the bone marrow to nonyl phenol was maximised. Accordingly, a high level of confidence can be given to this negative result.

An earlier micronucleus test was conducted using the oral route of administration (Hüls, 1988). In accordance with the OECD guideline, groups of five male and five female mice of the NMRI strain received a single oral dose of nonylphenol at 500 mg/kg. The dose level was chosen as the maximum tolerated dose. No evidence was presented to support this choice, but it is noted that it is greater that a reported oral LD_{50} of 307 mg/kg/day for mice. Appropriate positive and negative controls were included. Bone marrow was sampled at 18, 48 and 72 hours. There were no increases in the frequency of micronuclei at any of the sampling times and the test was declared negative. The PCE/NCE ratio was not affected by nonylphenol, which raises concerns about adequacy of exposure of the bone marrow to the test substance. The toxicokinetic information suggests that the systemic bioavailability of nonylphenol following oral administration is

restricted, which adds to this concern. Overall, because of doubts regarding the extent of exposure of the target tissue, only limited significance can be given to this negative result.

4.1.2.7.3 Summary of mutagenicity

No human data are available. Nonylphenol tested negative in two bacterial assays and an *in vitro* mammalian cell gene mutation assay. An *in vivo* micronucleus test, conducted using the intraperitoneal route, was negative. A second in vivo micronucleus test, which used the oral route, was also negative, although there were methodological weaknesses in this study. These results show that nonylphenol is not mutagenic.

4.1.2.8 Carcinogenicity

Carcinogenicity has not been studied directly in humans or animals. However, some information on the carcinogenic potential can be derived from other data. On the basis of the information currently available it is considered unlikely that that nonylphenol is mutagenic, so concerns for cancer caused by a genotoxic mechanism are low. Considering the potential for carcinogenicity by a non-genotoxic mechanism, no evidence of sustained cell proliferation or hyperplasia was seen in the standard repeated exposure toxicity studies. Nonylphenol has been reported to induce cell proliferation in the mammary gland of the Nobel rat following subcutaneous exposure at levels down to 0.05 mg/kg/day, but this finding could not be reproduced in a duplicated study; furthermore there are doubts about the relevance of this model to humans because of the route of exposure and sensitivity of the strain selected. Overall, there are low concerns for carcinogenicity by a non-genotoxic mechanism.

4.1.2.9 Toxicity to reproduction

Only data from animals or *in vitro* test systems are available.

4.1.2.9.1 Studies investigating oestrogenic activity

The oestrogenic activity of nonylphenol has been investigated in a number of studies using either recombinant yeast, oestrogen sensitive MCF-7 cells or a rodent uterotrophic assay response. None of these assays have been validated as an internationally accepted toxicity test method, although the MCF-7 and uterotrophic assays have been established for a number of years as standard assays for oestrogenic activity. It should be noted that the significance to human health of oestrogenic activity detected in these assays has yet to be established.

<u>In vitro systems</u>

4-Nonylphenol was one of a number of alkyl phenols tested in a yeast assay in a study which looked at the structural features important for oestrogenic activity in this chemical group (Routledge and Sumpter, 1997). The assay uses a recombinant strain of yeast (*Saccharomyces cerevisiae*) which contains an oestrogen-inducible expression system. In the presence of oestrogens a reporter gene (Lac-Z) encoding for the enzyme ß-galactosidase is expressed, which can be monitored by measuring a colour change reaction in the culture medium. The oestrogenic activity of the test substances was expressed as a potency relative to 17ß-oestradiol by comparing the molar concentrations required to produce the same response. 17ß-oestradiol was found to be about 30 000 times more potent than nonylphenol. Tamoxifen, an oestrogen antagonist known to act via the oestrogen receptor, was shown to inhibit the activity of the alkyl

phenols, demonstrating that the assay response was due to interaction with the oestrogen receptor.

The oestrogenic activity of nonylphenol has also been assessed in an *in vitro* assay involving oestrogen sensitive human breast tumour MCF-7 cells (Soto et al., 1991). The cells are cultured in the presence of charcoal-stripped (to remove endogenous oestrogens) human serum which inhibits cell proliferation. Substances with oestrogenic activity can then overcome this inhibition. The MCF-7 cells were cultured 17ß-oestradiol and nonylphenol at several concentrations were each cultured in triplicate in multiwell plates and cell proliferation was assessed after a six-day exposure period by counting nuclei from lysed cells. Nonylphenol at a concentration of 10 µM elicited a similar proliferative response to oestradiol at a concentration of 30 pM; thus, on a molar basis the oestrogenic potency of oestradiol, as measured in this assay, is 3 000 000 times greater than that of nonylphenol. At concentrations of 1 and 0.1 µM the proliferative response produced by nonylphenol was similar to that observed in negative control cultures.

