テトラヒドロチオフェン-1,1-ジオキシドの細菌を用いる復帰突然変異試験

Reverse Mutation Test of Tetrahydrothiophen 1,1-dioxide on Bacteria

要約

既存化学物質安全性調査事業の一環として，テトラヒドロチオフェン-1,1-ジオキシドについて，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/プレートの用量で実施したところ，すべての検定菌において抗菌性は認められなかった．したがって，本試験はS9 mix無添加試験および添加試験を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，復帰変異コロニー数は，2回の本試験とも，用いた5種類の検定菌の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，テトラヒドロチオフェン-1,1-ジオキシドは，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．

材料および試験方法

〔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，試験に際して，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〕

テトラヒドロチオフェン-1,1-ジオキシド(CAS No. 126-33-0)は，分子量120.16の無色固体である．試験には，新日本理化㈱製［ロット番号8050074，純度99.9％以上(不純物：水分0.1％以下)］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，使用した．

テトラヒドロチオフェン-1,1-ジオキシドは，水に溶解性がよいことから，用量設定試験においては純水，本試験においては注射用蒸留水に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，テトラヒドロチオフェン-1,1-ジオキシドの注射用蒸留水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(3.00 mg/ml)溶液は当研究所で実施した染色体異常試験で調製したものについて，高濃度(50.0 mg/ml)溶液は当該試験の本試験Ⅰで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，99.1および99.3％であった．また，本試験Ⅰ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，低濃度(3.13 mg/ml)溶液は，97.9％，高濃度(50.0 mg/ml)溶液は102％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はジメチルスルホキシド(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混液の組成〕

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，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およびトップアガー2 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注射用蒸留水，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，すべての検定菌においてS9 mix無添加試験および添加試験のいずれも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．テトラヒドロチオフェン-1,1-ジオキシドの用量を，S9 mix無添加試験および添加試験でともに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，テトラヒドロチオフェン-1,1-ジオキシドは，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．

文献

1) D. M. Maron, B. N. Ames, *Mutat. Res.*, 113, 173, (1983).
2) M. H. L. Green, "Handbook of Mutagenicity Test Procedures," eds.by B. J. Kilbey, M. Legator, W. Nichols, C. Ramel, Elsevier, Amsterdam, New York, Oxford, 1984, pp.161-187.

連絡先

試験責任者：澁谷　徹
試験担当者：原　巧，坂本京子，川上久美子，清水ゆり，松木容彦，中込まどか，飯田さやか，北嶋美似子
㈶食品薬品安全センター　秦野研究所
〒257　秦野市落合 729-5
Tel 0463-82-4751　Fax 0463-82-9627

Correspondence

Authors: Tohru Shibuya (Study Director)
Takumi Hara, Kyoko Sakamoto, Kumiko Kawakami, Yuri Shimizu, Yasuhiko Matsuki, Madoka Nakagomi, Sayaka Iida and Miiko Kitashima.
Hatano Research Institute, Food and Drug Safety Center
729-5 Ochiai, Hadano-shi, Kanagawa 257, Japan
Tel +81-463-82-4751　Fax +81-463-82-9627

Table 1. Mutagenicity of tetrahydrothiophene 1,1-dioxide** 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	90 88 89 (89± 1.0)	13 10 17 (13± 3.5)	20 22 24 (22± 2.0)	23 25 21 (23± 2.0)	9 10 10 (10± 0.6)	
	313	124 107 103 (111±11.2)	10 8 14 (11± 3.1)	25 35 36 (32± 6.1)	22 14 27 (21± 6.6)	11 7 8 (9± 2.1)	
	625	103 131 120 (118±14.1)	15 10 13 (13± 2.5)	36 33 27 (32± 4.6)	21 16 15 (17± 3.2)	9 11 6 (9± 2.5)	
	1250	109 118 97 (108±10.5)	7 13 15 (12± 4.2)	27 23 31 (27± 4.0)	15 18 22 (18± 3.5)	8 7 12 (9± 2.6)	
	2500	87 142 124 (118±28.0)	9 10 13 (11± 2.1)	27 27 23 (26± 2.3)	26 19 15 (20± 5.6)	6 6 11 (8± 2.9)	
	5000	109 123 106 (113± 9.1)	9 12 9 (10± 1.7)	23 28 28 (26± 2.9)	24 26 15 (22± 5.9)	9 10 9 (9± 0.6)	
S9mix (+)	0	134 135 129 (133± 3.2)	9 16 18 (14± 4.7)	36 28 24 (29± 6.1)	30 32 38 (33± 4.2)	15 12 10 (12± 2.5)	
	313	95 85 117 (99±16.4)	7 10 10 (9± 1.7)	42 35 35 (37± 4.0)	46 40 33 (40± 6.5)	8 10 16 (11± 4.2)	
	625	134 134 110 (126±13.9)	13 11 12 (12± 1.0)	39 38 31 (36± 4.4)	33 38 33 (35± 2.9)	18 20 18 (19± 1.2)	
	1250	114 131 119 (121± 8.7)	14 14 13 (14± 0.6)	27 19 24 (23± 4.0)	34 31 25 (30± 4.6)	16 8 14 (13± 4.2)	
	2500	122 127 159 (136±20.1)	5 13 5 (8± 4.6)	28 28 30 (29± 1.2)	30 33 42 (35± 6.2)	14 12 14 (13± 1.2)	
	5000	149 136 128 (138±10.6)	10 16 17 (14± 3.8)	19 17 23 (20± 3.1)	30 36 30 (32± 3.5)	12 10 14 (12± 2.0)	
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	858 879 838 (858±20.5)	193 193 206 (197± 7.5)	122 209 194 (175±46.5)	735 788 820 (781±42.9)	1767 2148 2578 (2164±405.7)	
Positive control S9 mix (+)	Chemical	2AA	2AA	2AA	2AA	2AA	
	Dose (μg/plate)	1	2	10	0.5	2	
	Number of colonies/plate	1370 1381 1290 (1347±49.7)	298 281 294 (291± 8.9)	1243 1223 1506 (1324±157.9)	337 335 335 (336± 1.2)	287 280 283 (283± 3.5)	

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

**: Purity was above 99.9% and water (below 0.1 %) was contained as impurity.

Table 2. Mutagenicity of tetrahydrothiophene 1,1-dioxide** in reverse mutation test (II) 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	112 123 125 (120± 7.0)	11 18 16 (15± 3.6)	17 18 33 (23± 9.0)	22 24 32 (26± 5.3)	12 7 8 (9± 2.6)	
	313	131 127 156 (138±15.7)	15 17 17 (16± 1.2)	23 31 32 (29± 4.9)	21 14 23 (19± 4.7)	9 17 5 (10± 6.1)	
	625	137 130 128 (132± 4.7)	18 16 25 (20± 4.7)	29 24 22 (25± 3.6)	21 19 22 (21± 1.5)	12 8 10 (10± 2.0)	
	1250	105 132 129 (122±14.8)	20 16 15 (17± 2.6)	23 22 18 (21± 2.6)	27 24 27 (26± 1.7)	11 15 6 (11± 4.5)	
	2500	137 145 133 (138± 6.1)	9 18 12 (13± 4.6)	28 20 23 (24± 4.0)	38 21 20 (26±10.1)	14 9 14 (12± 2.9)	
	5000	156 129 123 (136±17.6)	25 20 15 (20± 5.0)	28 17 28 (24± 6.4)	27 23 31 (27± 4.0)	17 12 5 (11± 6.0)	
S9mix (+)	0	148 120 141 (136±14.6)	15 12 21 (16± 4.6)	16 25 25 (22± 5.2)	35 39 20 (31±10.0)	15 14 17 (15± 1.5)	
	313	127 137 146 (137± 9.5)	22 26 14 (21± 6.1)	30 30 25 (28± 2.9)	42 31 39 (37± 5.7)	12 20 20 (17± 4.6)	
	625	137 146 133 (139± 6.7)	15 10 16 (14± 3.2)	24 29 25 (26± 2.6)	29 38 42 (36± 6.7)	14 18 18 (17± 2.3)	
	1250	137 142 143 (141± 3.2)	17 16 19 (17± 1.5)	33 25 26 (28± 4.4)	26 35 27 (29± 4.9)	20 19 13 (17± 3.8)	
	2500	138 172 166 (159±18.1)	17 15 18 (17± 1.5)	29 22 22 (24± 4.0)	38 39 29 (35± 5.5)	23 25 15 (21± 5.3)	
	5000	134 132 159 (142±15.0)	23 17 19 (20± 3.1)	19 22 28 (23± 4.6)	47 30 36 (38± 8.6)	9 16 13 (13± 3.5)	
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	728 781 743 (751±27.3)	207 153 128 (163±40.4)	85 81 91 (86± 5.0)	767 780 762 (770± 9.3)	908 890 965 (921±39.2)	
Positive control S9 mix(+)	Chemical	2AA	2AA	2AA	2AA	2AA	
	Dose (μg/plate)	1	2	10	0.5	2	
	Number of colonies/plate	1228 1435 1409 (1357±112.8)	184 198 197 (193± 7.8)	1211 1206 1296 (1238±50.6)	340 323 309 (324±15.5)	204 192 221 (206±14.6)	

AF2: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, SA: Sodium azide, 9AA: 9-Aminoacridine, 2AA: 2-Aminoanthracene
**: Purity was above 99.9% and water (below 0.1%) was contained as impurity.

テトラヒドロチオフェン-1,1-ジオキシドの 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 Tetrahydrothiophene 1,1-dioxide 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，テトラヒドロチオフェン-1,1-ジオキシド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)，短時間処理(6時間)ともに 1.2 mg/ml(10 mM)の濃度においても50%を明らかに越える増殖抑制は認められなかったことから，すべての試験において1.2 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時間および48時間連続処理したいずれの処理群においても，染色体の構造異常や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，テトラヒドロチオフェン-1,1-ジオキシドは，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

テトラヒドロチオフェン-1,1-ジオキシド(略号：THTD，CAS No.:126-33-0，ロット番号：8050074，新日本理化㈱製造，㈳日本化学工業協会提供)は，25℃において無色固体で，水に対して易溶，芳香族炭化水素に対しても易溶で，融点28℃，沸点287℃，分子式$C_4H_8O_2S$，分子量120.16，純度99.9%以上(不純物として水分0.1%以下)の物質である．

被験物質原体は安定であり，溶媒中(注射用水)では，3.0～50.0 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．溶媒は注射用水(㈱大塚製薬工場)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10%(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，短時間処理ともに，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作用は認められなかった(Fig. 1)．

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，短時間処理とも1.2 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.)

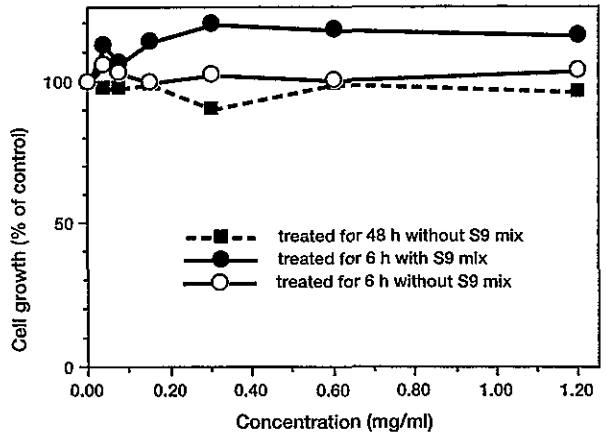


Fig. 1 Growth inhibition of CHL/IU cells treated with tetrahydrothiophene 1,1-dioxide

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．テトラヒドロチオフェン-1,1-ジオキシドを加えて24時間および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．テトラヒドロチオフェン-1,1-ジオキシドを加えて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，テトラヒドロチオフェン-1,1-ジオキシドは，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．

文献

1) 日本環境変異原学会・哺乳動物試験分科会編，“化学物質による染色体異常アトラス，”朝倉書店，東京，1988.
2) 林　真，変異原性試験，1，255(1992).
3) 吉村　功　編著，“毒性・薬効データの統計解析，事例研究によるアプローチ，”サイエンティスト社，東京，1987.
4) 吉村　功，大橋靖夫　編，“毒性試験講座14，毒性試験データの統計解析，”地人書館，東京，1992.

連絡先
試験責任者：田中憲穂
試験担当者：山影康次，日下部博一，橋本恵子，長尾哲二，太田　亮
㈶食品薬品安全センター秦野研究所
〒257　神奈川県秦野市落合729-5
Tel 0463-82-4751　Fax 0463-82-9627

Correspondence
Authors: Noriho Tanaka (Study director)
Kohji Yamakage, Hirokazu Kusakabe, Keiko Hashimoto, Tetsuji Nagao, Ryo Ohta
Hatano Research Institute, Food and Drug Safety Center
729-5 Ochiai, Hadano, Kanagawa, 257, Japan
Tel +81-463-82-4751　Fax +81-463-82-9627

Table 1 Chromosome analysis of Chinese hamster cells (CHL/IU) continuously treated with tetrahydrothiophene 1,1-dioxide (THTD)* 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	1	0	1	0	0	0	2	0	2	(1.0)	1	(0.5)	0.13		
Solvent[1]	0	24	200	0	0	0	0	1	0	1	1	1	(0.5)	1	(0.5)	0.25		
THTD	0.30	24	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.13		
THTD	0.60	24	200	0	1	0	0	1	0	2	0	2	(1.0)	2	(1.0)	0.13	NT	NT
THTD	1.2	24	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.63		
MC	0.00005	24	200	10	67	140	0	1	0	218	0	119	(59.5)	116	(58.0)	0.00		
Solvent[1]	0	48	200	1	0	1	2	0	0	4	0	3	(1.5)	2	(1.0)	0.50		
THTD	0.30	48	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
THTD	0.60	48	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00	NT	NT
THTD	1.2	48	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.25		
MC	0.00005	48	200	9	86	200	1	10	90	396	9	138	(69.0)	138	(69.0)	0.38		

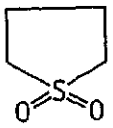
Abbreviations: gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring etc.), mul: multiple aberrations, TAG: total no. of cells with aberrations, TA: total no. of cells with aberrations except gap, SA: structural aberration, NA: numerical aberration, MC: mitomycin C, NT: not tested.
1) Water for injection 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. *: Purity was more than 99.9%, and water was contained (≦0.1%).

Table 2 Chromosome analysis of Chinese hamster cells (CHL/IU) treated with tetrahydrothiophene 1,1-dioxide (THTD)* 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	2	0	1	0	0	0	3	0	3	(1.5)	1	(0.5)	0.63		
Solvent[1]	0	-	6-(18)	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
THTD	0.30	-	6-(18)	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.13		
THTD	0.60	-	6-(18)	200	1	1	0	0	0	0	2	0	2	(1.0)	1	(0.5)	0.25	NT	NT
THTD	1.2	-	6-(18)	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.63		
CPA	0.005	-	6-(18)	200	1	0	1	0	0	0	2	0	2	(1.0)	1	(0.5)	0.50		
Solvent[1]	0	+	6-(18)	200	1	1	1	0	0	0	3	0	3	(1.5)	2	(1.0)	0.63		
THTD	0.30	+	6-(18)	200	1	1	0	0	0	0	2	0	2	(1.0)	1	(0.5)	0.38		
THTD	0.60	+	6-(18)	200	0	0	0	0	1	0	1	0	1	(0.5)	1	(0.5)	0.13	NT	NT
THTD	1.2	+	6-(18)	200	2	3	3	0	0	0	8	0	3	(1.5)	1	(0.5)	0.00		
CPA	0.005	+	6-(18)	200	2	161	311	0	4	440	918	0	186	(93.0)	186	(93.0)	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) Water for injection 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 99.9%, and water was contained (≦0.1%).

SIDS INITIAL ASSESSMENT PROFILE

CAS No.	126-33-0
Chemical Name	Tetrahydrothiophene 1,1-dioxide
Structural Formula	

SUMMARY CONCLUSIONS OF THE SIAR

Human Health

In rats dosed intravenously at 500 mg/kg, 28%, 36% and 37% of the dose was excreted unchanged between days 0-2, 0-4 and 0-7, respectively. At 1000 mg/kg, 50% and 67.2% of the dose was excreted unchanged between days 0-2 and 0-4, respectively. The observation that the proportion of the dose recovered increased with dosage suggests that the metabolic pathway is saturable. In rabbits, dogs and squirrel monkeys given a single iv injection, this chemical was rapidly distributed throughout the body and was slowly removed from plasma with a half-life of 3.5-5 hours. One major metabolite with 85% of the urinary radioactivity was found in male rats injected intraperitoneally with this chemical. In a follow up study, the metabolite was identified as 3-hydroxysulfolane in the urine of rabbits injected intraperitoneally with this chemical.

LD_{50} values by gavage [OECD TG 401] were 2006 mg/kg (males) and 2130 mg/kg (females) in rats. Dermal LD_{50} in male and female rats was greater than 2000 mg/kg [84/449/EEC, B3]. Inhalation LC_{50} in male and female rats(four hours) was greater than 12,000 mg/m^3. Acute behavioural studies in rats indicated that hypothermia contributed to the behavioural effect of an interperitoneal injection of 800 mg/kg of sulfolane. Rabbits became hyperthermic, at 28°C, upon subcutaneous injection of 600 mg/kg sulfolane.

The chemical is not irritating to guinea pig and rabbit skin or to rabbit eyes. The chemical was not sensitising (0/20) in a guinea pig maximisation test [84/449/EEC, B6].

In a 28 day repeat dose toxicity study [Japanese TG] conducted under GLP, male and female rats were dosed by gavage with this chemical at 0, 60, 200 and 700 mg/kg/day. At 700 mg/kg some females showed transient reduction in locomotor activity during the early administration period. Bodyweight gain and food consumption at this dose were decreased in both males and females. Blood chemistry revealed increases in cholinesterase activity and total bilirubin levels in males and GPT in females and decreases of chloride levels in males and glucose levels in females. Histopathological examination in males dosed at 700 and 200 mg/kg/day revealed increases of hyaline droplets and eosinophilic bodies in the renal tubules which was accompanied by an increase in relative kidney weight. There was a decrease of splenic weight in females at 700 mg/kg/day, but no histological abnormalities were detected. No changes considered to be attributable to sulfolane were observed on urinary and haematological examinations at any dose. Kidney lesions tended to recover and the other changes related to the chemical disappeared after a 14 day recovery period. The NOAEL was 60 mg/kg/day for male rats and 200 mg/kg/day for female rats.

The chemical was not mutagenic in bacteria [OECD TG 471 and 472] and did not induce chromosome aberrations in mammalian cells in vitro [OECD TG 473] either with or without metabolic activation.

In a reproduction/developmental toxicity screening test [OECD 421]) rats were dosed at 0, 60, 200, or 700 mg/kg/day

by gavage for 41 to 50 days from 14 days prior to mating to day 3 of lactation. Some mortality occured in the high-dose group. There was a decrease in body weight gain and food consumption of males and females during the pre-mating period, at 700 mg/kg. The number of oestrus cycles was decreased in the 700 mg/kg group. Four dams lost all their pups during the lactation period in the 700 mg/kg group. Birth index, live index, number of pups on days 1 and 4 of lactation, viability index and body weights of pups of both sexes on days 0 and 4 of lactation decreased, and the number of still births increased in the 700 mg/kg group. Birth index and the number of pups on day 0 and 4 of lactation decreased in the 200 mg/kg group. The NOAEL for reproductive and developmental toxicity was 60 mg/kg/day. There were no treatment-related findings in the external appearance, general conditions and necropsy findings in offspring.

Environment

The chemical has a log Pow of -0.77, a vapour pressure of 0.0083 hPa at 20°C and a water solubility of greater than 100 g/l. Fugacity model Mackay level III calculations suggest that the chemical will distribute almost completely to water if released to the aquatic compartment and equally to soil and water if released into air or soil separately or simultaneously to all three compartments.. The chemical is not readily biodegradable (10% after 14 days), and is hydrolytically stable (t1/2 greater than 1 year at pH 4, 7 and 9, 25°C). It can be biodegraded after acclimatisation of activated sludge and by a variety of bacterial cultures, and may be substantially biodegradable. Inorganic sulphate has been identified as the final degradation product of sulfolane metabolism. The chemical has been shown to have low potential for bioaccumulation. Indirect photo-oxidation by hydroxy radicals is predicted to occur with a half-life estimated at 9.7 h (calculated using AOPWIN rate constant, 1.328×10^{-11} cm^3/molecule/sec).

In an acute fish toxicity study [OECD TG 203, *Oryzias latipes*] a 96-hLC_{50} > 100 mg/l was reported. In *Daphnia magna* [OECD TG 202], an acute toxicity value of 48h EC_{50} = 852 mg/l was reported. The results in algae [OECD 201] were an E_rC_{50} (72h) >1000 mg/l,E_bC50 = 500mg/ l and a $NOEC_r$ (72 h) = 556 mg/l, $NOEC_b$ = 171 mg/l. The chronic toxicity to *Daphnia magna* [OECD 211] was a NOEC (21d, reproduction) of 25 mg/l and an LC_{50} (21d, parental) > 100 mg/l.

In a study to determine plant toxicity [Environment Canada protocol, lettuce (*Lactuca sativa*), carrot (*Daucus carota*), alfalfa (*Medicago sativa*) and timothy (*Phleum pratense*)] it was determined that plants were generally most sensitive to sulfolane in till and least sensitive in loam. A five day seed germination/root elongation test conducted using lettuce (*Lactuca sativa*) reported NOEC values of 290 mg/kg (root elongation) and 570 mg/kg (seed germination) for lettuce grown in fine-textured soil.

Exposure

Production of the chemical during 2003 was 1100 t/year in Japan. Global production in 2003 was approximately 13,300 t/year. Geographically, production was divided between sites in the Americas (35-45%), Asia (20-30%) and Europe/Africa (35-45%).

The major use of Sulfolane is as a solvent for extraction of aromatic hydrocarbons from oil refinery streams and acid gas purification. These uses account for approximately 80% of production. A number of minor uses (accounting for 20% of production) include fractionation of wood tars, tall oil and other fatty acids, electronic applications, textile manufacturing and finishing, as a plasticizer and as a solvent in pharmaceutical manufacturing. Other uses mentioned in the literature include solvent for jet printing inks, a component of hydraulic fluid, a curing agent for epoxy resins and medicinal application (although this latter application is thought to exist in the patent literature only).

Monitoring studies performed in the vicinity of gas processing facilities in Canada have shown that environmental release of sulfolane during its use in these facilities is possible. Sulfolane was detected in soil, bedrock and shallow till aquifers, wetlands and creeks near these facilities. It was also detected in wetland vegetation.

There is low potential for exposure to workers during production of the chemical. It is manufactured in a closed

system and transferred directly from the reactors into storage tanks. There is potential exposure to workers during drum filling. This operation is performed on 16 days per year for 7 hours each day. The concentration of sulfolane close to the drums has been measured at 0.2 ppm. There is potential exposure to workers at user sites. Since the predominant use of sulfolane is as a solvent in commercial extraction processes there is little potential for direct consumer exposure, however there is a potential for indirect human exposure via drinking water and food crops in areas surrounding processing plants.

RECOMMENDATION AND RATIONALE FOR THE RECOMMENDATION AND NATURE OF FURTHER WORK RECOMMENDED

Human health: The chemical is a candidate for further work. The chemical possesses properties indicating a hazard for human health (reproductive and developmental toxicity). Based on data presented by the Sponsor country worker exposure in sites manufacturing the chemical is controlled. No information is available for occupational exposure in industries using the chemical nor for indirect human exposure via drinking water and food crops in areas surrounding processing plants. It is therefore recommended that member countries perform an exposure assessment for industrial users and indirect human exposure, and if then indicated, risk assessments be performed.

Environment: The chemical is currently of low priority for further work because of its low hazard potential.

3 HUMAN HEALTH HAZARDS

3.1 Effects on Human Health

3.1.1 Toxicokinetics, Metabolism and Distribution

Studies in Animals

In vivo Studies

Groups of 3 male rats were dosed intravenously at 500 or 1000 mg/kg with non-radiolabelled sulfolane and the amount of sulfolane excreted unchanged in the urine was measured for 7 days after administration using gas-liquid chromatography. At 500 mg/kg, 28%, 36% and 37% of the dose was excreted unchanged between days 0-2, 0-4 and 0-7, respectively. At 1000 mg/kg, 50% and 67.2% of the dose was excreted unchanged between days 0-2 and 0-4, respectively. The observation that the proportion of the dose recovered increased with dosage suggests that the metabolic pathway is saturable. In a follow up to this study, blood-sulfolane decay curves were obtained following intravenous injections of sulfolane to a single rabbit, dog and squirrel monkey. Sulfolane was rapidly distributed throughout the animals and being slowly removed from plasma with a half-life of 3.5-5 hours (Andersen, 1976). No information is given on which tissues sulfolane distributes to in this study.

In a second study 3 male Wistar rats were injected intraperitoneally with ^{35}S-sulfolane (100 mg/rat in 2 ml water) and the 24 hour urinary samples analysed. One major metabolite was found, constituting 85% of the urinary radioactivity. Subsequently, 3 rabbits were injected intraperitoneally with a mixture of unlabelled sulfolane (1g) and ^{35}S-sulfolane (100 mg) and the urine samples collected and extracted with chloroform. The metabolite was identified as 3-hydroxysulfolane (Roberts, 1961).

3.1.2 Acute Toxicity

Studies in Animals

Inhalation

There is one reliable study reported. Andersen et al (1977) examined the acute inhalation toxicity of sulfolane. No rats died after 4 hours exposure to sulfolane at 12,000 mg/m^3 (the highest concentration that could be maintained as a stable aerosol) or during a subsequent 2-week observation period. Exposures at these high concentrations were continued until all all rats died. It was calculated that a mean survival time of 24 hours would be observed in atmospheres containing 4700 mg/m^3 of sulfolane. In a second experiment, nine rats were exposed to sulfolane at a concentration of 3600 mg/m^3 for 17.5 hours (when all the rats had convulsed and were *in extremis*). Significant decreases in white blood cell counts were observed, but haemocrit and haemoglobin were unchanged. At necropsy, all rats exhibited varying degrees of pulmonary haemorrhage. In a third experiment, two squirrel monkeys exposed to 4850 mg/m^3 vomited and convulsed during exposure and were sacrificed after 18.5 hours. Both had a greater than 25% decrease in white blood cells and a greater than 15% reduction in haemoglobin and haematocrit. Once again, pulmonary haemorrhage was evident. The LC_{50} (4h, rat) was > 12,000 mg/m^3.

Table 3 Acute inhalation toxicity to experimental animals

Species	Exposure time	Result	Reference
Rat	4 h	LC_{50} >12 000 mg/m^3 (combined)	(Andersen et al.,

Rat	17.5 h	Total mortality: LC_{50} <3600 mg/m^3 (males)	1977)
Monkey	18.5 h	Total mortality: LC_{50} <4850 mg/m^3 (males)	

Dermal

There are four acute dermal toxicity studies, one of which is considered reliable. In this study, conducted to GLP [Directive 84/449/EEC, B3], sulfolane was applied directly to the intact skin of 5 male and 5 female rats at a dose of 2000 mg/kg and covered with an occlusive dressing for 24 hours. There were no deaths or signs of systemic toxicity during the 14-day observation period and no macroscopic changes were apparent at necropsy (Gardner, 1993). The LD_{50} was > 2000 mg/kg.

Oral

There are 10 acute oral toxicity studies. In a reliable study [OECD TG 401, GLP] male and female rats (5 animals of each sex per dose) were dosed by gavage at doses of 0, 892, 1204, 1626, 2191, 2963 and 4000 mg/kg. Clinical signs of convulsion as well as decreased locomotion activity, ptosis, salivation, piloerection, chromodacryorrhea and perineal region soiling with urine were observed in the treated groups. Body weights of the treated animals were lower than those of the control group on the day after dosing. All deaths occurred on the day of dosing at doses of 2195 mg/kg and above. Dead animals showed haemorrhagic black spots in their glandular stomach mucosa. The LD_{50} was 2006 mg/kg (males) and 2130 mg/kg (females) (MHW Japan, 1996a).

In a second reliable study [Directive 84/449/EEC, B1, GLP] male and female rats (5 animals of each sex per dose) were dosed by gavage at 1600, 2240 and 3136 mg/kg. Deaths occurred from two hours after dosing until Day 2 among rats treated at the intermediate and high dose levels. Clinical signs included fasciculation, tremor, twitching, splayed gait, hunched posture, piloerection, unkempt appearance and yellow staining of the anogenital fur. Convulsions and salivation developed among the rats dosed at 2240 and 3136 mg/kg. Isolated cases of hypersensitivity to stimuli, hyperactivity, lethargy, hypothermia, diarrhoea, lachrymation, pallor of the eyes and blood around the mouth were also observed. Onset of the principal clinical signs was generally apparent within four hours of dosing. All surviving rats had gained weight relative to their Day 1 bodyweights by the end of the of the 14 day observation period. Necropsy findings amongst the decedents were lung congestion, exaggerated lobular pattern or dark patches on the liver, darkening of the spleen or kidneys and abnormal contents (colourless liquid or gaseous) of the gastrointestinal tract, especially the stomach and small intestine. Rats killed at completion of the observation period showed no macroscopic changes other than a single case of hepatic pallor. The LD_{50} was 2489 mg/kg (males), 2324 mg/kg (females) and 2473 mg/kg (combined) (Gardner, 1993).

In a third reliable study male and females rats (5 animals of each sex per dose) were dosed by gavage at 0, 1000, 1500, 2000, 3000 and 5000 mg/kg (males) and 0, 1000, 2000, 2500, 3000 and 5000 mg/kg (females). One male and five females dosed at 1000 mg/kg and four males dosed at 1500 mg/kg appeared normal from normal to termination. Clinical signs noted in the remaining animals included depression, slight depression, rough coat, salivation, hunched appearance, tremors, ataxia, urine stains, soft faeces and red stains on the nose and/or eyes. All surviving rats that showed clinical signs appeared normal by Day 3 through to termination of the study. All surviving rats gained weight relative to their Day 1 bodyweights. No gross pathological findings were observed in rats surviving to termination. Alterations of the stomach and/or intestines were the most common findings amongst animals that died. These alterations included compound like material, dark red material, reddish fluid or yellowish fluid in the stomach and/or intestines. Findings in the

lung and liver were noted at the 5000 mg dose only. The LD_{50} was 2739 mg/kg (males), 2108 mg/kg (females) and 2363 mg/kg (combined) (Phillips Petroleum Company, 1983a)

Table 4 Acute oral toxicity in experimental animals

Species	LD_{50}	Reference
Rat	2006 (males); 2130 mg/kg (females)	(MHW Japan, 1996a)
Rat	2489 (males); 2324 (females); 2473 mg/kg (combined)	(Gardner, 1993)
Rat	2739 (males); 2108 (females); 2363 mg/kg (combined)	(Phillips Petroleum Company, 1983a)

Other Routes of Exposure

Ruppert and Dyer (1985) studied the influence of hypothermia on the acute behavioural toxicity of sulfolane. Adult male rats (Long-Evans), 10 per group, received a single interperitoneal injection of saline, 200, 400 or 800 mg/kg sulfolane. Separate groups of rats at each dose were housed in rooms maintained at 32.3±0.7°C (warm ambient temperature) or 20.8±0.2°C (cool ambient temperature). Motor activity was assessed in figure of eight mazes one hour after dosing. Immediately after testing (one hour), body temperatures were recorded.

At the cool ambient temperature, the body temperature of rats receiving 400 and 800 mg/kg was lower than that of the controls. At the warm ambient temperature, hypothermia in the rats receiving 400 and 800 mg/kg was attenuated, if not prevented. One animal receiving 800 mg/kg at the warm ambient temperature died during testing. At both ambient temperatures, 400 and 800 mg/kg sulfolane produced a decrease in motor activity. At the cool ambient temperature, 800 mg/kg sulfolane produced a decrease in movement throughout the maze. It was concluded that a behavioural change could be detected at sublethal dosages of sulfolane in the absence of hypothermia.

Another similar study was conducted by Mohler and Gordon (1988) to investigate the thermoregulatory responses of the rabbit. Nine male rabbits were subcutaneously injected with 0, 100, 200, 400, 600 and 750 mg/kg sulfolane at an ambient temperature of 10°C. This caused a dose-dependent decrease in colonic temperature of the rabbits. Metabolic rate remained unchanged during the initial phase of the hypothermia for all dose groups; but peripheral vasodilation, as indicated by an increase in ear skin temperature, was seen at the higher dose levels. The highest doses of sulfolane caused behavioural deficits in the rabbits. Two to three hours after exposure to 600 mg/kg sulfolane, when the rabbits were removed from the environmental chamber and first observed, the animals exhibited a slight postural tremor similar to shivering. Both rabbits receiving 750 mg/kg sulfolane exhibited tonic seizures characterised by gross muscle contraction, forceful urination, and some vocalisation. These episodes were followed by exhaustion, panting, loss of postural control, and near catatonia. All rabbits in these experiments survived the sulfolane exposure, even at the highest dose levels. Seven male rats were subcutaneously injected with 600 mg/kg sulfolane at ambient temperatures of 10, 20 and 28°C. At ambient temperatures of 10 and 20°C there was a significant decrease in colonic temperature, however metabolic rate did not change significantly prior to or during peak hypothermia. At an ambient temperature of 28°C, there was a significant increase in colonic temperature and metabolic rate following administration of sulfolane.

Conclusion

The oral LD_{50} (rat) was 2006 mg/kg (males) and 2130 mg/kg (females) [OECD TG 401]. The dermal LD_{50} (rat) was > 2000 mg/kg [Directive 84/449/EEC, B3]. The inhalation LC_{50} (4h, rat) was > 12,000 mg/m^3.

Acute behavioural studies indicated hypothermia contributed to the behavioural effect of 800 mg/kg sulfolane in rats, however, the rabbits became hyperthermic, at 28°C, upon injection of 600 mg/kg sulfolane.

3.1.3 Irritation

Skin Irritation

Studies in Animals

In a reliable study (Brown et al., 1966) undiluted sulfolane (1 ml) was applied to the shaved backs of 4 male and 4 female rabbits on 3 consecutive days and covered with an occlusive bandage for 6 hours each day. The final visual assessment was made on day 7. No signs of skin irritation were observed in any of the rabbits used. Histopathological examination of the skins taken post mortem revealed no evidence of skin damage.

Brown et al. (1966) also applied undiluted sulfolane (0.5 ml) daily, five days per week for four and a half weeks to the shaved backs of 10 guinea pigs. Application areas were left uncovered during the test. In findings similar to those in the rabbit, no signs of skin irritation were observed. Histopathological examination of the skins taken post mortem revealed no evidence of skin damage.

Eye Irritation

Studies in Animals

In a reliable study [US Federal Register 29 FR 13009] undiluted sulfolane (0.2 ml) was instilled into the right eyes of rabbits. Only a mild conjunctivitis was produced, which cleared within a few hours (Brown et al., 1966).

Conclusion

Sulfolane is not considered to be a skin or eye irritant.

3.1.4 Sensitisation

Studies in Animals

Skin

There are 3 sensitisation studies, one of which is reliable. In a guinea pig maximisation test [Directive 84/449/EEC, B6, GLP] a group of 10 male and 10 female guinea pigs were induced intradermally using 2% m/v sulfolane in water/Freunds Complete Adjuvant followed a week later by topical induction using undiluted sulfolane (0.3 ml) which was applied over the sites of the intradermal injections and covered occlusively for 48 hours. Challenge was carried out 3 weeks after the intradermal induction. Undiluted sulfolane (0.1ml) was applied to the shaven backs of the test animals and covered with occlusive tape for 24 hours. Dermal reaction to the challenge was assessed after removal of the bandages and at 24 hours and 48 hours after challenge. None of the test animals showed any positive response at either 24 or 48 hours after removal of the challenge patches and therefore sulfolane is not considered to be a skin sensitiser in guinea pigs (Gardner, 1993).

Conclusion

Sulfolane is not considered to be a skin sensitiser in guinea pigs.

3.1.5 Repeated Dose Toxicity

There are 8 studies for repeat dose inhalation toxicity and one study for repeat dose oral toxicity. None of the inhalation studies are considered to be reliable due to the non-standard test methods used. The oral study is considered to be reliable.

Studies in Animals

Oral

In a 28 day repeat dose toxicity study [Japanese TG] conducted to GLP (MHW Japan, 1996b) male and female rats were administered doses of 0, 60, 200 and 700 mg/kg/day of the chemical by gavage. There were 12 animals per dose for the group at 60, 200 mg/kg/day and 24 per dose for the group at 0, 700 mg/kg/day. The recovery period was 14 days.

At 700 mg/kg some females showed transient reduction of locomotor activity at the early stage of the administration period. Bodyweight gain and food consumption at this dose were decreased in both males and females. Blood chemistry revealed increases in cholinesterase and total bilirubin in males and GPT in females and decreases of chloride in males and glucose in females. Pathological examination revealed increases of hyaline droplets and eosinophilic bodies in the renal tubules and an increase in the relative weight of the kidney in males. There was a decrease of splenic weight in females, but no histological abnormalities were detected.

At 200 mg/kg pathological examination revealed increases of hyaline droplets and eosinophilic bodies in the renal tubules of males.

No changes considered to be attributable to sulfolane were observed on urinary and haematological examinations at any dose. Kidney lesions tended to recover and the other changes related to the chemical disappeared after a 14 day recovery period. The NOAEL is considered to be 60 mg/kg/day for males and 200 mg/kg/day for females.

Conclusion

The oral NOAEL is 60 mg/kg/day (males) and 200 mg/kg/day (females).

3.1.6 Mutagenicity

There are 8 *in vitro* mutagenicity studies, seven of which are considered to be reliable. There are no *in vivo* studies available.

In vitro Studies

Sulfolane has been tested for reverse mutation in *Salmonella typhimurium* and *Escherichia coli* with and without exogenous metabolic activation by standard Japanese test methods in full compliance with OECD TG 471 and 472 (MHW, 1996c). No cytotoxicity was observed at 5000 µg/plate in any of the 5 strains. The tests were negative, in both the presence and absence of a metabolising system. An *in vitro* chromosome aberration study in CHL cells was conducted in accordance with Japanese guidelines similar to OECD TG 473 (MHW, 1996d). The highest dose level was cytotoxic. Structural chromosomal aberrations and polyploidy were not induced up to the maximum dose either in the presence or absence of a metabolising system.

Several other bacterial mutagenicity tests, a chromosome aberration study using rat liver RL4, a sister chromatid exchange study and a yeast gene mutation assay are also reported as negative.

In a mouse lymphoma assay (Phillips Petroleum Company, 1982b) exposure to sulfolane in the presence and absence of metabolic activation increased the induction of forward mutations in L5178Y mouse lymphoma cells at the T/K locus. Sulfolane was considered to be mutagenic in this test system by the authors. However, there was no dose response and the survival percentage was not affected by increasing doses, therefore it is considered that this interpretation of the data is incorrect.

Table 5 Genotoxicity studies of sulfolane

Type of test	Test system	Dose	Result	Reference
Bacterial test (reverse mutation)	*S. typhimurium* TA 98, TA100, TA 1535, TA 1537. *E. coli* WP2uvrA	5 doses between 313 to 5000 µg/plate	Negative, with and without metabolic activation	(MHW, 1996c)
Bacterial test (reverse mutation)	*S. typhimurium* TA 98, TA100, TA 1535, TA 1537, TA 1538. *E. coli* WP2, WP2uvrA	8 doses between 31.25 to 4000 µg/plate	Negative, with and without metabolic activation	(Thorpe, 1982)
Bacterial test (reverse mutation)	*S. typhimurium* TA 98, TA100, TA 1535, TA 1537, TA 1538	5 doses between 642 to 52000 µg/plate	Negative, with and without metabolic activation	(Phillips Petroleum Company, 1982a)
In vitro chromosome aberration assay	CHL/IU	-S9: (continuous exp.24 or 48 h) 0.3, 0.6, 1.2 mg/ml +/- S9: (6h exp.) 0.3, 0.6, 1.2 mg/ml	Negative with and without metabolic activation	(MHW, 1996d)
Sister chromatid exchange	CHO	70, 210, 700, 2100, 6400 µg/ml	Negative with and without metabolic activation	(Phillips Petroleum Company, 1983b)
Yeast gene mutation assay	*Saccharomyces cerevisiae*	0.01, 0.1, 0.5, 1.0, 5.0 mg/ml	Negative with and without metabolic activation	(Thorpe, 1982)
In vitro chromosome aberration assay	Rat liver RL4	250, 500, 1000 µg/ml	Negative without metabolic activation	(Thorpe, 1982)
Mouse lymphoma assay	L5178Y T/K locus	60, 90, 135, 202, 301, 449, 670, 1000 µg/mL	Positive*	(Phillips Petroleum Company, 1982b)

* There was no dose response and the survival percentage was not affected by increasing doses, therefore it is considered that this interpretation of the data is incorrect.

In vivo Studies

No data available

Conclusion

Sulfolane was not mutagenic in bacteria [OECD TG 471 and 472] and did not induce chromosomal aberrations in mammalian cells *in vitro* [OECD TG 473] either with or without metabolic activation.

3.1.7 Carcinogenicity

No data available.

3.1.8 Toxicity for Reproduction

There is one study available for reproductive/developmental toxicity.

Studies in Animals

Effects on Fertility

A reproduction/developmental toxicity screening test [OECD TG 421] was performed (MHW Japan, 1999). Twelve animals of each sex were dosed once daily by gavage (0, 60, 200, 700 mg/kg b.w./day). Males were dosed for 49 days (from 14 days prior to mating) and females for 41-50 days (from 14 days prior to mating to day 3 of lactation). One male and one female in the 700 mg/kg group died. There was a decrease in body weight gain and food consumption amongst males, and females during the pre-mating period, at 700 mg/kg. The number of oestrus cases was decreased in the 700 mg/kg group. Four dams lost all their pups during the lactation period in the 700 mg/kg group. Birth index, live index, number of pups on days 1 and 4 of lactation, viability index and body weights of pups of both sexes on days 0 and 4 of lactation decreased, and the number of still birth increased in the 700 mg/kg group. Birth index and the number of pups on day 0 and 4 of lactation decreased in the 200 mg/kg group. Parental NOAEL was 200 mg/kg/day. NOAEL for offspring was 60 mg/kg/day.

Developmental Toxicity

In the above study, there were no treatment-related findings in the external appearance, general conditions and necropsy findings of the offspring.

Conclusion

The reproductive toxic effects, such as decreased number of oestrus stages and an increased number of litters totally died, in female parents were found at 700 mg/kg bw/day. Developmental toxic effects, such as decreased birth index and number of pups were observed at 200 mg/kg bw/day and higher. The NOAEL for reproductive and developmental toxicity was 60 mg/kg/day. There were no treatment-related findings in the external appearance, general conditions and necropsy findings in offspring.

3.2 Initial Assessment for Human Health

Groups of 3 male rats were dosed intravenously at 500 or 1000 mg/kg of non-radiolabelled sulfolane and the amount of sulfolane excreted unchanged in the urine was measured for 7 days after administration using gas-liquid chromatography. At 500 mg/kg, 28%, 36% and 37% of the dose was excreted unchanged between days 0-2, 0-4 and 0-7, respectively. At 1000 mg/kg, 50% and 67.2% of the dose was excreted unchanged between days 0-2 and 0-4, respectively. The observation that the proportion of the dose recovered increased with dosage suggests that the metabolic pathway is saturable. In a follow up to this study, blood-sulfolane decay curves were obtained following intravenous injections of sulfolane to a single rabbit, dog and squirrel monkey. Sulfolane was

rapidly distributed throughout the animals and was slowly removed from plasma with a half-life of 3.5-5 hours.

In a second study 3 male Wistar rats were injected intraperitoneally with ^{35}S-sulfolane (100 mg/rat in 2 ml water) and the 24 hour urinary samples analysed. One major metabolite was found, constituting 85% of the urinary radioactivity. Subsequently, 3 rabbits were injected intraperitoneally with a mixture of unlabelled sulfolane and 35S-sulfolane (1 g: 100 mg) and the urine samples collected and extracted with chloroform. The metabolite was identified as 3-hydroxysulfolane.

The Oral LD_{50} (rat) was 2006 mg/kg (males) and 2130 mg/kg (females) [OECD TG 401]. The dermal LD_{50} (rat) was > 2000 mg/kg [Directive 84/449/EEC, B3]. The inhalation LC_{50} (4h, rat) was > 12,000 mg/m^3. The chemical is not a skin irritant or eye irritant [US Federal Register 29 FR 13009] or a skin sensitiser in guinea pigs [Directive 84/449/EEC, B6]. The acute behavioural studies showed that hypothermia contributed to the behavioural effect of 800 mg/kg sulfolane in rats, however, the rabbits became hyperthermic, at 28°C, upon injection of 600 mg/kg sulfolane.

Based on the results of a valid repeat dose study [Japanese TG], the NOAEL for repeat dose toxicity (oral) is 60 mg/kg/day (males) and 200 mg/kg/day (females).

Sulfolane was not mutagenic in bacteria [OECD TG 471 and 472] and did not induce chromosomal aberrations in mammalian cells *in vitro* [OECD TG 473]. There is no information on carcinogenicity, however in the absence of significant mutagenic effects *in vitro* there is no immediate concern.

In a reproduction/developmental toxicity screening test [OECD TG 421] males were dosed for 49 days (from 14 days prior to mating) and females for 41-50 days (from 14 days prior to mating to day 3 of lactation) at 0, 60, 200 and 700 mg/kg. One male and one female in the 700 mg/kg group died. There was a decrease in body weight gain and food consumption amongst males, and females during the pre-mating period, at 700 mg/kg. The number of oestrus cases was decreased in the 700 mg/kg group. Four dams lost all their pups during the lactation period in the 700 mg/kg group. Birth index, live index, number of pups on days 1 and 4 of lactation, viability index and body weights of pups of both sexes on days 0 and 4 of lactation decreased, and the number of still births increased in the 700 mg/kg group. Birth index and the number of pups on day 0 and 4 of lactation decreased in the 200 mg/kg group. Parental NOAEL was 200 mg/kg/day. The NOAEL for reproductive and developmental toxicity was 60 mg/kg/day. There were no treatment-related findings in the external appearance, general conditions and necropsy findings of the offspring.

4 HAZARDS TO THE ENVIRONMENT

4.1 Aquatic Effects

Acute and Chronic Toxicity Test Results

There are a number of studies reported on determination of acute aquatic effects of sulfolane. However many of the study results are reported by a secondary source and have to be considered as unreliable.

Acute Toxicity Test Results

In a reliable acute fish toxicity study [OECD TG 203] *Oryzias latipes* were exposed under semi-static conditions to sulfolane at a nominal concentration of 0 and 100 mg/L for 96 hours. There were no mortalities or signs of toxicity during the study in either the control or test fish. The LC_{50} was > 100 mg/L. In a further reliable acute fish toxicity study (Stephenson, 1982), *Salmo gairdneri* were exposed under semi-static conditions to sulfolane at concentrations of 100-1000 mg/L for 96

6 REFERENCES

Alexander WM, Arbeu BE, Weaver LC, Faith HE and Newberne JW (1959). Pharmacology of some monohalogenated derivatives of sulfolane (tetrahydrothiophene-1,1-dioxide). Arch. Int. Pharmacodyn. **119** (3-4), 423-434.

Andersen ME, Jones RA, Kurlansik L, Mehl RG and Jenkins LJ (1976). Sulfolane-induced convulsions in rodents. Research Communications in Chemical Pathology and Pharmacology **15** (3), 571-580.

Andersen ME, Jones RA, Mehl RG, Hill TA, Kurlansik L and Jenkins LJ (1977). The inhalation toxicity of sulfolane (Tetrahydrothiophene-1,1-dioxide). Toxicology and Applied Pharmacology **40**, 463-472.

Brown VKH, Ferrigan LW and Stevenson DE (1966). Acute toxicity and skin irritant properties of sulfolane. Brit. J. Industr. Med. **23**, 302-304.

Chou CC and Swatloski RA (1983). Biodegradation of sulfolane in refinery wastewater. Proc. Ind. Waste Conf. **37**, 559-66.

Daubert TE and Danner RP (1989). Physical and Thermodynamic Properties of Pure Chemicals: Data Compilation. Design Institute for Physical Property Data. American Institute of Chemical Engineers, New York, NY. Hemisphere Publishing Corp. Volume 4; Reported in Federal Register 56 (44), 9568, March 6, 1991.

Gardner JR (1993). Sulfolane: Acute Oral and Dermal Toxicity in Rat and Skin Sensitisation Potential in the Guinea Pig. Shell Group Research Report No. SBGR.92.289.

Hansch C and Leo AJ (1981). Medchem Project. Issue 19. Pomona College, Claremont, CA.

Kamalova EG, Davydova VA, Lazareva DN, Tolstikov GA, Novitskaya NN and Flekter BV (1979). Anti-inflammatory activity of some sulfolane derivatives. Russian Pharmacol. and Toxic. **42** (3), 97-101.

Komex International Ltd (2001). Soil and Water Quality Guidelines for Sulfolane and Diisopropanolamine (DIPA): Environmental and Human Health. Report Prepared for Canadian Association of Petroleum Producers.

Lewis RJ (2003). Sax's Dangerous Properties of Industrial Materials. 10th Edition, Volume 3, 3325. John Wiley & Sons.

Lindstrom M and Williams R (1983). Sulfolanes and Sulfones. **In:** Kirk-Othmer, Encyclopaedia of Chemical Technology. 3rd Edition, Volume 21, 961. Edited by M Grayson. John Wiley & Sons.

METI, Japan (1975). Biodegradability Test of Tetrahydrothiophene-1,1-dioxide (Test Material No. K-132) in Microorganisms. Chemicals Evaluation and Research Institute (CERI, Japan).

METI, Japan (1977). Bioaccumulation Test of Tetrahydrothiophene-1,1-dioxide (Test Material No. K-132) in Common Carp (*Cyprinus carpio*). Chemicals Evaluation and Research Institute (CERI, Japan).

MHW, Japan (1996a). Ministry of Health and Welfare, Toxicity Testing Reports of Environmental Chemicals. Volume 4, 435-436.

MHW, Japan (1996b). Ministry of Health and Welfare, Toxicity Testing Reports of Environmental Chemicals Volume 4, 437-445.

MHW, Japan (1996c). Ministry of Health and Welfare, Toxicity Testing Reports of Environmental Chemicals Volume 4, 447-450.

MHW, Japan (1996d). Ministry of Health and Welfare, Toxicity Testing Reports of Environmental Chemicals Volume 4, 451-453.

MHW, Japan (1999). Ministry of Health and Welfare, Toxicity Testing Reports of Environmental Chemicals Volume 7, 473-481.

MITI, Japan (2001). Measurement of Physicochemical Properties of Tetrahydrothiophene-1,1-dioxide (Test material No. K-132). Chemicals Evaluation and Research Institute (CERI, Japan) Test No. 80132K.

Mohler FS and Gordon CJ (1988). Thermoregulatory responses of the rabbit to subcutaneous injection of sulfolane. Arch. Toxicol. **62**, 216-219.

Phillips Petroleum Company (1982b). Hazleton Laboratories America Inc. *Salmonella typhimurium* Mammalian Microsome Plate Incorporation Assay: Sulfolane. Project No. 652-132.

Phillips Petroleum Company (1982c). Hazleton Laboratories America Inc. Mouse Lymphoma Forward Mutation Assay: Sulfolane. Project No. 652-133.

Phillips Petroleum Company (1983a). Hazleton Laboratories America Inc. Acute Oral Toxicity Study in Rats: Sulfolane. Project No. 652-135.

Phillips Petroleum Company (1983b). Hazleton Laboratories America Inc. *In Vitro* Sister Chromatid Exchange in Chinese Hamster Ovary Cells: Sulfolane. Project No. 652-134.

Roberts JJ and Warwick GP (1961). The mode of action of alkylating agents – III. The Formation of 3-Hydroxytetrahydrothiophene-1:1-dioxide from 1:4-Dimethanesulphonyloxybutane (Myleran), S-β-L-Alanyltetrahydrothiophenium Mesylate, Tetrahydrothiophene and Tetrahydrothiophene-1:1-dioxide in the Rat, Rabbit and Mouse. Biochemical Pharmacology **6**, 217-227.

Ruppert PH and Dyer RS (1985). Acute behavioural toxicity of sulfolane: Influence of hypothermia. Toxicology Letters **28**, 111-116.

Safepharm Laboratories (2004) Sulfolane: EPIWIN v3.12 Calculations. Unpublished data.

Smyth HF, Carpenter CP, Weil CS, Pozzani UC, Streigel JA and Nycum JS (1969). Range-Finding Toxicity Data: List VII. American Industrial Hygiene Association. September - October, 470-476.

Stephenson RR (1982). Shell Research Limited. Sulfolane: Acute Toxicity to *Salmo gairdneri*, *Daphnia magna* and *Selenastrum capricornutum*. Document No. SBTR.82.039.

Sumitomo Seika (2004a). Unpublished Data.

Sumitomo Seika (2004b). Unpublished Data.

The Environment Agency, Japan (1999a). Acute Swimming Inhibition Test of Sulfolane in Water Fleas (Daphnia magna). Chemicals Evaluation and Research Institute (CERI, Japan) Test No. 92058.

The Environment Agency, Japan (1999b). Acute Toxicity Test of Sulfolane in killifish (*Oryzias latipes*). Chemicals Evaluation and Research Institute (CERI, Japan) Test No. 92060.

The Environment Agency, Japan (1999c). Growth Inhibition Study of Sulfolane in Algae (*Selenastrum capricornutum*). Chemicals Evaluation and Research Institute (CERI, Japan) Test No. 92057.

The Environment Agency, Japan (1999d). Reproduction Test of Sulfolane in Water fleas *(Daphnia magna*). Chemicals Evaluation and Research Institute (CERI, Japan) Test No. 92059.

Thorpe E (1982). Toxicity Studies with Fine Chemicals: *In Vitro* Genotoxicity Studies with Sulfolane. Shell Research Limited Report No. SBTR.81.098.

4-クロロ-o-クレゾ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 4-Chloro-o-cresol in Rats

要約

消毒剤や除草剤の合成原料等に用いられている既存化学物質，4-クロロ-o-クレゾールの28日間反復経口投与毒性試験をSD系[Crj:CD(SD)]ラットを用い，0(対照)，15，60，250および1000 mg/kg/day用量の投与により実施した．動物数は1群雌雄各5匹とし，7群を設け，5群は投与期間終了時屠殺群，2群は対照および1000 mg/kgの14日間回復群とした．

15および60 mg/kg群では，被験物質の投与に起因する変化は認められなかった．250 mg/kg群では，膀胱粘膜に上皮の過形成が雌雄に，前胃粘膜に扁平上皮の過形成が雄に認められた．また，雄の血清総ビリルビンおよび雌の腎臓相対重量は増加した．1000 mg/kg群では，250 mg/kg群で認められた変化に加えて，前胃粘膜の扁平上皮過形成が雌にも認められた．さらに，自発運動低下，深大呼吸，筋の弛緩，腹臥姿勢，流涎などの一般状態の変化および盲腸の拡張が雌雄に，体重増加抑制，血清GPTの増加，副腎皮質細胞の空砲化が雄に，血清コリンエステラーゼの減少，肝細胞の肥大が雌に認められ，雄1匹と雌3匹は死亡した．これらの変化は，回復群においては回復あるいは回復傾向を示し，可逆的であることが確認された．

以上の結果から，4-クロロ-o-クレゾールは，ラットへの28日間反復経口投与により，主な毒性影響として，投与経路である消化管，特に胃，排泄経路である膀胱，および肝臓に変化が認められ，腎臓および副腎に対する影響も認められた．無影響量は，雌雄とも60 mg/kg/dayと推定された．

方法

1. 被験物質

4-クロロ-o-クレゾールは，分子量142.60，融点43-46℃の水に溶けにくく，エタノール，エーテルには溶け易いフェノール様の臭いのする褐色がかった結晶で，東京化成工業㈱製造の試薬［ロット番号FBY01，純度93.6％(不純物として異性体3.3％，クレゾール1.5％等を含む)］を入手し，冷暗所(4℃)で密栓保管し，使用した．投与液は，局方ゴマ油(宮澤薬品)を用い，純度換算で所定の投与用量になるような濃度の溶液として調製し，使用時まで冷所遮光下で密栓保管した．被験物質の原体および投与液中の被験物質は，安定であることを確認した．

2. 使用動物および飼育条件

日本チャールス・リバー㈱より搬入したSD系[Crj:CD(SD)]ラットを，雄は6日，雌は7日間検疫・馴化飼育し，5週齢(雄145-159 g，雌133-147 g)で，1群雌雄各5匹として試験に用いた．ラットは，温度22±3℃，湿度55±10％，換気回数10回以上/時，照明12時間(6-18時)に設定された飼育室で，金網ケージに個別に収容し，固型飼料［日本農産工業㈱，ラボMRストック］および水を自由に摂取させた．

3. 投与量および投与方法

4-クロロ-o-クレゾールの単回経口投与におけるLD_{50}値は，1194 mg/kgと報告されている[1]．投与量設定試験を，ラットを1群雌雄各4匹とし，0，10，30，100，300および1000 mg/kg/day用量の14日間反復経口投与により実施した．剖検で，前胃粘膜の肥厚が300 mg/kg以上の群の雌雄に認められた．1000 mg/kg群では，自発運動低下，流涎，深大呼吸，筋弛緩，腹臥姿勢などの症状，体重増加の抑制，摂餌量の減少傾向，肝臓重量の増加が雌雄に認められた．尿検査，血液学検査，血液生化学検査では，明らかな変化は認められなかった．したがって，本試験における投与量は，1000 mg/kg/dayを最高用量とし，以下250，60，15 mg/kg/dayの4用量および対照を設定した．試験群は，以上の5群の他に，1000 mg/kgおよび対照の14日間回復群を設けた．投与は，胃ゾンデを装着した注射筒を用いて，投与液を1日1回，28日間にわたって経口投与した．投与液量は，体重100 g当たり0.5 mlとした．対照群には局方ゴマ油を同様に投与した．

4. 観察および検査項目

1) 一般状態観察

投与および回復期間中毎日，生死および外観，行動等を観察した．

2) 体重および摂餌量測定

体重は，投与1日(投与初日の投与直前)，3日およびその後は週2回，3あるいは4日ごと，ならびに屠殺日あるいは死亡発見日に測定した．摂餌量は，ケージごとに週1回(雄は投与3，10，17，24日および投与終了後3，10日，雌は投与2，9，16，23日および投与終了後2，9日)，翌日までの24時間の飼料消費量を測定した．

3) 尿検査

投与27日および投与終了後10日にラットを代謝ケージに約3時間収容して採尿し，pH，潜血，タンパク，糖，ケトン体，ビリルビン，ウロビリノーゲン［以上，マルティスティックス，マイルス・三共㈱］，外観および沈渣(URI-CEL液で染色，ケンブリッジケミカルプロダクト社)を検査した．

4) 血液学検査

供試血液の採取は，投与期間および回復期間終了翌日における屠殺剖検時に行った．動物は採血前日の午後5時より除餌し，水のみを給与した．採取した血液は3分割し，その一部はEDTA-2Kで凝固防止処理し，多項目自動血球計数装置［東亜医用電子㈱，E-4000］により，赤血球数(電気抵抗検出方式)，血色素量(ラウリル硫酸ナトリウム-ヘモグロビン法)，ヘマトクリット値(パルス検出方式)，平均赤血球容積，平均赤血球血色素量，平均赤血球血色素濃度(以上，計算値)，白血球数および血小板数(以上，電気抵抗検出方式)を，また塗抹標本を作製して網状赤血球数(Brilliant cresyl blue染色)および白血球百分率(May-Giemsa染色)を測定した．さらに一部は3.8％クエン酸ナトリウム液で処理して血漿を得，血液凝固自動測定装置(アメルング社，KC-10A)により，プロトロンビン時間(Quick一段法)および活性化部分トロンボプラスチン時間(エラジン酸活性化法)を測定した．

5) 血液生化学検査

採取した血液の一部から血清を分離し，生化学自動分析装置［日本電子㈱，JCA-VX-1000型クリナライザー］により，総タンパク(Biuret法)，アルブミン(BCG)，A/G比(計算値)，血糖，トリグリセライド，総コレステロール(以上，酵素法)，総ビリルビン(Jendrassik法)，尿素窒素(Urease-UV法)，クレアチニン(Jaffé法)，GOT，GPT，γ-GTP，LDH(以上，SSCC法)，アルカリホスファターゼ(GSCC法)，コリンエステラーゼ(BTC-DTNB法)，カルシウム(OCPC法)および無機リン(酵素法)を，電解質自動分析装置［東亜電波工業㈱，NAKL-1］により，ナトリウム，カリウムおよび塩素を測定した．

6) 病理学検査

死亡動物は発見後速やかに，計画屠殺動物は所定の投与期間あるいは回復期間終了翌日の採血に続いて放血屠殺し，剖検した．また，脳，心臓，胸腺，肝臓，腎臓，脾臓，副腎，精巣，卵巣を秤量した．病理組織学検査は，採取した器官を10％中性リン酸緩衝ホルマリン液で固定後，対照群および1000 mg/kg群では脳，下垂体，眼球，甲状腺(上皮小体を含む)，胸腺，心臓，肺，肝臓，腎臓，脾臓，副腎，胃，小腸(十二指腸・空腸・回腸)，大腸(盲腸・結腸・直腸)，膵臓，精巣，卵巣，膀胱，骨髄について，15，60および250 mg/kg群ならびに回復群では，毒性影響がうかがわれた雄では胃，副腎，膀胱，雌では肝臓，胃，膀胱について，常法によりパラフィン切片を作製し，ヘマトキシリン・エオジン染色を施して鏡検した．

5. 統計処理

得られた平均値あるいは頻度について，Dunnettあるいは Scheffé(群の大きさが異なる場合)の多重比較検定を行った．ただし，回復群については，t検定およびU検定を行った．

結果

1. 一般状態および死亡

死亡については，1000 mg/kgにおいて，回復群の5匹を含む雌雄各10匹中雄の1匹(投与11日，回復群)および雌の3匹(投与7，16および22日，2匹は回復群)が死亡した．一般状態については，1000 mg/kg群の雌雄で，自発運動低下，筋弛緩およびそれによると思われる無力性の歩行異常，腹臥姿勢，深大呼吸，流涎がほぼ全例に認められた．これらの症状は毎日の投与直後から概ね1～3時間認められたが，翌日の投与時には回復していた．さらに，1000 mg/kg群では，立毛，腹部膨満，眼瞼下垂，削痩が雌雄に，下腹部被毛の尿による汚染が雌に，いずれも低い頻度で認められた．回復期間においては，回復群に異常は認められなかった．

2. 体重(Fig. 1)

1000 mg/kg群の雄で体重増加の抑制が認められ，投与7日以降の体重に対照群との間に有意差が認められた．250 mg/kg群の雄および1000 mg/kg群の雌にも体重増加の抑制傾向がうかがわれたが，有意な変化ではなかった．1000 mg/kgの回復群においては，雄で投与終了後3日までは有意差が残るものの，それ以降は対照群に比べ有意差は認められなくなった．

3. 摂餌量

各群の雌雄とも，有意な変化は認められなかった．1000 mg/kg群の雄の摂餌量は，投与1および2週において対照群に比べてやや少なかったが，統計学的有意差は認められなかった．

4. 尿所見

タンパクおよびケトン体濃度の減少が1000 mg/kg群の雄に認められたが，毒性学的に有意と思われる変化は，雌雄とも認められなかった．

5. 血液学所見(Table 1，2)

各群の雌雄とも，各検査項目において，統計学的に有意な変化は認められなかった．

6. 血液生化学所見(Table 3，4)

有意な総ビリルビンの増加が250 mg/kg以上の群の雄に用量依存的に，GPTの増加が1000 mg/kg群の雄に，

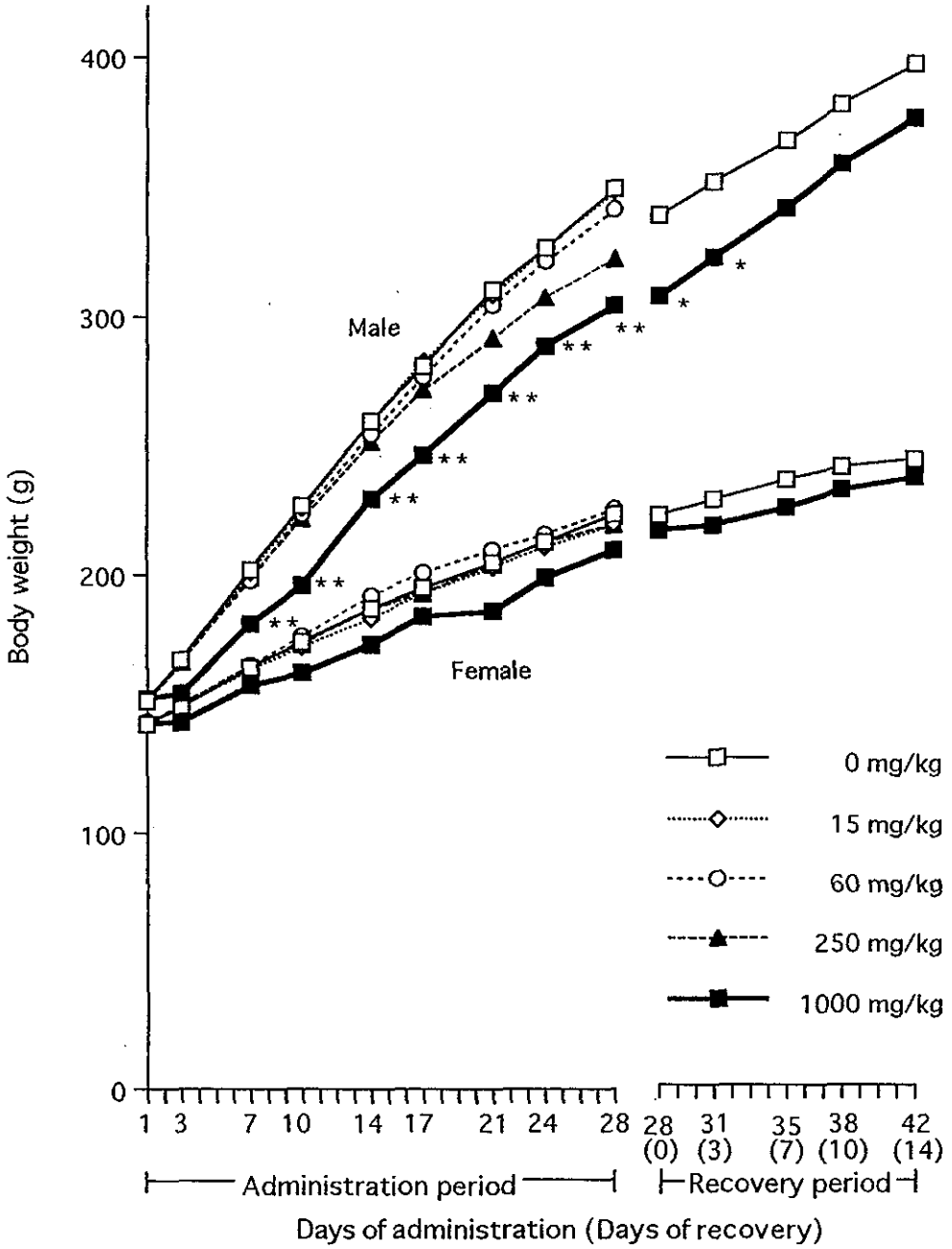


Fig. 1 Body weight changes in rats treated orally with 4-chloro-o-cresol in the 28-day repeat dose toxicity test
Significantly different from control group (*: $p<0.05$; **: $p<0.01$)

コリンエステラーゼの減少が1000 mg/kg群の雌に認められた．1000 mg/kgの回復群においては各検査項目に有意な変化は認められず，投与期間終了時屠殺動物で認められた変化は回復していた．

7. 剖検所見

投与期間終了時生存動物においては，1000 mg/kg群で前胃粘膜の軽度～中等度な肥厚が雄5匹，雌4匹の全例に認められ，雌雄各1匹は表面が粗造であった．また，盲腸の軽度な拡張が雄4匹，雌1匹に認められた．投与期間中に死亡した1000 mg/kg群の雄1匹および雌3匹では，胃および腸のガス貯留による膨満が共通した所見で，特に雄と雌の2匹では風船様の外観を呈する重度な変化であった．さらに，前胃に粘膜の肥厚，表面粗造あるいは褐色点散在，胸腺，脾臓あるいは雄の副生殖腺の萎縮などが認められた．回復群においては，異常は認められなかった．

8. 器官重量(Table 5, 6)

雄においては，剖検直前の体重が対照群に比べて250 mg/kg群は12%，1000 mg/kg群は19%少なく，それに伴って両群の肝臓，腎臓，心臓および1000 mg/kg群の脾臓は絶対重量が減少する傾向にあり，1000 mg/kg群の心臓重量は有意に減少した．しかし，これらの器官の

Table 1 Hematological examination in male rats after the oral administration of 4-chloro-*o*-cresol for 28 days and a recovery period for 14 days

	After administration period					After recovery period	
Dose level (mg/kg)	0	15	60	250	1000	0	1000
No. of animals	5	5	5	5	5	5	4
Erythrocyte (10^4/mm^3)	740 ± 38	758 ± 34	773 ± 26	779 ± 35	790 ± 38	812 ± 53	763 ± 22
Hemoglobin (g/dl)	15.5 ± 0.5	15.4 ± 1.1	15.3 ± 0.3	15.7 ± 0.4	16.3 ± 0.5	15.6 ± 0.6	14.7 ± 0.4
Hematocrit (%)	43.9 ± 1.5	44.2 ± 2.4	44.0 ± 0.8	45.1 ± 1.2	45.6 ± 1.4	45.2 ± 1.6	43.1 ± 0.8
MCV (fl)	60 ± 2	59 ± 1	57 ± 2	58 ± 2	58 ± 1	56 ± 2	57 ± 1
MCH (pg)	21.0 ± 0.5	20.3 ± 0.7	19.8 ± 0.7	20.3 ± 1.1	20.7 ± 0.8	19.2 ± 0.7	19.3 ± 0.6
MCHC (%)	35.4 ± 0.2	34.8 ± 0.8	34.8 ± 0.5	34.9 ± 0.8	35.8 ± 0.9	34.4 ± 0.4	34.2 ± 0.6
Reticulocyte (‰)	29 ± 9	31 ± 8	27 ± 6	27 ± 3	32 ± 8	26 ± 8	30 ± 7
PT (sec)	12.8 ± 0.2	12.9 ± 0.3	12.7 ± 0.3	13.0 ± 0.3	13.1 ± 0.5	13.2 ± 0.6	13.1 ± 0.4
APTT (sec)	16.8 ± 0.8	16.3 ± 0.4	16.8 ± 0.7	17.1 ± 0.5	17.3 ± 1.0	16.9 ± 1.2	17.3 ± 0.7
Leukocyte (10^2/mm^3)	70 ± 16	80 ± 23	82 ± 37	77 ± 21	90 ± 14	74 ± 20	77 ± 27
Differential count (%)							
Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Eosinophil	2 ± 1	0 ± 1	0 ± 1	0 ± 1	0 ± 1	0 ± 1	0 ± 1
Neutrophil band	0 ± 0	0 ± 0	1 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
segmented	15 ± 5	20 ± 13	16 ± 8	13 ± 4	12 ± 5	17 ± 6	13 ± 5
Lymphocyte	82 ± 7	79 ± 12	81 ± 9	86 ± 4	86 ± 5	82 ± 6	86 ± 5
Monocyte	1 ± 2	1 ± 1	2 ± 2	0 ± 1	1 ± 1	1 ± 1	1 ± 1
Platelet (10^4/mm^3)	160 ± 12	159 ± 17	152 ± 17	140 ± 19	147 ± 12	141 ± 13	140 ± 14

Values are expressed as Mean ± S.D.

Table 2 Hematological examination in female rats after the oral administration of 4-chloro-*o*-cresol for 28 days and a recovery period for 14 days

	After administration period					After recovery period	
Dose level (mg/kg)	0	15	60	250	1000	0	1000
No. of animals	5	5	5	5	4	5	3
Erythrocyte (10^4/mm^3)	771 ± 25	767 ± 26	756 ± 38	763 ± 37	783 ± 31	793 ± 28	762 ± 66
Hemoglobin (g/dl)	15.7 ± 0.6	15.7 ± 0.6	15.3 ± 0.5	15.2 ± 0.6	15.5 ± 0.1	15.4 ± 0.3	15.0 ± 1.0
Hematocrit (%)	43.4 ± 1.8	43.8 ± 1.6	43.0 ± 1.4	42.7 ± 1.4	43.5 ± 0.5	44.1 ± 0.8	42.6 ± 2.8
MCV (fl)	56 ± 1	57 ± 1	57 ± 2	56 ± 1	56 ± 2	56 ± 1	56 ± 1
MCH (pg)	20.4 ± 0.4	20.5 ± 0.4	20.3 ± 0.8	20.0 ± 0.4	19.8 ± 0.6	19.4 ± 0.4	19.7 ± 0.6
MCHC (%)	36.2 ± 0.7	35.9 ± 0.4	35.6 ± 0.2	35.7 ± 0.6	35.7 ± 0.1	34.9 ± 0.2	35.2 ± 0.2
Reticulocyte (‰)	20 ± 6	20 ± 7	21 ± 5	23 ± 5	22 ± 11	25 ± 6	26 ± 2
PT (sec)	13.2 ± 0.4	13.0 ± 0.1	13.2 ± 0.2	13.3 ± 0.1	13.2 ± 0.4	13.2 ± 0.2	13.2 ± 0.4
APTT (sec)	16.9 ± 1.7	16.0 ± 0.9	17.1 ± 0.4	16.4 ± 0.8	17.7 ± 0.4	15.7 ± 0.3	16.7 ± 1.0
Leukocyte (10^2/mm^3)	42 ± 14	45 ± 13	38 ± 7	43 ± 15	37 ± 8	49 ± 8	47 ± 9
Differential count (%)							
Basophil	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Eosinophil	2 ± 1	2 ± 2	2 ± 1	1 ± 1	0 ± 0	1 ± 2	2 ± 3
Neutrophil band	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
segmented	14 ± 6	15 ± 7	12 ± 3	16 ± 4	14 ± 11	10 ± 2	10 ± 3
Lymphocyte	82 ± 5	82 ± 8	86 ± 4	83 ± 4	86 ± 11	87 ± 3	87 ± 6
Monocyte	1 ± 1	1 ± 0	1 ± 1	1 ± 1	1 ± 1	2 ± 1	1 ± 1
Platelet (10^1/mm^3)	135 ± 17	126 ± 8	152 ± 15	138 ± 11	145 ± 6	138 ± 8	134 ± 26

Values are expressed as Mean ± S.D.

Table 3 Blood chemical examination in male rats after the oral administration of 4-chloro-*o*-cresol for 28 days and a recovery period for 14 days

	After administration period					After recovery period	
Dose level (mg/kg)	0	15	60	250	1000	0	1000
No. of animals	5	5	5	5	5	5	4
LDH (IU/l)	295 ± 105	278 ± 112	386 ± 287	268 ± 24	258 ± 57	284 ± 117	218 ± 15
GOT (IU/l)	61 ± 7	61 ± 10	59 ± 6	64 ± 5	62 ± 9	61 ± 9	61 ± 2
GPT (IU/l)	29 ± 3	29 ± 7	31 ± 2	35 ± 6	41 ± 6**	30 ± 2	30 ± 4
ALP (IU/l)	431 ± 103	406 ± 86	558 ± 185	451 ± 117	552 ± 91	354 ± 18	325 ± 49
γ-GTP (IU/l)	0.26 ± 0.19	0.39 ± 0.34	0.19 ± 0.08	0.36 ± 0.25	0.38 ± 0.25	0.58 ± 0.22	0.34 ± 0.37
ChE (IU/l)	54 ± 28	32 ± 8	33 ± 15	36 ± 18	40 ± 18	24 ± 9	38 ± 11
T.protein (g/dl)	6.01 ± 0.18	5.98 ± 0.29	6.05 ± 0.24	6.03 ± 0.13	5.98 ± 0.23	6.29 ± 0.12	6.13 ± 0.16
Albumin (g/dl)	3.05 ± 0.11	2.87 ± 0.35	2.95 ± 0.10	3.03 ± 0.14	3.03 ± 0.20	2.98 ± 0.25	2.99 ± 0.16
A/G ratio	1.03 ± 0.07	0.94 ± 0.20	0.95 ± 0.07	1.02 ± 0.13	1.04 ± 0.12	0.91 ± 0.12	0.95 ± 0.06
T.cholesterol (mg/dl)	92 ± 14	82 ± 16	83 ± 11	90 ± 24	90 ± 20	96 ± 19	89 ± 10
Triglyceride (mg/dl)	94 ± 19	56 ± 8	103 ± 39	125 ± 54	85 ± 57	100 ± 35	73 ± 33
Glucose (mg/dl)	142 ± 15	160 ± 26	141 ± 11	139 ± 7	144 ± 13	162 ± 26	145 ± 4
T.bilirubin (mg/dl)	0.33 ± 0.02	0.30 ± 0.03	0.35 ± 0.02	0.38 ± 0.01*	0.41 ± 0.04**	0.29 ± 0.02	0.30 ± 0.04
Urea nitrogen (mg/dl)	14.6 ± 1.7	14.3 ± 1.3	13.8 ± 2.0	13.3 ± 1.7	15.4 ± 1.4	16.8 ± 3.6	16.5 ± 0.1
Creatinine (mg/dl)	0.54 ± 0.02	0.52 ± 0.07	0.50 ± 0.02	0.49 ± 0.03	0.52 ± 0.03	0.54 ± 0.04	0.53 ± 0.04
Ca (mg/dl)	10.0 ± 0.1	9.9 ± 0.4	10.0 ± 0.3	10.1 ± 0.3	10.4 ± 0.3	10.1 ± 0.2	10.2 ± 0.1
I. phosphorus (mg/dl)	8.5 ± 0.3	8.8 ± 0.5	8.9 ± 0.3	8.4 ± 1.0	8.9 ± 0.3	7.5 ± 0.6	7.7 ± 0.2
Na (mEq/l)	140 ± 1	141 ± 1	141 ± 1	140 ± 1	141 ± 1	141 ± 1	140 ± 1
K (mEq/l)	4.59 ± 0.18	4.80 ± 0.37	4.72 ± 0.09	4.68 ± 0.11	4.48 ± 0.39	4.74 ± 0.27	4.79 ± 0.32
Cl (mEq/l)	104 ± 1	104 ± 1	105 ± 1	105 ± 2	103 ± 1	105 ± 1	104 ± 1

Vales are expressed as Mean ± S.D.
Significantly different from control group (*: $p<0.05$; **: $p<0.01$)

Table 4 Blood chemical examination in female rats after the oral administration of 4-chloro-*o*-cresol for 28 days and a recovery period for 14 days

	After administration period					After recovery period	
Dose level (mg/kg)	0	15	60	250	1000	0	1000
No. of animals	5	5	5	5	4	5	3
LDH (IU/l)	501 ± 267	343 ± 197	399 ± 210	332 ± 99	274 ± 98	279 ± 132	198 ± 42
GOT (IU/l)	63 ± 6	63 ± 9	62 ± 8	68 ± 10	61 ± 6	57 ± 5	58 ± 5
GPT (IU/l)	22 ± 4	25 ± 6	27 ± 3	26 ± 5	28 ± 3	24 ± 2	25 ± 3
ALP (IU/l)	269 ± 44	270 ± 78	366 ± 90	339 ± 52	333 ± 67	192 ± 38	202 ± 45
γ-GTP (IU/l)	0.54 ± 0.37	0.40 ± 0.15	0.44 ± 0.38	0.42 ± 0.20	0.33 ± 0.18	0.43 ± 0.31	0.25 ± 0.22
ChE (IU/l)	263 ± 34	230 ± 69	267 ± 51	219 ± 33	131 ± 42*	337 ± 149	325 ± 85
T.protein (g/dl)	6.45 ± 0.14	6.45 ± 0.15	6.37 ± 0.27	6.23 ± 0.22	6.25 ± 0.14	6.77 ± 0.24	6.69 ± 0.07
Albumin (g/dl)	3.32 ± 0.12	3.26 ± 0.17	3.20 ± 0.30	3.10 ± 0.10	3.14 ± 0.22	3.49 ± 0.29	3.40 ± 0.09
A/G ratio	1.06 ± 0.07	1.03 ± 0.13	1.02 ± 0.12	0.99 ± 0.06	1.02 ± 0.13	1.07 ± 0.11	1.03 ± 0.06
T.cholesterol (mg/dl)	85 ± 28	108 ± 14	93 ± 19	91 ± 28	103 ± 14	101 ± 11	102 ± 7
Triglyceride (mg/dl)	27 ± 5	55 ± 16	42 ± 24	38 ± 16	39 ± 19	45 ± 14	61 ± 19
Glucose (mg/dl)	129 ± 12	139 ± 29	124 ± 12	130 ± 12	129 ± 7	143 ± 12	134 ± 6
T.bilirubin (mg/dl)	0.25 ± 0.02	0.22 ± 0.03	0.24 ± 0.03	0.21 ± 0.02	0.26 ± 0.02	0.25 ± 0.01	0.24 ± 0.01
Urea nitrogen (mg/dl)	18.7 ± 2.0	16.5 ± 2.0	18.7 ± 2.9	15.6 ± 2.2	18.2 ± 2.3	19.5 ± 0.6	21.3 ± 3.5
Creatinine (mg/dl)	0.59 ± 0.07	0.50 ± 0.04	0.53 ± 0.07	0.51 ± 0.04	0.50 ± 0.07	0.61 ± 0.03	0.63 ± 0.05
Ca (mg/dl)	10.0 ± 0.4	10.1 ± 0.1	9.9 ± 0.2	9.6 ± 0.2	10.0 ± 0.3	10.1 ± 0.3	10.2 ± 0.1
I. phosphorus (mg/dl)	6.7 ± 0.9	6.7 ± 0.4	6.3 ± 0.6	6.4 ± 1.0	7.0 ± 1.4	6.0 ± 0.6	6.0 ± 0.2
Na (mEq/l)	142 ± 1	142 ± 1	141 ± 1	141 ± 1	140 ± 2	142 ± 1	142 ± 0
K (mEq/l)	4.32 ± 0.18	4.41 ± 0.18	4.51 ± 0.21	4.39 ± 0.20	4.30 ± 0.15	4.73 ± 0.24	4.56 ± 0.22
Cl (mEq/l)	107 ± 1	106 ± 2	106 ± 1	106 ± 1	104 ± 2	105 ± 2	105 ± 1

Vales are expressed as Mean ± S.D.
Significantly different from control group (*: $p<0.05$)

Table 5 Absolute and relative organ weights in male rats after the oral administration of 4-chloro-*o*-cresol for 28 days and a recovery period for 14 days

	After administration period					After recovery period	
Dose level (mg/kg)	0	15	60	250	1000	0	1000
No. of animals	5	5	5	5	5	5	4
Body weight (g)	339 ± 13	321 ± 30	319 ± 15	300 ± 11**	274 ± 13**	366 ± 11	348 ± 28
Absolute weight							
Brain (g)	1.96 ± 0.05	1.91 ± 0.10	1.96 ± 0.08	1.94 ± 0.06	1.91 ± 0.05	1.99 ± 0.06	1.90 ± 0.06
Liver (g)	10.24 ± 0.66	10.67 ± 1.70	10.36 ± 0.80	9.72 ± 0.84	9.32 ± 1.00	10.55 ± 0.73	10.16 ± 0.85
Kidneys (g)	2.39 ± 0.16	2.54 ± 0.35	2.44 ± 0.12	2.29 ± 0.23	2.17 ± 0.17	2.55 ± 0.16	2.58 ± 0.23
Spleen (g)	0.62 ± 0.04	0.64 ± 0.09	0.59 ± 0.12	0.62 ± 0.15	0.53 ± 0.05	0.66 ± 0.08	0.64 ± 0.07
Heart (g)	1.10 ± 0.06	1.17 ± 0.14	1.15 ± 0.10	1.03 ± 0.06	0.93 ± 0.10*	1.19 ± 0.11	1.24 ± 0.17
Thymus (g)	0.63 ± 0.09	0.54 ± 0.04	0.58 ± 0.09	0.51 ± 0.09	0.57 ± 0.09	0.45 ± 0.16	0.47 ± 0.03
Adrenals (mg)	46.8 ± 5.8	54.8 ± 3.9	50.1 ± 4.7	45.9 ± 6.2	51.9 ± 5.3	53.8 ± 5.5	55.6 ± 7.8
Testes (g)	2.91 ± 0.25	2.97 ± 0.36	3.02 ± 0.06	2.98 ± 0.15	2.88 ± 0.05	3.26 ± 0.29	3.19 ± 0.20
Relative weight							
Brain (g%)	0.58 ± 0.04	0.60 ± 0.04	0.62 ± 0.01	0.65 ± 0.02**	0.70 ± 0.02**	0.55 ± 0.03	0.55 ± 0.03
Liver (g%)	3.02 ± 0.25	3.30 ± 0.26	3.25 ± 0.20	3.24 ± 0.20	3.40 ± 0.28	2.88 ± 0.16	2.92 ± 0.06
Kidneys (g%)	0.70 ± 0.03	0.79 ± 0.08	0.76 ± 0.05	0.76 ± 0.06	0.79 ± 0.05	0.70 ± 0.03	0.75 ± 0.05
Spleen (g%)	0.18 ± 0.02	0.20 ± 0.03	0.18 ± 0.03	0.21 ± 0.04	0.19 ± 0.01	0.18 ± 0.02	0.18 ± 0.01
Heart (g%)	0.32 ± 0.03	0.37 ± 0.02	0.36 ± 0.02	0.34 ± 0.03	0.34 ± 0.04	0.33 ± 0.03	0.36 ± 0.02
Thymus (g%)	0.18 ± 0.03	0.17 ± 0.02	0.18 ± 0.03	0.17 ± 0.02	0.20 ± 0.03	0.12 ± 0.05	0.14 ± 0.01
Adrenals (mg%)	13.77 ± 1.31	17.11 ± 1.13*	15.77 ± 2.18	15.29 ± 1.92	18.99 ± 2.26**	14.71 ± 1.47	16.13 ± 2.81
Testes (g%)	0.86 ± 0.07	0.93 ± 0.11	0.95 ± 0.03	0.99 ± 0.05*	1.05 ± 0.04**	0.89 ± 0.06	0.93 ± 0.13

Values are expressed as Mean ± S.D.
Significantly different from control group (*: $p<0.05$, **: $p<0.01$)

Table 6 Absolute and relative organ weights in female rats after the oral administration of 4-chloro-*o*-cresol for 28 days and a recovery period for 14 days

	After administration period					After recovery period	
Dose level (mg/kg)	0	15	60	250	1000	0	600
No. of animals	5	5	5	5	4	5	3
Body weight (g)	206 ± 15	205 ± 12	209 ± 14	202 ± 10	191 ± 15	224 ± 10	217 ± 6
Absolute weight							
Brain (g)	1.87 ± 0.11	1.80 ± 0.05	1.89 ± 0.07	1.91 ± 0.08	1.78 ± 0.05	1.84 ± 0.03	1.78 ± 0.04*
Liver (g)	6.42 ± 0.90	6.36 ± 0.68	6.29 ± 0.58	6.45 ± 0.44	6.76 ± 0.79	6.25 ± 0.51	6.40 ± 0.30
Kidneys (g)	1.54 ± 0.13	1.62 ± 0.05	1.61 ± 0.07	1.68 ± 0.13	1.63 ± 0.11	1.67 ± 0.09	1.63 ± 0.15
Spleen (g)	0.45 ± 0.08	0.44 ± 0.02	0.44 ± 0.05	0.43 ± 0.05	0.42 ± 0.05	0.47 ± 0.04	0.44 ± 0.04
Heart (g)	0.74 ± 0.08	0.76 ± 0.04	0.80 ± 0.07	0.76 ± 0.03	0.70 ± 0.04	0.82 ± 0.05	0.77 ± 0.05
Thymus (g)	0.48 ± 0.10	0.45 ± 0.04	0.46 ± 0.04	0.47 ± 0.09	0.37 ± 0.04	0.42 ± 0.07	0.38 ± 0.01
Adrenals (mg)	58.2 ± 9.4	59.8 ± 7.8	60.5 ± 8.9	65.2 ± 10.4	52.0 ± 4.9	62.4 ± 9.0	56.0 ± 2.6
Ovaries (mg)	92.5 ± 19.5	83.5 ± 11.6	82.6 ± 22.3	83.8 ± 7.5	75.6 ± 6.9	82.3 ± 11.5	81.9 ± 11.4
Relative weight							
Brain (g%)	0.91 ± 0.03	0.88 ± 0.07	0.91 ± 0.05	0.94 ± 0.05	0.94 ± 0.09	0.82 ± 0.04	0.82 ± 0.01
Liver (g%)	3.10 ± 0.27	3.10 ± 0.21	3.01 ± 0.18	3.18 ± 0.11	3.55 ± 0.19*	2.78 ± 0.14	2.95 ± 0.12
Kidneys (g%)	0.75 ± 0.02	0.79 ± 0.04	0.77 ± 0.04	0.83 ± 0.04**	0.86 ± 0.03	0.74 ± 0.03	0.75 ± 0.05
Spleen (g%)	0.22 ± 0.03	0.22 ± 0.02	0.21 ± 0.02	0.21 ± 0.02	0.22 ± 0.02	0.21 ± 0.02	0.20 ± 0.02
Heart (g%)	0.36 ± 0.02	0.37 ± 0.03	0.38 ± 0.03	0.38 ± 0.02	0.37 ± 0.02	0.37 ± 0.03	0.35 ± 0.02
Thymus (g%)	0.23 ± 0.05	0.22 ± 0.01	0.22 ± 0.01	0.23 ± 0.04	0.19 ± 0.03	0.19 ± 0.03	0.17 ± 0.01
Adrenals (mg%)	28.16 ± 3.86	29.30 ± 4.24	28.97 ± 3.73	32.09 ± 3.54	27.26 ± 0.95	27.77 ± 3.07	25.88 ± 1.53
Ovaries (mg%)	44.5 ± 7.1	40.9 ± 6.2	39.1 ± 9.0	41.4 ± 2.5	39.9 ± 5.1	36.7 ± 4.8	37.9 ± 6.1

Values are expressed as Mean ± S.D.
Significantly different from control group (*: $p<0.05$, **: $p<0.01$)

相対重量には有意な変化は認められなかった．250 mg/kgおよび1000 mg/kg群の脳および精巣は，絶対重量に変化は認められなかったが，相対重量では有意に増加した．また，副腎は，1000 mg/kg群で相対重量の有意な増加が認められた．15 mg/kg群においても有意な副腎の相対重量増加が認められたが，変化は軽度で，60および250 mg/kg群では有意な変化は認められず，用量依存的でなかった．一方，雌においては，体重に有意な変化は認められなかったが，肝臓相対重量の有意な増加が1000 mg/kg群，腎臓相対重量の有意な増加が250および1000 mg/kg群に認められた．1000 mg/kgの回復群においては，これらの変化は認められず回復していた．以上の変化とは別に，1000 mg/kgの回復群の雌の脳絶対重量が対照群の平均値1.84 gに対し1.78 gとわずかに下回り，有意差が認められたが，これは両群とも測定値のバラツキが極めて小さかったためで，その差は約3％のわずかなものであった．

9. 病理組織学所見(Table 7, 8)

被験物質の投与に起因すると考えられる変化が，胃，膀胱，肝臓および副腎に認められた．投与期間終了後屠殺動物において，胃では，前胃の粘膜に扁平上皮の過形成が，250 mg/kg群の雄1匹および1000 mg/kg群の雌雄全例に認められた．膀胱では，上皮の過形成が，250および1000 mg/kg群の雌雄の半数以上ないし全例に認められた．以上の変化に加えて，1000 mg/kg群で，小葉中心性の軽度な肝細胞の肥大が雌に，副腎皮質細胞の軽度な空胞化が雄に，いずれも約半数の動物で認められた．1000 mg/kg群の投与期間中死亡した雄1匹および雌3匹においては，投与期間終了後屠殺動物で認められた変化のほか，雄の例の肝臓には肝細胞の単細胞壊死，雌の全例の前胃には粘膜上皮の変性および1例に潰瘍が認められた．また，雌雄とも全身諸器官に萎縮性の変化が認められた．

回復期間終了後屠殺動物においては，雌雄とも前胃および膀胱の変化は認められたが，投与期間終了後屠殺動物に比べて軽減しており，また雄の副腎および雌の肝臓には異常は認められなかった．

以上の所見のほかにも，検査した各器官に異常が認められたが，いずれも散発的あるいは用量相関性のない変化で，自然発生病変と考えられる所見であった．

考察

クロロクレゾールの毒性について，4-クロロ-m-クレゾールについては，ラットを用いた28日間の反復経口投与毒性試験の結果が報告されており，400 mg/kgで体重の有意な増加抑制が認められるが，一般状態，血液学検査，血液生化学検査および病理学検査では異常は認められず，NOELは200 mg/kgと推定されている[2]．

今回実施した，4-クロロ-o-クレゾールのラットを用いた28日間の反復経口投与毒性試験では，消化管，特に胃および膀胱の粘膜，肝臓，腎臓，副腎などに変化が発現した．

消化管に対する影響としては，前胃粘膜に扁平上皮の過形成が，250 mg/kg群の雄および1000 mg/kg群の雌雄に認められた．投与期間中の死亡動物においては，粘膜上皮の変性および潰瘍を認める例があった．また，盲腸の拡張が1000 mg/kg群の雌雄に認められ，死亡動物では胃および腸がガスで膨満していた．

o-クレゾールには腐食性があり[3]，4-クロロ-o-クレゾールについても，刺激性が強いことが知られている[4]．さらに，4-クロロ-o-クレゾールは殺菌剤として用いられている物質で，これの経口投与により腸内細菌叢に対し影響し，盲腸などの拡張を引き起こすことは十分考えられる．したがって，消化管に対する影響は，主に本被験物質の局所刺激性および殺菌作用が関与して発現したものと考えられる[5,6]．

クロロクレゾールの代謝について，大部分がグルクロン酸および硫酸抱合され，尿中排泄されることが，4-クロロ-m-クレゾールにおいて確認されている[7]．

膀胱粘膜にも上皮の過形成が250および1000 mg/kg群の雌雄に認められた．この変化は排泄器官である腎臓を経て濃縮された尿中の主に代謝物の，膀胱粘膜への直接的な作用に対する組織の反応性増殖と解せられる．

なお，腎臓においては，相対重量の増加が250および1000 mg/kg群の雌に認められたが，病理組織学的には著変は認められず，影響としては，軽度なものと考えられる．

肝臓に対する影響については，肝細胞の肥大および肝臓相対重量の増加が1000 mg/kg群の雌に認められた．雄の血清GPTおよび総ビリルビンの増加，雌のコリンエステラーゼの減少も，肝機能の異常を示唆する変化と考えられる．

4-クロロ-o-クレゾールは，NAD依存の脱水素酵素を抑制することにより，ラット肝ミトコンドリアの電子伝達系を阻害する[7]．これを投与したラットにおける組織内残留量と病理組織学的変化の関連性をみたHattulaら[1]の報告では，1200 mg/kgの単回経口投与により，肝細胞に核濃縮や単細胞壊死が認められている．本試験で雄の死亡動物の肝臓に認めれた単細胞壊死も，被験物質の投与に起因するものと判断される．

副腎では，1000 mg/kg群の雄で相対重量が増加し，病理組織学的には皮質細胞の軽度な空胞化が観察された．空胞化は細胞内のリポイドの増加によるもので，被験物質の全般的な毒性によるストレスを反映した二次的な変化と推察される．

臨床観察で，1000 mg/kg群の雌雄に一般状態の変化が認められ，自発運動低下，深大呼吸，腹臥姿勢，流涎などに加えて，筋弛緩およびそれによると思われる無力性の歩行異常が特徴的な症状として観察された．しかし，これらの症状の発現時間は毎日の投与後1～3時間と短く，投与の反復につれて蓄積的に症状が増強する傾向は認められなかった．体重は，1000 mg/kg群の雄で増加抑制が認められ，同群の雌雄各10匹中雄1匹および雌3匹が死亡した．15および60 mg/kg群では，各観察およ

Table 7 Incidence of histopathological findings in male rats after the oral administration of 4-chloro-*o*-cresol for 28 days and a recovery period for 14 days

Organ:Findings	Dose level (mg/kg)	After administration period						After recovery period	
		0	15	60	250	1000		0	1000
	Fate	KT	KT	KT	KT	KT(FD)	T	KR	KR
	No. of animals	5	5	5	5	5(1)#	6	5	4
Heart:Myocardial degeneration/fibrosis	+	1	-	-	-	0(0)	0	-	-
Lung:Arterial wall mineraliztion	+	2	-	-	-	3(0)	3	-	-
Foamy cell accumulation	+	1	-	-	-	0(0)	0	-	-
Liver:Single cell necrosis	+	0	-	-	-	0(1)	1	-	-
Forestomach:Squamous hyperplasia	+	0	0	0	1	1(1)	2	0	3
	++	0	0	0	0	3(0)	3	0	0
	+++	0	0	0	0	1(0)	1	0	0
Kidney:Basophilic tubules	+	3	-	-	-	3(0)	3	-	-
	++	0	-	-	-	1(0)	1	-	-
Hyaline cast	+	2	-	-	-	0(0)	0	-	-
Cellular infiltration	+	1	-	-	-	0(0)	0		
Urinary bladder:Epithelial hyperplasia	+	0	0	0	4	3(1)	4	0	3
	++	0	0	0	0	2(0)	2	0	0
Adrenal:Cortical cell vacuolization	+	0	0	0	0	2(0)	2	0	0
Thymus:Atrophy	+	0	-	-	-	0(1)	1	-	-
Spleen:Atrophy	+	0	-	-	-	0(1)	1	-	-
Seminal vesicle:Atrophy	++	0	-	-	-	0(1)	1		
Prostate:Atrophy	++	0	-	-	-	0(1)	1	-	-

KT:Killed by design after the administration period;FD:Found dead;T:Total;KR:Killed by design after a recovery period
+:Slight;++:Moderate;+++:Marked;-:Not examined
#:Animal supposed to be killed after a recovery period
No abnormalities detected in the pancreas, glandular stomach, testis, pituitary, thyroid, parathyroid, bone marrow, brain and eye ball from animals of control and 1000 mg/kg groups.