In another similar *in vitro* assay, MCF-7 and ZR-75 human breast cancer cell lines were used (White et al., 1994). Cells were cultured in quadruplicate in the presence of nonylphenol at concentrations ranging from 0.1 nM to 10 µm or 17ß-oestradiol at 10 nM. No oestrogenic activity was detected at nonylphenol concentration of 100 nM and less. At 1 and 10 µM nonylphenol elicited a proliferative response which at the higher concentration was similar to that produced by oestradiol. Thus, 17ß-oestradiol was 1000 times more potent than nonylphenol in this assay. In a further investigation, the ability of nonylphenol to stimulate transcriptional activity was determined in MCF-7 and chicken cell fibroblasts (CEFs) transfected with reporter gene pEREBLCAT and a mouse oestrogen receptor. Nonylphenol stimulated transcription at culture concentrations of 1 and 10 µM.

To summarise the *in vitro* oestrogenic data, there is evidence that nonylphenol has oestrogenic activity, of 3-6 orders of magnitude less potent than oestradiol.

In vivo systems

The oestrogenic activity of nonylphenol has been assessed in several studies using an assay based upon the uterotrophic response in the rat.

In the first study, five groups of immature (aged 20 - 22 days) female rats (six in each group) of a Wistar derived strain received single oral gavage doses of nonylphenol in corn oil on each of three consecutive days (ICI, 1996). The dose levels ranged from 9.5 to 285 mg/kg/day. Vehicle and positive (oestradiol benzoate 8 µg/kg, by subcutaneous route) groups were included. One day after the final dose the females were killed and the uterus was removed from each animal and weighed. Absolute uterus weight and bodyweight related uterus weight were statistically significantly increased, in a dose-dependent manner, at levels of 47.5 mg/kg/day and above. The NOAEL was 9.5 mg/kg/day. The uterine response seen in the positive control group was much greater than that of the nonylphenol groups, although a direct comparison of potency is not possible given the differing exposure routes. Similar data from the same laboratory have also been presented in peer-review literature (Odum et al., 1997). This latter report also included oral positive control groups (17ß-oestradiol, 10-400 µg/kg), which indicated that oestradiol was about 1000 times more potent in this assay than nonylphenol.

In a similar assay, groups of ten ovariectomised female Sprague-Dawley rats were dosed once daily for three consecutive days by the oral route with ethanol/oil suspensions of nonylphenol at

levels of 0 (vehicle control), 30, 100 and 300 mg/kg/day (Chemical Manufacturers Association 1997b). Positive control groups received ethynyloestradiol in ethanol at levels of 10, 30 and 80 µg/kg/day according to the same dosing regimen. One day after the final dose the females were killed and the uterus was removed from each animal and weighed. Uterus weights at 300 mg/kg/day were significantly increased (1.5-fold) in comparison with the vehicle control group. A slightly greater response (a 2-fold increase) was seen in the 30 and 80 µg/kg/day positive control groups.

In another uterotrophic assay, groups of three immature (aged 20 - 21 days) Sprague-Dawley rats each received a single intraperitoneal injection of nonylphenol at dose levels of 0, 1, 2 or 4 mg/animal (approximately 25, 50 or 100 mg/kg) (Lee and Lee, 1996). Oestradiol, administered by the same route, served as a positive control. The animals were killed 24 hours later and each uterus was removed, weighed and analysed for protein and DNA content and peroxidase (thought to be a uterotrophic marker enzyme) activity. There was a dose-dependent and statistically significantly increase in uterine weight at all levels, with associated increases in uterine protein and DNA content and uterine peroxidase activity. In further experiments, the uterotrophic activity of nonylphenol was found to be blocked by the co-administration ICI 182,780, an oestrogen antagonist, providing evidence that the effect of nonylphenol is mediated through the oestrogen receptor. Also, the potency was compared with oestradiol; in this assay oestradiol was found to be about 1000 - 2000 times more potent than nonylphenol.

Overall, these *in vitro* and *in vivo* studies show that nonylphenol has oestrogenic activity of a potency that is between 3 to 6 orders of magnitude less than that of oestradiol.