Table 8 Incidence of histopathological findings in female rats after the oral administration of 4-chloro-*o*-cresol for 28- days and a recovery period for 14 days

Organ : Finding	Dose level (mg/kg)	After administration period						After recovery period	
		0	15	60	250	1000		0	1000
	Fate	KT	KT	KT	KT	KT(FD)	T	KR	KR
	No. of animals	5	5	5	5	4(3)#	7	5	3
Heart:Myocardial degeneration/fibrosis	+	1	-	-	-	0(0)	0	-	-
Lung:Arterial wall mineraliztion	+	3	-	-	-	1(0)	1	-	-
Foamy cell accumulation	+	0	-	-	-	1(0)	1	-	-
Liver:Hepatocellular hypertrophy	+	0	0	0	0	2(1)	3	0	0
Forestomach:Epithelial degenaration	+	0	0	0	0	0(3)	3	0	0
Squamous hyperplasia	+	0	0	0	0	1(0)	1	0	1
	++	0	0	0	0	3(0)	3	0	0
Ulcer	++	0	0	0	0	0(1)	1	0	0
Kidney:Basophilic tubules	+	3	-	-	-	1(2)	3	-	-
Distal tubular dilatation	+	0	-	-	-	1(0)	1	-	-
Hyaline cast	+	0	-	-	-	1(0)	1	-	-
Urinary bladder : Epithelial hyperplasia	+	0	0	0	3	2(3)	5	0	2
	++	0	0	0	0	1(0)	1	0	0
Thymus:Atrophy	+	0	-	-	-	0(1)	1	-	-
Spleen:Atrophy	+	0	-	-	-	0(1)	1	-	-

KT:Killed by design after the administration period;FD:Found dead;T:Total;KR:Killed by design after a recovery period
+:Slight;++:Moderate;+++:Marked;-:Not examined
#:One animal supposed to be killed after the administration period and two after a recovery period were found dead during the administration period
No abnormalities detected in the pancreas, glandular stomach, ovary, adrenal, pituitary, thyroid, parathyroid, bone marrow, brain and eye ball from animals of control and 1000 mg/kg groups.

び検査を通じて，被験物質の投与に起因すると考えられる変化は認められなかった．

以上の被験物質投与期間中あるいは投与期間終了後屠殺動物の観察および検査で認められた変化は，1000 mg/kgの回復群においてはいずれも回復あるいは回復傾向にあり，可逆的な変化であることが確認された．

なお，投与期間終了後の解剖で，最終体重が対照群に比べて少なかった250あるいは1000 mg/kg群の雄に，心臓の絶対重量減少や脳および精巣の相対重量増加が認められたが，これらの器官には病理組織学的な異常は認められず，低体重に伴う変化と判断された．

以上の結果から，4-クロロ-o-クレゾールは，ラットへの反復経口投与により，主な毒性影響として，投与経路である消化管特に胃，排泄器官である膀胱，および肝臓に変化が認められ，腎臓および副腎に対する影響も認められた．無影響量は，60 mg/kg/dayと推定された．

文献

1) M. L. Hattula, H. Reunanen, A. V. Arstila, *Bull. Environm. Contam. Toxicol.*, **21**, 492 (1979).
2) C. Madsen, P. H. Andersen, O. Meyer, G. Wurtzen, *Bull. Environm. Contam. Toxicol.*, **37**, 651 (1986).
3) W. B. Deichmann, *J. Pharmacol. Exp. Ther.*, **80**, 233 (1944).
4) R. A. Lenga, "Sigma-Aldrich Library of Chemical Safety Data," 2, Vol.1, Sigma-Aldrich, Milwaukee, 1988, p.807.
5) 今井清，"毒性試験法講座5-毒性病理学，" 前川昭彦，林裕造編，地人書館，東京，1991，pp.127-135.
6) 小野寺威，"毒性試験法講座15-医薬品，" 福田英臣，秋元健，坂口孝編，地人書館，東京，1991，pp.37-38.
7) M. L. Richardson, "The Dictionary of Substance and their Effects," 2-C, Royal Society of Chemistry, Cambridge, 1993, pp.332-334.

連絡先

試験責任者：伊藤義彦

試験担当者：藩栗緒，福田苗美，杉本忠美
伊藤雅也，鈴木昭雄

(財)畜産生物科学安全研究所

〒229-11　神奈川県相模原市橋本台3-7-11

Tel 0427-62-2775　Fax 0427-62-7979

Correspondence

Authors: Yoshihiko Ito (Study director)
Cleo Pan, Naemi Fukuda,
Tadami Sugimoto, Masaya Ito,
Teruo Suzuki

Research Institute for Animal Science in Biochemistry and Toxicology

3-7-11 Hashimotodai, Sagamihara-shi, Kanagawa, 229-11, Japan

Tel +81-427-62-2775　Fax +81-427-62-7979

4-クロロ-*o*-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-Chloro-*o*-cresol 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，4-クロロ-*o*-クレゾールについて*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．

予備試験における抗菌性の結果をもとに，本試験ではS9 mix非共存下のWP2 *uvrA*については1250～39.1 μg/プレート，その他の菌株については625～19.5 μg/プレートのそれぞれ公比2の6濃度を設定し，S9 mix共存下についてはいずれの菌株も1250～39.1 μg/プレートの公比2で6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．また，抗菌性がS9 mix非共存下では625 μg/プレート以上で，S9 mix共存下ではTA1535, WP2 *uvrA*は1250 μg/プレートで，その他の菌株は625 μg/プレート以上で認められた．従って，4-クロロ-*o*-クレゾールの変異原性は，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, 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〕

4-クロロ-*o*-クレゾール(CAS No.：1570-64-5，ロット番号：FBY01, 純度：93.9%；東京化成工業㈱)は分子量142.58，融点40℃，沸点220～225℃の白色結晶塊で通常の取り扱い条件では安定である．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．

4-クロロ-*o*-クレゾール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．また，調製に際しては純度換算(93.9%)を実施した．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非共存下ではすべての菌株の625 μg/プレート以上で，S9 mix共存下ではWP2 *uvrA*の1250 μg/プレート以上および他の菌株の625 μg/プレート以上でそれぞれ抗菌性が認められた．ただし，S9 mix非共存下におけるWP2 *uvrA*の625 μg/プレートでの抗菌性は他の菌株に比べ弱いものであった．従って，本試験ではS9 mix非共存下のWP2 *uvrA*については1250, 625, 313, 156, 78.1, 39.1 μg/プレートの6濃度を，その他の菌株については625, 313, 156, 78.1, 39.1, 19.5 μg/プレートの6濃度を設定した．S9 mix共存下ではすべての菌株について1250, 625, 313, 156, 78.1, 39.1 μg/プレートの6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，抗菌性がS9 mix非共存下では625 μg/プレート以上で，S9 mix共存下ではTA1535, WP2 *uvrA*は1250 μg/プレートで，その他の菌株は625 μg/プレート以上で認められた．

以上の結果から，4-クロロ-*o*-クレゾールの変異原性は陰性と結論した．

参考文献

1) D. M. Maron and B. N. Ames, *Mutation Research*, 113, 173-215 (1983).
2) M. H. L. Green and W. J. Muriel, *Mutation Research*, 38, 3-32 (1976).

連絡先

試験責任者：西冨　保
試験担当者：水野文夫，榎本佳明，石毛裕子
藤代洋子，村田久美，鈴木美江
㈱三菱化学安全科学研究所　鹿島研究所
〒314-02　茨城県鹿島郡波崎町砂山14
Tel 0479-46-2871　Fax 0479-46-2874

Correspondence

Authors: Tamotsu Nishitomi (Study director)
Fumio Mizuno, Yoshiaki Enomoto,
Yuko Ishige, Yoko Fujishiro,
Kumi Murata, Yoshie Suzuki
Mitsubishi Chemical Safety Institute Ltd.,
Kashima Laboratory
14 Sunayama, Hasaki-machi, Kashima-gun,
Ibaraki, 314-02 Japan
Tel +81-479-46-2871　Fax +81-479-46-2874

Table 1 Results of reverse mutation test (I) of 4-chloro-*o*-cresol 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	86 75 (84) 90 (± 8)	11 7 (9) 8 (± 2)	30 27 (33) 42 (± 8)	12 22 (16) 15 (± 5)	3 7 (6) 8 (± 3)
	19.5	71 79 (77) 81 (± 5)	7 11 (7) 4 (± 4)		14 9 (13) 16 (± 4)	3 3 (3) 4 (± 1)
	39.1	84 81 (79) 71 (± 7)	11 7 (9) 10 (± 2)	28 36 (35) 41 (± 7)	18 18 (16) 11 (± 4)	2 9 (5) 5 (± 4)
	78.1	79 92 (85) 85 (± 7)	3 7 (6) 8 (± 3)	33 39 (38) 43 (± 5)	24 12 (16) 12 (± 7)	4 7 (6) 6 (± 2)
	156	75 83 (83) 91 (± 8)	16 7 (10) 8 (± 5)	25 27 (29) 36 (± 6)	9 15 (13) 15 (± 3)	5 3 (5) 7 (± 2)
	313	66 68 (65) 61 (± 4)	13 7 (10) 10 (± 3)	28 31 (31) 33 (± 3)	9 11 (9) 7 (± 2)	6 6 (5) 4 (± 1)
	625	0* 0* (0) 0* (± 0)	0* 0* (0) 0* (± 0)	12* 4* (8) 7* (± 4)	0* 0* (0) 0* (± 0)	0* 0* (0) 0* (± 0)
	1250			0* 0* (0) 0* (± 0)		
S9 mix (+)	0	84 74 (84) 95 (± 11)	9 9 (10) 11 (± 1)	28 28 (33) 42 (± 8)	24 28 (22) 15 (± 7)	13 8 (10) 8 (± 3)
	39.1	102 85 (92) 89 (± 9)	7 8 (8) 10 (± 2)	53 28 (39) 35 (± 13)	14 20 (21) 28 (± 7)	3 7 (5) 6 (± 2)
	78.1	103 95 (104) 115 (± 10)	9 6 (7) 7 (± 2)	36 45 (35) 25 (± 10)	19 19 (18) 17 (± 1)	9 11 (8) 4 (± 4)
	156	86 91 (91) 96 (± 5)	13 5 (9) 10 (± 4)	39 22 (36) 47 (± 13)	27 21 (25) 26 (± 3)	7 10 (8) 7 (± 2)
	313	76 96 (88) 92 (± 11)	10 14 (11) 9 (± 3)	36 43 (39) 37 (± 4)	27 15 (20) 19 (± 6)	11 4 (6) 4 (± 4)
	625	53* 41* (41) 30* (± 12)	6 8 (6) 5 (± 2)	27 26 (26) 25 (± 1)	21* 10* (15) 13* (± 6)	4* 2* (3) 2* (± 1)
	1250	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)
Positive control S9 mix (−)	Name	AF-2	NaN_3	ENNG	AF-2	9-AA
	Concentration (μg/plate)	0.01	0.5	2	0.1	80
	Number of revertants	508 411 (455) 446 (± 49)	307 264 (293) 307 (± 25)	467 471 (491) 536 (± 39)	426 472 (449) 448 (± 23)	814 716 (753) 729 (± 53)
Positive control S9 mix (+)	Name	2-AA	2-AA	2-AA	2-AA	2-AA
	Concentration (μg/plate)	1	2	10	0.5	2
	Number of revertants	893 829 (838) 792 (± 51)	316 312 (304) 283 (± 18)	764 788 (784) 799 (± 18)	334 387 (364) 371 (± 27)	173 155 (159) 150 (± 12)

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.)

*: Microbial toxicity was observed.

Table 2 Results of reverse mutation test (II) of 4-chloro-*o*-cresol 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	87 77 (80) 76 (± 6)	13 9 (11) 12 (± 2)	20 32 (26) 25 (± 6)	9 11 (11) 14 (± 3)	3 4 (5) 7 (± 2)
	19.5	73 80 (75) 73 (± 4)	7 9 (8) 8 (± 1)		16 12 (14) 14 (± 2)	4 3 (4) 5 (± 1)
	39.1	80 77 (79) 80 (± 2)	11 13 (11) 8 (± 3)	29 17 (27) 36 (± 10)	15 15 (14) 13 (± 1)	3 3 (3) 2 (± 1)
	78.1	85 81 (78) 69 (± 8)	10 9 (9) 8 (± 1)	18 26 (21) 19 (± 4)	13 21 (14) 8 (± 7)	6 9 (6) 2 (± 4)
	156	74 76 (78) 85 (± 6)	10 9 (8) 6 (± 2)	28 25 (25) 21 (± 4)	13 15 (13) 11 (± 2)	4 2 (4) 5 (± 2)
	313	72 79 (73) 68 (±6)	10 8 (9) 9 (± 1)	22 23 (21) 19 (± 2)	8 10 (9) 9 (± 1)	4 7 (5) 4 (± 2)
	625	0* 0* (0) 0* (± 0)	0* 0* (0) 0* (± 0)	10* 5* (6) 4* (± 3)	0* 0* (0) 0* (± 0)	0* 0* (0) 0* (± 0)
	1250			0* 0* (0) 0* (± 0)		
S9 mix (+)	0	84 84 (85) 86 (± 1)	15 11 (12) 9 (± 3)	23 26 (25) 25 (± 2)	17 17 (20) 26 (± 5)	8 8 (7) 4 (± 2)
	39.1	83 86 (90) 101 (± 10)	10 9 (9) 8 (± 1)	36 28 (30) 25 (± 6)	16 19 (18) 20 (± 2)	5 10 (6) 3 (± 4)
	78.1	95 92 (97) 103 (± 6)	9 9 (8) 7 (± 1)	32 24 (31) 37 (± 7)	20 33 (26) 24 (± 7)	4 6 (6) 8 (± 2)
	156	97 92 (93) 91 (± 3)	8 15 (10) 8 (± 4)	32 25 (26) 21 (± 6)	26 16 (19) 14 (± 6)	11 9 (11) 12 (± 2)
	313	112 96 (106) 109 (± 9)	9 8 (8) 7 (± 1)	28 35 (30) 28 (± 4)	20 18 (22) 27 (± 5)	10 6 (8) 8 (± 2)
	625	13* 13* (12) 9* (± 2)	2* 1* (2) 2* (± 1)	27 12 (19) 17 (± 8)	4* 4* (3) 2* (± 1)	0* 0* (0) 0* (± 0)
	1250	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)
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	578 513 (535) 513 (± 38)	326 352 (344) 353 (± 15)	539 519 (510) 473 (± 34)	460 390 (417) 402 (± 37)	467 501 (456) 399 (± 52)
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	1088 1098 (1048) 959 (± 78)	424 450 (454) 488 (± 32)	647 712 (704) 753 (± 53)	388 334 (374) 401 (± 36)	161 154 (152) 140 (± 11)

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.)

*: Microbial toxicity was observed.

4-クロロ-o-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-Chloro-o-cresol 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，4-クロロ-o-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，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48時間処理において約50%の増殖抑制を示す濃度は，それぞれ0.053，0.017 mg/ml，短時間処理法のS9 mix存在下および非存在下では，それぞれ0.072，0.121 mg/mlであった．従って染色体異常試験において，連続処理法の24時間処理では0.060 mg/ml，48時間処理では0.020 mg/ml，短時間処理法のS9 mix存在下では0.080 mg/ml，非存在下では0.125 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．

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，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のいずれの処理群においても，倍数性細胞の出現頻度は5%未満であった．しかし，短時間処理法のS9 mix存在下の高濃度群(0.080 mg/ml)で，観察した細胞の7.5%に染色体構造異常が誘発され，判定は疑陽性であった．従って，再現性を確認するため，0.040，0.080，0.120 mg/mlの濃度で確認試験を実施した．その結果，0.080，0.120 mg/mlで観察した細胞のそれぞれ7.0，21.0%に染色体構造異常が観察され，染色体異常誘発の再現性が確認されると共に構造異常細胞の明らかな増加が認められたため，短時間処理法のS9 mix存在下で陽性と判定した．

以上の結果より4-クロロ-o-クレゾールは，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すると結論した．

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

4-クロロ-o-クレゾール(CAS No.:1570-64-5，ロット番号:FBY01，純度:93.9%;東京化成工業㈱)は，分子量142.58，融点40℃，沸点220～225℃の白色結晶塊で通常の取り扱い条件では安定である．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．溶媒はジメチルスルホキシド(DMSOと略す．関東化学㈱，ロット番号:603E2089)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．また，調製に際しては純度換算(93.9%)を実施した．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，陰性対照群に対する細胞増殖の比をもって指標とした．

その結果，4-クロロ-o-クレゾールの約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24時間処理および48時間処理ではそれぞれ0.053，0.017 mg/ml，短時間処理法のS9 mix存在下および非存在下ではそれぞれ0.072，0.121 mg/mlであった(Fig. 1)．

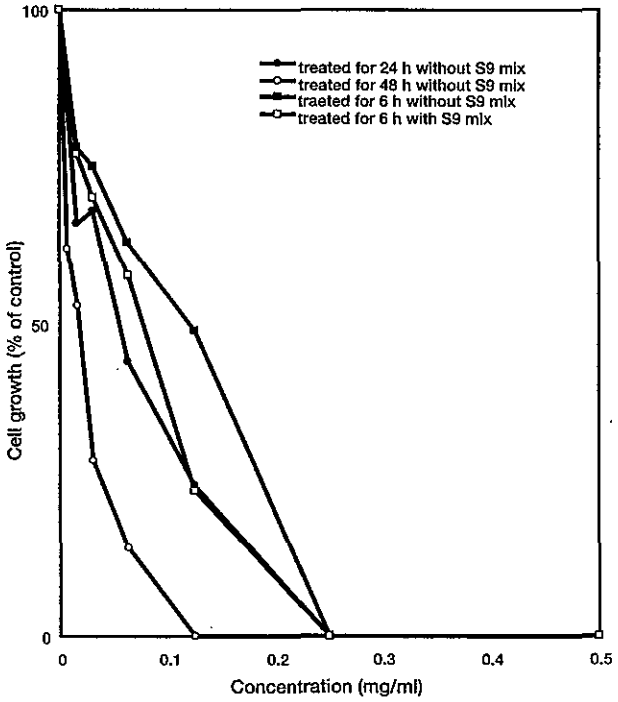


Fig. 1 Inhibition of cell growth treated with 4-chloro-*o*-cresol

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の24時間処理では0.060 mg/m*l*，48時間処理では0.020 mg/m*l*，短時間処理法のS9 mix存在下では0.080 mg/m*l*，非存在下では0.125 mg/m*l*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て設定した．

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/m*l*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．4-クロロ-*o*-クレゾールを加えて，24時間および48時間処理した各濃度群で，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．4-クロロ-*o*-クレゾールを加えてS9 mix存在下および非存在下で6時間処理した各濃度群で，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存在下の高濃度群(0.080 mg/m*l*)で，観察した細胞の7.5%に染色体構造異常(gapを含む)が誘発され，判定は疑陽性であった．

従って，再現性を確認するため，0.040，0.080，0.120 mg/m*l*の濃度で確認試験を実施した(Table 3)．その結果，0.080，0.120 mg/m*l*で観察した細胞のそれぞれ7.0，21.0%に染色体構造異常が観察され，染色体異常誘発性の再現性が確認されると共に構造異常細胞の明らかな増加が認められたため，短時間処理法のS9 mix存在下で陽性と判定した．

文献

1) 日本環境変異原学会・哺乳動物試験分科会編，“化学物質による染色体異常アトラス，”朝倉書店，1988.
2) 石館　基　監修，“〈改訂〉染色体異常試験データ集”，エル・アイ・シー社，1987.

連絡先
試験責任者：西冨　保
試験担当者：水野文夫，太田絵律奈，中川宗洋，岩井由美子，鈴木美江
㈱三菱化学安全科学研究所　鹿島研究所
〒314-02　茨城県鹿島郡波崎町砂山14
Tel 0479-46-2871　Fax 0479-46-2874

Correspondence
Authors: Tamotsu Nishitomi (Study director)
Fumio Mizuno, Erina Ohta,
Munehiro Nakagawa, Yumiko Iwai,
Yoshie Suzuki
Mitsubishi Chemical Safety Institute Ltd.,
Kashima Laboratory
14 Sunayama, Hasaki-machi, Kashima-gun,
Ibaraki, 314-02 Japan
Tel +81-479-46-2871　Fax +81-479-46-2874

Table 1 Chromosomal analysis of Chinese hamster cells (CHL) continuously treated with 4-chloro-*o*-cresol without S9 mix (main test)

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	1	1	0	2	0	0	4	3 (1.5)	4 (2.0)	0.0		
C-*o*-C	0.015	24	200	0	3	0	1	0	0	4	4 (2.0)	4 (2.0)	0.0	-	-
	0.030	24	200	2	7	0	0	0	0	9	7 (3.5)	9 (4.5)	0.5	-	-
	0.060	24	200	0	9	0	0	0	0	9	9 (4.5)	9 (4.5)	0.0	-	-
MC	0.00003	24	200	0	24	11	3	0	0	38	37 (18.5)	37 (18.5)	0.0	+	-
Solvent	0	48	200	0	2	0	0	0	0	2	2 (1.0)	2 (1.0)	0.0		
C-*o*-C	0.005	48	200	0	0	0	1	0	0	1	1 (0.5)	1 (0.5)	0.0	-	-
	0.010	48	200	0	2	1	0	0	0	3	3 (1.5)	3 (1.5)	0.5	-	-
	0.020	48	200	0	6	1	1	0	0	8	8 (4.0)	8 (4.0)	0.0	-	-
MC	0.00003	48	200	0	24	34	8	0	0	66	56 (28.0)	56 (28.0)	0.0	+	-

Abberviations : 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, C-*o*-C : 4-chloro-*o*-cresol, MC : mitomycin C
1) Dimethylsulfoxide 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 4-chloro-*o*-cresol with and without S9 mix (main test)

Group	Concentration (mg/ml)	S9 mix	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	-	6-(18)	200	0	1	0	0	0	0	1	1 (0.5)	1 (0.5)	0.0		
C-*o*-C	0.0313	-	6-(18)	200	0	2	0	1	0	0	3	3 (1.5)	3 (1.5)	1.0	-	-
	0.0625	-	6-(18)	200	1	0	0	1	0	0	2	1 (0.5)	2 (1.0)	1.0	-	-
	0.125	-	6-(18)	200	1	3	3	0	0	0	7	6 (3.0)	7 (3.5)	1.5	-	-
BP	0.020	-	6-(18)	200	0	0	0	1	0	0	1	1 (0.5)	1 (0.5)	0.0	-	-
Solvent	0	+	6-(18)	200	0	1	0	0	0	0	1	1 (0.5)	1 (0.5)	0.5		
C-o-C	0.020	+	6-(18)	200	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.0	-	-
	0.040	+	6-(18)	200	1	1	0	1	0	0	3	2 (1.0)	3 (1.5)	0.5	-	-
	0.080	+	6-(18)	200	0	7	10	0	0	0	17	15 (7.5)	15 (7.5)	1.0	±	-
BP	0.020	+	6-(18)	200	2	69	118	1	0	0	190	132 (66.0)	133 (66.5)	1.5	+	-

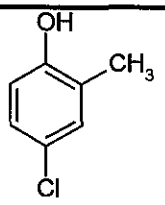
Abberviations : gap : chromatid gap and chromosome gap, ctb : chromatid break, cte : chromatid exchange, csb : chromosome break, cse : chromosome exchange (dicentric and ring etc.), f : acentric fragment (chromatid type), -g : total no. cells with aberrations except gap, +g : total no. of cells with aberrations, SA : structural aberration, NA : numerical aberration, C-*o*-C : 4-chloro-*o*-cresol, BP : benzo[a]pyrene
1) Dimethylsulfoxide 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 3 Chromosomal analysis of Chinese hamster cells (CHL) treated with 4-chloro-*o*-cresol with S9 mix (confirmation test)

Group	Concentration (mg/ml)	S9 mix	Time of exposure (h)	No. of cells analysed	No. of structrual 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	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.0		
C-*o*-C	0.040	+	6-(18)	200	1	5	4	0	0	0	10	6 (3.0)	7 (3.5)	1.0	-	-
	0.080	+	6-(18)	200	1	5	11	1	0	0	18	13 (6.5)	14 (7.0)	1.5	±	-
	0.120	+	6-(18)	200	0	24	28	1	0	0	53	42 (21.0)	42 (21.0)	0.5	+	-
BP	0.020	+	6-(18)	200	1	70	143	1	3	0	218	149 (74.5)	149 (74.5)	0.0	+	-

Abberviations : 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, C-*o*-C : 4-chloro-*o*-cresol, BP : benzo[a]pyrene
1) Dimethylsulfoxide 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).

SIDS INITIAL ASSESSMENT PROFILE

CAS Nr.	1570-64-5
Chemical Name	Phenol, 4-chloro-2-methyl
Structural formula	

CONCLUSIONS AND RECOMMENDATIONS

Environment

The chemical is very toxic to aquatic organisms. The chemical is considered as readily biodegradable and has a low bioaccumulative potential. The predicted environmental concentrations are lower than the predicted no effect levels for all environmental compartments. It is currently considered of low potential risk and low priority for further work.

Health

This chemical is corrosive and toxic by inhalation. Workers exposure is considered to be low because the substance is produced in a closed system as an intermediate for the manufacturing of phenoxyherbicides. Consumer exposure is considered to be negligible. It is currently considered of low potential risk and low priority for further work.

SHORT SUMMARY WHICH SUPPORTS THE REASONS FOR THE CONCLUSIONS AND RECOMMENDATIONS

The EU tonnage of (4-chloro-2-methylphenol) for the year 1989 has been estimated as a total of 15000 tons per annum based on the production volumes presented by the manufacturers and supported by the production and consumption figures of the herbicides MCPA (4-chloro-2-methylphenoxy acetic acid), MCPB (4-chloro-2-methylphenoxy butyric acid) and MCPP (mecoprop 2-4chloro-2-methylphenoxy-propionic acid). The main points of emissions are at manufacturing sites of the substance where PCOC is used as an intermediate for manufacturing of the phenoxyherbicides (i.e. PCOC processing and phenoxyherbicides formulation sites) and where these herbicides are used in agriculture (PCOC occurs as an impurity in the phenoxyherbicides). The environmental distribution of PCOC (using a Mackay fugacity level 1 calculation (Mackay & Paterson 1990) is expected to be 33% in air, 56% in water, 6% in soil and 5% in sediment.

The environmental exposure assessment is primarily based on monitoring data from the two main manufacturing sites in EU where all production and all processing of PCOC takes place, and where approximately 60% of the production volume in EU is formulated. A worst case environmental exposure scenario for a separate, but hypothetical, formulation site has also been considered. PEC local water is calculated as 0.0038 mg/l and 0.0014 mg/l for specific site and formulation, respectively. For the exposure assessment of PCOC in sewerage treatment plants (STP), the dissolved concentration of PCOC is assumed to be equal to the effluent concentration. The predicted environment concentrations for the sewerage treatment plant are: 0.004 mg/l [specific

site], 0.0013 mg/l [formulation]. The predicted environmental concentration for soil is calculated as 0.00000088 - 0.000002 mg/kg.

PCOC is very toxic to aquatic organisms. The acute toxicity to fish LC_{50} (96h) was observed to be 2.3-6.6mg/l. The EC_{50} (48h) to daphnids was 0.29-1.0 mg/l and the EC_{50} (96h) to algae was 8.2 mg/l and EC_{10} to algae (96h) was 0.89 mg/l. The NOEC (28 days) for fish was 0.5 mg/l for histopathological changes in kidneys and liver. NOEC (21 days) for Daphnia reproduction was 0.55 mg/l. The presence of an algae EC_{10}, a long term NOEC for fish and a Daphnia reproduction test suggest that use of an assessment factor of 10 may be appropriate. The predicted no effect concentration (PNEC) is 0.05 mg/l. The PNEC $STP_{microrgnaisms}$ is obtained by using the EC50 for inhibition of respiration of activated sludge microorganisms and an assessment factor of 100 (0.55 mg/l). Since no ecotoxicological data are available for soil organisms the equilibrium partitioning method has been applied ($PNEC_{soil}$ = 0.36 mg/kg).

A local risk for aquatic organisms is not anticipated as the predicted environment concentration is lower than the predicted no effect concentration(regardless of whether an assessment factor of 10 or 100 is employed). Similarly the risks for microorganisms in sewerage treatment plants and for soil organisms is not expected.

The most important sources of direct human exposure are assumed to be at production sites (with predicted exposures of up to 0.7 mg/kg/day) or in conjunction with the use of phenoxy herbicides where exposures of ca. 0.35 mg/kg/day is estimated. Indirect exposure is estimated as being several orders of magnitude lower than the above values at a regional level while consumer exposure to the substance as an impurity in lawn-treatment sprays may be as high as 0.07 mg PCOC /kg/event.

PCOC is corrosive and toxic by inhalation but is only moderately toxic in acute mammalian tests by other routes. The substance is not a skin sensitizer. In an OECD screening test 422, PCOC did not cause reproductive effects in rats. Tests for repeated dose toxicity suggest an NOAEL of 200 mg/kg and a LOAEL of 800/mg/kg (slight liver toxicity and decrease in haemoglobin concentration in the blood). PCOC was positive in an older mouse micronucleus test, but negative in a recent valid test performed according to the current OECD guideline. It did not give rise to genotoxicity in valid Ames tests. On the basis of current knowledge, the substance can not be considered a mutagen.

Repeat dose toxicity is not likely to present a major health problem. The margin of safety for workers based on a NOAEL of 200 mg/kg/day is 200/0.7 = 285. For the end-points irritation/corrosivity the concentration is below the level of concern.

For consumers exposure may be in the order of 0.07 mg/kg for each event corresponding to a daily dose of 9.6×10^{-4} mg/kg/day. With a NOAEL for repeat dose toxicity of 200 mg/kg/day the margin of safety is at least 20,000 for each single event.

IF FURTHER WORK IS RECOMMENDED, SUMMARISE ITS NATURE

4 HUMAN HEALTH

4.1 HUMAN HEALTH (TOXICITY)

4.1.1 Exposure assessment

4.1.1.1 General discussion

P-chloro-o-cresol (PCOC) is used in the chemical industry as an intermediate in the synthesis of chlorophenoxy herbicides, e.g. MCPA (4-chloro-2-methylphenoxy acetic acid), MCPB (4-chloro-2-methylphenoxy butyric acid), and mecoprop (2-(4-chloro-2-methyl-phenoxy)-propionic acid, MCPP). PCOC is no longer produced in Denmark.

PCOC is found as an impurity in the herbicides MCPA, MCPB, and mecoprop.
During production of PCOC and in the synthesis of other compounds (down stream uses) PCOC is released to the environment through emitted air and waste water. As a degradation product and as an impurity PCOC will also be found at the application sites of the herbicides mentioned above.

PCOC was detected upon branches sprayed with MCPA, 2 weeks post application, at concentrations of 8900 ppb; and upon potatoes, carrots, green lettuce and onions grown on fields adjacent to a treated railway bed in Northern Finland at concentrations of 0.2, 2.9, 52.9 and 593.0 ppb, respectively (Paasivirta *et al.*, 1983).

Concentrations in the environment are estimated in chapter 3.

The most important routes of direct exposure is by inhalation in occupational settings in the production of the substance itself or during use in the synthesis of other compounds (down stream uses). Oral or dermal exposure during production is assumed to be of relevance only in the case of accidents.

Exposure to PCOC as an impurity in herbicides such as MCPA can also occur during crop spraying.

In the Danish Product Register PCOC is only registered as a substance, but was formerly found in one product at a concentration of around 1% in a survey carried out in 1985. With reference to information from industry it is concluded, that no exposure takes place through use of ordinary (non-herbicidal) consumer products.

One potential source of indirect exposure is the consumption of food treated with the herbicides, of which PCOC is a degradation product or an impurity, and drinking of water contaminated by the substance.

4.1.1.2 Occupational exposure

Two companies in the U.K. are high volume producers of PCOC, which is also used as an intermediate for further synthesis at the same sites of the herbicides MCPA, mecoprop, and MCPB. In addition one Dutch high volume producer has been identified, producing PCOC as a non-isolated part of a continuous process which need not be reported under the Regulation. There are no data on occupational exposure available from this producer.

No occupational exposure limits for PCOC have been found but for related substances (cresols and chlorphenols) the values given below apply.

The occupational exposure limit (8-hour threshold limit value (TLV)) for cresols set by the UK and DK authorities (all isomers) is 22 mg/m^3 (HSE 1994, AT, 1994). For chlorphenols (all isomers) the TLV in e.g. Denmark is 0.5 mg/m^3 (AT, 1994).

Production: At one of the production sites, plant operators were monitored at the workplace and tank farm operators were monitored whilst offloading PCOC to the road tanker on four and three occasions, respectively (Road tanker is used to move the substance within the area). According to the manufacturer less than 20 people are involved in these operations (pers. communication, 1997). For the plant operators the monitoring period lasted from 183 to 238 minutes. For the tank farm operators the monitoring period lasted from 15 to 101 minutes. Concentrations for plant operation and offloading to road tankers ranged from below detection limit to about 5 mg/m^3 (equivalent 8 hour TWA's max. ca. 5 mg/m^3) (A.H. Marks, 1997b).

During cleaning operations, which were infrequent (twice per year) and where protective clothing and breathing apparatus was worn, a concentration of about 53.8 mg/m^3 was recorded (8 hr. TWA 1.2 mg/m^3). The monitoring was done at one occasion (A.H. Marks, 1997b). According to the manufacturer, less than 20 people are involved in this operation (pers. comm.). One of the main manufacturers has reported that the exposure actually only applies to one worker. (pers. comm., A.H. Marks, march 6 1998)

Beside the actual operator monitoring point location monitoring in working areas was performed showing TWA values of less than 5 mg/m^3 for all instances (e.g. control room, by reactor, by holding vessels, process scrubber, and whilst offloading PCOC to road tanker) except cleaning of the equipment where a concentration of 1,274.8 mg/m^3 was recorded (equivalent to 8 hr. TWA 18.6 mg/m^3)(A.H. Marks, 1997b).

For production of phenoxy herbicides, which was done at the same plant, monitoring data (operator and point location monitoring) was of a similar order of magnitude, less than 5 mg/m^3 at all occasions. Here cleaning of the equipment was not monitored (A.H. Marks, 1997b). PCOC is used in the molten state, which together with the corrosive nature of the substance, ensures that workers comply fully with PPE requirements (pers. Comm., A.H. Marks, march 6, 1998)

There are no monitoring data on PCOC available from the other U.K. production site. According to the producer the occupational exposure to PCOC is regarded as being minimal, because all vessels and sample points are enclosed and maintained under extraction with air being discharged via caustic scrubbing columns. In addition all employees are provided with appropriate Personal Protective Equipment which is laundered and maintained by the company (Nufarm, 1997b).

According to the producers most of the manufacture and use of PCOC do not require operator intervention. However, there are exceptions e.g. maintenance and tanker loading and unloading. For these operations as well as for emergency situations appropriate PPE are provided including suit (PVC or full body cotton overalls), full face mask, PVC gloves, boots (leather or PVC), safety helmet and glasses (A.H. Marks, 1997b; Nufarm, 1997b).

Assuming inhalation of 10 m^3 of air during an eight hour work shift, for a 70 kg person, 5 mg/m^3 would correspond to a realistic worst case dose for systemic toxicity of about 0.7 mg/kg/day. It can be noted that while this concentration is less that 0.25 of the TLV for cresols and thus meets U.K. regulatory standards, it is 10 times higher than the TLV for chlorphenols, which from a chemical-structural point of view are quite similar to PCOC.

The EASE estimation (app. 5) of inhalation exposure during production and further processing of PCOC assuming use pattern is closed system and the pattern of control is full containment resulted in exposures of 0 to 0.1 ppm corresponding to 0 to 0.6 mg/m^3. This range is much lower than monitored data.

While some degree of dermal exposure may also occur, the EASE model predicts this as being of no consequence when compared with the inhalation route (app. 5). Direct contact with the skin would only happen in the case of accidents, where it could result in systemic toxicity as well as severe burns.

In conclusion the known corrosive nature of PCOC together with its use in the molten form ensures that routine transfer and equipment cleaning amd maintenance operations are performed with strict adherence to PPE requirements, resulting in minimal exposure to workers via both dermal and inhalation routes.

Application[2]: In certain occupational settings such as municipal gardening, worst case exposures may be higher. Using a standard model for plan protection product use (Lundeher, 1992) which also incorporates exposure during mixing and loading, a geometric mean exposure of 0.047 mg/kg/day is calculated for hand-held (knapsack) spraying of 1 ha assuming application of 2 kg/ha MCPA with a 1% content of PCOC and 100 % absorption. The 90th percentile exposure using the same inputs results in a total of 0.35 mg/kg/day.

4.1.1.3 Consumer exposure

PCOC is not found in any ordinary consumer products. It can occur as an impurity or breakdown product in herbicides used for controlling weeds in lawns of private gardens. One such product available in the vegetable section of a Danish super market contains MCPA in concentrations of 5.20 g/l in a one-liter plastic bottle provided with a hand pump for aerosol generation. As this form of dispensation can lead to the highest exposures, a realistic worst case for combined inhalation and dermal exposure of 10% is assumed. If PCOC is present as an impurity at 0.5%, and a further 0.5% is generated by exposure of the aerosol to sunlight, a total exposure to PCOC of 5.2 mg/event, or 0.07 mg/kg/event for a 70 kg person could result.

It is difficult to assess the frequency with which such consumer exposure might occur, directions for use on the particular product only state that it can be used during the entire growth period, but is most effective during periods of rapid growth in May, June, July and August (Source, "Toxan" - Labelling information, Distribution: Bayer Denmark A/S, Gammelager 1, 2605 Brøndby. In addition to MCPA, one liter of this product is also stated to contain 1.50 g Dichloprop-p and 0.32 g Dicamba as active ingredients). Assuming a really worst case of five times application per year the total yearly dose of PCOC would be 5 x 0.07 = 0.35 mg/kg/year (= 9.6x10^{-4} mg/kg/day).

2

During spraying, including mixing of pesticides, using sprayers on tractors the exposure is generally estimated to be around 0.00005% of the amount sprayed in a concentration of 15 g/ha using the best available technology. Using standard spraying equipment the exposure is 0.0002% of the amount sprayed (Lund & Kirknel, 1995).

Using a standard model for plant protection product use (Lundeher, 1992) which also incorporates exposure during mixing and loading, and assuming 2 kg MCPA per ha, with a 1% content of PCOC, a geometric mean exposure of 0.02 mg/kg body weight/day is derived, or for the 90th percentile, 0.28 mg/kg body weight/day for 20 ha of downward vehicle-mounted spraying.

4.1.1.4 Indirect exposure via the environment

Exposure of the environment can take place during the production of PCOC itself, as well as from the production and use of phenoxy herbicides.

At the production site the potential exposure would be through waste water and air effluent.

At sites of MCPA or other phenoxy herbicide applications, indirect exposure may occur, since PCOC is an impurity in the herbicide and has been identified as a degradation product of MCPA.

According to USES1.0 calculations involving local indirect exposure due to use of herbicides the following daily doses can be expected:

Intake air:	1.63×10^{-9} mg/kg/day
Intake drinking water:	8.95×10^{-8} mg/kg/day
Intake fish:	4.04×10^{-9} mg/kg/day
Intake stem of plant:	7.97×10^{-10} mg/kg/day
Intake root of plant:	3.38×10^{-12} mg/kg/day
Intake meat:	5.49×10^{-13} mg/kg/day
Intake milk:	5.47×10^{-13} mg/kg/day

Amounting to a total human dose of 9.60×10^{-8} mg PCOC/kg/day

The EUSES calculations (November 1997) for local indirect exposure resulting from production of PCOC are as follows:

	Ready degradability	Inherent biodegradability
Specific site*:	1.18×10^{-4} mg/kg/day	2.52×10^{-4} mg/kg/day
Formulation site**:	1.25×10^{-4} mg/kg/day	4.30×10^{-4} mg/kg/day

*: Specific site incl. production, processing and formulation. The values are based on average monitoring data on emissions from the two main manufacturers.

**: Formulation site is a generic site where it is assumed that 10 % of the total PCOC production is formulated (worst case).

We assume PCOC being readily biodegradable. However, knowing the substance may be a borderline case, the calculations for inherent biodegradability are included for comparison purposes only.

EUSES calculations (November 1997) for regional indirect exposure assuming ready or inherent (worst case) biodegradability. Again, inherent biodegradability has been included for comparison purposes only:

Daily human dose through:	Ready biodegradability	Inherent biodegradability
Intake air:	1.60×10^{-7} mg/kg/day	2.17×10^{-7} mg/kg/day
Intake drinking water:	4.49×10^{-6} mg/kg/day	8.05×10^{-6} mg/kg/day
Intake fish:	7.74×10^{-6} mg/kg/day	1.39×10^{-5} mg/kg/day
Intake from leaf crops:	2.44×10^{-7} mg/kg/day	3.31×10^{-7} mg/kg/day
Intake root of crops:	2.56×10^{-7} mg/kg/day	3.01×10^{-7} mg/kg/day

Intake meat:	$1.29x10^{-9}$ mg/kg/day	$2.25x10^{-7}$ mg/kg/day
Intake milk:	$7.60x10^{-10}$ mg/kg/day	$1.33x10^{-7}$ mg/kg/day
Regional total daily intake:	$1.29x10^{-5}$ mg/kg/day	$2.28x10^{-5}$ mg/kg/day

4.1.1.5 Combined exposure

Some parts of a population are exposed to PCOC both during work and during indirect exposure via the environment.

A person working at a production site for PCOC and/or phenoxy herbicides or a person spraying phenoxy herbicides on a field might apart from the occupational exposure also be exposed via the environment. However, the potential routes of exposure differs and as can be seen from 4.1.1.1., 4.1.1.2., and 4.1.1.3. the magnitude of the exposure varies greatly. In table 1 the calculated exposure data are given.

Table 1. Calculated exposure data excl. agricultural spraying[3]

Exposure	mg PCOC/kg/day
Occupational exposure during production	0.7
Spraying (municipal - hand spraying)	0.35
Consumer exposure	$9.5x10^{-4}$
Indirect regional exposure via the environment	$1.3x10^{-5}$
Local indirect exposure*	$1.2x10^{-4}$
Combined exposure, total	1.05 PCOC mg/kg/day

*: Local indirect exposure resulting from production, formulation or processing is estimated assuming ready biodegradability.

4.1.2 Effects assessment: hazard identification and dose (concentration) - response (effect) assessment

All the PCOC studies below that were performed by Scantox, Denmark and Teknologisk Institute were conducted in accordance with the OECD guidelines for testing of chemicals and GLP. The identity of the substance was as described in chapter 1 i.e. 97.09% 4-chloro-2-methylphenol, 1.21 % 6-chloro-2-methylphenol, 0.92% 2-methylphenol, and 0.78% 2,4-dichloro-6-methylphenol. The study by the Institute of Toxicology in Denmark (Hansen, 1996) used a 97% pure Aldrich PCOC batch no. C5.520-8. The study by Hattula *et al.* (1979) used 100% pure PCOC.

4.1.2.1 Toxico-kinetics, metabolism and distribution

Very little is known about the toxico-kinetics, metabolism, distribution, and excretion of PCOC in humans and experimental animals. However, from the acute toxicity studies it can be inferred that PCOC can be taken up in the body through the gastro-intestinal tract, the skin, and via inhalation. There is no information on the metabolism and excretion of PCOC.

[3] Table 1a. Calculated exposure data for agricultural spraying for comparison purposes only.

Exposure	mg PCOC/kg/day
Spraying (agricultural)	0.28

The concentrations of PCOC in liver, kidney, spleen, and muscle was studied in an acute and a repeat dose study (Hattula *et al.*, 1979). After 28 days of dosing by gavage with 100, 250, or 500 mg PCOC/kg, PCOC was found in the highest concentration 2.81 mg/kg in the spleen, and in the lowest concentration 0.27 mg/kg in muscle tissue in the high dose group. In the low dose group only traces of PCOC were found.

PCOC was found in concentrations of 47-31 µg/g in the liver of rats receiving 2-3 g/l MCPA in the drinking water for three months (Hattula *et al.*, 1977). A recent rat metabolism study with MCPA performed at Hazleton Lab. showed that PCOC was not a metabolite. It is therefore possible that the PCOC in the Hattula - study was a contaminant of MCPA (Jahanshahi J., 1995).

Acute toxicity

Animal data: Acute oral toxicity
In a guideline (401) study using five male and five female rats per group and dosing by gavage with the doses 1728, 2488, 3583 and 5160 mg/kg with oleum arachidis as vehicle, an LD_{50} of 3195 mg PCOC/kg (range 2698 - 3834 mg/kg) was found.

In the 5160 mg/kg group all animals died within one hour after dosing, in the 3583 mg/kg group 5 deaths occurred up to 6 hours after dosing, in the 2488 mg/kg group three deaths occurred within one day after dosing, and in the 1728 mg/kg group no deaths occurred. Symptoms observed just after dosing at all dose levels were paresis and depressions. On the second day, ruffled fur, which lasted to day five in the 3583 mg/kg group, was seen. Animals that died during the observation period showed bleeding in the mucous membrane of the stomach at autopsy. Animals sacrificed after the 14 days observation period showed no dose related macroscopic changes. However, two of the animals from the 2488 mg/kg group, sacrificed after the 14 days observation period, showed infiltrations between the oesophagus area of the ventricle and the diaphragm. In one animal from the high dose group, infiltrations between the oesophagus area of the ventricle and the liver were seen (Scantox, 1982b).

Groups of ten male Wistar rats, 2-3 months of age, were given 1000 , 1100, or 1200 mg PCOC/kg with the substance dissolved in olive oil. The animals were all killed 24 hours after dosing. A LD_{50} of 1190 mg/kg was derived (Hattula *et al.*, 1979). At the histopathological examination the following observations were made: At 1000 and 1100 mg/kg inflammatory mononuclear infiltration was seen in many glomeruli in the kidney. Inflammatory infiltrations were also seen in other parts of the kidney mostly around distal tubules. At 1200 mg/kg also histopathological alterations in the liver and spleen were seen. In the liver numerous pycnotic nuclei and hydropic degeneration of cytoplasm were observed. In the spleen the reaction centres were unusually large (Hattula *et al.*, 1979).

Further studies on the acute oral toxicity of PCOC to rats include BASF (1978) and Hazleton (1977). These test reports have not been available, but their results (see table 1) are in accordance with the results of the only guideline study available (Scantox, 1982b). It can be concluded that PCOC not only shows corrosive properties but also properties resulting in systemic effects i.e. effects on liver and kidney.

In rats the oral LD_{50} of PCOC is above 2.000 mg/kg in the most reliable study.

In mice, Schrötter *et al.* (1977) report the oral LD_{50} of PCOC as being 1330 mg/kg, but few experimental details are provided.

In range finding studies of PCOC in aqueous gum tragacanth emulsion, mice died consistently at lower doses (4/4 at 1200 mg/kg and 3/4 at 576 mg/kg) suggesting that the vehicle may play an important role in determining absorption following oral administration (Huntingdon, 1997).

Animal data: acute inhalation toxicity

Groups of five male and five female rats were exposed to an aerosol containing 0, 5.79, 8.33, 9.11, or 10% PCOC in 50% alcohol for 4 hours following OECD Guideline 403. All deaths during the study occurred during exposure or within the first hour after exposure. The deaths were distributed as follows between the groups: control 0 deaths, 5.79% 0 deaths, 8.33% two deaths, 9.11% four deaths, 10% 7 deaths. The LC_{50} was calculated as 900 mg/m^3 (0.9 mg/l range 0.83 - 1.08 mg/l) (Scantox, 1983a). The alcohol aerosol was used as it was not possible to generate a dust aerosol, as the test substance clumped. The LC_{50}-value is based on the nominal concentration in the experiment. The symptoms observed during and after exposure were respiration difficulties, depressions, ruffled fur and bleeding from the nose. These symptoms occurred in a dose related manner. Petechiae of the lungs were also observed.
At macroscopic examination of the animals that died up to the first hour after dosing bleeding of the lungs and a thin, mucous, yellowish content of the small intestine were found.

Another study was performed by Hazleton Lab in 1977 is cited from BUA (1994): The original report is not available and the study was carried out before guidelines were in general use. By inhalation of 2000 - 30.000 mg PCOC/m^3 (average particle size of 0.6 µm) for 4 hours no deaths occurred but swelling red noses and lips were seen. In one animal blood was found in the urine.

Animals sacrificed immediately after the exposure period or after 14 days of observation period showed no alterations in the lung or essential organs.

Animal data: acute dermal toxicity

Groups of five male and five female rats were dermally dosed with 1667, 2000, 2400, or 2880 mg PCOC in oleum arachidis in a guideline study (402). A LD_{50} of 2240 mg/kg (range 2023 -2484) was calculated from the observed deaths (Scantox, 1982c).

In the 2880 mg/kg group 9 animals died within 6 hours after dosing, in the 2400 mg/kg group six animals died within one day after dosing, in the 2000 mg/kg group four animals died within one day after dosing, and in the 1667 mg/kg group no animals died. At necropsy bleeding of the lungs, a mucous, red-yellow content of the jejunum, enlarged kidneys and blood or blood coagulum in the bladder plus bleeding of the bladder wall were observed.

During the first 24 hours after treatment blood was observed in the urine of all rats. From the day after treatment erythema and oedema at the application sites were seen. Paresis occurred in nearly all animals 1 to 6 hours after treatment. Depressions occurred up to 2 days after treatment, and ruffled fur up to 3 days after treatment. In animals sacrificed on day 14 weak bleeding of the intestine (jejunum) was observed in five of the rats (dose levels not stated).

In table 1 the acute toxicity data found for PCOC are given without any comments on quality of the studies.

Table 1. Data on acute toxicity of PCOC

species	application	dose	effect	literature

rat	oral	3.195 mg/kg	LD_{50}	Scantox, 1982b
rat	oral	1.190 mg/kg	LD_{50}	Hattula et al, 1979
rat	oral	2.650 mg/kg	LD_{50}	Hazleton Lab, 1977
rat	oral	2.700 mg/kg	LD_{50}	BASF AG, 1978 *
mouse	oral	1.330 mg/kg	LD_{50}	Schrötter et al, 1977
rat	i.p.	794 mg/kg	LD_{50}	Hattula et al, 1979
mouse	i.p.	570 mg/kg	LD_{50}	BASF AG, 1978 *
rat	inhal, 4h.	900 mg/m^3	LC_{50}	Scantox, 1983a
rat	inhal, 4h.	>30.000 mg/m^3	LC_{50}	Hazelton Lab., 1977
rat	dermal	2.240 mg/kg	LD_{50}	Scantox, 1982c
rat	dermal	>5.000 mg/kg	LD_{50}	Hazleton Lab,1977*

*: unpublished results sited in a BUA report (BUA, 1994).

In relation to acute oral, dermal and inhalation acute toxicity the Scantox Reports (1982b,c, 1983a) are found to be most reliable. For the acute oral toxicity the Hazleton and BASF studies support the oral LD_{50} found by Scantox. Poor reporting of the Hattula study makes its interpretation difficult. No details are available which would allow further interpretation of the Hazleton inhalation study.

The overall conclusion for acute toxicity is:

$LD_{50\ oral,rat}$ = 2650 - 3195 mg/kg
$LC_{50\ inh,rat}$ = 0.9 mg/l (as an EtOH aerosol)
$LD_{50\ dermal,rat}$ = 2240 mg/kg

4.1.2.2 Irritation

&

4.1.2.3 Corrosivity

Animal data: skin irritation

In a guideline (404) study 6 female rabbits were dermally exposed to 0.5 g PCOC in 0.1 ml oleum arachidis. A primarily irritation index of 8.0, the maximum value obtainable, was calculated. Immediately after removal of the test substance the skin was white as a sign of initial necrosis (Scantox, 1982d).

BUA (1994) reports a study of Hazleton labs (1977), where rabbits received 500 mg PCOC on the shaved back in a semi-occlusive bandage. It is not stated if a vehicle was applied, and what time of exposure was used. After 12 hours necroses were observed and after 24 hours pronounced erythema with light oedema was observed

BUA (1994) reported a study of BASF where occlusive exposure to 80% of PCOC in water was carried out (species used and amount applied not mentioned). It was concluded that PCOC was very corrosive. After only one minute of exposure necrosis was found. After 20 minutes the necrosis was very pronounced, and after 8 days it had not disappeared. On day 8 after application the skin was still scarred.

In the rabbits eye BUA (1994) citing BASF reports 50 mg PCOC in an 80% aqueous solution as strongly corrosive. The eye turned red and after 1 hour oedema and opacity of the cornea was found. After 8 days the clinical observations were the same and a staphyloma was found.

In conclusion, some of the studies concerning corrosive effects of PCOC are cited from secondary references, but together with the results of the irritation test (Scantox, 1982d), they indicate that PCOC, according to EU criteria, may be classified as corrosive with R35: causes severe burns, in agreement with classification by the manufacturers.

At least one human fatality attributed to PCOC poisoning has been reported following exposure of the face and neck to a momentary blast of PCOC and steam during a workplace accident. It was not possible to estimate the dose or concentration involved (Pers. comm., HSE, U.K. 1996).

4.1.2.4 Sensitization

In a Guinea Pig maximization test carried out according to OECD guidelines (Scantox, 1982e) PCOC caused no sensitization. 40 female albino Guinea Pigs were used in the study. As the provocation test with 30% solution of PCOC caused erythema, a further provocation test with 10% and 20% of PCOC applied on the left and right flank, respectively, was carried out a week later. No clear differences between the control group and the test group were found at this occasion. Some animals of both groups were reacting with erythema (score 1-2). The reactions in the two groups were of the same magnitude. Macroscopically none of the reactions appeared to be an allergic.

The report by BUA (1994) mentions another negative sensitization study. However, the study does not seem to have been reported properly.

4.1.2.5 Repeated dose toxicity

There are three available studies on repeated dose toxicity of PCOC.

In a guideline (407) study groups of five male and five female rats were given 0, 50, 200, or 800 mg PCOC/kg in oleum arachidis by gavage for 28 days (Scantox, 1982a). During the last three days of dosing three rats from the 800 mg/kg group showed salivation after dosing, and on the last day of dosing three rats from the group had ruffled fur. Body weight gain and feed consumption did not differ between groups. In blood parameters *the thromboplastin time and the number of leukocytes were statistically smaller in females from the 800 mg/kg group. In males from the same group the erythrocyte count was statistically significantly reduced. Serum alanine-aminotransferase (ALAT)* was statistically significantly increased *in males of the 800 mg/kg group, and marginally increased in females*. In females from the 800 mg/kg group relative and absolute liver weights were significantly increased.

No histopathological changes were seen in any organ at 800 mg/kg. The changes of ALAT and liver weights in the 800 mg/kg group indicated *mild toxicity to the liver*. It was concluded in the test report that 800 mg/kg is a LOAEL, and that *200 mg/kg is a NOAEL*.

Hattula *et al.* (1979) dosed groups of ten male Wistar rats with 0, 100, 250, or 500 mg PCOC/kg in olive oil for 28 days by injection (gavage). It is very difficult to interpret the results of this study, basically because of lack of tables and explanations to the few tables given. However, at 100 mg/kg all investigated organs were normal except for the small intestine, which had necrotic areas of the mucosa. The dose relationship of the other histopathological observations mentioned is obscure. It is stated that

blood analyses showed that leukocytes were decreased with larger doses. *At 500 mg/kg a clear-cut leucopenia was found.*

In a combined *repeated dose/reproduction screening test* carried out according *to OECD draft guideline 422* (Ernst Hansen, "4-Chloro-2-methylphenol," National Food Agency, 1996) groups of 10 male and 10 female rats per dose were given 0, 50, 200, or 600 mg PCOC/kg in soybean oil by gavage for two weeks prior to mating until day 20 of gestation i.e. dosing was for a total of 40-45 days.

Weight gain was slightly reduced, and water consumption increased in the highest dose groups. *Males in the 600 mg/kg group showed a decrease in haemoglobin concentration ($p<0.01$).* (A slight decrease in plasma creatine ($p<0.05$) in the middle dose group was considered to be without physiological significance.)

A dose-related decrease in the absolute and relative weight of the adrenals of female rats was seen ($p<0.05$ at 200mg/kg, $p<0.01$ at 600 mg/kg) but was unaccompanied by histopathological changes, and without obvious toxicological significance.
No effects were seen in other macroscopic and histological examinations of the organs. No behavioural changes were found by a functional observational battery, or in motor activity. It was concluded that the NOAEL was 200 mg/kg.

With regard to respiratory irritation and corrosivity after repeated dosing no data are available. However, due to the caustic properties of the substance it seems unlikely that an inhalation study would add any new information on the systemic toxicity. Further, it seems that the way the substance is handled and used in the existing productions do not lead to any respiratory problems. At both production sites health surveillance programmes including examination of the respiratory function have been undertaken for several years. According to the medical reports submitted by the producers no significant increase in any specific symptoms such as sore throats, coughs and changes of lung function and no significant group changes of lung function have been observed (A.H. Marks, 1997b; Nufarm, 1997b).

4.1.2.6 Mutagenicity

Genetic toxicity *in vitro*

According to Ames, et. al. 1975, and/or OECD guideline 471 four direct plate Ames tests (Räsänen *et al.*, 1977 ; Teknologisk Inst, 1982; Strobel & Grummt, 1987; BASF, 1988) and one pre-incubation Ames test (BASF, 1988) have been carried out to study the mutagenicity of PCOC in the dose range 1-500 µg/plate.

Ames direct plate test was performed with the *Salmonella typhimurium* strains TA1537, TA1535, TA100, and TA98 at 0, 1, 5, 10, 50, 100, and 500 µg/plate with and without metabolic activation. The identity of the substance was as described in chapter 1. There was clear general toxicity in all strains at 500 µg/plate, but none of the strains showed an increase in the number of revertants/plate (Teknologisk Institut, 1982).

Ames direct plate test was performed with the *Salmonella typhimurium* strains TA1537, TA1535, TA100, and TA98 at 0, 0.5, 5, 50, and 500 µg/plate with and without metabolic activation. None of the strains showed an increase in the number of revertants/plate (Räsänen *et al.* 1977).

Ames direct plate test was performed with the *Salmonella typhimurium* strains TA1537, TA1535, TA100, and TA98 at 0, 20, 100, 500, 2500, and 5000 µg/plate and at 0, 4, 20, 100, 500, and 1500

µg/plate with and without metabolic activation. There was clear general toxicity in all strains at and above 500 µg/plate, and none of the strains showed an increase in the number of revertants/plate (BASF, 1988).

An Ames direct plate test using the strains TA98, TA100, TA97 and TA104 at 10, 25, 50, 100, 250, 500, and 1000 µg/plate with and without metabolic activation showed a 4.4 fold dose related increase with TA97-S9 and a 5.4 fold dose related increase with TA97+S9. Only these results were significant. At the highest dose a toxic effect was found in all the strains (Strobel & Grummt, 1987). The report of these results in the literature leaves open some questions with regard to the interpretation of results. For this reason an additional test was performed.

In this new test, 97% PCOC (Aldrich lot no. 3302005) was dissolved in DMSO and tested according to the Salmonella/microsome standard plate assay in *S. typhimurium* strains TA-97 and TA-98 at doses of 500, 250, 100, 50, 25 and 10 ug/plate with and without S9-mix (at 2 and 4 mg S9 protein/plate). No mutagenic effect was seen with or without metabolic activation in either strain. The experiments were repeated again with the same results. (Binderup, "4-Chloro-2-methylphenol: Assessment of mutagenic potential," National Food Agency of Denmark, 1996:)

In the Ames test with pre-incubation (BASF, 1988) *Salmonella typhimurium* strains TA1535, TA100, TA1537, and TA98 was used with and without metabolic activation in concentrations of 0, 4, 20, 100, 500, and 1000 µg/plate and 0, 15, 30, 60, 125, and 250 µg/plate (two separated series). General toxicity occurred at dose levels of 125 µg/plate or higher. There was no increase in the number of revertants/plate.

Genetic toxicity *in vivo*

In a micronucleus assay performed according to the first version of OECD guideline 474 male and female mice were dosed by gavage with 1600 mg PCOC/kg in 10 ml of peanut oil, corresponding to the maximum tolerable dose. Bone marrow cells were harvested at 24, 48, and 72 hours post dosing. A significant ($p < 0.0007$) increase (4-6 times) in the frequency of micronuclei was observed in the dosed animals at all harvesting times (Scantox, 1982f). It was noted that there was no clear evidence of a time-course for these effects. The incidences of micronucleated cells in treated animals were not particularly high compared to published data for untreated mice, while the incidence in the control group was lower than what would usually be expected. It was not possible to re-examine concurrent control data to obtain information on background rates, as the records are no longer available.

A new mouse micronucleus assay was performed in 1997 according to current guidelines (EEC , 29 December 1992, Official Journal of the European Communities No. L358B: Methods for determination of toxicity, B12: Mutagenicity (Micronucleus test) p. 124), including the OECD guideline revision (OECD 1996) recommending use of aqueous suspending agents for poorly soluble substances. The test substance, 99.3% pure PCOC consisting of 50% of current production lots from each of the two U.K. producers was suspended in aqueous 0.5% gum tragacanth. A preliminary toxicity test indicated that in this vehicle, the maximum dose which did not induce excessive lethality was approximately 400 mg/kg. For the Micronucleus test, groups of 5 male and 5 female mice were dosed by gavage with 20 ml/kg suspensions of test substance corresponding to 100, 200 and 400 mg/kg body weight of PCOC, using the vehicle alone as the negative, and Mitomycin C as the positive control.

Severe lethargy was noted shortly after dosing at 400 mg/kg. One female in the high dose group died , and was replaced by another female from the concurrently-treated satellite group. No adverse clinical signs were observed for the positive or negative control groups during the duration of the test.

Bone marrow samples were examined (1000 erythrocytes per smear) after 24 hours and 48 hours and did not show any substantial increase in the incidence of micronucleated immature erythrocytes or decrease in the proportion of immature erythrocytes. It was concluded that PCOC did not show any evidence of causing chromosome damage or bone marrow cell toxicity in this test. The positive control caused highly significant (P<0.001) increases in the number of micronucleated immature erythrocytes at both 24 and 48 hours. Results for PCOC treated and control animals were within the expected range for unaffected mice based of published information and laboratory control data (Huntingdon, 1997).

While cytotoxic effects were not seen in the bone marrow, there is little to suggest that PCOC would not be absorbed, or would break down prior to reaching this site. Clear evidence of leukopenia seen in the two repeat-dose studies is highly suggestive bone marrow effects. *In vivo* mutagenicity studies of the meta isomer of chlorcresol (4-Chloro-3-methyl phenol, Cas. no. 59-50-7) showed a similar pattern, with no change in the observed PCE/NCE ratio and no clastogenic activity (mouse micronucleus test, oral, 200 and 400 mg/kg, 24 hours: mouse micronucleus test, single i.p. injection of 125 mg/kg - 10 % mortality - investigations at 24, 48 and 72 hours post dosing, stat. significant response in cyclophosphamide control) (BUA, 1993 - U.S. EPA 1997).

Conclusion on mutagenicity.

PCOC was negative in 3 Ames tests, equivocal in one, and negative in repeat tests of the equivocal strain. An oral mouse micronucleus performed in 1982 according to the first OECD guidelines was positive. A repeat of this test in 1997 using modern guideline recommendations and possibly a more suitable test vehicle was clearly negative. Using the best available data PCOC cannot be considered a mutagen.

4.1.2.7 Carcinogenicity

For 4-chloro-o-cresol (PCOC) no studies in humans or animals are available.

Human data on phenoxy herbicide production

A cohort study of workers employed in manufacturing of phenoxy herbicides, primarily MCPA, in Denmark before 1982 was carried out. The study seems to support the Swedish observation of an increased risk of soft tissue sarcomas following exposure to phenoxy herbicides. The purpose of the study was to shed further light on the potential carcinogenic effect indicated by a Swedish case control study of the 2,4-dichlorophenol and 4-chloro-o-cresol based phenoxy herbicides unlikely to be contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Cancer cases were identified by linkage with the National Cancer Register. Special attention was given to soft tissue sarcomas and malignant lymphomas. Five cases of soft tissue sarcomas were observed among male employees in contrast to 1.84 expected cases, RR=2.72, CI95 =0.88-6.34 (Lynge, 1985).

An update of the above mentioned cohort study (Lynge, 1993) adds data for the period 1983-87. Based on small numbers the study adds to the evidence for a possible association between phenoxy herbicide exposure and risk of soft tissue sarcomas. There are, however, a number of possible confounders in these studies, and the overall cancer incidence of workers employed in manufacturing and packaging of phenoxy herbicides was the same as for the Danish population (66 observed v. 64.27 expected, SIR 1.0, 95% CI 0.8-1.3).

IARC (1987) concluded that the chlorophenoxy herbicides should be placed in group 2B because of limited evidence for carcinogenicity to humans and because no adequate published data were available on the carcinogenicity of MCPA to animals.

While PCOC is a breakdown product and possible contaminant of (impurity in) MCPA, implications of these finding for the effects of PCOC itself can remain only speculative.

4.1.2.8 Toxicity for reproduction

In a combined repeated dose/reproduction screening test carried out according to OECD draft guideline 422 (Hansen, 1996) groups of 10 male and 10 female rats were given 0, 50, 200, or 600 mg PCOC/kg in soybean oil by gavage for two weeks prior to mating and until day 20 of gestation. No toxic effects on any reproductive or developmental parameters were observed, resulting in a no effect level for these endpoints of 600 mg/kg.

In a recently conducted *in vitro* assay for estrogenic effects using human breast cancer cells (Körner et al, 1996; Körner et al, 1997), PCOC was found to express activity corresponding to $1x10^{-6}$ that of 17-β-Estradiol. It is difficult to evaluate what possible influence this might have on reproductive parameters.

Risk characterisation

4.1.2.9 General aspects

Major effects of possible concern are corrosivity, acute inhalation toxicity and repeat dose toxicity. Direct exposure is possible for production workers, and indirect exposure for workers, consumers and the general population.

The human risk assessment according to the pesticide scenario is not conducted based on a decision at EU Technical meeting on risk assessment of existing substances (TM III, Nov.1996) referring to this part of the risk assessment being conducted by DGVI working group on risk assessment of plant protection products.

4.1.2.10 Workers[4]

Production facility workers (see 4.1.1.2. for exposure levels). Realistic worst case exposure is likely to be of the order of 0.7 mg/kg/day according to information provided by one of the producers (A.H. Marks, 1997b).

[4] Herbicide application workers. (see 4.1.1.1. for exposure levels).
The exposure PCOC as a 1% impurity in MCPA can be in the order 0.28 mg/kg/day (agricultural) or 0.35 mg/kg/day (municipal weed control).

For the end-points irritation/corrosivity the concentration is below the level of concern. For repeat dose toxicity this should not present a major health problem, e.g. for repeat dose toxicity the margin of safety based on a NOAEL of 200 mg/kg/day is 200/0.35= 571.

The margin of safety for effects is in the order of 300-600, thus workplace exposure to PCOC does not seem to present a major risk.

Repeat dose toxicity is not likely to present a major health problem. The margin of safety based on a NOAEL of 200 mg/kg/day (slight effect on liver enzyme (ALAT), haemoglobin conc.) is 200/0.7 = 285.

Also the end-point irritation/corrosivity does not seem to cause any health concern. In situations with possible contact with the substance safety measures, such as wearing appropriate PPE, are prescribed in the existing productions. Further, health surveillance programmes including examination of the respiratory function have been undertaken for several years. According to the medical reports submitted by the producers no significant effects on the respiratory system have been observed.

It should be stressed that direct skin contact with PCOC can lead to burns and/or irritation, but that adequate warning of this effect is given by the manufacturers classification (R-35) and that the wearing of approriate PPE is compulsary when exposure at the workplace is possible (according to the UK Control Of Substances Hazardous to Health regulation - referred to in (Marks A H , 1997a)).

Conclusion of the risk assessment for workers:

( ) i) There is need for further information and/or testing
(X) ii) There is at present no need for further information and/or testing or for risk reduction measures beyond those which are being applied already
( ) iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account

4.1.2.11 Consumers

For this group exposure may be in the order of 0.07 mg/kg for each event corresponding to a daily dose of $9.6x10^{-4}$ mg/kg/day (see 4.1.1.3. for further details). With a NOAEL for repeat dose toxicity of 200 mg/kg/day the margin of safety is at least 20,000 for each single event.

Conclusion of the risk assessment for consumers:

( ) i) There is need for further information and/or testing

(X) ii) There is at present no need for further information and/or testing or for risk reduction measures beyond those which are being applied already

( ) iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account

4.1.2.12 Man exposed indirectly via the environment

The exposure of man indirectly via the environment through herbicide use is likely to be $10x10^{-8}$ mg/kg/day via human intake media.

Regional exposure resulting from production of PCOC is estimated as being low ($1.3x10^{-5}$ mg/kg/day), while local indirect exposure estimates of $1.2x10^{-4}$ mg/kg/day does not give rise to immediate concern with regard to corrosivity or repeat dose toxicity.

Conclusions of the risk assessment for man exposed indirectly via the environment:

( ) i) There is need for further information and/or testing

(X) ii) There is at present no need for further information and/or testing or for risk reduction measures beyond those which are being applied already

( ) iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account

4.1.2.13 Combined exposure

On the basis of the conclusion made in 4.1.1.2. and 4.1.1.3. a consumer, who also works at a production site and sprays garden herbicides, will receive the highest dose of PCOC during work and during gardening activities of 1.05 mg/kg/day. The dose received indirectly via the environment is low compared to this, $1.2x10^{-4}$ mg/kg/day, but would occur regularly. A margin of safety of 190 (200 mg/kg /1.05 mg/kg) would not seem to present undue risk.

Conclusions of the risk assessment for man during combined exposure:
( ) i) There is need for further information and/or testing

(X) ii) There is at present no need for further information and/or testing or for risk reduction measures beyond those which are being applied already

( ) iii) There is a need for limiting the risks; risk reduction measures which are already being applied shall be taken into account

HUMAN HEALTH (PHYSICO-CHEMICAL PROPERTIES)

4.1.3 Exposure assessment

The substance PCOC gives no reason for concern in relation to the following physical-chemical properties. The tests performed all gave or were expected to give negative results.

4.1.3.1 Occupational exposure

4.1.3.2 Consumer exposure

Indirect exposure via the environment
Effects assessment: Hazard identification and Dose (concentration) - response (effect) assessment

4.1.3.3 Explosivity

Explosive properties have not been tested. No reports of explosive properties were found in the available literature, nor does the chemical structure contain any elements associated with explosivity.

4.1.3.4 Flammability

The substance does not burn according to methods used (EF 3.10 and EF 3.10 mod.), nor is it flammable in contact with water. (Quist Laboratory, 1983).

4.1.3.5 Oxidizing potential

The substance was classified as non oxidizing according to the test method from the working group PC II Annex V EEC/831/79, sixth amendment of Dir. 67/548/EEC. (Dantest, 1983).

4.1.4 Risk characterisation

It is not likely that any of the above mentioned adverse effects should occur under the conditions mentioned.

5 CONCLUSIONS

The documentation varies from original studies according to OECD test guidelines with GLP to literature references of varying quality. 4-Chloro-2-methylphenol (PCOC) is used in the industry as an intermediate in the synthesis of the phenoxy herbicides MCPA, MCPB and mecoprop (MCPP). From the industrial production, processing and formulation PCOC is emitted to air and waste water. The produced pesticides contain PCOC as impurity (normally < 1%, 0,5% estimated as realistic worst case). The use of the pesticides in the agriculture as herbicides results in exposure to soil of PCOC as an impurity and degradation product.

As MCPA is transformed to PCOC, and PCOC has a high vapour pressure, the atmosphere will receive a contribution from application of the above mentioned pesticides. PCOC has a low to medium adsorption and may be considered mobile in some soils.

PCOC is according to an experiment primarily degradable by photolysis in clean water with a half-life of 4 days. However, a re-estimation of photolysis to typical EU surface water resulted in an estimated photolytic degradation half-life of 300-700 days and therefore photolysis is considered negligible. The available biodegradation data are somewhat conflicting but based on a judgement of the balance of evidence the "realistic worst case" aerobic biodegradation half-life of PCOC in soil is estimated to be 21 days, whereas no biodegradation has been found under anaerobic conditions. The aerobic biodegradation half-life in surface waters is also estimated to be 21 days. The estimated half-life in biological waste water treatment plants is 0.7 hour resulting in an estimated removal of 88% which is in general accordance with simple mass balance estimations from one of the main manufacturers sites. The substance is therefore considered to be ready biodegradable (borderline).

PCOC has been found in water, soil, air and groundwater. In water occurs mainly around emission sources, in air near fields applied with MCPA or MCPP, and in soil and biota after the application of the herbicides. The findings in groundwater are assumed to be the result of mobility and reduced degradation under anaerobic conditions.

The exposure assessment is primarily based on monitoring data from the two main manufacturing sites where all production and all processing of PCOC takes place and where approximately 60% of the production volume is formulated. A worst case environmental exposure scenario for a separate formulation site is included in the risk assessment.

The emissions to surface water from production sites are local and the risk assessment based on monitoring data ($C_{STP + influent}$ and actual dilution in STPs) and TGD default environmental exposure assesment for formulation site where 10% of the production volume of phenoxy acids is formulated. Because only the STPs receiving waste water from one of the production sites and the formulation sites are using sludge application to soil, The sludge application is considered local.

PCOC is very toxic to aquatic organisms. The acute toxicity to fish LC_{50} (96h) was observed to be about 2.3-6.6 mg/l. The EC_{50} (48h) to daphnids were 0.29-1.0 mg/l. The EC_{50} (96h) to algae was 8.2 mg/l and the EC_{10} (96h) was 0.89 mg/l. The long term toxic effects were observed in fish to have a NOEC(28d) of 0.5 mg/l and the Daphnia reproduction NOEC(21d) was 0.55 mg/l.

The $PEC_{local(water)}/PNEC_{aquatic\ organisms}$ relationship is < 1. Model calculation using EUSES version 1.0 supports the assumption of no risks for adverse effects in the aquatic environment and for the microorganisms of STPs.

There are no data available on the terrestrial toxicity. The equilibrium partitioning method is applied as a conservative calculation, comparing $PEC_{soil,\ porewater}$ with $PNEC_{aquatic\ organisms}$: $PEC_{soil}/PNEC < 1$.

PEC_{air}: There are no effect data present and no relation PEC/PNEC can be calculated.

PCOC has a bioaccumulation potential based on log Kow 3.09, but BCF found in fish was low (≤ 30). The risk characterisation of secondary poisoning is therefore not performed.

The substance is considered to be of no concern to aquatic organisms and microorganisms of STPs, and no further information on environmental release from production and formulation facilities is required.

No current evidence was found for the use of PCOC as such in products, although it may formerly have been employed as a disinfectant. Direct exposure is therefore likely to be restricted to those involved in the manufacture and handling of PCOC, and in conjunction with its use in the manufacture of phenoxy herbicides. Based on limited information, exposures in the range of 0.02 - 0.7 mg/kg/day are estimated for these activities.

The main exposure of human beings to PCOC is likely to be via production, or use of phenoxy herbicides which may contain it as an impurity (< 1%), or as a breakdown product following exposure of herbicides to sunlight, or to their metabolic transformation to the substance. It is difficult to quantify exposure occurring through transformation, but this is assumed to be less than 1%. During production, a realistic worst case exposure of 0.7 mg/kg/day is indicated. In conjunction with agricultural application of herbicides, a worst-case estimate of exposure to PCOC of 0.28 mg/kg/day is obtained. Municipal gardeners may be exposed to higher levels with an estimate of 0.35 mg/kg/day suggested as a realistic worst case.

Similarly, some consumer exposure should also be expected, as the same herbicides can be used in lawn treatment and similar gardening activities. While no detailed information was found on such exposures, it may be amount to 0.07 mg/kg per event. Assuming a really worst case of five events per year, the total yearly dose of PCOC would be 0.35 mg/kg/year corresponding to 9.6×10^{-4} mg/kg/day.

Indirect exposure via the environment resulting from partitioning into air/water/soil and biomagnification in food sources is low at a regional level, combined secondary exposure estimate being in the range of 1.40×10^{-5} mg/kg/day of PCOC. Local indirect exposure estimates are about 1.2×10^{-4} mg/kg/day.

The acute toxicity of PCOC (LD_{50} oral rat 2650-3196 mg/kg, LD_{50} dermal rat 2240 mg/kg, LC_{50} inhal. rat 4h 0.9mg/l or >30 mg/l) does not give rise to immediate concern, particularly considering that the substance (crystalline needles) is unlikely to form aerosols or dusts, and that PPE is mandated during handling of the substance.

PCOC is corrosive in high concentrations, and has been assigned risk phrase R-35 by the manufacturers which should provide adequate warning to those handling it in industrial settings. No consumer exposure is expected at concentrations which could approach that required for corrosivity. No sensitization was observed in a Guinea pig maximization test and no case studies indicating sensitization of persons handling the substance were found.

There were no effects on reproduction according to OECD screening test 422 at doses of up to 600 mg/kg for a total of 40 days.

In 28-day repeat dose studies in rats, the best NOAEL appears to be 200 mg/kg, with a LOAEL of 800 mg/kg where salivation after dosing and ruffled fur was seen in some animals. At this dose, levels of serum alanine-aminotransferase were increased in males, and effects were seen on blood parameters (reduced thromboplastin times, reduction of leukocyte and erythrocyte counts). Liver weights in females were increased, but no histopathological changes were seen in this, or any other organs examined. Decreased adrenal weights were also seen in females at 200 mg/kg and above, but were unaccompanied by histopathological changes.

PCOC has not been investigated for carcinogenicity. Two older tests were positive for mutagenicity, one *in vivo* (mouse micronucleus test) and one *in vitro* in a single strain (TA97) of Salmonella in the Ames test (while showing no activity in other strains in a number of separate tests). Repeated testing with TA97 gave unequivocally negative results. A repeat of the micronucleus test according to current guidelines also gave clearly negative results. On the balance, it is not felt that there is evidence for PCOC being a mutagen.

The estimated human local indirect exposure of 1.2×10^{-4} mg/kg/day is well below the repeat dose toxicity (NOAEL 200 mg/kg/day).

For the population with the highest potential exposure (production workers assuming inhalation exposure at 5 mg/m^3 for eight hours) a margin of safety of 285 (200 mg/kg/0.7 mg/kg/day) is obtained with regard to the repeat dose NOAEL. For agricultural workers engaged in spraying phenoxy herbicides the ratio is 200 mg/kg /0.28 mg/kg, or 714. For municipal gardeners (0.35 mg/kg/day) a margin of safety of 571 is obtained. Consumers may be exposed to 0.07 mg/kg/day once, or a few times yearly. All other exposure scenarios result in much higher margins of safety.

6 REFERENCES

Ames B.N, J. McCann & E. Yamasaki (1975): Mutation Res. 31, 347-364.

AT: Arbejdstilsynet. (1994). Grænseværdier for stoffer og materialer. At-anvisning nr. 3.1.0.2. Direktoratet for Arbejdstilsynet, København, Denmark.

Atkinson R (1986): Kinetics and mechanisms of the gas-phase reaction of the hydroxyl radical with organic compounds under atmospheric conditions. Chem Rev 86: 69-201

BASF (1994). IUCLID Data Sheet Prepared by BASF 1994.

Benoit-Guyod JL, Andre C, Clavel K (1984): Chlorophenols: degradation and toxicity. J. Fr. Hydrol. 10(3): 249-63.

Benoit-Guyod JL, Crosby DG, Bowers JB (1986): Degradation of MCPA by ozone and light. Water. Res. 20(1): 67-72.

Bhamidimarri SMR, Petrie RA (1992):
Adsorption and biodegradation of herbicide chemicals in silt loam soil.
Proc. Ind. Waste Conf. 46: 591-5

Binderup, M-L, "4-choro-2-methylphenol," Mutagenicity Study, Institute of Toxicology, National Food Agency, Denmark, 1996.

Blackman GE, Parke MH, Garton G (1955): The physiological activity of substituted phenols. I. Relationships between chemical structure and physiological activity. Arch. Biochem. Biophys. 54: 45-54.

Bollag JM, Alexander M (1967): Enzymatic degradation of phenoxyalkanoate herbicides. Bacteriol Proc. 1967: 8

Bollag JM, Helling CS, Alexander M (1967): Metabolism of 4-chloro-2-methylphenoxyacetic acid by soil bacteria. Appl. Microbiol 15(6): 1393-8.

Boethling *et al.* (1995): Factors for intermedia extrapolation in biodegradability assessment, Chemosphere 30 , 741-52.

BUA (1993): 4-Chloro-3-methylphenol. BUA reprot 135, GDCh-Advisory Committee on Existing Chemicals of Environmental Relevance, Stuttgart, December 1993, p. 103.

BUA (1994): 4-Chlor-2-methylphenol (p-Chlor-o-Kreol, PCOC). BUA-Stoffbericht 134. Beratergremium für umweltrelevante Altstoffe (BUA) der Gesellschaft Deutscher Chemiker.

Buccafusco, R.J. (1981): Acute toxicity of priority pollutants to Bluegill *Lepomis macrochirus*. Bulletin of Environmental Contamination and Toxicology 26: 446-52.

Buisson, RSK. Kirk, PWW, Lester, JN (1984): Determination of chlorinated phenols in water, waste-water and waste-water sludge by capillary gas chromatography-electron capture detector. J. Chromatogr. Sci. 22(8): 339-42.