4.1.2.9.2 Effects on fertility

The effects of nonylphenol on fertility and reproductive performance have been investigated in a multigeneration study, and additionally, the testicular toxicity of nonylphenol has been studied in a repeated exposure study.

The multigeneration study was comprehensive, of good quality, and was conducted in compliance with GLP (NTP 1997). The overall study design was based on the OECD two-generation reproduction toxicity study guideline, with an extension to include the production of an F_3 generation. Groups of thirty male and thirty female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at concentrations of 0 (control) 200, 650 or 2000 ppm over three generations. Calculated nonylphenol intakes were, respectively, about 0, 15, 50 and 160 mg/kg/day during non-reproductive phases and rising to around 0, 30, 100 and 300 mg/kg/day during lactation.

Nonylphenol exposure commenced for the F_0 generation at about 7 weeks of age and continued until study termination when the F_3 generation were about 8 weeks old. F_0 animals were mated (one male with one female) within each dose group to produce the F_1 generation, selected F_1 animals were similarly mated to produce the F_2 generation and selected F_2 animals were mated to produce the F_3 generation. For the F_0 generation and retained F_1, F_2 and F_3 animals, clinical signs of toxicity, bodyweights and food consumption were reported. Oestrous cycles were monitored prior to mating. At the necropsy of adult animals, sperm samples were taken (but not from the F_3 generation) for analysis of density, motility (using a computer assisted sperm motion analysis system, only conducted on control and high dose group males) and morphology, a number of organs were weighed and selected organs were sampled for histopathology. Additionally, testicular spermatid counts were made. Parameters assessed in the young offspring included litter

size, bodyweights, survival, gross appearance, ano-genital distance, sexual development and, for animals killed at weaning, gross appearance of organs at necropsy and reproductive organ weights.

There was evidence of general toxicity in adults of all generations, seen as a reduction in bodyweight gain at 50 and 160 mg/kg/day and histopathological changes in the kidneys at all dose levels. These aspects are described in greater detail in section 4.1.2.6.1.

Considering the reproduction-related parameters, there were no adverse effects on fertility or mating performance. However, several other parameters were affected. Oestrous cycle length was increased by about 15% in the F_1 and F_2 females at 160 mg/kg/day, in comparison with controls. The timing of vaginal opening was accelerated by 1.5-7 days at 50 mg/kg/day and by 3-6 days at 160 mg/kg/day in females of the F_1, F_2 and F_3 generations. Also, absolute ovarian weights were decreased at 50 mg/kg/day in the F_2 generation and at 160 mg/kg/day in the F_1, F_2 and F_3 generations; however, no effect on ovarian weight was apparent in the F_1 and F_3 generations when analysed as an organ-to-bodyweight ratio. In males, changes in sperm endpoints were seen only in the F_2 generation; epididymal sperm density was decreased by about 10% at 50 and 160 mg/kg/day and spermatid count was decreased by a similar amount at 160 mg/kg/day. However, there may have been methodological problems with the epididymal sperm density measurements, because the density in all F_2 generation groups, including controls, was considerably greater (by about 25-40%) than reported for the F_0 and F_1 generation males; the age of each generation was similar at necropsy, so major differences in the sperm density would not be expected.

To summarise the reproductive aspects of this study, fertility and mating performance were not adversely affected by nonylphenol treatment. However, there were changes, albeit relatively slight, in the oestrous cycle length, timing of vaginal opening, ovarian weight and sperm/spermatid count. The effects on the oestrous cycle were seen in both the F_1 and F_2 generations (not assessed in F_3 females) and the timing of vaginal opening was influenced in all three generations; this consistency provides firm evidence of a relationship with treatment. These effects were possibly related to the oestrogenicity of nonylphenol. There is some uncertainty about the relationship to nonylphenol treatment with respect to the ovarian weight reduction because this effect was apparent after adjusting for bodyweight in only one generation and did not correlate with any histopathological changes; nevertheless, it is compatible with the anticipated direct effects of exogenous oestrogenic activity. Also, there is uncertainty regarding the cause of the apparent reduced sperm/spermatid numbers in the F_2 generation. It has been hypothesised that such changes could result from foetal or neonatal exposure to exogenous oestrogenic activity (Sharpe and Skakkebaek, 1993), but if the hypothesised mechanism were operating, semen/testicular changes should also have occurred in the F_1 generation. Furthermore, the possibility of methodological problems adds to the difficulty in interpreting the sperm/spermatid count data. However, the observation of impaired male reproductive tract development in an intraperitoneal study summarised in section 4.1.2.9.3 provides supporting evidence in favour of the sperm/spermatid count changes being causally related to nonylphenol treatment. Furthermore, the intraperitoneal study indicates that a critical window of exposure for this effect is likely to be the neonatal period. Overall, this study provided evidence that nonylphenol exposure over several generations can cause minor perturbations in the reproductive system of offspring, which are compatible with the predictable or hypothesised effects of exogenous oestrogenic activity, although these perturbations do not cause functional changes in reproduction of the rat at the dose levels tested. A clear NOAEL for these changes of 15 mg/kg/day was identified.