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルのラットを用いる単回経口投与毒性試験

Single Dose Oral Toxicity Test of Tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate in Rats

要約

既存化学物質の安全性を評価するため，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルを雌雄のCrj:CD(SD)系ラットに単回経口投与し，急性毒性を検討した．なお，雌雄とも投与量は2000 mg/kgの1用量とし，対照として媒体(コーン油)投与群を設けた．

一般状態の観察では，コーン油の影響と考えられる軟便が2000 mg/kg群の雌雄全例に認められた．観察期間における死亡例は，2000 mg/kg群の雌雄いずれにも認められなかった．体重は，2000 mg/kg群の雌雄ともに観察期間終了時まで順調に増加した．剖検では，2000 mg/kg群の雌雄いずれにも異常は認められなかった．

方法

1. 被験物質

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステル(CAS No.3319-31-1，大八化学工業㈱，Lot.No. N-60601，純度99.0%以上，分子量546.87，凝固点-30℃，沸点430℃)は淡黄色透明，油溶性の液体であり，使用時まで室温条件下で密閉遮光保管した．なお，投与液は調製後，冷蔵保存で7日間安定であることを確認した．

2. 供試動物

生後5週のCrj:CD(SD)系ラット(SPF)雌雄各15匹を日本チャールス・リバー(株)から購入した．8日間にわたり動物を検疫・馴化飼育した後，6週齢で試験に用いた．投与時の体重は，雄で149～163 g，雌で126～140 gであった．

3.飼　育

動物は，温度23±2℃，湿度55±10%，換気回数20回/時間，照度150～300 lux，照明時間12時間(午前7時点灯，午後7時消灯)に設定された飼育室で，㈱東京技研サービスの自動水洗式飼育機を使用し，ステンレス製網目飼育ケージに5匹ずつ収容して飼育した．飼育ケージおよび給餌器は週1回取り換えた．動物には，オリエンタル酵母工業㈱製造の固型飼料MFを自由に摂取させ，飲料水としては，水道水を自由に摂取させた．

4. 用量設定理由

200および2000 mg/kgの用量を雌雄各3匹のラットに投与した予備試験では，いずれの投与群にも死亡例は認められなかった．以上の結果を参考にして，本試験では雌雄ともに2000 mg/kgの1用量を設定し，さらにコーン油のみを投与する対照群を設けた．

5. 群分け

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．

6. 投与液の調製および投与方法

所定量の被験物質をコーン油(ナカライテスク㈱)に溶解し投与液を調製した．溶液の濃度は，2000 mg/kg群で40.0 w/v%であった．

投与経路は経口とし，16時間絶食させた動物に注射ポンプと胃ゾンデを用い，被験物質溶液を投与した．投与容量は体重100 gあたり0.5 mlとし，個体別に測定した体重に基づいて算出した．給餌は被験物質投与3時間後に行った．

7. 一般状態の観察

中毒症状および生死の観察は，投与後6時間までは1時間毎に，その後は1日2回(午前と午後，休日は午前のみ)の割合で，投与後14日まで実施した．

8. 体　重

体重は投与直前，投与後7および14日に測定した．

9. 病理学検査

観察期間終了時の生存例については，エーテル麻酔下で放血安楽死させ解剖した．肉眼的異常所見を記録した．

結果および考察

1. 死亡率およびLD_{50}値

2000 mg/kg群の雌雄いずれにも死亡例は認めらず，LD_{50}値は雌雄とも2000 mg/kg以上と推定された．

2. 一般状態

対照群および2000 mg/kg群の雌雄全例において，軟便が投与後1時間から認められたが，投与後4時間には消失した．軟便の発現および消失の時間については，対

照群と2000 mg/kg群の間で差は認められなかったことから，軟便は，媒体として用いたコーン油の投与によるものと考えられた．

3. 体重

対照群および2000 mg/kg群の雌雄全例において，投与後7および14日の測定で対照群とほぼ同様な増加が認められた．

4. 剖検所見

対照群および2000 mg/kg群の雌雄いずれにも，異常を示す所見は認められなかった．

連絡先
試験責任者：大庭耕輔
試験担当者：藤島　敦
㈶食品農医薬品安全性評価センター
〒437-12静岡県磐田郡福田町塩新田字荒浜582-2
Tel 0538-58-1266　Fax 0538-58-1393

Correspondence
Authors: Kousuke Oba (Study director)
Atsushi Fujishima
Biosafety Research Center, Foods, Drugs and Pesticides (An-Pyo Center)
582-2 Shioshinden Arahama, Fukude-cho, Iwata-gun, Shizuoka, 437-12, Japan
Tel +81-538-58-1266　Fax +81-538-58-1393

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステ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 Tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate in Rats

要約

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルは，塩化ビニル用可塑剤として使用される化合物である．本化合物の毒性については，ほとんど報告がないため，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，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．

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルは，コーンオイルに溶解し，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，雌雄いずれの群にも異常動物は観察されず，死亡例もなかった．

体重，摂餌量，飼料効率，および血液生化学検査および器官重量には，雌雄とも被験物質投与に起因すると考えられる変化は認められなかった．

血液学検査の結果，雌雄とも被験物質投与に起因すると考えられる変化は認められなかった．

尿検査の結果，雌雄の1000 mg/kg群で尿量が増加した動物が認められたが，平均尿量および尿比重に有意差は認められなかった．

病理学検査の結果，肉眼および組織学的検索ともに，被験物質投与の影響が示唆される病変は観察されなかった．なお，肉眼所見において肺の有色斑／区域が，組織所見において腎臓の好酸性小体が，対照群に比べ雄の投与群に多く観察されたが，いずれも自然発生性病変が偶発的に増加したものと考えられた．

以上の結果，雌雄とも無影響量は1000 mg/kg/dayと判断された．

材料および方法

1. 被験物質

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステル (CAS No.3319-31-1，大八化学工業㈱提供)は淡黄色透明の油溶性液体で，分子式$C_{33}H_{54}O_6$，分子量546.87の化合物である．本試験に用いたロットN-60601の純度は99.0％以上であった．

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130～151 g，雌で110～121 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0，100，300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1000 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〕

本試験に先立って用量設定のための2週間投与試験(投与量：0，200，600および1800 mg/kg)を実施した．その結果，一般状態，体重，摂餌量，血液学検査，血液生化学検査，器官重量および病理学検査において被験物質投与に起因すると考えられる変化は認められなかった．従って，28日間反復投与試験の高用量は，1000 mg/kgとし，以下公比3で除し，中用量を300 mg/kg，低用量を100 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98.0～102％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，精巣および卵巣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)および肉眼所見で変化が認められた雄の肺を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，心臓，肝臓，脾臓，腎臓，副腎および骨髄(大腿骨)については対照群と高用量群，雄の腎臓についてはすべての群について行った．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被験物質投与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，雌雄とも対照群を含むすべての試験群で死亡例は認められなかった．

また，回復期間中，雌雄とも対照群および1000 mg/kg群で死亡例は認められなかった．

2. 一般状態の観察

投与期間および回復期間を通じて，雌雄いずれの群にも異常動物は観察されなかった．

3. 体　重

投与期間および回復期間を通じて，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．

4. 摂 餌 量

投与期間および回復期間を通じて，雌雄とも対照群と被験物質投与群とで差が認められず，0～4週および5～6週の総摂餌量にも差は認められなかった．

5. 血液学検査(Table 1)

〔投与終了時の検査結果〕

血液学検査に関しては，雌雄とも検査したすべての項目について，対照群と被験物質投与群とで差が認められなかった．

血液凝固検査に関しては，雄では，1000 mg/kg群で対照群に比較してプロトロンビン時間が僅かに延長を示したが，生理的変動の範囲内の値であった．雌では，対照群と被験物質投与群とで3項目とも差が認められなかった．

〔回復試験終了時の検査結果〕

血液学検査に関しては，雌の1000 mg/kg群で対照群に比較してヘモグロビン量が僅かに高値を示したが，生理的変動の範囲内の値であった．その他の項目は雌雄とも対照群と差がなかった．

血液凝固検査に関しては，すべての検査項目について雌雄の1000 mg/kg群と対照群とで差がなかった．

6. 血液生化学検査(Table 2)

〔投与終了時の検査結果〕

雄では，検査を行ったすべての項目について対照群と被験物質投与群とで差が認められなかった．雌では，対照群に比較して300および1000 mg/kg群で塩素が低値を示した．

〔回復試験終了時の検査結果〕

雄の1000 mg/kg群で対照群に比較してカリウムの僅かな高値が，また，雌の1000 mg/kg群で対照群に比較してGOTの僅かな高値が認められたが，軽微な変化であり，投与終了時にはこれらの項目で変化が認められておらず意義のある変化ではなかった．その他の項目は雌雄とも対照群と差がなかった．

7. 尿 検 査(Table 3)

〔投与終了時の検査結果〕

雌雄とも1000 mg/kg群で尿量の増加した動物が認められたが，平均尿量および雌雄とも1000 mg/kg群で尿比重は対照群と差が認められなかった．

〔回復試験終了時の検査結果〕

雌雄とも1000 mg/kg群はすべての検査項目で対照群との間に明確な差が認められなかった．

8. 器官重量(Table 4)

〔投与終了時の検査結果〕

雌雄とも重量測定を行ったすべての器官について，対照群と被験物質投与群とで差は認められなかった．

〔回復試験終了時の検査結果〕

雌の1000 mg/kg群で対照群に比較して副腎重量が高値を示した．その他の器官は雌雄とも1000 mg/kg群と対照群とで差が認められなかった．

9. 器官重量・体重比(相対重量)(Table 4)

〔投与終了時の検査結果〕

雌の100 mg/kg群で対照群に比較して肝臓相対重量が高値を示したが，用量相関性のない変化であった．その他の器官は雌雄とも，対照群と被験物質投与群とで差が認められなかった．

〔回復試験終了時の検査結果〕

雄の1000 mg/kg群で腎臓相対重量の低値，雌の1000 mg/kg群で副腎相対重量の高値が認められた．

10. 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みられた所見として，肺の有色斑／区域が雄で，100 mg/kg群の1例，300 mg/kg群の2例，1000 mg/kg群の3例に観察された．その他は，雄に腎臓の嚢胞と肥大，上皮小体の肥大など，雌に胸腺の赤色斑，子宮の内腔拡大など，いづれも1ないし2例の発生にとどまった．回復試験終了時の検査結果において，対照群に比較して被験物質投与群で多くみられた所見は観察されなかった．観察された所見はいづれも1ないし2例に発生し，肺の黒色斑，子宮の内腔拡大などであった．

Table 1 Hematology of rats treated orally with tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate 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 (%)	42.2 ± 1.5	41.5 ± 1.6	41.1 ± 1.4	42.3 ± 1.5	44.4 ± 1.5	45.6 ± 3.5
HGB (g/dl)	14.3 ± 0.3	14.0 ± 0.3	14.1 ± 0.4	14.4 ± 0.4	15.2 ± 0.5	15.7 ± 1.0
RBC ($\times 10^6$/mm^3)	7.12 ± 0.15	7.06 ± 0.12	6.99 ± 0.39	7.24 ± 0.29	7.89 ± 0.15N	8.25 ± 0.75
MCV (μm^3)	59.3 ± 1.7	58.8 ± 1.6	58.9 ± 1.9	58.5 ± 1.0	56.4 ± 1.5	55.4 ± 2.2
MCH (pg)	20.1 ± 0.2	19.9 ± 0.3	20.2 ± 0.7	19.9 ± 0.4	19.3 ± 0.6	19.0 ± 0.6
MCHC (%)	34.0 ± 0.9	33.8 ± 0.6	34.3 ± 0.3	34.1 ± 0.6	34.2 ± 0.7	34.4 ± 0.7
PLT ($\times 10^3$/mm^3)	1082 ± 122N	1281 ± 295	1051 ± 68	1155 ± 72	1011 ± 47N	981 ± 201
WBC ($\times 10^3$/mm^3)	13.0 ± 2.7	13.1 ± 3.4	11.4 ± 2.4	12.9 ± 2.6	13.4 ± 3.9	11.9 ± 2.1
Differential leukocyte counts (%)						
NEUT	10 ± 2N	13 ± 7	10 ± 2	9 ± 1	9 ± 2	11 ± 3
LYMPH	86 ± 2N	83 ± 7	86 ± 3	88 ± 1	87 ± 3	85 ± 3
MONO	2 ± 0	2 ± 1	2 ± 1	2 ± 1	1 ± 1	2 ± 1
EOSN	1 ± 0	1 ± 0	1 ± 1	1 ± 0	1 ± 0	1 ± 0
BASO	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LUC	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Reticulocyte (‰)	23 ± 4	25 ± 8	27 ± 3	26 ± 8	22 ± 6	22 ± 4
PT (sec.)	13.1 ± 0.2N	13.0 ± 0.3	13.4 ± 0.3	14.1 ± 1.2*	14.4 ± 0.7	14.3 ± 1.5
APTT (sec.)	25.8 ± 1.2N	25.7 ± 0.5	26.5 ± 1.2	28.2 ± 3.2	26.8 ± 2.6	26.1 ± 3.2
Fibrinogen (mg/dl)	275 ± 9	268 ± 15	266 ± 14	255 ± 32	269 ± 21	272 ± 13
Female						
No. of animals	5	5	5	5	5	5
HCT (%)	41.1 ± 0.5	40.4 ± 0.9	40.6 ± 1.2	41.0 ± 0.8	40.6 ± 1.0	42.0 ± 1.4
HGB (g/dl)	14.5 ± 0.2	14.0 ± 0.4	14.3 ± 0.5	14.4 ± 0.3	14.5 ± 0.2	15.1 ± 0.4**
RBC ($\times 10^6$/mm^3)	7.20 ± 0.20	7.10 ± 0.19	7.11 ± 0.33	7.16 ± 0.12	7.39 ± 0.21	7.67 ± 0.29
MCV (μm^3)	57.1 ± 1.3N	56.9 ± 1.1	57.2 ± 1.0	57.3 ± 0.2	55.0 ± 0.4	54.7 ± 1.2
MCH (pg)	20.1 ± 0.6	19.7 ± 0.7	20.1 ± 0.4	20.2 ± 0.2	19.7 ± 0.7	19.7 ± 0.4
MCHC (%)	35.3 ± 0.3	34.7 ± 0.6	35.2 ± 0.4	35.2 ± 0.4	35.7 ± 1.2N	36.0 ± 0.3
PLT ($\times 10^3$/mm^3)	1086 ± 58	1115 ± 115	1142 ± 127	1233 ± 177	1046 ± 127	1105 ± 84
WBC ($\times 10^3$/mm^3)	5.7 ± 1.3	5.4 ± 1.4	5.6 ± 1.7	5.5 ± 1.6	8.4 ± 2.3	6.9 ± 1.1
Differential leukocyte counts (%)						
NEUT	11 ± 3	15 ± 4	13 ± 2	13 ± 4	11 ± 3	14 ± 5
LYMPH	85 ± 3	81 ± 4	82 ± 3	83 ± 4	84 ± 3	83 ± 6
MONO	1 ± 1	2 ± 0	2 ± 1	2 ± 1	2 ± 0	2 ± 1
EOSN	2 ± 1	1 ± 0	2 ± 0	2 ± 1	1 ± 0	1 ± 0
BASO	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LUC	1 ± 1	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Reticulocyte (‰)	13 ± 3	20 ± 7	20 ± 6	20 ± 10	23 ± 5	31 ± 9
PT (sec.)	14.3 ± 0.5	13.6 ± 0.2	13.9 ± 0.5	13.9 ± 0.4	14.1 ± 0.1N	14.0 ± 0.3
APTT (sec.)	23.0 ± 1.2	23.0 ± 1.2	22.5 ± 0.5	22.3 ± 1.4	20.5 ± 1.9	19.5 ± 2.1
Fibrinogen (mg/dl)	189 ± 20	202 ± 10	196 ± 14	191 ± 13	223 ± 15	226 ± 22

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

Table 2 Blood chemistry of rats treated orally with tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate 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)	10.0 ± 1.6N	12.7 ± 9.0	8.8 ± 1.0	10.3 ± 2.1	10.8 ± 1.1	12.2 ± 2.7
Creatinine (mg/dl)	0.61 ± 0.05	0.75 ± 0.08	0.66 ± 0.08	0.74 ± 0.10	0.69 ± 0.03	0.68 ± 0.07
T.cholesterol (mg/dl)	50 ± 8N	63 ± 30	44 ± 3	40 ± 13	39 ± 9	51 ± 22
T.protein (g/dl)	5.43 ± 0.16	5.41 ± 0.17	5.44 ± 0.19	5.39 ± 0.26	5.59 ± 0.19	5.76 ± 0.26
Albumin (g/dl)	3.12 ± 0.05	3.07 ± 0.21	3.13 ± 0.10	3.13 ± 0.11	3.16 ± 0.10	3.25 ± 0.19
A/G	1.35 ± 0.06	1.32 ± 0.14	1.36 ± 0.06	1.40 ± 0.13	1.30 ± 0.04	1.29 ± 0.07
Glucose (mg/dl)	138 ± 15	151 ± 15	145 ± 7	137 ± 13	145 ± 23	148 ± 10
Triglyceride (mg/dl)	65.0 ± 20.3	89.6 ± 30.9	63.2 ± 15.2	64.9 ± 47.8	69.6 ± 28.7	75.6 ± 15.0
GOT (U/l)	41 ± 9	53 ± 9	45 ± 3	47 ± 4	42 ± 5	42 ± 4
GPT (U/l)	12 ± 3	14 ± 2	11 ± 2	13 ± 2	12 ± 2	13 ± 4
ALP (U/l)	158 ± 27	146 ± 25	169 ± 34	184 ± 49	118 ± 31	133 ± 22
γ-GTP (U/l)	1.0 ± 0.4	1.1 ± 0.8	0.6 ± 0.3	0.6 ± 0.5	0.5 ± 0.1	0.7 ± 0.2
T.bilirubin (mg/dl)	0.14 ± 0.03N	0.21 ± 0.22	0.12 ± 0.03	0.15 ± 0.02	0.10 ± 0.03	0.13 ± 0.03
Sodium (mmol/l)	141.7 ± 0.9	142.6 ± 1.6	142.4 ± 1.1	142.7 ± 1.3	143.8 ± 0.5N	144.2 ± 2.1
Potassium (mmol/l)	4.75 ± 0.10	4.81 ± 0.21	4.66 ± 0.25	4.68 ± 0.12	4.61 ± 0.18	4.91 ± 0.20*
Chloride (mmol/l)	107.0 ± 1.4	107.1 ± 0.8	107.3 ± 1.7	107.5 ± 0.6	108.3 ± 1.9	105.6 ± 2.5
Calcium (mg/dl)	10.13 ± 0.32	10.00 ± 0.28	9.84 ± 0.36	9.81 ± 0.35	9.71 ± 0.25	9.77 ± 0.34
I.phosphate (mg/dl)	8.19 ± 0.48	8.43 ± 0.42	7.88 ± 0.65	8.48 ± 0.76	7.46 ± 0.69	7.37 ± 0.69
Female						
No. of animals	5	5	5	5	5	5
BUN (mg/dl)	12.8 ± 2.3	11.3 ± 1.0	12.1 ± 1.0	15.4 ± 3.3	13.7 ± 0.6N	14.2 ± 2.2
Creatinine (mg/dl)	0.55 ± 0.14	0.61 ± 0.05	0.58 ± 0.08	0.61 ± 0.10	0.68 ± 0.11	0.80 ± 0.07
T.cholesterol (mg/dl)	30 ± 16	40 ± 10	48 ± 12	39 ± 12	49 ± 13	54 ± 12
T.protein (g/dl)	5.44 ± 0.11	5.61 ± 0.22	5.54 ± 0.22	5.80 ± 0.29	5.69 ± 0.17	5.88 ± 0.20
Albumin (g/dl)	3.29 ± 0.07	3.45 ± 0.20	3.36 ± 0.14	3.59 ± 0.22	3.38 ± 0.15	3.49 ± 0.14
A/G	1.54 ± 0.08	1.60 ± 0.09	1.55 ± 0.07	1.62 ± 0.12	1.46 ± 0.07	1.46 ± 0.06
Glucose (mg/dl)	108 ± 8	121 ± 9	117 ± 10	108 ± 8	125 ± 17	126 ± 9
Triglyceride (mg/dl)	33.8 ± 6.6	34.3 ± 3.7	39.1 ± 7.3	29.9 ± 3.2	43.6 ± 6.2	45.5 ± 9.6
GOT (U/l)	50 ± 10	55 ± 7	51 ± 8	61 ± 12	52 ± 8	62 ± 12
GPT (U/l)	11 ± 2	10 ± 2	11 ± 2	11 ± 1	10 ± 1N	13 ± 4*
ALP (U/l)	100 ± 22	90 ± 20	102 ± 49	102 ± 40	79 ± 15	86 ± 34
γ-GTP (U/l)	0.7 ± 0.5	0.5 ± 0.3	0.7 ± 0.2	0.4 ± 0.2	0.8 ± 0.2	0.7 ± 0.4
T.bilirubin (mg/dl)	0.16 ± 0.03	0.16 ± 0.03	0.17 ± 0.04	0.19 ± 0.02	0.19 ± 0.04	0.19 ± 0.04
Sodium (mmol/l)	142.8 ± 0.7	142.3 ± 0.6	141.9 ± 1.0	142.7 ± 1.4	142.9 ± 0.8	143.0 ± 1.4
Potassium (mmol/l)	4.45 ± 0.12	4.55 ± 0.17	4.55 ± 0.24	4.66 ± 0.30	4.58 ± 0.37	4.68 ± 0.20
Chloride (mmol/l)	111.3 ± 1.5	110.3 ± 0.9	109.2 ± 1.8*	108.1 ± 0.5**	111.3 ± 1.4	110.8 ± 1.0
Calcium (mg/dl)	9.61 ± 0.17	9.67 ± 0.09	9.65 ± 0.07	9.75 ± 0.24	9.58 ± 0.15	9.77 ± 0.22
I.phosphate (mg/dl)	6.01 ± 0.76	6.23 ± 0.54	6.29 ± 0.57	6.41 ± 0.63	5.91 ± 0.63	6.30 ± 0.39

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 tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate 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)		13 ± 3	20 ± 10	16 ± 5	23 ± 14	16 ± 4	20 ± 10
Specific gravity		1.059 ± 0.011	1.044 ± 0.018	1.038 ± 0.023	1.040 ± 0.025	1.033 ± 0.010	1.033 ± 0.021
Color	Colorless	0	0	0	1	0	0
	Slignt yellow	5	5	5	4	5	5
Turbidity	Clear muddy	5	5	5	5	5	5
pH	6	0	1	0	0	0	0
	7	0	0	1	2	0	0
	7.5	1	1	0	0	0	1
	8	0	0	0	1	2	0
	8.5	1	0	1	0	1	1
	≧9	3	3	3	2	2	3
Occult blood	−	5	4	5	4	5	5
	+/−	0	0	0	1	0	0
	2+	0	1	0	0	0	0
Ketones	−	0	0	1	1	0	3
	+/−	2	3	3	2	2	0
	1+	2	2	1	2	3	2
	2+	1	0	0	0	0	0
Glucose (g/dl)	−	5	5	5	5	5	5
Protein	+/-	0	0	0	1	0	0
(mg/dl)	30	1	0	4	2	5	3
	100	1	3	0	0	0	1
	≧300	3	2	1	2	0	1
Bilirubin	−	3	5	4	4	5	4
	1+	2	0	1	1	0	1
Urobilinogen	0.1	1	0	3	3	3	2
(E.U./dl)	1.0	4	5	2	2	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	4	3	4	5	5
	+	0	1	2	1	0	0
others	−	0	2	2	2	4	1
	+	5	3	3	3	1	4

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)		12 ± 3N	11 ± 5	11 ± 4	17 ± 13	9 ± 4	12 ± 4
Specific gravity		1.043 ± 0.008	1.042 ± 0.020	1.053 ± 0.017	1.049 ± 0.028	1.068 ± 0.024N	1.037 ± 0.006
Color	Colorless	0	0	0	1	0	0
	Slight yellow	5	5	5	4	4	5
	Yellow-brown	0	0	0	0	1	0
Turbidity	Clear muddy	5	5	5	5	5	5
pH	6	0	0	0	2	2	0
	6.5	1	0	2	0	1	1
	7	1	1	0	2	0	0
	7.5	1	1	1	0	1	2
	8	0	1	0	1	0	0
	8.5	0	1	2	0	0	1
	≧9	2	1	0	0	1	1
Occult blood	−	5	5	5	5	5	3
	+/−	0	0	0	0	0	2
Ketones	−	1	2	1	2	1	2
	+/−	4	2	2	1	1	3
	1+	0	1	2	2	3	0
Glucose (g/dl)	−	5	5	5	5	5	5
Protein	−	0	2	1	2	0	1
(mg/dl)	+/−	1	0	1	0	1	2
	30	3	0	0	1	0	0
	100	1	3	1	1	1	2
	≧300	0	0	2	1	3	0
Bilirubin	−	5	5	2	4	1	4
	1+	0	0	3	1	4	1
Urobilinogen	0.1	0	2	1	2	0	2
(E.U./dl)	1.0	5	3	4	3	5	3
Erythrocytes	−	4	4	5	5	4	5
	1+	1	1	0	0	1	0
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	−	0	2	3	3	1	2
	+	5	3	2	2	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 tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate 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)	343 ± 22	329 ± 28	328 ± 25	334 ± 18	378 ± 29	378 ± 38
Absolute organ weight						
Brain (g)	2.00 ± 0.04	2.06 ± 0.06	2.08 ± 0.06	2.07 ± 0.09	2.16 ± 0.11	2.10 ± 0.07
Liver (g)	11.03 ± 0.48	11.37 ± 0.96	10.62 ± 0.75	11.24 ± 1.66	10.62 ± 0.95	11.17 ± 1.67
Kidneys (g)	3.02 ± 0.72N	4.24 ± 4.02	2.45 ± 0.15	2.52 ± 0.17	2.84 ± 0.43	2.54 ± 0.29
Spleen (g)	0.60 ± 0.06	0.59 ± 0.09	0.55 ± 0.06	0.59 ± 0.06	0.63 ± 0.06	0.63 ± 0.07
Adrenals (mg)	47 ± 7	45 ± 5	45 ± 9	47 ± 9	48 ± 6	46 ± 9
Testes (g)	2.79 ± 0.12	2.82 ± 0.28	2.90 ± 0.25	2.75 ± 0.11	3.00 ± 0.17	3.10 ± 0.25
Relative organ weight						
Brain (%)	0.585 ± 0.030	0.628 ± 0.047	0.637 ± 0.043	0.620 ± 0.037	0.574 ± 0.037	0.560 ± 0.053
Liver (%)	3.224 ± 0.136	3.471 ± 0.447	3.247 ± 0.153	3.356 ± 0.349	2.812 ± 0.177	2.943 ± 0.207
Kidneys (%)	0.891 ± 0.260N	1.358 ± 1.426	0.752 ± 0.084	0.757 ± 0.093	0.749 ± 0.063	0.671 ± 0.032*
Spleen (%)	0.176 ± 0.019	0.183 ± 0.044	0.170 ± 0.025	0.175 ± 0.014	0.166 ± 0.016	0.168 ± 0.023
Adrenals (%)	0.014 ± 0.002	0.014 ± 0.001	0.014 ± 0.003	0.014 ± 0.002	0.013 ± 0.001	0.012 ± 0.002
Testes (%)	0.816 ± 0.069N	0.862 ± 0.112	0.885 ± 0.028	0.825 ± 0.031	0.796 ± 0.068	0.823 ± 0.071
Female						
No. of animals	5	5	5	5	5	5
Body weight (g)	202 ± 14	204 ± 26	216 ± 18	209 ± 15	226 ± 14	228 ± 19
Absolute organ weight						
Brain (g)	1.87 ± 0.06	1.87 ± 0.06	1.91 ± 0.10	1.94 ± 0.10	1.95 ± 0.07	1.96 ± 0.11
Liver (g)	5.65 ± 0.78	6.51 ± 0.74	6.51 ± 0.66	6.27 ± 0.52	6.03 ± 0.35	6.20 ± 0.72
Kidneys (g)	1.62 ± 0.13	1.65 ± 0.09	1.66 ± 0.14	1.65 ± 0.22	1.65 ± 0.16	1.71 ± 0.25
Spleen (g)	0.39 ± 0.08	0.37 ± 0.09	0.40 ± 0.08	0.37 ± 0.06	0.42 ± 0.06	0.43 ± 0.07
Adrenals (mg)	59 ± 13	58 ± 5	63 ± 4	60 ± 12	58 ± 5	70 ± 8*
Ovaries (mg)	85 ± 20	95 ± 16	87 ± 14	77 ± 16	75 ± 7	91 ± 23
Relative organ weight						
Brain (%)	0.926 ± 0.077	0.924 ± 0.096	0.886 ± 0.067	0.931 ± 0.094	0.862 ± 0.062	0.861 ± 0.046
Liver (%)	2.781 ± 0.194	3.191 ± 0.072**	3.013 ± 0.193	2.995 ± 0.175	2.663 ± 0.066	2.711 ± 0.128
Kidneys (%)	0.802 ± 0.031	0.812 ± 0.067	0.770 ± 0.052	0.789 ± 0.080	0.728 ± 0.060	0.747 ± 0.059
Spleen (%)	0.192 ± 0.031	0.178 ± 0.019	0.184 ± 0.028	0.178 ± 0.024	0.187 ± 0.026	0.188 ± 0.016
Adrenals (%)	0.029 ± 0.005	0.029 ± 0.003	0.029 ± 0.003	0.029 ± 0.004	0.026 ± 0.003	0.031 ± 0.002**
Ovaries (%)	0.042 ± 0.007	0.046 ± 0.005	0.040 ± 0.005	0.037 ± 0.007	0.033 ± 0.003	0.039 ± 0.007

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

Table 5 Summary of gross findings in rats treated orally with tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate 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
RESPIRATORY SYSTEM							
lung	black patch/zone	0	0	0	0	1	1
	colored patch/zone	0	1	2	3	0	0
URINARY SYSTEM							
kidney	cyst	1	1	0	0	0	0
	enlarged	0	1	0	0	0	0
ureter	dilated lumen	0	1	0	0	0	0
ENDOCRINE SYSTEM							
parathyroid gland							
	hypertrophic	0	0	0	1	0	0
Female							
No. of animals necropsied		5	5	5	5	5	5
HEMATOPOIETIC SYSTEM							
thymus	red patch/zone	0	0	1	0	0	0
RESPIRATORY SYSTEM							
lung	colored patch/zone	0	0	0	0	1	0
DIGESTIVE SYSTEM							
liver	white patch/zone	0	1	0	0	0	0
REPRODUCTIVE SYSTEM							
uterus	dilated lumen	0	1	0	0	1	2

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い発生を示した所見として，腎臓の好酸性小体が雄の対照群，100 mg/kg群，300 mg/kg群および1000 mg/kg群の順に，0, 1, 1および3例と投与群にやや多く観察された．

その他，肺の出血および細胞浸潤，肝臓の肉芽巣，腎臓の好塩基化，石灰沈着，副腎の空胞化などが観察された．

回復試験終了時の検査結果において，対照群に比較して被験物質投与群で多くみられた所見は観察されなかった．腎臓の好酸性小体は対照群でも軽度の所見が1例観察された．

その他，肺の出血，腎臓の好塩基化，石灰沈着など，投与終了時計画屠殺動物に観察された所見とほぼ同様の所見が観察された．

考察および結論

一般状態の観察で，雌雄いずれの群にも異常動物は認められず死亡例も認められなかった．

体重および摂餌量は，雌雄とも対照群と被験物質投与群で差がなく，被験物質投与の影響は認められなかった．また，体重および摂餌量に変化が認められないことから，飼料効率にも被験物質投与の影響は認められなかった．

血液学検査の結果，雌雄とも被験物質投与に起因すると考えられる変化は認められなかった．また，凝固検査においても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．

血液生化学検査の結果，雄では被験物質投与に起因すると考えられる変化は認められなかった．雌では，対照群に比較して300および1000 mg/kg群で塩素の低値が認められたが，ナトリウムおよびカリウムに変化は認められず，塩素の変化自体も軽微であることから，毒性学

Table 6 Summary of histopathological findings in rats treated orally with tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate 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		
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		
RESPIRATORY SYSTEM																			
lung		(5)			(5)			(5)			(5)			(5)			(5)		
	hemorrhage	3	0	0	3	0	0	3	0	0	4	0	0	2	0	0	1	0	0
	accumulation of foamy cells	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	infiltration/cellular	3	0	0	1	0	0	4	0	0	4	0	0	1	0	0	1	0	0
	interstitial pneumonia	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	fibrosis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DIGESTIVE SYSTEM																			
liver		(5)			(0)			(0)			(5)			(0)			(0)		
	bile duct dilatation	0	1	0	-	-	-	-	-	-	0	0	0	-	-	-	-	-	-
	necrosis, focal	1	0	0	-	-	-	-	-	-	0	0	0	-	-	-	-	-	-
	granulation	3	0	0	-	-	-	-	-	-	4	0	0	-	-	-	-	-	-
	infiltration/cellular	1	0	0	-	-	-	-	-	-	0	0	0	-	-	-	-	-	-
	lymphocytic infiltration	2	0	0	-	-	-	-	-	-	2	0	0	-	-	-	-	-	-
	bile duct hyperplasia	0	1	0	-	-	-	-	-	-	0	0	0	-	-	-	-	-	-
	extramedullary hematopoiesis	1	0	0	-	-	-	-	-	-	0	0	0	-	-	-	-	-	-
URINARY SYSTEM																			
kidney		(5)			(5)			(5)			(5)			(5)			(5)		
	basophilic change	3	1	0	4	0	0	4	0	0	3	0	0	5	0	0	3	0	0
	cyst	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	deposit of calcium	1	0	0	1	0	0	0	0	0	2	0	0	2	0	0	0	0	0
	eosinophilic body	0	0	0	0	1	0	0	1	0	0	3	0	1	0	0	1	1	0
	protein cast	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	tubular dilatation	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	lymphocytic infiltration	5	0	0	4	0	0	2	0	0	2	0	0	2	0	0	2	0	0
	scarring	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
ENDOCRINE SYSTEM																			
adrenal gland		(5)			(0)			(0)			(5)			(0)			(0)		
	vacuolic change	3	0	0	-	-	-	-	-	-	4	0	0	-	-	-	-	-	-
Female																			
No. of animals necropsied		5			5			5			5			5			5		
DIGESTIVE SYSTEM																			
liver		(5)			(0)			(0)			(5)			(0)			(0)		
	granulation	4	0	0	-	-	-	-	-	-	5	0	0	-	-	-	-	-	-
	lymphocytic infiltration	2	0	0	-	-	-	-	-	-	0	0	0	-	-	-	-	-	-
URINARY SYSTEM																			
kidney		(5)			(0)			(0)			(5)			(0)			(0)		
	basophilic change	4	0	0	-	-	-	-	-	-	2	0	0	-	-	-	-	-	-
	deposit of calcium	1	0	0	-	-	-	-	-	-	1	0	0	-	-	-	-	-	-
	deposit of pigment	1	0	0	-	-	-	-	-	-	0	0	0	-	-	-	-	-	-
	lymphocytic infiltration	2	0	0	-	-	-	-	-	-	0	0	0	-	-	-	-	-	-

1:slight 2:moderate 3:marked

Numbers in parenthesis indicate No. of animals examined microscopically at this site.

的意義は乏しいと考えられる.

尿検査の結果,雌雄とも1000 mg/kg群で尿量の増加した動物が認められたが,その他の定性項目,沈渣に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.

器官重量測定の結果,投与終了時の測定では雌雄とも被験物質投与に起因すると考えられる変化は認められなかった.回復期間終了時の測定では,1000 mg/kg群の雄で腎臓相対重量の低値,雌で副腎の実重量および相対重量の高値が認められたが,いずれも軽微な変化で,被験物質投与とそれに続く投与の休止に関連した変化とは考えられなかった(背景値,雄腎臓相対重量:0.73±0.06%,n=50,雌副腎実重量;66±7 mg,n=50,雌副腎相対重量:0.028±0.004%,n=50).

病理学検査の結果,対照群と比較して投与群に多くみられた所見として,肉眼所見では投与終了時計画屠殺動物において肺の有色斑/区域が,また,組織所見では腎臓に好酸性小体が,ともに雄にそれぞれ観察された.肺の有色斑/区域については,直径1～2 mm程度の褐色あるいは黒色調の小さな斑点が,単一あるいは少数個観察された.組織学的には限局性の出血,その周囲間質への炎症細胞浸潤などで説明される変化と考えられた.これらの組織変化は,雄の全群を通じて発生率ならびに程度に差はみられなかったことから,被験物質投与による変化ではなく,自然発生的な変化と考えられた.

また,腎臓の好酸性小体については,hyalin bodyの1種とされており[1]d-Limoneneや無鉛ガソリンなどの化学物質の影響で特に雄の近位尿細管での顕著な発生が報告されている[2].しかし,本所見は雄に自然発生性にも観察され,過去に当センターで実施した同系統,同週齢の背景値(8試験,雄45匹)によると22.2%(0～100%)の発生率で観察されていること,雄の1000 mg/kg群に特に多く観察されたものの全例には観察されず,また全投与群を通じて明らかな用量相関もみられないこと,回復試験群の対照群にも観察されたことから,肺の肉眼所見同様,自然発生性病変が偶発的に投与群に多くみられたものと考えられた.また,その他に観察された所見も発生率および程度に用量相関性は認められずすべて自然発生病変と考えられた.

以上のことから,本被験物質は,最大投与可能量の1000 mg/kg投与でも明確な被験物質投与の影響は示唆されず無影響量は雌雄とも1000 mg/kg/dayと判断された.

参考文献

1) 渡辺満利,"泌尿器系,毒性病理学," 前川昭彦,林裕造編,,地人書館,東京,1991, p. 229.
2) Carl L. Alden and Charles H. Frith, "Urinary System, Handbook of toxicologic pathology," ed. by Wanda M. Haschek and Colin G. Rousseauk, Academic Press, Inc., San Diego, 1991, pp.340-342

連絡先
試験責任者:井上博之
試験担当者:各務 進;庄子明徳,渡 修明,
小林和雄,山本慎二
(財)食品農医薬品安全性評価センター
〒437-12 静岡県磐田郡福田町塩新田字荒浜582-2
Tel 0538-58-1266 Fax 0538-58-1393

Correspondence
Authors: Hiroyuki Inoue (Study director),
Susumu Kakamu, Akinori Shoji,
Nobuaki Watari, Kazuo Kobayashi,
Shinji Yamamoto
Biosafety Research Center, Foods, Drugs and Pesticides (An-pyo Center)
582-2 Shioshinden Aza Arahama, Fukude-cho,
Iwata-gun, Shizuoka, 437-12, Japan
Tel 81-538-58-1266 Fax 81-538-58-1393

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルのラットを用いる経口投与簡易生殖毒性試験

Preliminary Reproduction Toxicity Screening Test of Tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate by Oral Administration in Rats

要約

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの100，300および1000 mg/kgを雄ラットに対しては交配前，交配期間および交配後の計46日間，雌ラットに対しては交配前，交配および妊娠期間，ならびに哺育3日までの期間，経口反復投与し，雌雄ラットへの反復投与による影響，雌雄ラットの生殖および次世代の発生に及ぼす影響についてスクリーニング試験を実施して，以下の知見を得た．

反復投与毒性では，雄の精巣の病理組織学検査で300 mg/kg群の2例および1000 mg/kg群の12例全例に精母細胞および精子細胞の減少が認められた．雌雄の一般状態，体重推移，摂餌量，剖検所見，生殖器重量および卵巣の病理組織学所見にはいずれの投与群においても被験物質投与による影響は認められなかった．以上のことから，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの反復投与による無影響量は，雄で100 mg/kg/day，雌で1000 mg/kg/dayであると判断された．

生殖発生毒性では，上述の如く雄の精巣に病理組織学的変化が認められたが，生殖能検査，生殖器重量，分娩および母性行動，新生児の生存率，一般状態，体重推移および剖検所見に被験物質投与による影響は認められなかった．以上のことから，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの雄の生殖に対する無影響量は100 mg/kg/day，雌の生殖に対する無影響量は1000 mg/kg/day，次世代の発生に対する無影響量は1000 mg/kg/dayであると判断された．

方法

1. 被験物質

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルは，淡黄色透明液体であり，遮光気密容器に入れ，室温で保存した．本試験では，大八化学工業㈱製造のロット番号N-80301(純度:99.0 %)を使用した．なお，被験物質は投与期間中安定であったことが製造業者の分析により確認された．

投与には，被験物質を20，60および200 mg/mLの濃度となるようにトウモロコシ油(片山化学工業㈱)に溶解して調製した．調製頻度は7日間に1回以上とし，投与に用いるまで遮光気密容器に入れ，室温で保存した．各濃度の調製液は規定の濃度であり，かつ均一であることがエヌシー技研㈱により確認された．

2. 試験動物および飼育条件

生後8週齢のCrj:CD(SD)系のSPFラットを日本チャールス・リバー㈱から受け入れ，14日間の検疫・馴化を行い，順調な発育を示した動物を試験に用いた．雌については，10日間の性周期検査を併せて行い，性周期に異常の認められない動物を用いた．

動物は，温度23±3℃，湿度55±10 %，換気回数10～15回／時間および照明時間12時間に設定されたバリアシステムの飼育室において，ブラケット式金属製金網床ケージを用いて飼育した．雌は，妊娠17日から金網床のかわりに実験動物用床敷(ホワイトフレーク，日本チャールス・リバー㈱)を敷いたステンレス製受皿を使用した．ケージ当たりの収容匹数は，群分け前は2匹以内，群分け後は1匹，交配中は雌雄各1匹，妊娠期間中は1母動物，哺育期間中は1腹とした．飼料は固形飼料(CRF-1，オリエンタル酵母工業㈱)を金属製給餌器を用いて，飲料水は水道水(札幌市水道水)を自動給水装置を用いて，それぞれ自由に摂取させた．

3. 投与量の設定，試験群の構成および群分け

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの100，300および1000 mg/kgを雌雄各5例に14日間反復経口投与した予備試験では，雌の100および1000 mg/kg群で摂餌量の低値傾向，雌の1000 mg/kg群で体重の低値傾向がみられた．したがって，最高用量は親動物に対して毒性を与えるが死亡させない量として，予備試験と同じ1000 mg/kg/dayとし，以下，予備試験と同じく公比約3で，300および100 mg/kg群を設定した．さらに媒体であるトウモロコシ油を投与する対照群を設け，計4群とし，動物数は1群当たり雌雄各12匹を用いた．群分けは，投与開始前日に投与開始前々日の体重値をもとに各群の体重が均一になるように体重別層化無作為抽出法を用いて行った．

4. 投与方法

投与経路は経口投与とし，胃ゾンデを用いて強制的に胃内に投与した．

投与期間は，雄については交配前14日間および交配期間を含む46日間，雌については交配前14日間および交尾までの交配期間，さらに交尾例は妊娠期間および哺育3日までの期間とした．

投与容量は，体重1 kg当たり5 mLとして投与日に当日または最も近い日の体重に基づいて算出した．投与は10週齢から開始し，投与開始時の平均体重(体重範囲)

は雄で401.2 g(373～435 g)，雌で 237.4 g(217～257 g)であった．

5. 観察，測定および検査項目

(1) 一般状態

一般状態は，1日1回以上の頻度で，視診および触診により行動，外観を観察した．

(2) 体重測定

体重は，投与1日(投与前)，投与2，5，7，10および14日，その後は雄については7日毎(投与終了日を含む)および剖検日に，雌については妊娠0，1，3，5，7，10，14，17および20日，哺育0，1および4日に，また交配期間中(雄と同居中)は相手雄の測定日と同じ日に電子天秤を用いて体重を測定した．雄については投与1から46日の，雌については投与1から14日，妊娠0から20日および哺育0から4日の体重増加量および体重増加率を算出した．

(3) 摂餌量測定

摂餌量は，雄については交配期間および剖検日を除き，雌については妊娠0日および哺育0日を除き体重測定日と同じ日(投与終了日を除く)に電子天秤を用いて測定した．

(4) 剖検および器官重量測定

雄については交尾成立例は投与46日の翌日に，交尾不成立例は交配期間終了の翌日に，エーテル麻酔下で採血後，放血致死させ，全身の器官および組織を肉眼的に観察した．雌については交尾不成立例は交配期間終了の翌日に，哺育3日まで生存児のみられた例は哺育4日に，妊娠25日まで分娩の認められない例は妊娠26日に，エーテル麻酔下で放血致死させ，全身の器官および組織を肉眼的に観察し，子宮の着床痕および卵巣の妊娠黄体を計数した．さらに，雌雄の全例について，精巣，精巣上体および卵巣の重量を電子天秤を用いて測定するとともに，器官体重重量比を算出した．

(5) 病理組織学検査

雄全例の精巣および精巣上体について，パラフィン包埋後薄切し，ヘマトキシリン・エオジン染色標本あるいは精巣のセルトリ細胞を確認するため，ストレプトアビジン・ビメンチン法を用いた免疫酵素抗体染色標本を作製し，病理組織学検査を行った．また，Matsui et al. の方法[11]に従い，精細管上皮の減少傾向がみられた例を優先して各群5例を選び，精子形成サイクルの14ステージのうち，ステージⅠ～Ⅵ(Group 1)，ステージⅦ～Ⅷ(Group 2)，ステージⅨ～XI(Group 3)，ステージXII～XIV(Group 4)について各グループに属する精細管を5本ずつ任意に選択して，精上皮細胞数をカウントし，各グループ毎に1精細管あたりの〔生殖細胞(精子細胞および精母細胞)数／セルトリ細胞数〕を算出した．雌については，全例の卵巣をパラフィン包埋後薄切し，ヘマトキシリン・エオジン染色標本を作製し，病理組織学検査を行った．

(6) 生殖能検査

雌全例について，投与開始日の10日前から交尾までの連日，ギムザ染色による膣垢塗抹標本を作製し，光学顕微鏡下で性周期段階(発情前期，発情期前期，発情期後期，発情後期および発情休止期)の判定を行い，性周期の異常の有無を検索した．

投与14日の雌雄について，同試験群内で夕方から1対1(無作為組合わせ)で14日間を限度として同居させた．交尾成立は雌の膣垢中に精子が確認された場合とし，その日を妊娠0日とした．妊娠の成立は雌の子宮に着床痕が確認された場合とした．交尾率〔(交尾動物数／同居動物数)×100〕および受胎率〔(受胎動物数／交尾動物数)×100〕を算出した．

(7) 分娩および母性行動

交尾した雌全例について，妊娠21日から分娩終了日まで分娩状態を観察し，午前9時に分娩が終了していた動物を当該日分娩とし，その日を哺育0日とした．分娩終了が確認された母動物について母性行動，総出産児数，生児数および死亡児数，出産児の性別および外表を観察した．また，妊娠期間〔妊娠0日から哺育0日(分娩終了日)までの日数〕，出産率〔(生児出産雌数／妊娠雌数)×100〕，分娩率〔(総出産児数／着床痕数)×100〕，出生率〔(出産生児数／総出産児数)×100〕，哺育4日時哺育率〔(哺育4日時に哺育児の認められる雌動物数／正常に分娩した雌動物数)×100〕および性比〔雄生児数／雌生児数〕を算出し，解剖時の計測結果から着床率〔(着床痕数／妊娠黄体数)×100〕を算出した．

(8) 新生児の一般状態および生存率

全例について，哺育0日から哺育4日まで1日1回生存および死亡を確認し，一般状態および外表について観察した．観察結果から，新生児の哺育4日の生存率〔(哺育4日生児数／出産生児数)×100〕を1腹を単位として算出した．なお，喰殺を受け死亡あるいは不明例となった新生児は死亡例として扱った．

(9) 新生児の体重測定

測定対象となる全例について，哺育0および4日に電子天秤を用いて測定し，体重値は1腹ごとに雌雄別に1匹あたりの平均値で示した．得られた測定値から体重増加量〔(哺育4日体重－哺育0日体重)〕および体重増加率〔(体重増加量／哺育0日体重)×100〕を算出した．

(10) 新生児の剖検

死亡例は発見後直ちに剖検し，その他の例については哺育4日に体外表(口腔内を含む)を観察した後，二酸化炭素吸入法を用いて安楽致死させ，全身の器官および組織を肉眼的に観察した．死亡例および異常所見部位の認められた例については，whole bodyを10％中性緩衝ホルマリン液で固定し，保存した．

6. 統計解析

性周期，交尾率，受胎率，出産率および哺育率，ならびに病理組織学検査結果のうち1段階の陽性グレードがみられた所見については，多試料χ^2-検定を行い，有意な場合2試料χ^2-検定を行った．また，これらの検定に不適合の場合はFisherの直接確率検定法を用いた．

その他の項目および病理組織学検査の結果のうち2段

階以上の陽性グレードがみられた所見についてはBartlettの等分散性検定後，一元配置分散分析法あるいはKruskal-Wallis法により解析し，有意な場合，Dunnettの検定法あるいはMann-WhitneyのU-検定法により，対照群と1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステル各投与群との比較を行った．

対照群との検定に際しては，有意水準を5および1％とした．

結果

1. 反復投与毒性

(1) 一般状態

雌雄ともに，いずれの群にも異常は認められなかった．

(2) 体重推移(Table 1, 2)，摂餌量および器官重量

雌雄ともに，被験物質投与と関連した変化は認められなかった．

(3) 剖検

雄では，腎臓に腎盂の拡張が300 mg/kg群で1例に，嚢胞が1000 mg/kg群で1例に認められた．他に精巣上体に黄白色斑が1000 mg/kg群で1例に認められ，いずれも先天的な異常と考えられた．

雌では，頭頂骨の隆起が100 mg/kg群の交尾不成立の1例に，空腸の憩室が300 mg/kg群の1例に認められた．

(4) 病理組織学検査(Table 3, 4)

雄では，精巣の病理組織学検査で，精母細胞および精子細胞の軽度の減少が300 mg/kg群で2例，1000 mg/kg群で11例，中等度の同所見が1000 mg/kg群で1例に認められた．中等度の精母細胞および精子細胞の減少が認められた1000 mg/kg群の1例では精細管内に多核巨細胞の軽度の出現およびセルトリ細胞の軽度の空胞化も認められ，精巣上体には管腔内に中等度の細胞残屑ならびに精子の中等度の減少が認められた．また，剖検で精巣上体に黄白色斑が認められた1000 mg/kg群の1例では，精母細胞および精子細胞の軽度の減少の他に，精巣上体に軽度の精子肉芽腫が認められた．対照群でも2例に精巣で精細管の軽度の萎縮が認められ，これら2例では精巣上体の管腔内に軽度の細胞残屑が認められ，そのうち1例で精巣上体管内精子の軽度の減少が認められた．

精巣の精上皮細胞数を計数した結果，Group 1(ステージⅠ～Ⅵ)では300 mg/kg群で精子細胞(roundおよびelongate)の低値，1000 mg/kg群で精母細胞および精子細胞(roundおよびelongate)の低値が認められ，Group 2(ステージⅦ～Ⅷ)では1000 mg/kg群で精子細胞(round)および精子細胞(round)のセルトリ細胞比の低値が認められた．Group 3(ステージIX～XI)では1000 mg/kg群で精子細胞(elongate)および精子細胞の(elongate)のセルトリ細胞比の低値が認められ，Group 4(ステージXII～XIV)では1000 mg/kg群で精母細胞，精子細胞(elongate)および精子細胞(elongate)のセルトリ細胞比の低値が認められた．雌では，黄体嚢胞が300 mg/kg群の2例に認められた．100 mg/kg群の交尾不成立の1例，対照群および100 mg/kg群の各1例では，卵巣に異常は認められなかった．

2. 生殖発生毒性

(1) 生殖能検査(Table 5)

いずれの群にも交尾成立までの日数，交尾率および受胎率に有意差は認められなかった．雌の性周期観察では，被験物質投与との関連を示唆する変化は認められなかった．なお，発情休止期の継続が投与期間に100 mg/kg群の1例で認められ，同例は交尾不成立であった．不妊例が対照群および100 mg/kg群で各1組に認められた．

(2) 分娩および母性行動(Table 6)

いずれの群にも分娩および母性行動の観察項目に被験物質投与と関連した変化は認められなかった．

(3) 新生児の生存率(Table 6)

いずれの群にも被験物質投与と関連した変化は認められなかった．

(4) 新生児の一般状態

いずれの例にも被験物質投与との関連を示唆する症状は認められなかった．

(5) 新生児の体重推移(Table 6)

300 mg/kg群の雄で哺育4日体重および哺育4日までの体重増加量に低値が認められ，同群の雌でも哺育1および4日ならびに哺育4日までの体重増加量に低値が認められた．

しかし，100および1000 mg/kg群では対照群と比較して有意差は認められなかった．

(6) 新生児の剖検

死亡例および哺育4日に屠殺した新生児の剖検では，被験物質投与との関連を示唆する所見は認められなかった．

考察

1. 反復投与毒性

雄では，精巣の病理組織学検査で300 mg/kg群の2例，1000 mg/kg群の12例全例に精母細胞および精子細胞の減少が認められた．300 mg/kg群および1000 mg/kg群では体重増加抑制および副生殖器の萎縮性変化は認められないことから，栄養障害あるいはホルモンのアンバランスによるものではなく，被験物質が直接，精母細胞に影響を及ぼした可能性が考えられた．一方，本スクリーニング試験と同じく1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの100，300および1000 mg/kg/dayを28日間経口反復投与した試験[2]では精細胞に影響は認められていない．今回の試験で精巣上体に精子の減少が認められたのは1000 mg/kg群の1例のみであり，その他の例では精巣上体管内の精子数に異常はみられないことから，本スクリーニング試験において認められた精細胞への影響は46日間の投与期間の後期に発現したと考えられた．

精巣上体では1000 mg/kg群の1例で精子肉芽腫が認められたが，この所見は自然発生的にも認められ，被験物質投与によるものとは考えられなかった．

上記の他に，雌雄の一般状態，摂餌量，剖検所見および生殖器重量ならびに雌の卵巣の病理組織学所見に被験物質投与による影響は認められなかった．

以上のことから，本試験における1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの反復投与による無影響量は，雄で100 mg/kg/day，雌で1000 mg/kg/dayであると判断された．

2. 生殖発生毒性

生殖能検査では交尾率および受胎率などに影響は認められず，300 mg/kg群および1000 mg/kg群の全例で交尾および妊娠が成立した．このことから，前述のように46日間の投与で精細胞の減少がみられるものの，本試験における交配前14日間の投与では妊娠の成立に影響を及ぼすものではなかったと考えられた．

なお，100 mg/kg群で認められた交尾不成立例1組では雌で頭頂骨の隆起がみられたが，生殖器に交尾不成立の原因を示唆する病理組織学所見は認められず，発情休止期の継続により交尾不成立となったと考えられ，より高用量の300および1000 mg/kg群で性周期に異常は認められず，全例で交尾が成立していることから，100 mg/kg群の交尾不成立例は被験物質投与との関連はないものと考えられた．

その他，雌の性周期に対して被験物質投与による影響は認められなかった．

新生児の観察では，300 mg/kg群で新生児の雌雄に体重の低値がみられたが，用量依存性は認められないことから，被験物質投与による影響とは考えられなかった．

他に，新生児の生存性，一般状態および剖検所見に被験物質投与による影響は認められなかった．

以上のことから，本スクリーニング試験における1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの親世代の生殖に対する無影響量は雄で100 mg/kg/day，雌で1000 mg/kg/day，次世代の発生に対する無影響量は1000 mg/kg/dayであると判断された．

文献

1) H. Matsui *et al.*, *J. Toxicol. Pathol.*, 9, 285(1996).
2) 井上博之ら，化学物質毒性試験報告，4，701(1996).

連絡先
試験責任者：吉村浩幸
試験担当者：茂野　均，古川正敏，河村公太郎，武田みよ子，引地のゆみ
㈱化合物安全性研究所
〒004-0839　北海道札幌市清田区真栄363番24号
Tel 011-885-5031　Fax 011-885-5313

Correspondence
Authors: Hiroyuki Yoshimura (Study director)
Hitoshi Shigeno, Masatoshi Furukawa, Kohtaro Kawamura, Miyoko Takeda, Noyumi Hikichi
Safety Research Institute for Chemical Compounds Co., Ltd.
363-24 Shin-ei, Kiyota-ku, Sapporo, Hokkaido, 004-0839, Japan
Tel +81-11-885-5031　Fax +81-11-885-5313

Table 1 Body weight changes of male rats treated orally with tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate in preliminary reproduction toxicity screening test

Item	0 mg/kg	100 mg/kg	300 mg/kg	1000 mg/kg
No. of animals	12	12	12	12
Day 1	400.3 ± 17.6	402.2 ± 16.7	400.0 ± 15.1	402.5 ± 14.4
2	400.2 ± 16.9	400.2 ± 19.1	399.5 ± 14.9	401.3 ± 17.5
5	420.7 ± 20.8	419.8 ± 23.0	419.8 ± 18.6	421.3 ± 18.1
7	430.4 ± 21.9	429.3 ± 23.7	430.7 ± 19.4	432.9 ± 20.0
10	443.3 ± 23.6	444.3 ± 28.4	445.8 ± 21.7	446.9 ± 20.6
14	461.1 ± 24.3	463.5 ± 33.0	463.0 ± 23.1	463.7 ± 20.0
21	486.3 ± 24.7	491.3 ± 38.3	489.2 ± 26.9	484.6 ± 21.0
28	511.4 ± 27.2	519.3 ± 45.2	514.1 ± 27.0	511.3 ± 22.5
35	537.6 ± 31.2	554.0 ± 46.5(11)	542.1 ± 28.2	538.2 ± 25.0
42	555.8 ± 34.3	571.7 ± 50.7(11)	561.6 ± 35.3	552.8 ± 27.4
46	566.4 ± 33.8	585.5 ± 51.1(11)	576.6 ± 34.9	560.2 ± 29.0
Day 1-46, gain	166.2 ± 24.5	181.5 ± 38.5(11)	176.6 ± 26.5	157.7 ± 21.4
Body weight gain [a] (%)	41.5 ± 5.9	44.7 ± 8.3(11)	44.1 ± 6.2	39.2 ± 5.1

Values are expressed as Mean ± S.D. (gram).
Values in parentheses are no. of animals examined.
a) : (Body weight gain/body weight on day 1)×100

Table 2 Body weight changes of female rats treated orally with tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate in preliminary reproduction toxicity screening test

Item	0 mg/kg	100 mg/kg	300 mg/kg	1000 mg/kg
Before gestation period				
No. of animals	12	12	12	12
Day 1	238.3 ± 14.0	239.0 ± 9.2	236.8 ± 11.6	235.4 ± 11.1
2	238.6 ± 11.1	240.2 ± 10.8	237.3 ± 13.7	237.5 ± 12.5
5	245.6 ± 15.6	248.7 ± 10.7	243.5 ± 13.8	242.3 ± 12.7
7	251.8 ± 12.5	252.6 ± 11.1	250.7 ± 12.0	247.6 ± 12.4
10	254.3 ± 15.0	258.3 ± 13.6	252.8 ± 16.2	251.4 ± 16.7
14	261.3 ± 18.0	264.1 ± 15.8	258.5 ± 17.9	257.8 ± 16.9
Day 1-14, gain	22.9 ± 10.4	25.1 ± 10.3	21.8 ± 10.8	22.3 ± 8.8
Body weight gain [a] (%)	9.6 ± 4.3	10.5 ± 4.2	9.2 ± 4.6	9.4 ± 3.6
During gestation period				
No. of animals	11	10	12	12
Day 0	267.0 ± 16.7	274.7 ± 16.5	265.6 ± 17.4	266.7 ± 18.4
1	270.8 ± 17.1	280.1 ± 16.7	273.3 ± 15.4	271.5 ± 17.3
3	285.9 ± 20.0	291.3 ± 17.6	286.8 ± 17.2	285.7 ± 18.4
5	296.2 ± 20.7	302.0 ± 17.0	295.0 ± 15.3	296.1 ± 17.7
7	304.9 ± 21.5	309.9 ± 16.7	305.0 ± 15.4	304.9 ± 19.1
10	319.2 ± 23.2	323.3 ± 18.5	319.3 ± 16.1	318.7 ± 21.7
14	343.1 ± 25.7	348.3 ± 20.0	342.7 ± 17.8	344.3 ± 20.6
17	372.1 ± 24.8	378.4 ± 23.9	372.3 ± 22.1	375.2 ± 21.6
20	420.1 ± 27.9	428.9 ± 27.0	422.2 ± 28.7	426.1 ± 22.0
Day 0-20, gain	153.1 ± 15.2	154.2 ± 15.5	156.6 ± 17.8	159.4 ± 14.9
Body weight gain [b] (%)	57.4 ± 4.9	56.2 ± 5.4	59.1 ± 6.4	60.1 ± 7.3
During lactation period				
No. of animals	11	10	12	12
Day 0	328.9 ± 28.1	327.6 ± 22.3	321.6 ± 19.6	329.5 ± 27.6
1	325.5 ± 25.4	331.5 ± 23.3	323.8 ± 23.0	326.1 ± 22.0
4	337.6 ± 26.9	343.2 ± 25.5	336.8 ± 20.6	336.6 ± 23.5
Day 0-4, gain	8.7 ± 10.1	15.6 ± 8.1	15.3 ± 8.5	7.1 ± 17.8
Body weight gain [b] (%)	2.7 ± 3.3	4.7 ± 2.4	4.8 ± 2.8	2.4 ± 5.3

Values are expressed as Mean ± S.D. (gram).
a) : (Body weight gain/body weight on day 1)×100
b) : (Body weight gain/body weight on day 0)×100

Table 3 Number of cells in seminiferous tubules of male rats treated orally with tris (2-ethylhexyl) 1,2,4-benzenetricarboxylate in preliminary reproduction toxicity screening test

Item	0 mg/kg	100 mg/kg	300 mg/kg	1000 mg/kg
No. of animals examined	5	5	5	5
Group 1 (Stage I - VI)				
No. of Sertoli cells	20.12 ± 3.18	19.08 ± 1.49	18.52 ± 1.45	18.08 ± 1.45
Spermatogonia				
No.	16.80 ± 5.65	20.52 ± 2.58	18.48 ± 3.17	15.76 ± 2.61
ratio a)	0.85 ± 0.29	1.08 ± 0.19	1.01 ± 0.21	0.87 ± 0.11
Spermatocytes				
No.	50.80 ± 7.44	51.80 ± 4.84	43.64 ± 2.63	40.84 ± 5.63*
ratio	2.53 ± 0.13	2.72 ± 0.26	2.37 ± 0.24	2.25 ± 0.16
Round spermatids				
No.	138.36 ± 17.20	128.00 ± 8.89	117.68 ± 5.59*	112.60 ± 3.11**
ratio	6.91 ± 0.35	6.75 ± 0.84	6.39 ± 0.70	6.26 ± 0.48
Elongate spermatids				
No.	130.00 ± 21.71	132.32 ± 11.17	103.28 ± 12.34*	95.36 ± 8.44**
ratio	6.53 ± 1.15	6.98 ± 0.88	5.62 ± 0.90	5.30 ± 0.69
Group 2 (Stage VII - VIII)				
No. of Sertoli cells	16.96 ± 2.63	17.04 ± 2.17	16.64 ± 2.73	16.52 ± 2.23
Spermatogonia				
No.	2.92 ± 1.06	2.40 ± 0.93	2.04 ± 0.68	2.60 ± 1.10
ratio	0.18 ± 0.09	0.14 ± 0.05	0.12 ± 0.03	0.16 ± 0.06
Spermatocytes				
No.	91.68 ± 10.37	94.68 ± 6.55	84.44 ± 6.99	82.32 ± 6.70
ratio	5.45 ± 0.56	5.60 ± 0.51	5.16 ± 0.79	5.03 ± 0.54
Round spermatids				
No.	142.08 ± 13.39	131.64 ± 13.72	123.96 ± 8.23	118.76 ± 8.28*
ratio	8.45 ± 0.62	7.75 ± 0.39	7.66 ± 1.66	7.25 ± 0.62*
Elongate spermatids				
No.	129.24 ± 17.37	128.32 ± 16.88	114.72 ± 9.80	105.64 ± 13.47
ratio	7.78 ± 1.54	7.56 ± 0.72	7.09 ± 1.62	6.46 ± 1.05
Group 3 (Stage IX - XI)				
No. of Sertoli cells	19.28 ± 1.92	20.52 ± 1.55	19.20 ± 1.58	19.32 ± 2.18
Spermatogonia				
No.	4.52 ± 1.32	4.20 ± 1.50	4.92 ± 1.63	3.32 ± 1.02
ratio	0.23 ± 0.05	0.21 ± 0.08	0.26 ± 0.11	0.18 ± 0.05
Spermatocytes				
No.	102.52 ± 10.83	99.08 ± 8.42	97.56 ± 4.50	89.04 ± 9.00
ratio	5.34 ± 0.56	4.85 ± 0.50	5.10 ± 0.36	4.62 ± 0.32
Elongate spermatids				
No.	145.24 ± 11.01	130.64 ± 9.90	131.68 ± 19.71	119.24 ± 15.90*
ratio	7.56 ± 0.61	6.37 ± 0.23	6.88 ± 1.04	6.21 ± 0.83*
Group 4 (Stage XII - XIV)				
No. of Sertoli cells	19.16 ± 2.81	20.92 ± 1.73	18.64 ± 1.72	16.72 ± 0.92
Spermatogonia				
No.	4.04 ± 0.89	3.72 ± 0.72	3.64 ± 0.48	3.64 ± 0.71
ratio	0.21 ± 0.05	0.18 ± 0.03	0.20 ± 0.02	0.22 ± 0.05
Spermatocytes				
No.	109.80 ± 13.15	110.36 ± 9.22	99.44 ± 4.54	88.76 ± 4.33**
ratio	5.76 ± 0.29	5.28 ± 0.12	5.36 ± 0.34	5.32 ± 0.46
Elongate spermatids				
No.	159.76 ± 15.91	150.28 ± 18.99	137.08 ± 17.70	105.16 ± 18.34**
ratio	8.39 ± 0.63	7.19 ± 0.71	7.35 ± 0.62	6.33 ± 1.31**

Values are expressed as Mean±S.D.
Significantly different from 0 mg/kg group ; *: $p \leqq 0.05$, **: $p \leqq 0.01$.
a) : (No. of spermatogenic cells/no. of Sertoli cells in a seminiferous tubule)

Table 4 Histopathological findings in rats treated orally with tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate in preliminary reproduction toxicity screening test

Item		0 mg/kg	100 mg/kg	300 mg/kg	1000 mg/kg
No. of male animals examined		12	12	12	12
Organ: Findings					
	Grade				
Testis:					
Decrease, spermatocyte and spermatid	Total	0	0	2	12**
	+	0	0	2	11
	++	0	0	0	1
Multinuclear giant cell, seminiferous tubule	+	0	0	0	1
Vacuolization, Sertoli cell	+	0	0	0	1
Atrophy, seminiferous tubule	+	2	0	0	0
Epididymis:					
Cell debris, lumen	Total	2	0	0	1
	+	2	0	0	0
	++	0	0	0	1
Decrease, sperm	Total	1	0	0	1
	+	1	0	0	0
	++	0	0	0	1
Granuloma, spermatic	+	0	0	0	1
No. of female animals examined		12	12	12	12
Organ: Findings					
Ovary:					
Cyst, corpus luteum	<+>	0	0	2	0

Values are no. of animals with findings.
Grade: +=slilght, ++=moderate change and <+>=detected.
Significantly different from 0 mg/kg group; **: $p \leq 0.01$.

Table 5 Influence of tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate on reproductive performances of rats in preliminary reproduction toxicity screening test

Item	0 mg/kg	100 mg/kg	300 mg/kg	1000 mg/kg
No. of pairs mated	12	12	12	12
No. of pairs with successful copulation	12	11	12	12
Duration of mating (days, Mean±S.D.)	2.1 ± 1.2	2.3 ± 1.3	2.7 ± 1.2	2.7 ± 1.1
Copulation index[a] (%)	100.0	91.7	100.0	100.0
No. of pregnant animals	11	10	12	12
Fertility index[b] (%)	91.7	90.9	100.0	100.0

a): (No. of pairs with successful copulation/no. of pairs mated)×100
b): (No. of pregnant animals/no. of pairs with successful copulation)×100

Table 6 Influence of tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate on developmental performances of rats in preliminary reproduction toxicity screening test

Item		0 mg/kg	100 mg/kg	300 mg/kg	1000 mg/kg
No. of pregnant animals		11	10	12	12
No. of corpora lutea		16.8 ± 1.5	17.3 ± 1.3	17.0 ± 2.3	17.9 ± 2.2
No. of implantation sites		15.5 ± 1.7	16.6 ± 1.3	16.0 ± 2.0	16.3 ± 2.3
Implantation index[a] (%)		92.5 ± 7.2	96.2 ± 6.6	94.5 ± 8.4	91.3 ± 8.8
No. of pups born (%)		13.7 ± 3.1	15.0 ± 1.7	15.0 ± 1.8	15.1 ± 2.7
Delivery index[b] (%)		87.6 ± 15.4	90.3 ± 6.8	94.1 ± 7.2	92.2 ± 9.6
Live pups born					
No.		13.3 ± 2.9	14.7 ± 2.0	14.9 ± 2.0	15.0 ± 2.7
Live birth index[c] (%)		97.1 ± 5.6	97.8 ± 3.6	99.2 ± 2.6	99.4 ± 2.1
Sex ratio (M/F)		1.09 ± 0.69	1.05 ± 0.50	1.17 ± 0.75	0.76 ± 0.44
Dead pups born					
No.		0.5 ± 0.9	0.3 ± 0.5	0.1 ± 0.3	0.1 ± 0.3
Gestation length (day)		22.7 ± 0.5	22.7 ± 0.5	22.5 ± 0.5	22.6 ± 0.5
Gestation index[d] (%)		100.0	100.0	100.0	100.0
Nursing index[e] (%)		100.0	100.0	100.0	100.0
Live pups on day 4					
No.		13.2 ± 2.8	14.6 ± 2.1	14.4 ± 2.9	14.5 ± 2.9
Viability index[f] (%)		99.5 ± 1.8	99.3 ± 2.3	95.6 ± 11.5	96.7 ± 6.7
Body weight of pups (g)					
Male	Day 0	7.32 ± 0.77	7.13 ± 0.52	6.69 ± 0.55	6.87 ± 0.84
	Day 4	11.71 ± 1.76	11.09 ± 0.93	10.23 ± 0.98*	10.60 ± 1.47
	Day 0-4, gain (g)	4.39 ± 1.04	3.96 ± 0.53	3.54 ± 0.77*	3.73 ± 0.80
	Body weight gain[g] (%)	59.41 ± 8.87	55.54 ± 6.16	53.19 ± 11.91	54.39 ± 9.50
Female	Day 0	6.93 ± 0.83	6.63 ± 0.64	6.33 ± 0.58	6.58 ± 0.62
	Day 4	11.08 ± 1.71	10.28 ± 1.01	9.48 ± 1.01*	10.03 ± 1.46
	Day 0-4, gain (g)	4.16 ± 1.00	3.65 ± 0.56	3.14 ± 0.79*	3.46 ± 0.96
	Body weight gain (%)	59.63 ± 10.42	55.24 ± 8.07	49.95 ± 13.09	52.17 ± 11.10

Values are expressed as Mean ± S.D.
Significantly different from 0 mg/kg group ; *: $p \leq 0.05$.
a) : (No. of implantation sites/no. of corpora lutea)×100
b) : (No. of pups born/no. of implantation sites)×100
c) : (No. of live pups born/no. of pups born)×100
d) : (No. of females with live pups delivered/no. of pregnant females)×100
e) : (No. of females nursing live pups/no. of females with normal delivery)×100
f) : (No. of live pups on day 4/no. of live pups born)×100
g) : (Body weight gain/body weight on day 0)×100

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの細菌を用いる復帰突然変異試験

Reverse Mutation Test of Tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate on Bacteria

要約

既存化学物質安全性調査事業の一環として，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルについて，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/プレートの用量で実施したところ，いずれの検定菌においても，抗菌性は認められなかった．したがって，本試験はS9 mix無添加試験および添加試験を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，2回の本試験とも，用いた検定菌のいずれについて，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，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルは，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

〔被験物質〕

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステル(CAS No. 3319-31-1)は，分子量546.87の淡黄色透明液体である．試験には，大八化学工業㈱製〔ロット番号：N-60601，純度99.0％以上(不純物：不明)〕を，㈳日本化学工業協会から供与された，使用時まで室温遮光保管して用いた．

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルは，アセトンに溶解性がよいことから，アセトンに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，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルのアセトン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(3.13 mg/ml)溶液は本試験Ⅰで調製したものについて，また高濃度(500 mg/ml)溶液は染色体異常試験で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，ともに初期値(0時間)の平均値に対して，101％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，低濃度は91.4％，高濃度は91.7％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はジメチルスルホキシド(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1l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,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を混合したのちトップアガー2 mlを加えて混和し,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アセトン,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,すべての検定菌においてS9 mix無添加試験および添加試験のいずれも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.1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルの用量を,S9 mix添加試験および添加試験でともに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,1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルは,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.

文献

1) D. M. Maron, B. N. Ames, *Mutation Res.*, **113**, 173 (1983).
2) M. H. L. Green, "Handbook of Mutagenicity Test Procedures," eds. by B. J. Kilbey, M. Legator, W. Nichols, C. Ramel, Elsevier, Amsterdam, New York, Oxford, 1984, pp. 161-187.

連絡先

試験責任者:澁谷 徹

試験担当者:原 巧,坂本京子,川上久美子,清水ゆり,松木容彦,中込まどか,飯田さやか

(財)食品薬品安全センター 秦野研究所

〒257 秦野市落合729-5

Tel 0463-82-4751 Fax 0463-82-9627

Correspondence

Authors: Tohru Shibuya (Study Director), Takumi Hara, Kyoko Sakamoto, Kumiko Kawakami, Yuri Shimizu, Yasuhiko Matsuki, Madoka Nakagomi and Syaka Iida

Hatano Research Institute, Food and Drug Safety Center

729-5 Ochiai, Hadano-shi, Kanagawa 257 Japan

Tel +81-463-82-4751 Fax +81-463-82-9627

Table 1. Mutagenicity of tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate** 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	96 111 91 (99±10.4)	20 11 14 (15± 4.6)	20 21 22 (21± 1.0)	26 10 22 (19± 8.3)	6 10 5 (7± 2.6)	
	313 #	117 104 125 (115±10.6)	13 11 20 (15± 4.7)	26 34 31 (30 ±4.0)	21 26 19 (22± 3.6)	12 7 10 (10± 2.5)	
	625 #	155 112 119 (129±23.1)	15 12 26 (18± 7.4)	26 21 20 (22 ±3.2)	25 36 22 (28± 7.4)	10 9 8 (9± 1.0)	
	1250 #	117 130 130 (126± 7.5)	21 14 15 (17± 3.8)	19 24 25 (23 ±3.2)	22 23 25 (23± 1.5)	8 11 11 (10± 1.7)	
	2500 #	143 142 148 (144± 3.2)	15 21 12 (16± 4.6)	23 28 20 (24 ±4.0)	21 27 23 (24± 3.1)	3 5 11 (6± 4.2)	
	5000 #	132 121 138 (130± 8.6)	13 20 12 (15± 4.4)	25 36 32 (31 ±5.6)	14 28 19 (20± 7.1)	5 3 9 (6± 3.1)	
S9mix (+)	0	107 125 125 (119±10.4)	16 19 9 (15± 5.1)	23 24 28 (25± 2.6)	25 32 28 (28± 3.5)	12 13 9 (11± 2.1)	
	313	144 118 116 (126±15.6)	12 13 11 (12± 1.0)	31 27 33 (30± 3.1)	38 33 42 (38± 4.5)	19 18 17 (18± 1.0)	
	625	168 137 137 (147±17.9)	18 13 12 (14± 3.2)	35 33 23 (30± 6.4)	38 29 32 (33± 4.6)	19 14 14 (16± 2.9)	
	1250 #	130 120 166 (139±24.2)	8 20 15 (14± 6.0)	35 42 35 (37± 4.0)	36 30 36 (34± 3.5)	18 16 12 (15± 3.1)	
	2500 #	138 129 107 (125±15.9)	12 15 8 (12± 3.5)	32 29 25 (29± 3.5)	37 39 37 (38± 1.2)	10 12 8 (10± 2.0)	
	5000 #	149 144 128 (140±11.0)	12 7 11 (10± 2.6)	24 15 25 (21± 5.5)	37 39 23 (33± 8.7)	10 11 7 (9± 2.1)	
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	499 483 527 (503±22.3)	576 533 509 (539±33.9)	107 100 111 (106± 5.6)	665 569 726 (653±79.1)	798 714 804 (772±50.3)	
Positive control S9 mix(+)	Chemical	2AA	2AA	2AA	2AA	2AA	
	Dose(μg/plate)	1	2	10	0.5	2	
	Number of colonies/plate	1091 1292 1423 (1269±167.2)	326 304 322 (317±11.7)	1391 1251 1387 (1343±79.7)	438 455 387 (427±35.4)	252 243 261 (252± 9.0)	

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

#:Precipitate was observed on the surface of agar plates.

**:Purity was above 99.0 % and impurity was unknown.

Table 2. Mutagenicity of tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate** in reverse mutation test (II) 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	124 106 135 (122±14.6)	10 11 15 (12± 2.6)	29 28 25 (27± 2.1)	24 28 20 (24± 4.0)	9 3 12 (8± 4.6)	
	313 #	125 130 142 (132± 8.7)	14 18 18 (17± 2.3)	22 21 23 (22± 1.0)	26 21 25 (24± 2.6)	11 10 15 (12± 2.6)	
	625 #	125 116 141 (127±12.7)	18 10 16 (15± 4.2)	22 31 30 (28± 4.9)	33 13 19 (22±10.3)	9 7 10 (9± 1.5)	
	1250 #	134 125 127 (129± 4.7)	17 18 7 (14± 6.1)	21 16 23 (20± 3.6)	27 29 24 (27± 2.5)	9 11 6 (9± 2.5)	
	2500 #	129 120 124 (124± 4.5)	11 9 12 (11± 1.5)	21 17 13 (17± 4.0)	19 22 25 (22± 3.0)	7 10 8 (8± 1.5)	
	5000 #	145 147 141 (144± 3.1)	13 13 12 (13± 0.6)	18 21 21 (20± 1.7)	18 12 21 (17± 4.6)	12 15 13 (13± 1.5)	
S9mix (+)	0	138 130 126 (131± 6.1)	9 13 12 (11± 2.1)	23 26 16 (22± 5.1)	26 25 41 (31± 9.0)	23 16 15 (18± 4.4)	
	313	153 125 140 (139±14.0)	15 20 18 (18± 2.5)	32 20 25 (26± 6.0)	33 38 34 (35± 2.6)	26 18 16 (20± 5.3)	
	625	131 140 138 (136± 4.7)	13 17 16 (15± 2.1)	26 28 24 (26± 2.0)	25 32 26 (28± 3.8)	14 15 19 (16± 2.6)	
	1250 #	129 156 161 (149±17.2)	18 12 16 (15± 3.1)	30 23 12 (22± 9.1)	32 33 40 (35± 4.4)	17 17 15 (16± 1.2)	
	2500 #	122 138 160 (140±19.1)	9 12 13 (11± 2.1)	19 18 20 (19± 1.0)	37 32 33 (34± 2.6)	16 22 22 (20± 3.5)	
	5000 #	156 158 164 (159± 4.2)	14 25 15 (18± 6.1)	21 29 18 (23± 5.7)	29 39 30 (33± 5.5)	16 15 15 (15± 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	682 650 670 (667±16.2)	232 245 242 (240± 6.8)	108 151 190 (150±41.0)	954 935 930 (940±12.7)	1103 1071 999 (1058±53.3)	
Positive control S9 mix(+)	Chemical	2AA	2AA	2AA	2AA	2AA	
	Dose(μg/plate)	1	2	10	0.5	2	
	Number of colonies/plate	1262 1111 1309 (1227±103.5)	299 270 290 (286±14.8)	1510 1537 1546 (1531±18.7)	455 457 566 (493±63.5)	300 295 294 (296± 3.2)	

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

#:Precipitate was observed on the surface of agar plates.

**:Purity was above 99.0 % and impurity was unknown.

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルのチャイニーズ・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 Tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate 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，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステ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)，短時間処理(6時間)ともに5.0 mg/mlの濃度においても50%を越える増殖抑制は認められなかったことから，すべての試験において5.0 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時間および48時間連続処理したいずれの処理群においても，染色体の構造異常や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，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルは，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

1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステル(略号：TEBTC，CAS No.：3319-31-1，ロット番号：N-60601，大八化学工業㈱製造，㈳日本化学工業協会提供)は，淡黄色透明液体，水に対しては不溶で，DMSOおよびアセトンには極めてよく溶け，凝固点-30℃，沸点430℃(760 mmHg)，蒸気圧0.01 mmHg以下(25℃)，分子式$C_{33}H_{54}O_6$，分子量546.87，純度99.0%以上(不純物は不明)の物質である．

被験物質原体は高温時，水により加水分解を受ける．溶媒中(アセトン)では，3.13～500 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．溶媒はアセトン(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，被験物質処理群の陰性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，短時間処理ともに，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作用は認められなかった(Fig. 1)．

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，短時間処理とも5.0 mg/mlとし，それぞれ高濃度群の1/2の濃度を中濃

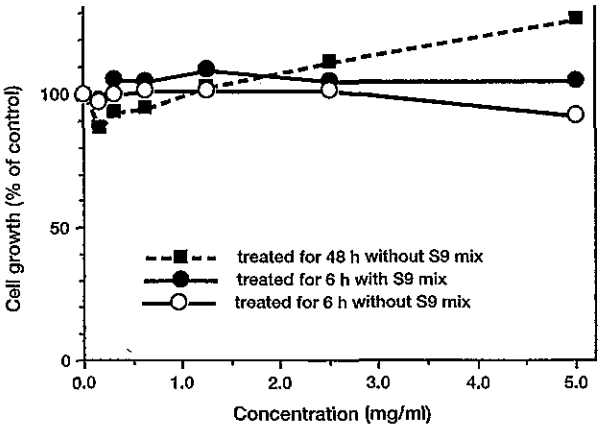


Fig. 1 Growth inhibition of CHL/IU cells treated with tris(2-ethylhexyl) 1,2,4-benzenetricarboxylate

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，陰性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．1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルを加えて24時間および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．1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルを加えて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，1,2,4-ベンゼントリカルボン酸トリス(2-エチルヘキシル)エステルは，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 tris (2- ethylhexyl) 1,2,4-benzenetricarboxylate (TEBTC) * 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	1	3	0	0	0	0	4	0	4 (2.0)	3 (1.5)	0.13		
Solvent[1]	0	24	200	0	0	0	1	0	0	1	0	1 (0.5)	1 (0.5)	0.50		
TEBTC	1.3	24	200	0	1	0	0	0	0	1	0	1 (0.5)	1 (0.5)	0.63		
TEBTC	2.5	24	200	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.25	NT	NT
TEBTC	5.0	24	200	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.38		
MC	0.00005	24	200	4	43	84	0	1	0	132	1	92 (46.0)	92 (46.0)	0.13		
Solvent[1]	0	48	200	0	0	0	1	0	0	1	0	1 (0.5)	1 (0.5)	0.25		
TEBTC	1.3	48	200	0	0	0	2	0	0	2	0	1 (0.5)	1 (0.5)	0.13		
TEBTC	2.5	48	200	0	1	0	0	0	0	1	0	1 (0.5)	1 (0.5)	0.25	NT	NT
TEBTC	5.0	48	200	0	0	0	0	0	0	0	2	0 (0.0)	0 (0.0)	0.25		
MC	0.00005	48	200	1	29	63	1	8	0	102	4	65 (32.5)	64 (32.0)	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. 1) Acetone 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 99.0%.

Table 2 Chromosome analysis of Chinese hamster cells (CHL/IU) treated with tris (2- ethylhexyl) 1,2,4-benzenetricarboxylate (TEBTC) ** 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	3	0	0	0	0	4	0	4 (2.0)	3 (1.5)	0.38		
Solvent[1]	0	-	6-(18)	200	0	0	1	2	0	0	3	0	2 (1.0)	2 (1.0)	0.38		
TEBTC	1.3	-	6-(18)	200	1	2	0	0	0	0	3	0	3 (1.5)	2 (1.0)	0.13		
TEBTC	2.5	-	6-(18)	200	0	0	0	0	0	0	0	0	0 (0.0)	0 (0.0)	0.50	NT	NT
TEBTC	5.0	-	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	2	0	0	0	0	0	2	0	2 (1.0)	0 (0.0)	0.25		
Solvent[1]	0	+	6-(18)	200	0	2	5	0	0	0	7	0	4 (2.0)	4 (2.0)	0.25		
TEBTC	1.3	+	6-(18)	200	1	1	0	0	0	0	2	1	2 (1.0)	1 (0.5)	0.25		
TEBTC	2.5	+	6-(18)	200	2	1	1	0	0	0	4	0	4 (2.0)	2 (1.0)	0.50	NT	NT
TEBTC	5.0	+	6-(18)	200	2	2	1	0	0	0	5	0	5 (2.5)	3 (1.5)	0.25		
CPA	0.005	+	6-(18)	200	5	34	112	1	0	0	152	8	89 (44.5)	86 (43.0)	0.25		

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) Acetone 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 99.0%.

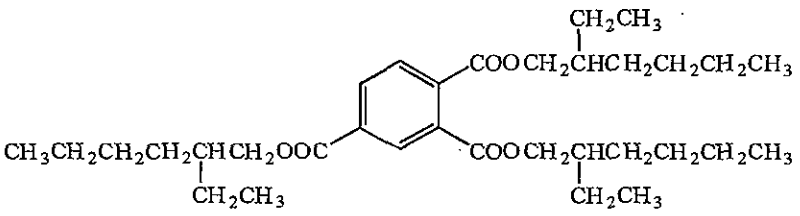
文献

1) 日本環境変異原学会・哺乳動物試験分科会編，“化学物質による染色体異常アトラス，” 朝倉書店，東京，1988.
2) 林　真，変異原性試験，1，255 (1992).
3) 吉村　功　編著，“毒性・薬効データの統計解析，事例研究によるアプローチ，” サイエンティスト社，東京，1987.
4) 吉村　功，大橋靖夫　編，“毒性試験講座14，毒性試験データの統計解析，” 地人書館，東京，1992.

連絡先
試験責任者：田中憲穂
試験担当者：山影康次，若栗　忍，日下部博一，橋本恵子，長尾哲二
(財)食品薬品安全センター秦野研究所
〒257　神奈川県秦野市落合729-5
Tel 0463-82-4751　Fax 0463-82-9627

Correspondence
Authors: Noriho Tanaka (Study director)
Kohji Yamakage, Shinobu Wakuri, Hirokazu Kusakabe, Keiko Hashimoto, Tetsuji Nagao
Hatano Research Institute, Food and Drug Safety Center
729-5 Ochiai, Hadano, Kanagawa, 257, Japan
Tel +81-463-82-4751　Fax +81-463-82-9627

SIDS INITIAL ASSESSMENT PROFILE

CAS No.	3319-31-1
Chemical Name	Tris(2-ethylhexyl)benzene-1,2,4-tricarboxylate
Structural Formula	

RECOMMENDATIONS

The chemical is currently of low priority for further work.

SUMMARY CONCLUSIONS OF THE SIAR

Human Health

In a single dose study of rats, 75 % of the orally administered chemical at 100 mg/kg bw was excreted in an unchanged form in the feces, 16 % as metabolites in the urine and 1.9 % was expired as CO_2.

The acute toxicity of the chemical is low because it showed no toxic signs at 2,000 mg/kg bw by oral route in rats [OECD TG 401] and at 2 mL/kg by dermal route in rabbits. During exposure by inhalation at 2600 mg/m^3, no death occurred in rats, but reddening patches in the lungs were observed after 14 days post exposure.. In an irritation-test for animals, the chemical was slightly irritating to the skin and the eyes. A sensitization test on guinea pigs showed no sensitization [OECD TG 406].

A feeding study with rats for 28 days showed a decrease of hemoglobin and an increases of leucocyte counts and serum cholesterol as well as an increased liver weight in the mid and high dose groups (0.67 and 2.0 %). Liver biochemistry revealed increases in palmitoyl CoA oxidation (increased in both sexes at 2.0% and males at all dose levels) and catalase activity (increased in males at 2.0%), suggesting the induction of peroxisome proliferation. Further analysis by an electron microscope indicated slight increased number of peroxisomes in hepatocytes at the high dose. It is generally accepted that the induction of peroxisome proliferation occurs specifically in rodents but much less in other species including humans. There were no dose-related histopathological changes in any treated groups. The NOAEL in this study was considered to be 0.2 % (184 mg/kg bw/day).

The OECD reproductive/developmental toxicity screening test [TG 421] for at least 46 days at doses of 100, 300 and 1,000 mg/kg/day demonstrated a decrease of spermatocytes and spermatids in testis in the 300 and 1000 mg/kg groups but not in the 100 mg/kg group.

Based on the testicular toxicity, the NOAEL for repeated dose toxicity is considered to be 100 mg/kg bw/day.

As for reproductive/developmental toxicity, the chemical showed no adverse effects on copulation, fertility, delivery and nursing of females nor on the viability, body weight and morphology of offspring in the above screening test [OECD TG 421]. However, the NOAEL for reproductive toxicity in males was considered to be 100 mg/kg bw/day

because of the testicular toxicity described above. Both NOAELs for reproductive toxicity in females and developmental toxicity of offspring were considered to be 1,000 mg/kg bw/day.

The genotoxicity of this chemical was evaluated in many *in vitro* assay systems. It was neither mutagenic in bacteria [OECD TG 471 & 472] nor clastogenic in mammalian cells [Guidelines for Screening Mutagenicity Testing of Chemicals (Japan)].

Environment

The Mackay level III fugacity Model was employed to estimate the environmental distribution of this chemical in air, water, soil and sediment. If released to air, this chemical will exist solely in the particulate phase in the ambient atmosphere. If released to soil, this chemical is not expected to be distributed to other compartments.

This chemical has to be considered as weakly toxic against aquatic organisms and is not biodegradable. This chemical has a high logPow value (5.94), the measured BCF is reported as less than 1 to 2.7 in carp for 6 weeks, but some uncertainty still remains regarding the bioaccumulation potential of this chemical. This result indicates that the bioavailability of this chemical is low. The toxicity results to aquatic plants (algae; *Selenastrum capricornutum*) were >100 mg/L for EC_{50} (72hr). The acute toxicity data in fish (medaka; *Oryzias latipes*) were >100 mg/L (96h, LC_{50}) and >75 mg/L (14d, LC_{50}). In *Daphnia magna*, the acute toxicity was >180mg/L (48hr: EC_{50}) and the chronic toxicity was >55.6 mg/L (21d, reproduction). All these data were obtained in supersaturated solution with the aid of solubilizer (HCO-40). The test solution was considered to be homogeneous. Another chronic toxicity data in *Daphnia magna* (NOEC >0.082mg/L) was reported (Procedure of ASTM and USEPA). Though this value is lower than the saturation point, the measured concentration data were less reliable.

Based on the description of the test results above, it can be concluded that Tris(2-ethylhexyl)benzene-1,2,4-tricarboxylate does not show any toxic effects at the limit of solubility towards those aquatic organisms, which were tested in the laboratory. Though it is difficult to determine a PNEC, this substance is not toxic at its water solubility (OECD TG105; 0.13 mg/L 25 C).

Exposure

This chemical is manufactured as a plasticizer for PVC.

The production volume in Japan is approximately 20,000 tonnes/year and there are 5 manufacturers in Japan. Estimated global production is 40,000-100,000 tonnes/year. This chemical is mainly used as a plasticizer for PVC electrical cable and wire.

Occupational exposure may occur through dermal contact and inhalation of mist. This chemical is produced in closed system and workers wear protective gloves and goggles during the operation, so actual exposure in the work place is considered to be low.

Since this chemical is difficult to extract from the polymeric matrix, consumer and environmental exposure are considered to be low.

NATURE OF FURTHER WORK RECOMMENDED

There is no recommendation for further work. The hazards of this chemical towards the environment and human health are considered to be low. Both occupational and consumer exposure are considered to be low.

これまで合計21種の化合物について検索を行ったが，1,2-dichloroethane のみが TA100 において S9 mix 共存下で陽性の結果を示し，他はすべて陰性であった．これらの検体については哺乳動物培養細胞に対する染色体異常誘発性をも検討しており，その結果を本誌に併せて発表してあるので参照されたい．

文 献

1) 佐谷戸安好：トキシコロジーフォーラム，7，443 (1984)
2) 矢部禎明：水，1，86 (1983)
3) Ames, B. N. *et al.*: *Mutation Res.*, **31**, 347 (1975)
4) Galli, A. *et al.*: *Boll.-Soc. Ital. Biol. Sper.*, **58**, 860 (1982)
5) Shahin, M. and R. C. von Borstel: *Mutation Res.*, **48**, 173 (1977)
6) Abe, S. and M. Sasaki: *J. Natl. Cancer Inst.*, **58**, 1635 (1977)
7) Guengerich, F. P. *et al.*: *Toxicol. Appl. Pharmacol.*, **55**, 303 (1980)
8) Ozawa, N. and F. P. Guengerich: *Proc. Natl. Acad. Sci. USA*, **80**, 5266 (1983)

水道水汚染有機化合物およびその関連物質の変異原性に関する研究
II．哺乳動物培養細胞による染色体異常試験

祖父尼俊雄・林　真・松岡厚子・沢田　稔
畑中みどり・石館　基

Mutagenicity Tests on Organic Chemical Contaminants in City Water and Related Compounds
II. Chromosome Aberration Tests in Cultured Mammalian Cells

Toshio Sofuni, Makoto Hayashi, Atsuko Matsuoka, Minoru Sawada,
Midori Hatanaka and Motoi Ishidate, Jr.

The clastogenic potential of organic chemical contaminants in city water and related compounds was examined using Chinese hamster cells (CHL) in culture. Out of 25 chemicals tested without a metabolic activation system, two chemicals, acrylonitrile and acrylamide, significantly induced chromosome aberrations. By the metabolic activation system, 27 chemicals were tested, and 5 of them, anthracene, pyrene, acetophenone, biphenyl and 1,2-dichloroethane, were positive only in the presence of S9 mix. Two chemicals, acrylonitrile and acrylamide, were also positive both with and without S9 mix. One chemical, benzaldehyde, was positive only in the absence of S9 mix. Other chemicals showed no significant increase in the incidence of chromosome aberrations when used with and without S9 mix. In summary, out of 32 chemicals examined with or without the metabolic activation system, 8 chemicals were positive in the presence and/or absence of S9 mix.

(Received May 31, 1985)

水道水中の微量有機化合物について，微生物による遺伝子突然変異試験に加えて，哺乳動物培養細胞による染色体異常試験を行ったので，その結果について報告する．

試 験 方 法

チャイニーズ・ハムスター肺由来の細胞株 (CHL) を用い，代謝活性化を行わない直接法[1]，あるいは S9 を用いる代謝活性化法[2] による染色体異常試験を行った．

直接法では 2×10^4 個の細胞をガラス培養瓶に播き，3日目に検体を加え，24時間および48時間後に染色体標本を作製した．検体濃度は，予備試験によって細胞増殖が約50%抑制される濃度を求めて，それを指標として3段階濃度（原則として公比2）を選択した．

代謝活性化法では，ガラス培養瓶に細胞を播種し，3日目に検体と S9 mix とで6時間処理した．その後，新しい培養液と交換し，さらに18時間培養し，染色体標本を作製した．S9 は Ames 試験に用いたものと同じく，PCB (KC 400) 処理したラットまたはマウスの肝から調製した．

染色体の観察は，培養瓶当たり 100 個のよく広がっ

Table 2. Chromosome aberration tests in cultured Chinese hamster cells (Direct method) (1982)

Compound	Solvent	Dose (mg/ml)	Treatment time (h)	Polyploid (%)	Frequency (%) of aberrant cells$ ctg	ctb	cte	csb	cse	Total	Judge
1,2,3-Trichloro-benzene	DMSO	0	24	1.0	2.0	0.0	0.0	0.0	0.0	2.0	
		0.0157	24	1.0	1.0	1.0	1.0	0.0	0.0	3.0	−
		0.0313	24	1.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.0625	24	0.0	0.0	1.0	1.0	0.0	0.0	1.0	−
		0	48	1.0	2.0	0.0	0.0	0.0	0.0	2.0	
		0.0157	48	1.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		0.0313	48	2.0	1.0	0.0	1.0	0.0	0.0	2.0	−
		0.0625	48	3.0	2.0	1.0	0.0	0.0	0.0	3.0	−
1,2,4-Trichloro-benzene	DMSO	0	24	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
		0.0313	24	3.0	0.0	2.0	1.0	0.0	0.0	2.0	−
		0.0625	24	3.0	1.0	0.0	1.0	0.0	0.0	2.0	−
		0.125	24	TOX	- - - - - TOX - - - - - -						
		0	48	0.0	1.0	0.0	0.0	0.0	0.0	1.0	
		0.0313	48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		0.0625	48	1.0	0.0	1.0	1.0	0.0	0.0	1.0	−
		0.125	48	TOX	- - - - - TOX - - - - - -						
1,3,5-Trichloro-benzene	DMSO	0	24	1.0	1.0	0.0	0.0	0.0	0.0	1.0	
		0.0157	24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		0.0313	24	4.0	1.0	1.0	0.0	0.0	0.0	2.0	−
		0.0625	24	1.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0	48	1.0	1.0	0.0	0.0	0.0	0.0	1.0	
		0.0157	48	1.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.0313	48	1.0	1.0	1.0	0.0	0.0	0.0	2.0	−
		0.0625	48	0.0	1.0	0.0	1.0	0.0	0.0	2.0	−
Biphenyl	DMSO	0	24	0.0	1.0	1.0	0.0	0.0	0.0	2.0	
		0.075	24	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.1	24	1.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		0.125	24	1.0	0.0	4.0	0.0	0.0	0.0	4.0	−
		0	48	0.0	0.0	0.0	1.0	0.0	0.0	1.0	
		0.075	48	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.1	48	2.0	1.0	0.0	0.0	0.0	1.0	2.0	−
		0.125	48	2.0	2.0	0.0	0.0	0.0	0.0	2.0	−
l-Menthol	DMSO	0	24	0.0	1.0	0.0	0.0	0.0	0.0	1.0	
		0.0313	24	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.0625	24	0.0	1.0	1.0	1.0	0.0	0.0	3.0	−
		0.125	24	0.0	2.0	0.0	0.0	0.0	1.0	3.0	−
		0	48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		0.0313	48	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.0625	48	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.125	48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
dl-Menthol	EtOH	0	24	1.0	1.0	1.0	0.0	0.0	0.0	2.0	
		0.1	24	2.0	3.0	1.0	1.0	0.0	0.0	5.0	±
		0.15	24	1.0	0.0	1.0	0.0	0.0	0.0	1.0	−
		0.2	24	0.0	3.5	2.5	0.5	0.0	0.0	6.0	±
		0	48	1.0	2.0	1.0	0.0	0.0	1.0	3.0	
		0.1	48	0.0	0.0	1.0	1.0	0.0	0.0	2.0	−
		0.15	48	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.2	48	0.0	2.0	1.0	2.0	0.0	1.0	4.0	−

$,TOX: See the foot-notes in Table 1.