The testicular toxicity of nonylphenol was investigated in Sprague Dawley rats in a briefly reported repeated dose study (de Jager et al., 1999a). Groups of 20 male rats were dosed once daily by the oral (gavage) route at doses levels of 0 (vehicle control, cotton seed oil), 100, 250 or 400 mg/kg/day for a period of 10 weeks, from the age of 12 weeks. The animals were killed at the end of the dosing period and a detailed evaluation of the reproductive organs was conducted. Testes and epididymal weight were recorded. The total cauda epididymal sperm numbers were determined. The testes were stored in Bouin's fixative and processed for histological examination, which included the identification of the stages of spermatogenesis present and the measurement of the seminiferous tubule diameter, lumen diameter and epithelial thickness.

Three, 15 and 18 animals from the 100, 250 and 400 mg/kg/day groups, respectively, died during the dosing period; no further information on these deaths was presented. Clinical signs of toxicity were not reported. The bodyweight gain of surviving animals was not affected by treatment, although bodyweight gain was reduced among the decedents. In comparison with the control group, lower testicular and epididymal weight, tubule and lumen diameter and seminiferous epithelial diameter were seen in surviving animals at 250 and 400 mg/kg/day and the sperm count was reduced at 400 mg/kg/day, but because of the very small groups sizes due to mortality, little toxicological significance can be accorded to these findings. At 100 mg/kg/day, testes and epididymal weight were not affected, but tubule and lumen diameter and seminiferous epithelial diameter were statistically significantly lower than found in the control group; the mean tubule diameter was reduced by 10%, but data for the other two parameters were not presented. Testicular abnormalities were identified by histopathology at both 250 and 400 mg/kg/day. In one animal at 250 mg/kg/day vacuolization and cell necrosis with sloughing of the epithelium was seen in about 40% of tubules. Both surviving animals at 400 mg/kg/day had tubular vacuolization, cell necrosis and derangement, with very few secondary spermatocytes and sperm being present.

This study provides evidence of nonylphenol-related testicular toxicity at exposure levels which also cause mortality. A LOAEL for testicular toxicity of 100 mg/kg/day can be designated. The observation of mortality at 100, 250 and 400 mg/kg/day in this gavage study contrasts with the findings of studies involving dietary administration summarised in the Repeated Dose Toxicity section (Hüls, 1989; Chemical Manufacturers Association, 1997a; NTP, 1997). This difference can probably be accounted for by the method of administration; gavage dosing is likely to produce higher peak concentrations of nonylphenol in the blood than dietary administration.

4.1.2.9.3 Developmental toxicity

A good quality standard oral rat developmental toxicity study and two studies, one using the intraperitoneal route and one using the oral route, looking specifically at the potential effects on the developing male reproductive tract are available.

The standard rat developmental toxicity study is well-reported, conducted according to OECD guideline 414 and in compliance with GLP (Initiative Umweltrelevante Altstoffe, 1992). Groups of timed-mated females of the Wistar strain were administered by oral gavage corn oil solutions of nonylphenol from days 6 to 15 of pregnancy at dose levels of 0, 75, 150 and 300 mg/kg/day. A further group receiving 600 mg/kg/day was terminated prematurely because many females died during the first few days of treatment. Sufficient females were allocated to the study to produce at least 21 pregnant females in each group. Surviving females were killed on day 20 of pregnancy and the foetuses were subjected to routine external, visceral and skeletal examinations.