Table 3. (continued)

Compound	Solvent	Dose (mg/ml)	Treatment time (h)	Polyploid (%)	Frequency (%) of aberrant cells‡ ctg	ctb	cte	csb	cse	total	Judge
Di(2-ethylhexyl)-phthalate	DMSO	0	24	0.0	1.0	0.0	0.0	0.0	0.0	1.0	
		0.0625	24	1.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		0.125	24	2.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		0.25	24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		0	48	1.0	1.0	0.0	0.0	0.0	0.0	1.0	
		0.0625	48	2.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		0.125	48	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		0.25	48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−

‡,TOX,MM: See the foot-notes in Table 1.

た分裂中期像について行い，染色分体ギャップ (ctg)，染色分体切断 (ctb)，染色分体交換 (cte)，染色体切断 (csb)，染色体交換 (cse) などの構造異常をもつ細胞の出現頻度を記録した．さらに，いずれかの構造異常を1個以上もつ異常細胞の出現頻度を求めると共に，倍数性細胞の出現頻度も記録した．判定は異常細胞の出現頻度が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．結果が疑陽性になった場合には，検体濃度を調節して，再度実験を繰り返した．

結　　果

1. 直接法

代謝活性化を行わない直接法の結果を Table 1～3 に示す．1981年度の11種類の検体（2種の chlordane の異性体を含む）のうち，acrylonitrile は 0.02 mg/ml で，acrylamide は 0.15 mg/ml 以上で陽性と判定されたが，他の9検体はいずれも陰性であった (Table 1)．1982年度は6種類の検体（3種の trichlorobenzene の異性体，2種の menthol の異性体を含む）について直接法で検討したが，いずれも陰性の結果に終わっており (Table 2)，直接染色体異常を誘発する性質をもたないものと判断された．1983年度の8種類の検体（2種の dichloroethane の異性体を含む）も直接法ではいずれも染色体異常誘発性を示さなかった(Table 3)．

2. 代謝活性化法

代謝活性化法による結果を Table 4～6 に示す．検体処理時の S9 の最終濃度は5％とし，S9 mix の添加，不添加にかかわらず処理時間は6時間とした．1981年度の検体のうち，α- および γ-chlordane は検体量が少なかったため実験はできなかった，Acrylonitrile および acrylamide は直接法と同様 −S9 で共に陽性の結果を示したが，直接法に比べて約2倍の濃度が必要であった．また，両検体は +S9 でも陽性の結果で示したが，−S9 の時とほぼ同じ濃度範囲であった (Table 4)．一方，anthracene および pyrene は +S9 の時にのみ陽性の結果を示し，しかも，細胞致死効果も強まる傾向にあった．ただし，異常の出現頻度には明確な濃度依存性はみられていない．尚，他の5検体は代謝活性化法によっても染色体異常誘発性は認められなかった (Table 4)．

代謝活性化法での S9 の最終濃度は5％にしてあるが，これを徐々に20％まで増加させると，1,1-dichloroethylene が染色体異常を誘発することが判明した．Acrylonitrile でも異常の増強効果が認められたが，trichloroethylene, tetrachloroethylene, 1,1,1-trichloroethane, carbon tetrachloride では S9 の割合を増加しても染色体異常の誘発は認められなかった．1,1-dichloroethylene の染色体異常誘発性の詳細については他の報告[3]を参照されたい．

1982年度の10種類の検体の結果を Table 5 に示す．Benzaldehyde は −S9 の時に陽性の結果を示し，+S9 では疑陽性の範囲にとどまり，しかも細胞致死効果も幾分低下の傾向を示した．一方，acetophenone および biphenyl は +S9 の時にのみ陽性の結果を示し，染色体異常誘発効果は後者の方が著しかった．なお，benzyl cyanide および naphthalene は +S9 の時に最高濃度で疑陽性となったにとどまった．他の5検体はいずれも染色体異常誘発性は認められなかった．

Table 6 に1983年度の結果を示す．8検体（2種の dichloroethane の異性体を含む）のうち 1,2-dichloroethane のみが +S9 の時に明らかな染色体異常誘発性を示し，しかも，濃度に依存して異常頻度が増加した．−S9 の時は最高濃度でのみ幾分異常細胞が増加したが，疑陽性の範囲にとどまった．1,2-dichloroethane の異性体である 1,1-dichloroethane は染色体異常を誘発せず，細胞毒性も 1,2- 体より弱かった．他の6検体はいずれも陰性の結果を示した．

Table 4. (continued)

Compound	Solvent	S9	Dose (mg/ml)	Polyploid (%)	Frequency (%) of aberrant cells$ ctg	ctb	cte	csb	cse	total	Judge
Acrylamide	Saline	−	0	3.0	2.0	1.0	1.0	0.0	0.0	3.0	
		−	0.13	0.0	1.0	4.0	0.0	0.0	0.0	4.0	−
		−	0.25	1.0	2.0	2.0	0.0	0.0	0.0	3.0	−
		−	0.5	1.0	13.0	18.0	9.0	0.0	0.0	28.0	+
		−	1.0	0.0	47.0	70.0	46.0	0.0	1.0	95.0	+
		+	0	0.0	3.0	0.0	0.0	0.0	0.0	3.0	
		+	0.13	1.0	2.0	0.0	1.0	0.0	0.0	3.0	−
		+	0.25	4.0	3.0	0.0	1.0	0.0	0.0	4.0	−
		+	0.5	0.0	8.0	10.0	8.0	0.0	0.0	20.0	+
		+	1.0	0.0	38.0	70.0	47.0	0.0	0.0	100.0	+
Anthracene	DMSO	−	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		−	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		−	0.015	0.0	0.0	0.0	1.0	0.0	0.0	1.0	−
		−	0.02	1.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		−	0.025	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		+	0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
		+	0.01	1.0	0.0	1.0	3.0	0.0	0.0	4.0	−
		+	0.015	1.0	1.0	2.0	8.0	0.0	0.0	9.0	±
		+	0.02	1.0	4.0	7.0	9.0	0.0	0.0	14.0	+
Pyrene	DMSO	−	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		−	0.005	2.0	2.0	2.0	1.0	0.0	0.0	4.0	−
		−	0.01	1.0	2.0	0.0	0.0	0.0	0.0	2.0	−
		−	0.015	1.0	1.0	0.0	1.0	0.0	0.0	2.0	−
		−	0.02	1.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		+	0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
		+	0.005	1.0	1.0	2.0	1.0	0.0	0.0	3.0	−
		+	0.01	3.0	15.0	13.0	10.0	0.0	0.0	24.0	+

$ See the foot-notes in Table 1.

考　　察

1981年度の11種類の検体のうちacrylonitrileとacrylamideは直接法で陽性の結果が得られた．一方，サルモネラ菌によるAmes試験ではacrylonitrileは代謝活性化を行った時（+S9）にのみ陽性の結果が報告されている[4]．本研究の培養細胞による染色体異常試験でも+S9で陽性となったが，その異常誘発効果は−S9の時と同程度であった．これらの結果は微生物での突然変異誘発と哺乳動物細胞での染色体異常誘発とではその発現機構に若干の差異があることが考えられる．なお，哺乳動物細胞による姉妹染色分体交換試験，体細胞突然変異試験においても本研究の染色体異常試験と同様に陽性結果が報告されている[5]．

Anthraceneとpyreneは+S9の時にのみ陽性の結果が得られた．Anthraceneの微生物による変異原性試験では一般に陰性の結果が報告されているが，なかには陽性の結果もみられ，逆にpyreneでは比較的陽性の結果が報告されている[6]．ただし，一般的には代謝活性化の有無にかかわらず変異原性はないとの判断がなされている[7]．しかし，両検体については発癌性を示唆する報告もあり[8]，今後慎重に検討する必要がある．

1,1-dichloroethyleneは本研究の通常の代謝活性化法（5% S9）では陰性であったが，S9の濃度を上げることによって染色体異常が誘発された[3]．本検体は微生物による変異原性試験でも代謝活性化法によって陽性の結果が報告されており[9]，さらに，哺乳動物培養細胞での姉妹染色分体交換試験でも+S9の時に陽性と判定されている[3]．ただし，マウスによる小核試験では陰性の結果となっており[3]，生体内での作用機序についてはさらに詳細に検討すべきである．

1982年度の検体の一つであるbiphenylは微生物による突然変異試験および哺乳動物培養細胞による染色体異常試験（直接法）で共に陰性の結果が得られた．チャイニーズ・ハムスター培養細胞（Don）による染色体異常および姉妹染色分体交換（SCE）試験でも陰性の結果が報告されており[10]，また，シリアン・ハムスター（BHK21）による細胞形質転換試験でも陰性の結果であった[11]．一方，本研究での代謝活性化法によ

Table 5. Chromosome aberration tests in cultured Chinese hamster cells (Metabolic activation method) (1982)

Compound	Solvent	S 9	Dose (mg/ml)	Polyploid (%)	Frequency (%) of aberrant cells* ctg	ctb	cte	csb	cse	total	Judge
Benzaldehyde	DMSO	−	0	0.0	1.0	0.0	1.0	0.0	0.0	2.0	
		−	0.8	11.0	0.0	0.0	2.0	0.0	0.0	2.0	−
		−	1.0	3.0	1.0	13.0	18.0	0.0	0.0	24.0	+
		+	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		+	0.8	11.0	1.0	0.0	1.0	0.0	0.0	2.0	−
		+	1.0	5.0	3.0	3.0	4.0	0.0	0.0	9.0	±
		+	1.2	0.0	0.0	5.0	4.0	0.0	0.0	9.0	±
Acetophenone	DMSO	−	0	0.0	0.0	1.0	1.0	0.0	0.0	2.0	
		−	0.8	2.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		−	1.0	0.0	1.0	1.0	0.0	0.0	0.0	2.0	−
		−	1.2	0.0	1.0	1.0	3.0	0.0	0.0	4.0	−
		+	0	0.0	0.0	0.0	2.0	0.0	0.0	2.0	
		+	0.6	2.0	0.0	3.0	5.0	0.0	0.0	7.0	±
		+	0.8	2.0	1.0	7.0	12.0	0.0	0.0	17.0	+
		+	1.0	1.0	1.0	9.0	12.0	0.0	0.0	16.0	+
Benzyl cyanide	DMSO	−	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		−	0.25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		−	0.5	3.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		−	1.0	4.0	0.0	1.0	1.0	0.0	0.0	2.0	−
		+	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		+	0.25	2.0	0.0	0.0	1.0	0.0	0.0	1.0	−
		+	0.5	1.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		+	1.0	1.0	0.0	3.0	5.0	0.0	0.0	7.0	±
p-Dichlorobenzene	DMSO	−	0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
		−	0.05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		−	0.1	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		−	0.2	0.0	1.0	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	
		+	0.05	1.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		+	0.1	0.0	2.0	0.0	0.0	0.0	0.0	2.0	−
		+	0.2	0.0	2.0	0.0	0.0	0.0	0.0	2.0	−
1,2,3-Trichloro-benzene	DMSO	−	0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	
		−	0.1	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		−	0.125	2.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		+	0	1.0	1.0	0.0	0.0	0.0	0.0	1.0	
		+	0.1	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		+	0.125	1.0	1.0	0.0	0.0	0.0	0.0	1.0	−
1,3,5-Trimethyl-benzene	DMSO	−	0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
		−	0.025	0.0	0.0	1.0	0.0	0.0	0.0	1.0	−
		−	0.05	2.0	0.0	1.0	0.0	0.0	0.0	1.0	−
		−	0.1	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		+	0	0.0	2.0	0.0	0.0	0.0	0.0	2.0	
		+	0.05	2.0	1.0	0.0	1.0	0.0	0.0	2.0	−
		+	0.1	0.0	0.0	1.0	0.0	0.0	0.0	1.0	−
		+	0.2	1.0	1.0	2.0	0.0	0.0	0.0	3.0	−
Naphthalene	DMSO	−	0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
		−	0.01	0.0	1.0	0.0	1.0	0.0	0.0	2.0	−
		−	0.02	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		−	0.04	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	
		+	0.01	1.0	1.0	0.0	2.0	0.0	0.0	3.0	−
		+	0.02	2.4	2.4	2.4	1.2	0.0	0.0	4.9	−
		+	0.04	1.0	2.0	3.0	7.0	0.0	0.0	8.0	±

Table 5. (continued)

Compound	Solvent	S9	Dose (mg/ml)	Polyploid (%)	Frequency (%) of aberrant cells* ctg	ctb	cte	csb	cse	total	Judge
Biphenyl	DMSO	−	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		−	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		−	0.015	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		−	0.02	1.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		+	0	1.0	1.0	0.0	1.0	0.0	0.0	2.0	
		+	0.01	4.0	2.0	0.0	3.0	0.0	0.0	5.0	±
		+	0.015	1.0	0.0	10.0	31.0	0.0	0.0	35.0	+
		+	0.02	1.0	4.0	35.0	28.0	0.0	0.0	51.0	+
Dibenzofuran	DMSO	−	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
		−	0.025	0.0	0.0	1.0	0.0	0.0	0.0	1.0	−
		−	0.05	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		−	0.1	0.0	2.0	0.0	0.0	0.0	0.0	2.0	−
		+	0	1.0	1.0	0.0	1.0	0.0	0.0	2.0	
		+	0.025	0.0	1.0	0.0	0.0	0.0	0.0	1.0	−
		+	0.05	1.0	0.0	0.0	1.0	0.0	0.0	1.0	−
		+	0.1	0.0	0.0	0.0	1.0	0.0	0.0	1.0	−
l-Menthol	DMSO	−	0	0.0	1.0	0.0	0.0	0.0	0.0	1.0	
		−	0.1	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	1.0	1.0	0.0	0.0	0.0	0.0	1.0	
		+	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−
		+	0.2	0.0	0.0	0.0	1.0	0.0	0.0	1.0	−
		+	0.3	0.0	0.0	0.0	1.0	0.0	0.0	1.0	−

* See the foot-notes in Table 1.

る染色体異常試験では S9 mix を添加すると明らかな染色体異常誘発性が認められており，biphenyl の代謝産物の中に染色体異常誘発物質が含まれているものと考えられる．

1983年度の8検体についての培養細胞による染色体異常試験の結果は，サルモネラ菌を用いる Ames 試験の結果と同一であった．つまり，1,2-dichloroethane のみが代謝活性化法で陽性の結果を示し，他はすべて陰性であった．

Cis-1,2-dichloroethylene の *Saccharomyces cerevisiae* による変異原性試験（組換試験，遺伝子突然変異試験，遺伝子変換試験）では S9 の共存，非共存下にかかわらず，陰性の結果が報告されている[12]．また，dibutylphthalate の *S. cerevisiae* による変異原性試験[13]，培養細胞による染色体異常試験および姉妹染色分体交換試験[10]の報告でも，陽性の結果は示されていない．これらの報告は本研究結果と一致している．

Di (2-ethyhexyl) phthalate は NTP (National Toxicology Program) の実験で発癌性が実証された[14]が，変異原性試験では，マウスによる優性致死試験[15]で陽性となった以外は S9 を併用しても，*in vitro* の変異原性試験は陰性に終わっている[10]．Di(2-ethylhexyl) phthalate の加水分解物である monoethylhexylphthalate は，姉妹染色分体交換を起こさないが，染色体異常誘発性を示すという報告がある[16]．Di(2-ethylhexyl) phthalate は S9 mix で加水分解されない[17]ため，本研究の染色体異常試験でも陰性に終わったものと考えられる．

これまで直接法あるいは代謝活性化法のいずれかまたは両法で，合計32種の化合物について染色体異常誘発性を検討してきたが，8種の化合物 (acrylonitrile, acrylamide, anthracene, acetophenone, pyrene, biphenyl, 1,2-dichloroethane, benzaldehyde) が S9 mix 共存下あるいは非共存下で陽性となった．1984年度以降も引き続き水道水汚染化学物質とその関連化合物について検索を行っており，それらの結果についても追って報告する予定である．

文　献

1) Ishidate, M., Jr. and S. Odashima: *Mutat. Res.*, **48**, 337 (1977)
2) 祖父尼俊雄，松岡厚子：環境変異原研究，**5**，4 (1984)
3) 沢田　稔，祖父尼俊雄，石館　基：環境変異原研究：**6**，149 (1984)
4) IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, **19**, 84

Table 22. Toxicity of chlorobenzenes after long-term exposures

Compound; Reference	Species[a]	Dose[b,c]		
1,2,3-TCB 1,2,4-TCB 1,3,5-TCB (≥99%) Côté et al. (1988)	Sprague-Dawley rat (weanling); 5 animals each sex/group	0, 1, 10, 100, 1000 mg/kg diet mixed with corn oil in the diet	(males: 0, 0.07-0.08, 0.78-0.81, 7.5-7.8, 78-82 mg/kg per day;	females: 0, 0.11-0.13, 1.3-1.5, 12-17, 101-146 mg/kg per day)

Results[d]	Effect Levels[e]
no clinical signs of toxicity; deaths of one high-dose female (not treatment related) and one male control (no apparent cause); statistically significant growth suppression in males exposed to 10 or 1000 mg 1,2,3-TCB/kg; nephrosis in one male exposed to 1000 mg 1,2,4-TCB/kg; significant increase in liver/body weight ratios in males exposed to high dose of all 3 TCBs; significant increase in serum AH (males) and APDH (both sexes) in animals exposed to 1000 mg 1,2,4-TCB/kg; "qualitatively similar" changes, mild to moderate (all TCB isomers) observed in the liver, thyroid, and kidney, significant at high-dose levels only and more severe in males; histopathological changes include: liver: mild to moderate increase in cytoplasmic volume and anisokaryosis of hepatocytes; all isomers produced fatty infiltration-most severe at 1000 mg 1,2,4-TCB/kg; thyroid: mild to moderate changes (high-dose groups) including reduction in follicular size, increased epithelial height and reduced colloid density; kidney: moderate changes in the convoluted tubules of males exposed to 1000 mg 1,3,5-TCB/kg	1,2,3,-TCB: 7.7 mg/kg per day; 1,2,4-TCB: 7.8 mg/kg per day; 1,3,5-TCB: 7.6 mg/kg per day (NOAEL)

DRUG AND CHEMICAL TOXICOLOGY, 11(1), 11-28 (1988)

TRICHLOROBENZENES: RESULTS OF A THIRTEEN WEEK FEEDING STUDY IN THE RAT

M. Côté[1], I. Chu[2]*, D.C. Villeneuve[2], V.E. Secours[2] and V.E. Valli[3]

[1]Department of Pharmacology
University of Montreal
Montreal, Quebec Canada
H3C 3J7
[2]Environmental and Occupational Toxicology Division
Bureau of Chemical Hazards
Environmental Health Directorate
Ottawa, Ontario
[3]Biopath Analysts Ltd.,
Guelph, Ontario
N1E 2X7

ABSTRACT

Trichlorobenzenes (TRCBs) are industrial chemicals and environmental pollutants found in Great Lakes fish. The present study was carried out to provide information on the toxic effects of these chemicals in mammals. Groups of male and female weanling rats were fed diets containing TRCB isomers at 1, 10, 100 or 1000 ppm for 13 weeks. The group of males fed 1000 ppm 1,2,3-TRCB diet had reduced weight gain. No other clinical signs of toxicity were observed. Increased relative liver and kidney weights occurred in the highest dose groups of males for all 3 TRCBs. Of the 3 isomers, only 1,2,4-TRCB at 1000 ppm caused increases in hepatic aminopyrine demethylase and aniline hydroxylase activities in male rats, and aminopyrine demethylase in females. Serum biochemical and hematological parameters measured were not affected. All

*To whom all correspondence and requests for reprints should be addressed.

three TRCBs produced histological changes of a moderate degree in the liver and thyroid of male rats at 1,000 ppm. Only 1,3,5-TRCB elicited moderate renal changes in male rats fed 1,000 ppm. Microscopic changes in the females were generally milder than those of the corresponding
males. Based on these data it was concluded that the non-observable adverse effect levels for the three TRCBs were 100 ppm in the diet, or 7.6 ~ 7.8 mg/kg b.w./day depending on the dietary consumption.

INTRODUCTION

Trichlorobenzenes (TRCBs) are industrial chemicals that are used as chemical intermediates in the synthesis of herbicides. These chemicals are also used as dye carriers and as a dielectrical fluid in electrical insulators and transformers[1]. Trichlorobenzenes have been detected in Great Lakes fish, drinking water and waste-water effluents around ng/L levels[2,3]. Because of the wide use of these chemicals together with subsequent release into the environment, concern has been raised over the potential health hazard to humans associated with occupational and environmental exposure. Previous studies have demonstrated that 1,2,4-TRCB caused biochemical changes and hepatomegaly in rats, dogs[4] and rabbits[5]. Metabolism studies in the monkey and rat have shown that 1,2,4-TRCB is biotransformed to a trichlorophenol, a cysteine conjugate and dihydrodiol derivatives[6]. A review of the literature reveals a lack of adequate toxicity data to permit the assessment of health risks in humans exposed to these chemicals. The present study was designed to investigate the toxic effects in the rats treated with trichlorobenzenes in the diet for 90 days.

MATERIALS AND METHODS

1,2,3-, 1,2,4- and 1,3,5-Trichlorobenzene were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). 1,2,3- and 1,3,5-TRCB were purified by recrystallization in hexanes to a purity of >99%. The 1,2,4-isomer, which had a purity of >99%, was used without further purification. The purities of these compounds were confirmed by thin layer chromatography and gas chromatography-mass spectrometry. All other chemicals and solvents were of reagent grade obtained commercially.

Preparation of Diet

The diet consumed by the control groups was prepared by blending thoroughly ground cubes (Ralston Purina) with corn oil (Mazola, 4% w/w). The test diets were made by mixing ground cubes with corn oil solutions containing appropriate amounts of test chemicals to give dietary levels of 1, 10, 100 or 1,000 ppm. Fresh diets were made every fourth week throughout the study, and kept in air-tight steel containers. Selection of dose levels was based on a range-finding study in which the LD_{50} of the three TRCB in rats were found to be 1,2,3-TRCB, 1.83 g/kg; 1,2,4-TRCB, 0.88 g/kg and 1,3,5-TRCB, 2.1 g/kg.

Animal Treatment

Weanling Sprague-Dawley rats (130 males and 130 females), obtained from Charles River, Montreal, were divided into 13 groups of 10 animals per group of each sex. The animals were housed individually in stainless-steel mesh cages and acclimatized to laboratory conditions for one week before initiation of the study.

The room temperature and relative humidity were kept at 22-24°C and 40-60% respectively. A 12 hr alternated light/dark cycle was maintained. The diet that each group of animals of both sexes received is as follows:

Group 1	Control (4% corn oil in ground cubes)
Group 2	1,2,4-Trichlorobenzene (TRCB) 1 ppm in ground cubes
Group 3	1,2,4-TRCB 10 ppm
Group 4	1,2,4-TRCB 100 ppm
Group 5	1,2,4-TRCB 1000 ppm
Group 6	1,2,3-TRCB 1 ppm
Group 7	1,2,3-TRCB 10 ppm
Group 8	1,2,3-TRCB 100 ppm
Group 9	1,2,3-TRCB 1000 ppm
Group 10	1,3,5-TRCB 1 ppm
Group 11	1,3,5-TRCB 10 ppm
Group 12	1,3,5-TRCB 100 ppm
Group 13	1,3,5-TRCB 1000 ppm

Body weight was determined weekly on all animals. In order to avoid possible loss of test chemical by volatility, the diet left in the feeder was replaced with fresh ones on a weekly basis. Food consumption was recorded at Weeks 1, 4, 8 and 12 of study on 5 animals per group of both sexes. Urinalysis (pH, protein and nitrite) was carried out on 5 animals per group per sex at Weeks

4, 8 and 12 of study. Clinical examinations were performed on all animals on a daily basis.

At the end of the 13 week feeding period all animals were anesthetized to surgical level with ether, and exsanguinated via the abdominal aorta. All animals were examined grossly at the time of necropsy. The brain, heart, liver, spleen, and kidneys were excised and weighed. Blood samples collected at necropsy were analyzed for the following parameters: hemoglobin, packed cell volume, erythrocyte count (Baker Model 7000), total and differential leukocyte counts, platelet count, and prothrombin time. Mean corpuscular hemoglobin concentration and mean corpuscular hemoglobin values were calculated. Serum biochemical profiles were determined in a Technicon microanalyzer (Model 12/60) and included sodium, potassium, inorganic phosphate, total bilirubin, alkaline phosphatase, aspartate aminotransferase, total protein, calcium, cholesterol, glucose, uric acid, and lactic dehydrogenase.

Hepatic microsomal aniline hydroxylase (AH)[7] and aminopyrine demethylase (APDM)[8] activities were determined based on the previously reported methods adapted to automated instruments. Liver protein content was determined by the biuret method[9]. A section of femoral bone marrow was aspirated, spread on the slide from which thin films were made, and stained with May-Grünwald-Giemsa stain for cytological evaluation. The following tissues were taken and fixed in 10% buffered formalin (pH 7.4) for routine histological examination: eye, optic nerve, spinal cord, skin, tongue, brain, pituitary, liver, adrenal, thyroid, parathyroid,

thymus, lungs, trachea, bronchi, thoracic aorta, esophagus, gastric cardia, fundus and pylorus, duodenum, jejunum, ileum, pancreas, colon, cecum, kidneys, spleen, bone marrow, mesenteric and mediastinal lymph nodes, skeletal muscle, ovaries, uterus, vagina or testes, prostate, epididymis, sciatic nerve, urinary bladder, salivary gland, mammary gland, and heart. Potential fatty change in the liver was determined in frozen sections as previously described[10].

Sections of liver and perirenal fat were excised and kept at -70°C pending residue analysis using a gas chromatographic method as described previously[10]. Data were analyzed by one-way analysis of variance followed by Duncan's multiple range test to indicate the groups which were significantly different from the control ($P \leq 0.05$)[11].

RESULTS

Clinical Observation

Clinical examination of animals revealed no signs of toxicity other than reduced weight gain of some groups. One male rat from the control group died without any apparent cause during the first week. A female rat in the 1,000 ppm 1,3,5-TRCB group died during the 4th week. The cause of death was unknown but did not appear to be treatment-related. The remaining animals survived until the termination of the 13 week feeding period.

Weight Gain and Food Consumption

There was a general trend towards lower weight gain in the

growth suppression was only observed in groups fed 1,2,3-TRCB at 10 or 1,000 ppm. The weight gain and food consumption data and the amount of test compounds ingested by male rats are presented in Table 1. There was no decrease in growth rate or food consumption in female rats. The approximate amount of test compounds ingested by the females, expressed in the form of mg/kg b.w./day are presented below:

Gross Changes

Fatty liver was observed in some of the male rats but the incidence was of a sporadic nature, occurring in both control and treated groups. One male rat receiving 1000 ppm 1,2,4-TRCB diet had nephrosis. No gross changes were observed in female rats.

Organ Weights

Organ weight data are presented in Table 3. The liver/body weight ratios of males receiving the highest dose of 3 TRCBs were significantly greater than those of the control group. The wet kidney weights as well as kidney/body weight ratios of males receiving 1000 ppm 1,2,4-TRCB; 10 ppm or 100 ppm 1,3,5-TRCB diet were higher compared to control values. The kidney/body weight ratios of the groups of males fed diet containing 1, 10 or 1000 ppm 1,2,3-TRCB or 1000 ppm 1,3,5-TRCB were also significantly greater than the control.

Biochemical Changes

None of the serum biochemical constituents were affected by exposure to TRCBs up to and including 1000 ppm in diet; these

TABLE 1 - Food Consumption and Weight Gain of Male Rats Fed TRCB

Treatment (ppm in the diet)	Food Consumption (g/rat)	Initial Weight (g)	Weight Gain (g)	Amount of[a] Chemical Ingested (mg/kg b.w./day)
Control	25 ± 1.1(5)[b]	85 ± 8(9)	471 ± 54(9)	0
1,2,4-TRCB				
1	23 ± 0.7(5)	85 ± 5	453 ± 27	0.07
10	25 ± 1.2(5)	80 ± 5	474 ± 47	0.78
100	25 ± 1.1(5)	85 ± 10	472 ± 42	7.8
1000	25 ± 2.2(5)	84 ± 10	444 ± 33	82
1,2,3-TRCB				
1	24 ± 1.8(5)	80 ± 5	447 ± 37	0.08
10	23 ± 1.4(5)	82 ± 6	425 ± 43*	0.78
100	24 ± 2.5(5)	84 ± 6	458 ± 47	7.6
1000	23 ± 0.8(5)	80 ± 4	423 ± 42*	78
1,3,5-TRCB				
1	26 ± 1.4(5)	87 ± 9	482 ± 51	0.08
10	27 ± 0.5(5)	89 ± 6	490 ± 34	0.81
100	24 ± 0.9(5)	85 ± 5	450 ± 60	7.7
1000	26 ± 1.8(5)	84 ± 6	450 ± 38	82

[a] The amount of TRCB ingested is an approximate figure derived from the following formula:

$$\frac{\text{food consumption (g)} \times \text{dietary concentration (ppm)}}{\text{average body weight}}$$

[b] Unless otherwise given in parentheses results represent mean ± S.D. derived from 10 animals. The food consumption was determined in 5 animals per group for all groups.

Table 2 Amount of Chemical Ingested by Female Rats

Compound	mg/kg b.w./day		Compound	mg/kg b.w./day	
1,2,4-TRCB	1 (ppm)	0.11	1,3,5-TRCB	1 (ppm)	0.13
	10	1.4		10	1.5
	100	15		100	17
	1000	101		1000	146
1,2,3-TRCB	1	0.13			
	10	1.3			
	100	12			
	1000	113			

included sodium, potassium, inorganic phosphate, total bilirubin, alkaline phosphatase, aspartate aminotransferase, total protein, calcium, cholesterol, glucose, uric acid and lactate dehydrogenase.

Measurements of hepatic mixed function oxidase activities revealed that 1,2,4-TRCB at the 1000 ppm level caused a significant elevation in AH and APDM activities in males, and APDM activity in females.

AH:	male	μmole PAP/hr/mg protein
	Control:	8.0 ± 3.4
	Treated:	15.6 ± 6.2
APDM:	male	μmole HCHO/hr/mg protein
	Control:	23 ± 3.6
	Treated:	53 ± 19
	female	μmole HCHO/hr/mg protein
	Control:	24 ± 3.1
	Treated:	41 ± 7.4.

The remaining 2 TRCB isomers at the dose levels studied had no effects on these hepatic microsomal enzymes.

TABLE 3 - Organ Weights of Male Rats Fed Diet Containing TRCB (mean ± S.D.)

Treatment (ppm in the diet)	n[a]	Liver		Kidney	
		Wet Weight (g)	Liver/b.w. ratio (% of b.w.)	Wet Weight (g)	Kidney/b.w. ratio (% of b.w.)
Control	9	19.6 ± 2.7	3.5 ± 0.30	1.56 ± 0.20	0.28 ± 0.02
1,2,4-TRCB					
1	10	17.8 ± 2.5	3.3 ± 0.43	1.57 ± 0.11	0.29 ± 0.02
10	10	20.5 ± 2.3	3.7 ± 0.29	1.68 ± 0.18	0.30 ± 0.04
100	10	20.8 ± 2.0	3.7 ± 0.20	1.72 ± 0.10	0.31 ± 0.02
1000	10	22.2 ± 1.5	4.2 ± 0.22*	2.04 ± 0.44*	0.38 ± 0.06*
1,2,3-TRCB					
1	10	18.8 ± 1.7	3.6 ± 0.22	1.68 ± 0.10	0.32 ± 0.03*
10	10	19.3 ± 2.5	3.8 ± 0.29	1.64 ± 0.14	0.32 ± 0.02*
100	10	19.4 ± 3.1	3.6 ± 0.31	1.60 ± 0.20	0.29 ± 0.02
1000	10	20.1 ± 2.5	4.0 ± 3.4*	1.72 ± 0.25	0.34 ± 0.04*
1,3,5-TRCB					
1	10	20.5 ± 2..	3.6 ± 0.24	1.75 ± 0.16	0.31 ± 0.03
10	10	21.8 ± 1.9	3.8 ± 0.21	1.87 ± 0.27*	0.32 ± 0.04*
100	10	20.1 ± 4.2	3.7 ± 0.41	1.85 ± 0.22*	0.35 ± 0.03*
1000	10	21.0 ± 3.4	3.9 ± 0.60*	1.69 ± 0.17	0.32 ± 0.04*

[a] Number of animals.

* Significantly different from the control ($P < 0.05$).

Hematological Changes

Treatment with 3 TRCBs had no effects on any hematological parameters monitored in this study.

Histopathology

The liver, thyroid and kidney were the organs which had treatment-related changes. The changes were qualitatively similar for all 3 TRCB isomers, and for the most part, were mild in nature and judged to be significant only at the highest dose level. In general, morphological alterations in males were more severe than those of females.

Microscopic changes in the livers of most treated groups consisted of a mild to moderate increase in cytoplasmic volume and anisokaryosis of hepatocytes observed mostly in perivenous and midzone areas. Animals treated with 1000 ppm 1,2,4-TRCB had marked changes characterized by aggregated basophilia as well as widespread midzonal vacuolation due to fatty infiltration. The 1,2,3- and 1,3,5-isomers, showed the same changes but were milder.

Changes in the thyroid were characterized by reduction in follicular size, increased epithelial height from flattened cuboidal cells to columnar shape; and reduced colloid density. Depending on the dose, the severity of these changes varied from minimal in the low dose groups to mild and moderate changes in the high dose groups.

Of the three isomers tested, only 1,3,5-TRCB resulted in mild to moderate renal changes in the convoluted tubules. The changes were characterized by eosinophilic inclusions, enlargement and

anisokaryosis of the epithelial lining cells and hyperplasia of renal tubular epithelial cells. The renal changes for most groups were considered to be mild, and only those changes associated with the 1000 ppm dose level were considered to be biologically significant.

Urinalysis

No significant treatment-related changes were observed in any of the urinary parameters monitored.

Residue Analysis

Table 4 presents the residue data in fat and liver of rats. There was a dose-dependent accumulation in both fat and liver in the following order:

1,3,5-TRCB > 1,2,4-TRCB > 1,2,3-TRCB.

The levels of TRCB in fat were one order of magnitude higher than those found in liver.

DISCUSSION

Treatment with TRCBs resulted in growth suppression, increased organ weight, hepatic microsomal enzyme induction and mild histological changes; most of these only occurred in the male rats exposed to the highest dose of TRCBs. The growth suppression observed in the 10 and 1,000 ppm 1,2,3-TRCB but not in the 100 ppm group would indicate that the effect at 10 ppm is probably an incidental finding, since it is not clearly dose-dependent.

TABLE 4 - Trichlorobenzene Residues (ppm) in Liver and Fat[a]

Treatment (ppm in the diet)	Males		Female	
	Liver	Fat	Liver	Fat
Control	ND[b]	ND	ND	ND
1,2,4-TRCB				
1	ND	ND	ND	ND
10	ND	0.37 ± 0.12	ND	0.26 ± 0.03
100	0.25 ± 0.07	3.5 ± 1.1	0.1 ± 0.03	2.7 ± 0.39
1000	2.5 ± 0.71	36 ± 12	1.0 ± 0.83	19 ± 2.7
1,2,3-TRCB				
1	ND	ND	ND	ND
10	ND	0.1 ± 0.04	ND	ND
100	0.24 ± 0.07	1.2 ± 0.31	ND	0.63 ± 0.15
1000	1.4 ± 0.55	15.5 ± 7.4	0.73 ± 0.37	7.8 ± 2.8
1,3,5-TRCB				
1	ND	0.21 ± 0.07	ND	0.1 ± 0.02
10	ND	1.3 ± 0.26	ND	0.43 ± 0.11
100	0.37 ± 0.11	7.3 ± 2.1	0.14 ± 0.03	4.0 ± 0.86
1000	4.3 ± 1.4	76 ± 13	1.9 ± 0.86	49 ± 17

[a] Data represent mean ± S.D. obtained from 5 animals

[b] Non-detectable; the limit of detection is 0.01 ppm.

However there is a general trend towards a lower weight gain in the 1000 ppm groups of males.

In our study, dietary exposure of rats to 3 TRCBs at the 1,000 ppm level resulted in increases of relative liver and kidney weights. Similar findings have been documented by Kociba et al (1981)[4] who observed that rats exposed to 100 ppm of 1,2,4-TRCB by inhalation, 7 h/day for 30 days had increased liver and kidney weights.

Although the relative kidney weights of the males exposed to 1, 10 and 1,000 ppm 1,2,3-TRCB or 1000 ppm 1,2,4-TRCB were statistically higher than those of the controls (Table 3), histopathological examination of this organ failed to reveal any abnormal changes, neither did the urinalysis. However, the increased renal weight at 10, 100 and 1000 ppm 1,3,5-TRCB was accompanied by histological changes.

Of the 3 TRCBs examined in the present study, only the 1,2,4-isomer resulted in elevated hepatic microsomal AH and APDM activities. These data are consistent with those of Goldstein et al (1982)[12] who reported an increase in rat hepatic microsomal APDM activity following administration of chlorinated benzenes including 1,2,4-TRCB at 250 mg/kg b.w./day for 7 days. The hepatic microsomal enzyme induction observed in the present study is consistent with the morphological changes in the liver characterized by increased eosinophilia and cytoplasmic volume. Ariyoshi et al (1975 a,b)[13,14] have shown that 1,3,5-TRCB is capable of inducing hepatic microsomal AH activity in rat at 250 mg/kg b.w. Thus the

failure to observe increased AH activity in our study may be attributed to the lower dose used (82 mg/kg b.w./day, Table 1).

Tissue residue data in the present study revealed that 1,3,5-TRCB residues accumulated to a higher level in fat and liver than those of the other two isomers. These data confirmed the results of our earlier pharmacokinetic study which demonstrated that tissue ^{14}C-contents was the highest for rats dosed orally with 1,3,5-TRCB (Chu et al, 1987)[15].

Histopathological changes for the most part were mild in nature. Only those produced in the liver and thyroid at 1000 ppm of 3 TRCBs as well as those in the kidney by 1000 ppm 1,3,5-TRCB were considered to be biologically significant. At these dose levels, some functional changes were also observed.

In the present study the effects observed for TRCB isomers can be summarized as follows:

1,2,4-TRCB: Significant increases in liver and kidney weights, and microsomal enzyme activities at 1,000 ppm; mild to moderate histological changes in the liver and thyroid at 1,000 ppm.

1,2,3-TRCB: Growth suppression at 1000 ppm; mild to moderate morphological changes in the liver and thyroid at 1,000 ppm.

1,3,5-TRCB: Moderate histological changes in the kidney, liver and thyroid at 1,000 ppm; increased relative liver and kidney weights at 1,000 ppm.

Based on the data summarized above, we conclude that the non-observable adverse effect levels are 100 ppm for all three TRCBs or 7.8 mg/kg b.w./day (1,2,4-), 7.7 mg/kg b.w./day (1,2,3-) or 7.6 mg/kg b.w./day (1,3,5-) depending on the dietary consumption.

The highest TRCB (1,2,4-) level found in lake trout is 5 ppb[3]. Assuming the average fish consumption is 16.6 g/day/person[16], this would give rise to an estimated daily intake of 83 ng TRCB/person/day for a person of 60 kg b.w. or 1.4 ng TRCB/kg b.w./day. Since the non-observable adverse effect level has been found to be 7.8 mg/kg b.w./day (1,2,4-), there exists a 5.6×10^6 times safety factor between the potential human exposure and the effect level in the rat.

Acknowledgement

The authors are indebted to Mrs. N. Beament, Mrs. B. Reed, Ms. M. Morrisett, Mr. J. Kelly, Mr. A. Viau and Mr. A. Yagminas for technical assistance; Mrs. M. Beaudette for data handling and Mrs. J. Ireland for typing the manuscript. We also thank Drs. L. Whitehouse and R. Moody for useful discussions and a review of the manuscript.

REFERENCES

1. US Environmental Protection Agency, Health Assessment Document for Chlorinated Benzenes. (No. 600/8-84-015A) (1984).

2. D.W. Kuehl, K.L. Johnson, B.C. Butterworth, E.N. Leonard and G.D. Veith. Quantification of octachlorostyrene and related compounds in Great Lakes fish by gas chromatography-mass spectrometry. J. Great Lakes Res. 7, 330 (1981).

3. B.G. Oliver, and K.D. Nicol. Chlorobenzenes in sediments, water and selected fish from Lakes Superior, Huron, Erie and Ontario. Environ. Sci. Technol. 16, 532 (1982).

4. R.J. Kociba, B.K.J. Leong and R.E. Hefner, Jr. Subchronic toxicity of 1,2,4-trichlorobenzene in the rat, rabbit and beagle dog. Drug. Chem. Toxicol. 4, 229 (1981).

5. K.S. Rao, K.A. Johnson and J.W. Henck. Subchronic toxicity of trichlorobenzene in the rabbit. Drug Chem. Toxicol. 5, 249 (1982).

6. R.D. Lingg, W.H. Kaylor, S.M. Pyle, F.C. Kopfler, C.C. Smith, G.F. Wolfe and S. Cragg. Comparative metabolism of 1,2,4-trichlorobenzene in the rat and rhesus monkey. Drug Metab. Dispos. 10, 134 (1982).

7. J.R. Fouts. Factors influencing the metabolism of drugs in liver microsomes. Ann. N.Y. Acad. Sci. 104, 875 (1963).

8. J. Cochin and J. Axelrod. Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine. J. Pharmacol. Exp. Therap. 125, 105 (1959).

9. A.G. Gornall, C.J. Bardawill and M.M. David. Determination of serum proteins by means of biuret reaction. J. Biol. Chem. 177, 751 (1948).

10. D.C. Villeneuve, V.E. Valli, I. Chu, V. Secours, L. Ritter and G.C. Becking. Ninety-day toxicity of photomirex in the male rat. Toxicology, 12, 235 (1979).

11. N.H. Nie, C.H. Hull, J.G. Jenkins, K. Steinbrenner and D.H. Bent. Statistical Programs for the Social Sciences, SPSS, Inc., Chicago, (1977).

12. J.A. Goldstein, P. Linko, J.N. Huckins and D.L. Stalling. Structure activity relationship of chlorinated benzenes as inducers of different forms of cytochrome P-450 in rat liver. Chem-Biol. Interactions, 41, 131 (1982).

13. T. Ariyoshi, K. Ideguchi, Y. Ishizuka, K. Iwasaki and M. Arakaki. Relationship between chemical structures and activity I. Effects of number of chlorinated atoms in chlorinated benzenes on the components of drug-metabolizing system and the hepatic constituents. Chem. Pharm. Bull. 23, 817 (1975a).

14. T. Ariyoshi, K. Ideguchi, K. Iwasaki and M. Arakaki. Relationship between chemical structure and activity II. Influences of isomers in dichlorobenzene, trichlorobenzene and tetrachlorobenzene on the activities of drug-metabolizing enzymes. Chem. Pharm. Bull. 23, 824 (1975b).

15. I. Chu, D.J. Murdoch, D.C. Villeneuve and A. Viau. Tissue distribution and elimination of trichlorobenzenes in the rat. J. Environ. Health Sci. (B). in press (1987).

16. Statistics Canada Report No. 32-230 (1983) Apparent per capita food consumption in Canada.

8.7 Developmental and Reproductive Effects

The results of studies concerning the embryotoxicity, fetotoxicity, teratogenicity, and reproductive effects of the chlorobenzenes are presented in Table 24. Effect levels derived in these studies for both mothers and offspring are also presented in this table. In contrast to the lack of information on acute, short-term, long-term, and carcinogenic effects, comparatively more data are available on the potential embryotoxic, fetotoxic, and teratogenic effects of the higher chlorinated benzenes. Furthermore, the TCBs, TeCBs, and PeCB have been better studied in this regard than MCB and the isomers of DCB.

There has been no evidence in any of the studies conducted to date that the chlorobenzenes (mono- to penta-) are teratogenic in animal species. Some relatively minor embryotoxic and fetotoxic effects have been observed for the lower chlorinated benzenes (MCB and DCBs), but only at dose levels that were toxic for the mother. For example, there was a slight delay in skeletal development (ossification) in the fetuses of pregnant rats exposed to 2864 mg MCB/m^3, a dose that induced decreases in body weight gain in the mothers (John et al., 1984). Similarly, the ossification of cervical vertebrae in fetuses of pregnant rats exposed to 2400 mg 1,2-DCB/m^3 was delayed; this dose also induced decreases in body weight gain and food consumption in the mothers (Hayes et al., 1985). However, in both of these studies, there was no convincing evidence of embryotoxic, fetotoxic, or teratogenic effects in rabbits exposed to MCB or 1,2-DCB via inhalation, even at dose levels that were maternally toxic (John et al., 1984; Hayes et al., 1985). In fetuses of pregnant rabbits exposed to 4720 mg 1,4-DCB/m^3, there was an increase in the incidence of retro-oesophageal right subclavian artery, a minor variation in the circulatory system that is often observed in control litters; at this dose, the maternal body weight gain was also decreased (Hayes et al., 1985). In an additional study, Giavini et al. (1986)

administered 1,4-DCB, in corn oil, by gavage, at doses of 250, 500, 750, or 1000 mg/kg body weight per day, between days 6 and 15 of gestation. Effects were noted only at doses greater than 500 mg/kg per day. These included an increased frequency of extra ribs, a reduction in fetal weight, and an increase in skeletal abnormalities. These dose levels also induced decreases in body weight gain and food consumption in the mothers.