There was clear evidence of maternal toxicity at 300 mg/kg/day, manifested as a reduction in bodyweight gain and food consumption, mortality of two females and the macroscopic organ changes in the kidney (pale or irregular shape in seven mothers) or spleen (reduced size in two mothers). Similar macroscopic changes were seen occasionally at 150 mg/kg/day and at a high incidence in females from the prematurely terminated 600 mg/kg/day group. No maternal toxicity was seen at 75 mg/kg/day. Post-implantation loss, litter size, foetal weights and incidence of both major and minor foetal abnormalities were not affected by treatment. To conclude, this study provides no evidence of developmental toxicity in the rat at exposure levels which are toxic to the mother; thus the maternal NOAEL was 75 mg/kg/day and the foetal NOAEL was 300 mg/kg/day.

In the intraperitoneal study, which was briefly reported, the effects of nonylphenol on male reproductive tract development were investigated in neonatal Sprague-Dawley rats (Lee, 1998; additional information was obtained by personal communication with the author). Age-matched male pups were randomly allocated to either the control or treated groups. Daily doses of nonylphenol were administered by the intraperitoneal route at a dose volume of 5-10 µg/injection, for varying schedules between the day of birth (day 0) and 30 days of age. Control animals received the vehicle (dimethylsulfoxide) only, by the same route. The pups were killed at 31 days of age; terminal observations included external appearance of genital area, ano-genital distance, the presence of undescended testes, and reproductive organ weights (which were reported as bodyweight-related values).

In the initial experiment, groups of at least three pups were dosed at 0, 0.08, 0.8 and 8 mg/kg/day, from birth to 15 days of age. At 0.8 and 8 mg/kg/day there was a statistically significant, dose-dependent, reduction in testes, epididymis, seminal vesicle and prostate weight; typically weights were about 15 to 25% less than found in the control group. Additionally, ano-genital distance was reduced at 8 mg/kg/day, only. Reproductive organ weights were not affected at 0.08 mg/kg/day. Next, groups of three or four pups received nonylphenol at 0 or 8 mg/kg/day, either from days 1 to 18 of age, days 6 to 24 or days 13 to 30, to see if there is a vulnerable phase of development. Reproductive organ weights were significantly reduced in the groups for which dosing commenced on day 1 or 6, but not in the group dosed from day 13. In a third experiment, the influence of the oestrogen receptor antagonist ICI 182,780 on nonylphenol-impaired reproductive organ weight development was investigated in groups of six or seven pups dosed with nonylphenol at 8 mg/kg/day from days 1 to 5 of age. The antagonist was administered by the intraperitoneal route at a dose of 0.5 mg/kg and dose volume of 5-10 µg/injection, 10 minutes after the nonylphenol dose. It was found that ICI 182,780 blocked the effects of nonylphenol on organ weights. Administration of ICI 182,780 alone had no effect on reproductive organ weight. The incidence of undescended testes was reported in groups of between 6 and 34 pups dosed with nonylphenol at 8 mg/kg/day, days 1 to 5, days 1 to 10 or days 1 to 18; this was 33%, 55% and 62%, respectively. Undescended testes were not observed in vehicle control pups, in pups receiving a single dose of nonylphenol on day 1, or when ICI 182,780 was administered concurrently with nonylphenol.

In a final experiment, eight male pups, selected from two litters, were dosed by the intraperitoneal route from days 1 to 15 of age with nonylphenol at 8 mg/kg/day and then reared to sexual maturity. Their fertility was assessed by serial pairing with either six or twenty untreated female rats and recording the number of females which became pregnant. Vehicle control male pups, selected from the same two litters, were used for comparison. Among the controls, pregnancies resulted from almost all pairings. In contrast, in the nonylphenol treated group, two males were completely infertile, failing to impregnate any females; three were

initially fertile, but failed to impregnate females in later pairings; two showed comparable fertility to the controls; the remaining male died near the start of the fertility trial. Necropsy findings were reported for five of the nonylphenol-treated males; all were observed with undescended testes and/or either slight or marked testicular atrophy.

There are a number of design weaknesses to this study: the group sizes were generally very small; the pups were apparently not weight-matched at the start of treatment; and the intraperitoneal route of administration, which could result in unrealistically high exposure of the reproductive organs, is of questionable relevance to the human risk assessment involving the inhalation, dermal and oral routes. Nevertheless, the consistent observation throughout the series of experiments of reduced reproductive organ weight or undescended testes, supported by observations of reduced ano-genital distance and, in animals reared to sexual maturity, reduced fertility, provide evidence that nonylphenol exposure during the neonatal period impairs male reproductive tract development in the rat. The period of maximum vulnerability to this effect appears to be prior to the age of 13 days. The blocking influence of the oestrogen receptor antagonist ICI 182,780 suggests that the effect of nonylphenol on the male reproductive tract may be mediated through action on the oestrogen receptor. However, in view of corrosive properties of nonylphenol and use of the intraperitoneal route of administration, it is possible that non-specific irritation of the undescended testes may have contributed to the observed effects. The author has stated that about 50% of the nonylphenol treated pups had peritoneal cavity adhesions, while none were seen in control animals, which supports this hypothesis. Although adhesions were seen, there were no treatment-related clinical signs of toxicity or increased mortality. The blocking influence of ICI 182,780 may possibly have resulted from dilution of the injected nonylphenol (this alternative explanation was not tested as the study did not include a control group receiving nonylphenol followed by a vehicle only injection). It should be noted that precise information on clinical signs, mortality and general macroscopic necropsy findings were not available from the author. No effects were seen in pups dosed at 0.08 mg/kg/day but, because of the very small numbers of animals receiving doses other than 8 mg/kg/day, information on the NOAEL and dose-response relationship can be gained from this study. Overall, because of the design weaknesses and the possibility that non-specific irritation may have contributed to the observed effects on the male reproductive tract, it is not possible to draw any firm conclusions from this study with respect to specific reproductive toxicity of relevance to humans. Consequently, this study carries little weight in the overall assessment of the available reproductive toxicity data base.

In the third study, which was briefly reported, the effects of nonylphenol exposure from the *in utero* period to sexual maturity of nonylphenol exposure were investigated in an oral (gavage) study (de Jager et al., 1999b). Groups of 10 mated females were dosed once daily with nonylphenol at levels of 0 (vehicle control, cotton seed oil), 100, 250 and 400 mg/kg/day from day 7 of pregnancy to weaning of their litters. Twenty F_1 generation males were randomly selected from each group for dosing as for the mother until 10 weeks of age. The selected F_1 males were then killed. Testes and epididymal weight were recorded. The total cauda epididymal sperm numbers were determined. The testes were stored in Bouin's fixative and processed for histological examination, which included the identification of the stages of spermatogenesis present and the measurement of the seminiferous tubule diameter, lumen diameter and epithelial thickness.

Concerning maternal toxicity, no information was presented on maternal bodyweights, but it was stated that no females showed any physical or behavioural abnormalities. No offspring were born from the mothers receiving 400 mg/kg/day; it is not clear from the report if this was because of maternal deaths or embryonic/foetal resorption.

There were no malformations or still births among the F_1 offspring. No physical or behavioural abnormalities were seen among the selected F_1 males, although possibly two animals at 250 mg/kg/day died since the group size at termination of the study was reduced to 18; this contrasts with the de Jager (1999a) study conducted in adult males in which 15 out of 20 animals died at 250 mg/kg/day (see section on Effects on Fertility). F_1 bodyweight gain over the course of the study was significantly reduced at both 100 and 250 mg/kg/day (by 11 and 20%, respectively), relative to the control group. F_1 absolute testicular and epididymal weights were less than the controls at both 100 and 250 mg/kg/day, but this effect was not evident when organ weights were expressed relative to bodyweight; the differences in absolute organ weight are thought likely to be related to the intergroup bodyweight differences. Total cauda epididymal sperm count was reduced at 250 mg/kg/day (by 36%, relative to controls), but at 100 mg/kg/day sperm counts were similar to those of the control group. Seminiferous tubule diameter was slightly lower in both nonylphenol treated groups (by about 10%); surprisingly, these slight differences were declared to be highly statistically significantly different from the control group. The authors also stated that the tubule lumen diameter and seminiferous epithelium thickness were highly statistically significantly less than the control group in both nonylphenol groups, but the data were not presented. Although these quantitative tubular changes were consistent with those of the de Jager (1999a) study, in the present study these may be related to the fact that testicular weight was lower in these groups. Histopathology revealed pathological changes in the testes of one F_1 male from the 100 mg/kg/day group; in the tubules, cell necrosis, vacuolation and sloughing of the germinal epithelium were described. However, no such histopathological abnormalities were seen at 250 mg/kg/day, so the changes outlined above cannot be attributed to nonylphenol treatment.

This study provides evidence of a reduction in sperm count at 250 mg/kg/day, a dose level which may have caused mortality, although it is not possible to state whether this is a developmental effect or as a result of direct exposure to the males after weaning. It is not clear if the changes in the tubular measurements represent specific reproductive toxicity or non-specific secondary consequences of the reduction in bodyweight gain.

4.1.2.9.4 Summary of toxicity to reproduction

No human data are available. Nonylphenol has been shown to have oestrogenic activity in a number of *in vitro* and *in vivo* assays. The potency of this oestrogenic activity in these assays ranged from 3 to 6 orders of magnitude less than that of oestradiol. The effects of nonylphenol on fertility and reproductive performance have been investigated in a good quality oral (dietary administration) multigeneration study in the rat. This study provided evidence that nonylphenol exposure over several generations can cause minor perturbations in the reproductive system of offspring, namely slight changes in the oestrous cycle length, the timing of vaginal opening and possibly also in ovarian weight and sperm/spermatid count, although functional changes in reproduction were not induced at the dose levels tested. The NOAEL for these changes was 15 mg/kg/day. The observed perturbations in offspring are compatible with the predictable or hypothesised effects of exogenous oestrogenic activity. Evidence of testicular toxicity, seen as seminiferous tubule vacuolation, cell necrosis and a reduction in tubule diameter, was reported at exposure levels which also cause mortality in a repeated dose gavage study in rats. The LOAEL for testicular toxicity was 100 mg/kg/day. The toxicity of nonylphenol appears to be enhanced by gavage administration in comparison to dietary administration, presumably because higher peak blood concentrations of nonylphenol are achieved by gavage.

No evidence that nonylphenol is a developmental toxicant was seen in a standard oral developmental toxicity study in the rat; maternal and foetal NOAELs were 75 and

300 mg/kg/day, respectively. In contrast, in a gavage study involving *in utero*, lactational and direct post-weaning exposure, there was a reduction in sperm count at 250 mg/kg/day, although it is not possible to state whether this is a developmental effect or as a result of direct exposure after weaning. In an intraperitoneal study designed to investigate the effects of nonylphenol on male reproductive tract development of neonatal rats, evidence of impaired development was observed. However, this study was difficult to interpret, such that these results carry little weight in the overall assessment of the available data.

Overall, the observations of oestrogenic activity in the *in vitro* and *in vivo* assays, minor perturbations in the reproductive system of offspring in the multigeneration study, and testicular changes in gavage studies collectively raise concerns for reproductive toxicity, possibly mediated through action on the oestrogen receptor. These concerns for reproductive toxicity are addressed in the risk characterisation, although there are uncertainties. The oestrogenic activity assays are merely screening tests. The effects on reproduction-related parameters in the multigeneration study were marginal and there was no evidence of functional changes in reproduction; furthermore any changes that were seen occurred at exposure levels in excess of the LOAEL for repeated dose toxicity (LOAEL for renal toxicity is 15 mg/kg/day, NOAEL for reproductive changes is 15 mg/kg/day). Evidence of testicular toxicity was reported in two repeated exposure studies designed specifically to investigate the effects on this organ, but only at doses which also caused mortality. No evidence of testicular toxicity was seen in standard repeated dose studies involving dietary administration. Development was not affected in a standard rat oral developmental toxicity study.

4.1.3 Risk characterisation

The risk characterisation below is divided into two parts. The first provides an overview of the toxicological assessment, pointing out the effects of nonylphenol (and the concentrations at which they occur) and making clear where there are critical gaps in the data. The second part contrasts the effects data with measured and modelled exposures. From the effects and exposure information available it is clear that not all of the possible effects will be expressed. The risk characterisation therefore concentrates on the key effects and the circumstances under which they are likely to occur.

4.1.3.1 General aspects

Few significant human data are available so this assessment of the hazardous properties of nonylphenol is based mainly on animal data.

Most of the information on the toxicokinetics of nonylphenol concerns oral exposure and is based on a small number of limited rat and human studies, supported by a read across from data relating to octylphenol, an alkyl phenol with a close structural relationship to nonylphenol. The available data, though sparse, do provide the basis for a general understanding of the main features of the toxicokinetic profile. Absorption from the gastrointestinal tract is initially rapid, and probably extensive. The major metabolic pathways are likely to involve glucuronide and sulphate conjugation, and there is evidence of extensive first pass metabolism of nonylphenol absorbed through the gastrointestinal tract. Because of first pass metabolism, the bioavailability of unconjugated nonylphenol is probably limited following oral exposure, at no more than 10-20% of the administered dose. Nonylphenol is distributed widely throughout the body, with the highest concentration in fat. The major routes of excretion are via the faeces and urine.

表 8-5　ノニルフェノールの生殖・発生毒性試験結果

動物種等	投与方法	投与期間	投与量	結　　果	文献
ラット SD 雌 9 –11匹/群	混餌 (大豆フリー)	F_0:妊娠7日- 離乳(出産後21日) F_1: 生後21日–77日	NP (Schenectady International Inc.) 0、25、500、2,000 ppm	F_0: 25 ppm以上： 摂餌量減少 いずれの群においても妊娠期間、F_1の出生時体重、性比、同腹生児数に影響なし F_1: 雄25 ppm以上及び雌2,000 ppm： 体重増加抑制 雄2,000 ppm： 摂餌量減少 雄雌2,000 ppm： 水及び食塩水の摂取量増加 母動物：LOEL=25 ppm 次世代：(雄) LOEL=25 ppm (雌) LOEL=2,000 ppm	Ferguson et al., 2000
ラット SD 雌雄	混餌	3世代	4-NP (混合物) 0、200、650、2,000 ppm (0、9 - 35、30 - 100、100 - 350 mg/kg/日 相当著者換算) (0、15、50、160 mg/kg/日 相当EU換算) (EU, 2001)	F_0： 雌雄：影響なし F_1： 650 ppm以上： 雌：子宮重量増加 2,000 ppm： 雄：体重増加抑制 雌：体重増加抑制、膣開口6日早期化 F_2： 650 ppm以上： 雄：体重増加抑制、精巣上体精子濃度低下 雌：体重増加抑制、膣開口2日早期化、卵巣相対重量減少 2,000 ppm： 雄：精巣精子細胞数減少 雌：膣開口6日早期化 F_3： 650 ppm以上： 雄：体重増加抑制 雌：体重増加抑制、膣開口2日早期化 2,000 ppm： 雌：膣開口6日早期化 次世代：(雌雄) NOAEL=200 ppm (15 mg/kg/日 相当 EU換算)	NTP, 1997; Chapin et al., 1999

動物種等	投与方法	投与期間	投与量	結　　果	文献
ラット SD 雌雄 雄：6週齢 雌：13週齢 25匹/性/群	強制経口 (コーン油)	F_0雄は交配前12週間、F_0雌は交配前2週間、交配は最大2週間 F_0雄は交配後剖検、F_0雌は妊娠、出産、哺乳期を通じて投与、F_1の離乳後剖検 F_1は離乳後投与、同じ投与群内で交配、F_1雌雄の剖検はF_0に準じる	NP（三井化学） 0、2、10、50 mg/kg/日	F_0： 50 mg/kg/日： 雄：腎臓の絶対及び相対重量の増加、胸腺の絶対及び相対重量の減少、肝臓の相対重量の増加、下垂体相対重量増加 肝細胞小葉中心性肥大、上皮管の好酸性小体の減少 TSH（甲状腺刺激ホルモン）濃度上昇、F_1生存率低下（生後0-4日のみでそれ以降の成長に影響なし） 雌：卵巣の絶対及び相対重量の減少（組織病理学的変化なし）、 F_1生存率低下（生後0-4日のみでそれ以降の成長に影響なし） F_1： 50 mg/kg/日： 雄：腎臓及び肝臓の相対重量の増加、血清中FSH（卵胞刺激ホルモン）濃度上昇、T3（トリヨードチロニン）濃度低下（生後22日）、着床数及びF_2生存児数の減少 雌：卵巣の絶対及び相対重量の減少、膣開口早期化、LH（黄体化ホルモン）及びTSH濃度低下、T3濃度上昇（生後22日）、着床数及びF_2生存児数の減少 親世代(雌雄)：NOAEL=50 mg/kg/日以上 (但し、一般毒性はNOAEL=10 mg/kg/日) 次世代(雌雄)：NOAEL=10 mg/kg/日	Nagao et al., 2001

NP: nonylphenol
太字はリスク評価に用いたデータを示す。

8.3.6　遺伝毒性

ノニルフェノールの遺伝毒性試験結果を表 8-6に示す。*in vitro* 試験では、ネズミチフス菌及び大腸菌を用いた復帰突然変異試験並びにチャイニーズ・ハムスター肺線維芽細胞株 (CHL)を用いる染色体異常試験で代謝活性化の有無に関わらず陰性と報告されている (GDCh BUA, 1988;Shimizu et al., 1985;厚生省, 1996)。

調査した範囲内では *in vivo* 試験の報告はない。