There is some evidence that the higher chlorinated benzenes (TCBs, TeCBs, PeCB) are embryotoxic or fetotoxic at dose levels that are not maternally toxic. However, available data are not consistent and the toxicities of the various isomers of the TCBs and TeCBs for the mother and fetus vary considerably. For example, the 1,2,4-isomer was the most maternally toxic of the 3 TCB isomers administered by ingestion to pregnant rats in a study conducted by Black et al. (1988); changes in haematological parameters occurred at doses as low as 150 mg/kg per day. At a lower dose (75 mg/kg), there were mild histological changes in the lenses of pups of exposed mothers; however, these changes were not observed at higher doses (150 or 300 mg/kg) and were unlikely, therefore, to be treatment-related. Kitchin & Ebron (1983a) observed growth retardation in the embryos of pregnant rats administered 360 mg 1,2,4-TCB/kg body weight on days 9-13, a dose that caused some lethality, reduced body weight gain, and produced moderate hepatocellular hypertrophy in mothers.

Although less toxic for the mothers than 1,2,4-TCB, the 1,3,5-isomer of trichlorobenzene induced mild changes in the lenses of pups of

pregnant rats administered doses as low as 150 mg/kg body weight by gavage; there was no significant maternal toxicity at this dose (Black et al., 1988). For the 1,2,3-isomer, there were no effects in offspring at any dose level (up to 600 mg/kg body weight), even though a level of 300 mg/kg was toxic to the mothers. The authors of this study did not discuss the significance of the observed histological changes in the lenses (areas of cellular disorientation and disaggregation with ballooning and granular degeneration) of the pups of mothers administered the 1,3,5-isomer, but concluded that there was no evidence that any of the TCB congeners were teratogenic or fetotoxic.

Kacew et al. (1984) reported that the 1,2,4,5-isomer was the most toxic of the TeCBs for both mothers and fetuses, in a study in which all 3 isomers were administered to pregnant rats by gavage (death of 9/10 animals at 200 mg/kg and increase in serum cholesterol at 50 mg/kg body weight). The toxicity was well correlated with the greater accumulation of 1,2,4,5-TeCB in maternal and fetal tissues. There was a decrease in the number of live fetuses in pregnant rats administered 50 mg 1,2,4,5-TeCB/kg body weight, a dose that induced minor changes (increases in serum cholesterol) in exposed mothers.

Table 24. Developmental and reproductive studies on chlorobenzenes

Compound; Reference	Species[a]	Dose[b,c]	Results[d]	Effect Levels[e]
1,2,3-TCB 1,2,4-TCB 1,3,5-TCB (99.5%) Ruddick et al. (1983); Black et al. (1988)	Sprague-Dawley rat (pregnant); 14/group	1,2,4-TCB: 0, 75, 150, or 300 mg/kg per day; 1,2,3-TCB and 1,3,5-TCB: 0, 150, 300 or 600 mg/kg per day on days 6-15 of gestation; gavage in corn oil	maternal toxicity - reduced body weight gain at 600 mg/kg (1,3,5-TCB), increased liver weight at 600 mg/kg (1,2,3- and 1,3,5-TCB) and 300 mg/kg (1,2,4-TCB), decreased haemoglobin levels and haematocrit, generally at highest dose (all 3 isomers), decreased RBC at 300 mg/kg (1,2,3-TCB), 150 and 300 mg/kg (1,2,4-TCB), and 150 and	1,2,3-TCB: 300 mg/kg per day (F); *600 mg/kg per day (O) (NOEL) 1,2,4-TCB:

Bull. Environ. Contam. Toxicol. (1988) 41:719–726

Environmental Contamination and Toxicology

Assessment of Teratogenic Potential of 1,2,3- 1,2,4- and 1,3,5-Trichlorobenzenes in Rats

W. D. Black,[1] V. E. O. Valli,[1] J. A. Ruddick,[2] and D. C. Villeneuve[2]

[1]Ontario Veterinary College, Guelph, Ontario N1G 2W1 and [2]Bureau of Chemical Hazards, Environmental Health Directorate, Health Directorate, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario

Trichlorobenzenes (TrCB) are chemicals that are used as chemical intermediates, dye carriers and as dielectric fluids (U.S. EPA, 1983). They have low water solubility but are freely soluble in organic solvents. Of the isomers (1,2,3-, 1,2,4- and 1,3,5-) 1,2,4-TrCB has been produced in the largest quantities, an estimated annual production of 1.3 - 7 million kg in the U.S. (U.S. EPA, 1983). TrCB have also been shown to arise from the degradation of the pesticide lindane (Mathur and Saha, 1977 and Engst et al., 1976). TrCB are demonstrated environmental contaminants. Gulls sampled from the Great Lakes basin are known to contain residues of TrCB (Hallett et al., 1982) and they were also identified in freshwater fish taken from the Saginaw River, Michigan (Veith et al., 1979) and from the Great Lakes (Oliver and Nichol, 1982).

The present study was initiated to screen the three TrCB congeners for their teratogenic potential and to determine their ability to cross the placenta and accumulate in the fetus.

METHODS AND MATERIALS

Female Sprague-Dawley (175 - 122 g) rats, purchased from Woodlyn Farm Laboratories, Guelph, Ontario were acclimatized for one week prior to mating. Timed pregnancies were obtained by placing two females into a cage overnight containing a male rat. The following morning vaginal swabs were examined to check for mating. The morning on which sperm was detected in the female was designated as day 1 pregnancy. Mated females were randomly assigned to ten groups and housed in wire-top plastic cages containing corn cob bedding. Each group consisted of approximately 14 dams.

Trichlorobenzene congeners (1,2,3-, 1,2,4- and 1,3,5- TrCB, 97-99% pure) purchased from Aldrich Chemical Company (Montreal, Quebec) were recrystallized from ethanol and confirmed as 99.5% pure by gas chromatography. The purified congeners were dissolved in corn oil and administered as follows: 1,2,4- TrCB - 75, 150 and 300 mg/kg

Send reprint requests to W.D. Black

b.w.; 150, 300 and 600 mg/kg b.w. for both the 1,2,3- and 1,3,5-TrCB. These preparations were administered by gavage (0.5 ml/100 g b.w.) to dams on the 6th to 15th day of gestation. Controls received an equal volume of corn oil. On the 22nd day of gestation, the dams were weighed, anesthetized with sodium pentobarbital and exsanguinated via the abdominal aorta. The uterus was transected anterior to the cervix and removed with the ovaries. Dams were reweighed and their liver, kidney, spleen, heart and brain removed and weighed. The pups were removed and weighed individually.

Maternal blood was examined hematologically using a Baker 7000 blood analyzer. Parameters measured were hemoglobin concentration, hematocrit value, erythrocyte count, total and differential counts of leucocytes, mean corpuscular volume, mean corpuscular hemoglobin concentration and mean corpuscular hemoglobin.

Serum from each dam was analyzed using a SMA 12/60 microanalyzer (Technicon, Montreal, Quebec). Measured were; sodium, potassium, inorganic phosphorus, total bilirubin, alkaline phosphatase, glutamic oxaloacetic transaminase (GOT), total protein, calcium, cholesterol, glucose, uric acid and lactic dehydrogenase (LDH).

Portions of the central lobe of maternal liver were homogenized in 0.15 M KCl, pH 7.4 (1 g/2.5 ml) and centrifuged at 10,000 x g for 15 min at 0°C. The supernatant obtained was used for enzymatic analysis. Aniline hydroxylase activity (AH) was determined by an automated method based on the procedure of Fouts (1963); aminopyrine-N-demethylase (APDM) was determined by an automated method based on the procedure of Cochin and Axelrod (1959), and protein concentrations were determined by the biuret method of Gornall et al. (1948) as modified by Becking (1973).

At necropsy, portions of maternal tissues were selected for TrCB residue analysis; kidney, brain, spleen, heart, liver and perirenal fat. In addition, one fetus/litter and the liver and brain of a litter mate were removed for TrCB analysis. Tissues were stored frozen at -20°C until analyzed. TrCB congeners were extracted according to a modified method of Hallett et al. (1978). Samples of tissues were homogenized and extracted in 8 mL of acetonitrile: distilled water (1:1). The homogenate was mixed with 4 ml of n-hexane, shaken for 30 min then centrifuged at 1000 x g for 15 min at 22°C. An aliquot of the n-hexane layer was removed and washed with an equal amount of concentrated (95-98% pure) H_2SO_4. The washed solution was analyzed using a Microtech 220 gas chromatograph fitted with a ^{63}Ni electron-capture detector and a glass column (100 cm x 6.25 mm O.D.) packed with 4% SE-30 and 6% QF-1 on Suppleport matrix. Gas chromatographic conditions were: injector temperature, 250°C; oven temperature, 225°C; detector temperature, 325°C; carrier gas, nitrogen and flow rate, 35 ml/min. Recovery ranged from 60-95% depending on the isomer and tissue. The detection limit was 0.01 ppm. Retention times for 1,2,3, 1,2,4- and 1,3,5-TrCB were 0.8, 1.15 and 0.57 min., respectively.

Fetuses were examined grossly at necropsy for birth defects. Only live fetuses were counted and evaluated for terata. Approximately two-thirds of each litter were processed for skeletal examination, the remainder were fixed in Bouin's fluid for visceral examination. The visceral anomalies were searched for by dissecting and razor sectioning. The skeleton anomalies were detected by examining the cleared and stained skeletons stereoscopically.

The histological procedure used to examine the maternal heart, brain, pituitary, eye, thyroid, parathyroid, trachea, bronchi, lung, thymus, stomach, small and large intestine, pancreas, liver, kidney, spleen, adrenal, skeletal muscle, peripheral nerve, skin, bone marrow, ovary, uterus and bladder tissues has been previously described (Villeneuve et al., 1979). In addition, entire fetuses were embedded in paraffin, serial-sectioned, stained and examined microscopically.

The data from organ weight, body weights, hematology and biochemistry were subjected to a simple one way analysis of variance. When significant differences ($p < 0.05$) were indicated, the Duncan's Multiple Range Test (SPSS version 8.1) selected the groups that were significantly different.

RESULTS AND DISCUSSION

Three animals died during the study: one in control group, one dosed with 1,2,4-TrCB (150 mg/kg) and one dosed with 1,2,3-TrCB (300 mg/kg). Deaths were not judged to be treatment related.

None of the animals displayed any signs of toxicity, however mean body weight of the mothers tended to be lower in the high dose treatments. The effect on weight was significant for the 600 mg/kg 1,3,5-TrCB group only (35.4±6.6 g) when compared to controls (53.7±3.1 g).

Table 1 summarizes, numbers of pregnancies, fetal weight, litter size, resorptions and dead fetuses as well as the visceral and skeletal anomalies. A significant increase in the number of resorptions was observed in the group administered 1,3,5-TrCB (300 mg/kg). This was attributed to one animal having 12 resorption sites and was judged not treatment related since similar occurrences were not observed in other animals of that group, nor in the 600 mg/kg group. One dam from the 1,3,5-TrCB (300 mg/kg) group delivered a fetus with micrognathia. No other gross skeletal or visceral abnormalities were observed. A few minor skeletal deviations were present but none had teratological significance.

A significant ($p < 0.05$) increase in the liver weight (as a percent body weight) was observed in 1,2,3- (5.8±0.26%) 600 mg/kg, 124- (5.7±0.1%) 300 mg/kg and 1,3,5-TrCB (5.6±0.1%) 300 mg/kg and (6.4±0.3%) 600 mg/kg when compared to controls (5.1±0.2%).

The wet liver weights of 1,2,4-TrCB 300 mg/kg dosage (15.0±0.4g) and 1,3, 5-TrCB 300 mg/kg (14.8±0.5g) and 600 mg/kg dosages

(16.0±0.7g) were significantly increased ($p < 0.05$). All other organ weights were statistically normal.

ADPM activity was significantly increased at the high dose of 1,2,3-TrCB (600 mg/kg), 27.3±0.9 nano moles formaldehyde/hr/mg protein and the 2 high doses of 1,2,4-TrCB, (150 & 300 mg/kg), 28.9±1 and 28.8±0.9 nano moles formaldehyde/hr/mg protein respectively, compared to control 22.8±0.6. All 3 doses of 1,3,5-TrCB had APDM activity levels greater than the control (25.1±0.6 to 25.5±0.9 nano moles of formaldehyde/hr/mg protein) but the difference was not significant. Treatment did not affect other biochemical parameters.

Hemoglobin concentrations were decreased in animals dosed at the two highest levels of 1,2,3-TrCB (300 and 600 mg/kg), 11.4±0.2 and 11.3±0.2 g/dl respectively, the two highest doses of 1,2,4-TrCB (150 and 300 mg/kg) 11.2±0.1 and 11.3±0.1 g/dl respectively, and the highest dose of 1,3,5-TrCB (600 mg/kg) 10.8±0.2 g/dl compared to control 12.0±0.3 g/dl. Hematocrit levels were decreased by 600 mg/kg 1,2,3-TrCB (31.1±0.6%) 150 and 300 mg/kg 1,2, 4-TrCB (31.2±0.4 and 31.4±0.5% respectively) and at 600 mg/kg 1.3,5-TrCB (29.8±10.5%) compared to control (33.3%).

Mild degenerative renal changes were observed in some rats, however they could not be attributed to treatment. They consisted primarily of multifocal glomerular adhesions with a normal architectural pattern. Mild changes were observed in the thyroid gland of animals dosed with 300 mg/kg 1,2,4-TrCB and the two highest dose groups receiving 1,2,3- and 1,3,5-TrCB. The changes consisted of a reduction of follicle size which was often accompanied by angular collapse. In the highest dose groups of each compound there was increased epithelial height accompanied by cytoplasmic vacuolation. Mild hepatic changes were also observed in the mothers and these were generally restricted to the highest and second highest dose levels for each compound. Changes consisted largely of increased periportal cytoplasmic eosinophilia and mild anisokaryosis of hepatocellular nuclei. In addition increased splenic hematopoesis was observed in some females.

Histological lesions occurred in the lenses of eyes of pups treated with 1,3,5-TrCB (all dose levels) and 1,2,4-TrCB (intermediate dose group). The changes consisted of central areas of cellular disorientation and disaggregation with ballooning and granular degeneration. Autolysis and incomplete preservation made examination of other fetal tissues difficult but there did not appear to be any significant treatment-related changes.

TrCB residues were found only in fat tissues. The 1,2,3-isomer had trace levels at the 2 high doses (means of 0.02 and 0.4 ppm) and 1,3,5-TrCB residues were found in fat at all three dosage levels (means 1.6 low dose to 4.8 ppm high dose). No 1,2,4-TrCB residues were detected in tissues.

Table 1. Fetal data (mean ± S.E.) following oral administration of trichlorobenzene (TrCB) congeners to pregnant rats

	1,2,3-TrCB				1,2,4-TrCB			1,3,5-TrCB		
	Control	150(mg/kg)	300	600	75	150	300	150	300	600
Dams at term/ Inseminated	12/14	10/13	11/13	11/13	11/13	11/13	12/14	12/13	12/13	11/13
Resorption + dead fetuses	0.7±0.2	0.4±0.1	0.4±0.1	0.5±0.2	1.1±0.2	0.6±0.2	0.6±0.3	0.7±0.2	2.0±0.9*	0.4±0.1
Litter size	11.5±1.2	11.5±1.1	13.3±0.6	12.7±1.1	12.1±0.9	13.1±0.5	13.3±0.6	12.5±0.7	11.5±1.0	11.5±1.3
Fetal wt (g)	5.4±0.1	5.4±0.1	5.2±0.1	5.1±0.2	5.3±0.1	5.5±0.1	5.1±0.1	5.4±0.1	5.5±0.1	5.2±0.1
Visceral Observations[a]	31	27	37	37	28	40	42	33	33	31
Skeleton Observations[a]	67	49	61	65	50	76	82[b]	66	55	56
Sternal anomilies[c]	11/3	1/1	1/1	13/3	14/6	11/4	9/7	9/6	5/3	9/5
Wavy ribs[c]	1/1	0	1/1	1/1	1/1	0	0	1/1	0	1/1
Centrum fusion delay	0	0	0	0	0	3/2	1/1	5/3	2/2	0
14th rib	0	1/1	0	0	0	5/5	2/1	2/1	0	3/2
13th rib (short)	0	1/1	0	0	1/1	0	0	0	0	0

a no. of pups examined
b one pup has micrognathia; others normal
c no. of pups affected/no. of litters affected
* significant difference $P \leq 0.05$

There was no evidence that any of the TrCB congeners tested were teratogenic or fetotoxic. This confirms previous findings of Kitchin and Ebron (1983) who also failed to observe any teratogenic effects in rats treated with 1,2,4-TrCB at high levels. In that study all animals in the highest dose group died on the third day and 22% of the dams died in the 360 mg/kg dose group. These authors also reported a significant decrease in embryonic growth prior to term. In a preliminary study, leading up to the study reported here, we found that 1,2,4-TrCB 600 mg/kg caused maternal deaths. Consequently, 300 mg/kg was the highest amount of this isomer administered. Thus our maternal toxicity data, for the 1,2,4- isomer, is in agreement with Kitchin and Ebron's results and suggests that it is more toxic to the dam than the other isomers. The 1,3,5-isomer was the next most potent since it caused decreased maternal body weight gain at 600 mg/kg. It was not possible to compare the feto- toxicity of the three congeners at any of the dose levels since no changes were detected. In terms of the effects observed on liver, both 1,2,4- and 1,3,5-TrCB produced hepatomegaly at levels as low as 300 mg/kg. However, of the two, only the 1,2,4-isomer caused a significant increase in mixed function oxidase activity (APDM). The 1,2,3-isomer also increased APDM activity but only at the high dose level (600 mg/kg). Neither the increased liver weight nor APDM activity could be considered dramatic at any dose levels tested. Increased liver weights were reported in rats and dogs following inhalation of 1,2,4-TrCB at 100 ppm for 7 h/day, 5 day per week for a total of 30 daily exposures (Kociba et al., 1981). Increased liver weight was also observed in male rats dosed orally with 40 mg/kg 1,2,4-TrCB for 14 or 90 days (Carlson and Tardiff, 1976). Numerous investigators have reported that 1,2,4-TrCB is capable of inducing mixed function oxidase activity in rats (Carlson and Tardiff, 1976 Carlson, 1978; Smith and Carlson, 1980). Ariyoshi et al., (1975a,b) reported that 1,2,4-TrCB could stimulate APDM activity and that 1,3,5-TrCB could do the same but to a lesser extent. Our results failed to show that the 1,3,5-isomer could significantly induce APDM activity, but the fact that pregnant animals were used might have some bearing.

The changes observed in the hemoglobin content, and hematocrit of rats treated with all 3 isomers indicated a very mild anemia. Examination of bone marrow demonstrated large numbers of erythroid cells in this tissue. The absence of reticulocytes in the blood smears suggested newly developing cells are not being released into the circulation and we conclude that this was due to ineffective erythropoiesis. No hematological effects of TrCB's have been previously reported.

Histological changes were uniformly mild in all tissues. No TrCB related lesions were found in the kidney and an increased hematopoesis observed in the spleen was likely an attempt to compensate for the mild anemia. The changes detected in the thyroid and liver, while restricted to the high dose levels of each chemical however their severity was not clearly dose related. The major

hexachlorcyclohexan-isomere im menschlichen urin. Z Gesamte Hyg 22:495-498
Fouts JR (1963) Factors influencing the metabolism of drugs in liver microsomes. Ann NY Acad Sci 104:875-889
Gornall AG, Bardawill CJ, David MM (1948) Determination of serum proteins by means of the buiret reaction. J Biol Chem 177:751-766
Hallett DJ, Khera KS, Stoltz DR, Chu I, Villeneuve DC, Trivett GJ (1978) Photomirex synthesis and assessment of acute toxicity tissue distribution and mutagenicity. J Agric Food Chem 26:338-339
Hallett DJ, Norstrom RJ, Onuska FI, Camba ME (1982) Incidence of chlorinated benzenes and chlorinated ethylenes in Lake Ontario herring gulls. Chemosphere 11:277-285
Kacew S, Ruddick JA, Parulekar M, Valli VE, Chu I, Villeneuve DC (1984) A teratological evaluation and analysis of fetal tissue levels following administration of tetrachlorobenzene isomers to the rat. Teratology 29:21-28
Kitchin KT, Ebron MT (1983) Maternal hepatic and embryonic effects of 1,2,4-trichlorobenzene in the rat. Environ Res 31:362-373
Kociba RJ, Leong BKJ, Hefner RE (1981) Subchronic toxicity study of 1,2,4-trichlorobenzene in the rat rabbit and beagle dog. 4:229-249
Mathur SP, Saha JG (1977) Degradation of lindane-(14)C in a mineral soil and in an organic soil. Bull Environ Contam Toxicol 17: 424-430
Oliver BG, Nichol KD (1982) Chlorobenzenes in sediments water and selected fish from Lake Superior Lake Huron Lake Erie and Lake Ontario (USA, Canada). Enivron Sci Technol 16:532-536
Smith EN, Carlson GP (1980) Various pharmacokinetic parameters in relation to enzyme-inducing abilities of 1,2,4-trichlorobenzene and 1,2,4-tribromobenzene. J Toxicol Environ Health 6:737-750
US EPA (1983) Chlorinated benzene aggregates derived from information reported under TSCA section 8(a). Office of Toxic Substances Washington DC
Veith GD, Kuehl DW, Leonard EN, Puglisi FA, Lemke AE (1979) Polychlorinated biphenyls and other organic chemical residues in fish from major watersheds of USA. Pestic Monit J 13:1-11
Villeneuve DC, Khera KS (1975) Placental transfer of halogenated benzenes (pentachloro- pentachloronitro and hexabromo-) in rats. Environ Physiol Biochem 5:328-331
Villeneuve DC, Hierlihy KS (1975) Placental transfer of hexa-chlorobenzene in the rat. Bull Environ Contam Toxicol 13:489-491
Villeneuve DC, Valli VE, Chu I, Secours V, Ritter L, Becking GC (1979) Ninety-day toxicity of photomirex in the male rat. Toxicology 12:235-250

Received April 11, 1988; accepted June 4, 1988.

There was no evidence that any of the TrCB congeners tested were teratogenic or fetotoxic. This confirms previous findings of Kitchin and Ebron (1983) who also failed to observe any teratogenic effects in rats treated with 1,2,4-TrCB at high levels. In that study all animals in the highest dose group died on the third day and 22% of the dams died in the 360 mg/kg dose group. These authors also reported a significant decrease in embryonic growth prior to term. In a preliminary study, leading up to the study reported here, we found that 1,2,4-TrCB 600 mg/kg caused maternal deaths. Consequently, 300 mg/kg was the highest amount of this isomer administered. Thus our maternal toxicity data, for the 1,2,4- isomer, is in agreement with Kitchin and Ebron's results and suggests that it is more toxic to the dam than the other isomers. The 1,3,5-isomer was the next most potent since it caused decreased maternal body weight gain at 600 mg/kg. It was not possible to compare the feto- toxicity of the three congeners at any of the dose levels since no changes were detected. In terms of the effects observed on liver, both 1,2,4- and 1,3,5-TrCB produced hepatomegaly at levels as low as 300 mg/kg. However, of the two, only the 1,2,4-isomer caused a significant increase in mixed function oxidase activity (APDM). The 1,2,3-isomer also increased APDM activity but only at the high dose level (600 mg/kg). Neither the increased liver weight nor APDM activity could be considered dramatic at any dose levels tested. Increased liver weights were reported in rats and dogs following inhalation of 1,2,4-TrCB at 100 ppm for 7 h/day, 5 day per week for a total of 30 daily exposures (Kociba et al., 1981). Increased liver weight was also observed in male rats dosed orally with 40 mg/kg 1,2,4-TrCB for 14 or 90 days (Carlson and Tardiff, 1976). Numerous investigators have reported that 1,2,4-TrCB is capable of inducing mixed function oxidase activity in rats (Carlson and Tardiff, 1976 Carlson, 1978; Smith and Carlson, 1980). Ariyoshi et al., (1975a,b) reported that 1,2,4-TrCB could stimulate APDM activity and that 1,3,5-TrCB could do the same but to a lesser extent. Our results failed to show that the 1,3,5-isomer could significantly induce APDM activity, but the fact that pregnant animals were used might have some bearing.

The changes observed in the hemoglobin content, and hematocrit of rats treated with all 3 isomers indicated a very mild anemia. Examination of bone marrow demonstrated large numbers of erythroid cells in this tissue. The absence of reticulocytes in the blood smears suggested newly developing cells are not being released into the circulation and we conclude that this was due to ineffective erythropoiesis. No hematological effects of TrCB's have been previously reported.

Histological changes were uniformly mild in all tissues. No TrCB related lesions were found in the kidney and an increased hematopoesis observed in the spleen was likely an attempt to compensate for the mild anemia. The changes detected in the thyroid and liver, while restricted to the high dose levels of each chemical however their severity was not clearly dose related. The major

lesion found in the fetuses was restricted to the lenses of the eye possibly indicating early cataract development. This observation has not previously been made.

The residue data confirm previous ^{14}C-TrCB pharmacokinetic studies (Chu 1986) showing that none of the isomers accumulate to any great degree, but the 1,3,5-isomer gives higher residues than the other two compounds. Also significant is the fact that the fetus does not bioaccumulate these chemicals. Higher chlorinated benzenes i.e. hexachloro-, pentachloro-, and 1,2,4 - tetrachlorobenzene, do cross the placenta and accumulate rather significantly in the fetus (Villeneuve and Hierlihy, 1975; Villeneuve and Khera, 1975; Kacew et al., 1984).

In summary, none of the TrCB isomers tested in this study produced teratogenic or fetotoxic effects. The most toxic isomer, at least with respect to maternal toxicity was 1,2,4-TrCB. None of the isomers accumulated in maternal or fetal tissue to any significant amount; however, 1,3,5-TrCB did show up in maternal fat samples in low ppm levels.

Acknowledgments. The technical assistance of Cecilia Doig, Jean Claxton and Rob Valli of the University of Guelph was greatly appreciated. Similarly, we acknowledge the assistance of Nanette Beament, Barb Reed, Vic Secours, Jack Kelly, Andre Viau, Mary Beaudette and Al Yagminas of the Environmental Health Centre, Ottawa. We would also like to thank Annette Cservik for typing the manuscript.

REFERENCES

Ariyoshi T, Ideguchi K. ishizuka Y, Iwasaki K, and Arakaki M (1975a) Relationship between chemical structure and activity. I Effects of the number of chlorine atoms in chlorinated benzenes on the components of drug-metabolizing systems and the hepatic constituents. Chem Pharm Bull (Tokyo) 23:817-823

Ariyoshi T, Ideguchi K, Iwasaki K, and Arakaki M (1975b) Relationship between chemical structure and activity. II Influences of isomers in dichlorobenzene trichlorobenzene and tetrachlorobenzene on the activities of drug-metabolizing enzymes. Chem Pharm Bull (Tokyo) 23:824-830

Becking GC (1973) Vitamin A status and hepatic drug metabolism in the rat. Can J Physiol Pharmacol 51:6-11

Carlson GP (1978) Induction of cytochrome P-450 by halogenated benzenes. Biochem Pharmacol 27:361-363

Carlson GP, Tardiff RG (1976) Effect of chlorinated benzenes on the metabolism of foreign organic compounds. Toxicol Appl Pharmacol 36:383-394

Cochin J, Axelrod J (1959) Biochemical and pharmacological changes in the rat following chronic administration of morphine nalorphine and normorphine. J Pharmacol Expt Therap 125:105-115

Engst von R, Macholz M, Kujawa M (1976) Uber das vorkammen von beta-glucuronidisch gebundenen metaboliten der

Mutagenesis vol.2 no.2 pp.111–113, 1987

The bone marrow clastogenicity of eight halogenated benzenes in male NMRI mice

E.Mohtashamipur, R.Triebel, H.Straeter and K.Norpoth

Institute of Hygiene and Occupational Medicine, University Medical Centre, Essen University, Hufelandstrasse 55, D-4300 Essen, FRG

Eight widely used halogenated benzenes, including bromobenzene (BB), chlorobenzene (CB), three isomers of dichlorobenzene (DCB) and three isomers of trichlorobenzene (TCB) were tested for acute toxicity (LD_{50}) and clastogenicity in 8-week-old NMRI mice by intraperitoneal administration. Four doses of each chemical (up to 70% of LD_{50}) were tested for clastogenic activity. Each compound was administered in two equal doses, 24 h apart. Increased formation of micronucleated polychromatic erythrocytes, observed in femoral bone marrow, 30 h after the first injection, was considered to be due to the clastogenic activity of the test compound. All the halogenated benzenes tested were found to be clastogenic ($P < 0.01$). The highest clastogenic activities were induced by *m*-DCB and BB. Among the three isomers of DCB, *m*-DCB significantly ($P < 0.05$) induced more micronuclei than *o*-DCB or *p*-DCB. No significant differences were found between the clastogenic activities of TCB isomers.

Introduction

Halogenated benzenes are widely used in relatively large quantities in various industries. The total production of all chlorinated benzenes in western Europe is estimated to have been more than 208 million kg in 1980, half of which was located in the Federal Republic of Germany (IARC, 1982a). In addition to direct occupational exposure of man, halogenated benzenes are found in most parts of the ecosystem and have been detected in air, soil, food and water (Pellizzari, 1979; Zoeteman *et al.*, 1979; IARC, 1982a; Lai, 1984). Some of these chemicals are not limited to industrial use. For example, dichlorobenzene (*p*-DCB) is used as a fungicide, an air freshener, a moth repellent on clothes, and its crystals can be used to counteract odours in garbage and other refuse (IARC, 1982a; Giavini *et al.*, 1986).

Chlorobenzenes and their metabolites have been detected in human and animal tissues and body fluids indicating the extent of environmental pollution (IARC, 1982a; Jan, 1983). The mean levels of chlorobenzenes in human milk and adipose tissue are found to range from traces to 25 μg/kg in milk and from not detectable to 146 μg/kg in adipose tissue (Jan, 1983). Few biological data relevant to toxicity and genotoxicity of halogenated benzenes are available. Although the hepatotoxic compound, bromobenzene (BB), is found to bind covalently to DNA/RNA to an extent comparable to that of moderately oncogenic substances (Colacci *et al.*, 1985), no data are available on its carcinogenicity. The results of a host-mediated mutagenicity assay on BB were negative (Simmon *et al.*, 1979). No adequate information exists on the carcinogenicity/mutagenicity of chlorinated benzenes (IARC, 1982a). Wester *et al.* (1985) carried out a lifetime carcinogenicity study with a 'mixture' of 11 halogenated hydrocarbons including chlorobenzenes administered to rats in the drinking water, and found no significant carcinogenic effect. The results of some teratogenicity assays with chlorobenzenes were either negative (Hayes *et al.*, 1985), or if positive, they were considered to be the consequence of other factors rather than teratogenic effects (Giavini *et al.*, 1986).

This paper describes an investigation of the effects of a series of halogenated benzenes on the incidence of micronucleus induction within bone marrow polychromatic erythrocytes after intraperitoneal administration to mice.

Materials and methods

Chemicals

Fetal calf serum was obtained from Boehringer Mannheim (Mannheim, FRG). Benzene and halogenated benzenes were purchased from Merck Co. (Hohenbrunn, FRG). All test compounds were of synthetic grade and their purities are given in Table I. *p*-DCB, trichlorobenzene (1,2,3-TCB) and 1,3,5-TCB were supplied as solids which were dissolved in the minimum possible amount of dimethylsulphoxide (Merck, Darmdstadt, FRG).

Animals

Male 8-week-old NMRI mice were obtained from the Zentralinstitut für Versuchstiere (Hannover, FRG). Animals were housed five per cage and were given free access to water and laboratory Altromin chow (Altromin, Lage, FRG).

Micronucleus test

Median lethal doses (LD_{50}) of the test compounds administered by i.p. injection, were conducted according to the standard method (Fowler, 1983) as described elsewhere (Mohtashamipur *et al.*, 1985).

Since the dosing regime of Schmid (1977) for the micronucleus test implies that the highest dose used should be tolerated by the animals, the doses were chosen in such a way that they did not exceed 70% of the LD_{50}. Each single group of five animals was treated by subacute i.p. injection of half of each chosen dosage of a test chemical 24 h before the other half was administered. The chemicals were given in corn oil (when the doses were too small to be injected alone) or alone. The control group of 10 mice received i.p. injections of corn oil only. Benzene, as the mother compound for halogenated benzenes, was chosen as the positive control. The animals were killed by an overdose of ether 30 h after the first injection of the test chemical; the femora were removed and the marrow was suspended in serum. Two smears per femur were prepared and coded. The smears were studied by two different examiners. One-thousand polychromatic erythrocytes per smear were examined for the presence of micronuclei.

Statistical analysis

LD_{50} doses and their 95% confidence limits were calculated using the method of Cavalli-Sforza and Lorenz (1964); significant testing of the micronucleus results was done using the Student's *t* test (Hill, 1967).

Results

The highest toxicity was found with BB and the least toxicity with 1,3,5-TBC, when the median lethal doses were determined (Table I). In general, trichlorobenzenes were less toxic to the animals than dichlorobenzenes (Table I).

All the halogenated benzenes tested were found to be clastogenic ($P < 0.01$) (Table II). The highest clastogenic activities were observed in animals dosed with *m*-DCB and BB. Among the three isomers of DCB, *m*-DCB induced significantly ($P < 0.05$) more micronuclei than *o*-DCB or *p*-DCB (Table II). No significant differences were found between the clastogenic activities of TCB-isomers (Table II).

Discussion

Although the chromosome-damaging effects of benzene in marrow cells of mammals, including man, and the capacity of

Table I. Median lethal doses (LD_{50}) of benzene and eight halogenated benzenes for 25–30 g male NMRI mice by i.p. administration. Purities of the compounds are those given by the manufacturer

Compound	Purity (%)	LD_{50} mg/kg body weight
Benzene	99.7	1714.24 ± 101.37
Bromobenzene	99.0	817.11 ± 50.71
Chlorobenzene	99.0	1355.24 ± 250.55
m-Dichlorobenzene	99.0	1061.84 ± 38.73
o-Dichlorobenzene	99.0	1228.12 ± 262.99
p-Dichlorobenzene	99.0	2000.42 ± 49.70
1,2,3-Trichlorobenzene	99.0	1390.21 ± 225.98
1,2,4-Trichlorobenzene	98.0	1223.25 ± 320.54
1,3,5-Trichlorobenzene	98.0	2259.83 ± 76.96

Table II. Effects of eight halogenated benzenes on frequency of micronucleated polychromatic erythrocytes of femoral bone marrow of male NMRI mice (significant value for all doses: $P < 0.01$). Values represent means ± SD of five individual experiments per dose

Compound	mg/kg body weight	Micronuclei/ 1000 PCE
Vehicle (corn oil)		1.80 ± 0.748
Benzene	264 (2 × 132)	4.40 ± 0.800
	528 (2 × 264)	8.10 ± 0.943
	1056 (2 × 528)	12.40 ± 1.356
	528 (1 × 528)	10.83 ± 1.343
Bromobenzene	125 (2 × 62.5)	3.70 ± 0.640
	240 (2 × 125)	5.50 ± 0.806
	375 (2 × 187.5)	6.50 ± 1.024
	500 (2 × 250)	8.30 ± 1.100
Chlorobenzene	225 (2 × 112.5)	3.10 ± 0.830
	450 (2 × 225)	3.90 ± 0.830
	675 (2 × 337.5)	4.90 ± 0.943
	900 (2 × 450)	7.20 ± 0.871
m-Dichlorobenzene	175 (2 × 87.5)	3.40 ± 0.663
	350 (2 × 175)	4.40 ± 0.916
	525 (2 × 262.5)	6.30 ± 1.100
	700 (2 × 350)	9.20 ± 1.248
o-Dichlorobenzene	187 (2 × 93.5)	3.90 ± 0.943
	375 (2 × 187.5)	4.50 ± 1.204
	562 (2 × 281)	5.44 ± 1.257
	750 (2 × 375)	5.90 ± 1.135
p-Dichlorobenzene	355 (2 × 177.5)	4.00 ± 0.632
	710 (2 × 355)	4.90 ± 1.135
	1065 (2 × 532.5)	6.00 ± 1.000
	1420 (2 × 710)	6.60 ± 0.916
1,2,3-Trichlorobenzene	250 (2 × 125)	4.10 ± 0.943
	500 (2 × 250)	5.20 ± 1.249
	750 (2 × 375)	6.80 ± 0.979
	1000 (2 × 500)	7.30 ± 1.005
1,2,4-Trichlorobenzene	210 (2 × 105)	3.50 ± 0.806
	420 (2 × 210)	4.50 ± 0.806
	630 (2 × 315)	5.80 ± 0.871
	840 (2 × 420)	7.40 ± 0.916
1,3,5-Trichlorobenzene	425 (2 × 212.5)	3.60 ± 0.800
	850 (2 × 425)	4.40 ± 0.916
	1275 (2 × 637.5)	6.60 ± 0.894
	1700 (2 × 850)	7.20 ± 0.979

benzene to induce myelogenous leukaemia and other blood dyscrasias in rodents and supposedly in man have been well documented (Diaz *et al.*, 1980; Meyne and Legator, 1980; Snyder *et al.*, 1980; Goldstein and Snyder, 1982; Harper *et al.*, 1984; Cronkite *et al.*, 1985; Styles and Richardson, 1984; Erexson *et al.*, 1986; Dean, 1985; Infante and White, 1985), little adequate information exists on the carcinogenic/mutagenic effects of halogenated benzenes.

Our data demonstrate the clastogenic activity of these compounds (Table II) and indicate the desirability of further testing of benzene derivatives in order to assess the mutagenic hazard and carcinogenic potential associated with the use of this class of compounds.

The number of human cases of leukaemia associated with DCB-exposure is too small to assess a causal relationship (IARC, 1982b). This observation and the possibility of weak mutagenic activity of chlorinated benzenes (IARC, 1982a) suggest, however, that they may possess the ability to bind to DNA and cause genetic alterations in cells. BB has been found to bind covalently to DNA/RNA in mouse and rat liver cells at rates comparable to those of some moderate oncogenic compounds (Colacci *et al.*, 1985). In this connection, the negative results of Wester *et al.*, 1985) following lifetime carcinogenicity testing of chlorinated benzenes on rat may not be a very reliable assessment of the carcinogenicity of chlorobenzene (CB) isomers. This view is based on a fact that a mixture of 11 halogenated hydrocarbons (including six CB isomers) was tested together rather than testing each compound separately. Although Lai (1984), in a review article, cites a study conducted by the US National Toxicology Program implying that monochlorobenzene is a rat carcinogen, the results of this study do not appear to have been published.

Further studies are in progress to identify the metabolite(s) and the mechanism(s) involved in the induction of clastogenic effects in bone marrow cells of mice by halogenated benzenes.

Acknowledgements

Our thanks are due to Ms. I.Bäcker-Bödecker from the Institute of Informatic and Biomathematics of University Medical Center of Essen for her assistance in computer programming and analysis of data. We are also grateful to Karin Wöhrmeyer for her valuable technical assistance, and to Iris Funke-Kaiser and Doris Münz for expert typing of the manuscript.

References

Cavalli-Sforza,L. and Lorenz,R.J. (1964) Grundbegriffe der Biometrie insbesondere der statistischen Methoden bei der Wertbemessung biologisch wirksamer Substanzen. Gustav Fischer Verlag, Suttgart, pp. 171–177.

Colacci,A., Arfellini,G., Mazzullo,M., Prodi,G. and Grilli,S. (1985) The covalent binding of bromobenzene with nucleic acids. *Toxicol. Pathol.*, **13**, 276–282.

Cronkite,E.P., Drew,R.T., Inoue,T. and Bullis,J.E. (1985) Benzene hematotoxicity and leukemogenesis. *Am. J. Ind. Med.*, **7**, 447–456.

Dean,B.J. (1985) Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. *Mutat. Res.*, **154**, 153–181.

Diaz,M., Reiser,A., Bruier,L. and Diez,J. (1980) Studies on benzene mutagenesis. I. The micronucleus test. *Experientia*, **36**, 297–298.

Erexson,G.L., Wilmer,J.L., Steinhagen,W.H. and Klingerman,A.D. (1986) Induction of cytogenetic damage in rodents after short-term inhalation of benzene. *Environ. Mutagenesis*, **8**, 29–40.

Fowler,I.S.L. (1983) Short-term testing in drug development. In Balls,M., Riddell,R.J. and Worden,A.N. (eds), *Animals and Alternatives in Toxicity Testing*. Academic Press, London, pp.16–23.

Giavini,E., Broccia,M.L., Prati,M. and Vismara,C. (1986) Teratologic evaluation of *p*-dichlorobenzene in the rat. *Bull. Environ. Contam. Toxicol.*, **37**, 164–168.

Goldstein,B.D. and Snyder,C.A. (1982) Benzene leukemogenesis. *Environ. Sci.*

Res., 25, 2778–2789.
Harper,B.L., Sadagopa Ramanujam,V.C., Gad-El-Karim,M.M. and Legator,M.S. (1984) The influence of simple aromatics on benzene clastogenicity. *Mutat. Res.*, 128, 105–114.
Hayes,W.C., Hanley,T.R., Gushow,T.S., Johnson,K.A. and John,J.A. (1985) Teratogenic potential of inhaled dichlorobenzenes in rats and rabbits. *Fund. Appl. Toxicol.*, 5, 190–202.
Hill,A.B. (1967) The 't' test for small numbers. In Hill,A.B. (ed.), *Principles of Medical Statistics*. The Lancet Ltd., London, pp. 146–151.
IARC (1982a) Monographs on the evaluation of the carcinogenic risks of chemicals to humans. *Some Industrial Chemicals and Dyestuffs*. Vol. 29, pp. 213–238.
IARC (1982b) Monographs on the evaluation of the carcinogenic risk of chemicals to humans. *Chemicals, Industrial Processes and Industries Associated with Cancer in Human*. Vol. 29 (Suppl. 4), pp. 108–109.
Infante,P.F. and White,M.C. (1985) Projections of leukemia risk associated with occupational exposure to benzene. *Am. J. Ind. Med.*, 7, 403–413.
Jan,J. (1983) Chlorobenzene residues in human fat and milk. *Bull. Environ. Contam. Toxicol.*, 30, 595–599.
Lai,D.Y. (1984) Halogenated benzenes, naphthalenes, biphenyls and terphenyls in the environment: their carcinogenic, mutagenic and teratogenic potential and toxic effects. *J. Environ. Sci. Health*, 2, 135–184.
Meyne,J. and Legator,M.S. (1980) Sex-related differences in cytogenetic effects of benzene in the bone marrow of Swiss mice. *Environ. Mutagenesis*, 2, 43–50.
Mohtashamipur,E., Norpoth,K., Woelke,U. and Huber,P. (1985) Effects of ethylbenzene, toluene, and xylene on the induction of micronuclei in bone marrow polychromatic erythrocytes of mice. *Arch. Toxicol.*, 58, 106–109.
Pellizzari,E.D. (1979) Quantification of chlorinated hydrocarbons in previously collected air samples. *Gov. Rep. Announce. Index (US)*, 79(8), 68.
Schmid,W. (1975) The micronucleus test. *Mutat. Res.*, 31, 9–15.
Simmon,V.F., Rosenkranz,H.S., Zeiger,E. and Poirier,A. (1979) Mutagenic activity of chemical carcinogens and related compounds in the intraperitoneal host-mediated assay. *J. Natl. Cancer Inst.*, 62, 911–918.
Snyder,C.A., Goldstein,B.D., Sellakumar,A.R., Bromberg,I., Laskin,S. and Albert,R.E. (1980) The inhalation toxicology of benzene: incidence of hematopoietic neoplasms and hematotoxicity in AKR/J and C57BL/6J mice. *Toxicol. Appl. Pharmacol.*, 54, 323–331.
Styles,J.A. and Richardson,C.R. (1984) Cytogenetic effects of benzene: dosimetric studies on rats exposed to benzene vapour. *Mutat. Res.*, 135, 203–209.
Wester,P.W., van der Heijden,C.A., Bisschop,A., van Esch,G.J., Wegman,R.C. and de Vries,T. (1985) Carcinogenicity study in rats with a mixture of eleven volatile halogenated hydrocarbon drinking water contaminants. *Sci. Total Environ.*, 47, 427–432.
Zoeteman,B.C.J., Piet,G.J., Kussmaul,H. and Muehlhausen,D. (1979) Behavior of organic water components during drinking water preparation, especially in groundwater enrichment. *Reinhalt. Wasser*, 1, 89–106.

Received on December 17, 1986; accepted on January 29, 1987

Environmental Mutagenesis Supplement 1:3–142 (1983)

Salmonella Mutagenicity Test Results for 250 Chemicals

Steve Haworth, Timothy Lawlor, Kristien Mortelmans, William Speck, and Errol Zeiger

Genetic Toxicology Department, Microbiological Associates, Bethesda, Maryland (S.H., T.L.), Microbial Genetics Department, SRI International, Menlo Park, California (K.M.), Department of Pediatrics, Case Western Reserve University, Cleveland (W.S.), and Cellular and Genetic Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina (E.Z.)

Key words: Salmonella, mutagenicity, Ames, testing

INTRODUCTION

The Salmonella/mammalian microsome test developed by Ames and his associates [Ames et al, 1975] has become widely accepted as an initial test for the identification of chemicals with mutagenic activity. Substances that produce a positive mutagenic effect are considered to be potential animal mutagens and carcinogens and, by extension, potential human mutagens and carcinogens. This is because of the reported high correlations between mutagenicity in Salmonella, and genetic and carcinogenic effects in mammalian systems [McCann et al, 1975; Sugimura et al, 1976; Purchase et al, 1978; Rinkus and Legator, 1979; Bartsch et al, 1980]. However, chemicals that are not mutagenic in Salmonella cannot be considered benign. It has been well documented [McCann et al, 1975; Rinkus and Legator, 1979] that certain chemical classes, such as chlorinated hydrocarbons, contain a large number of carcinogens that are not mutagenic to Salmonella. Other chemicals are negative in the standard plate or preincubation protocol but are positive in protocols modified to achieve improved metabolism of the chemical to a mutagen or optimal exposure of the cells to the mutagen. Examples of these are the requirement for flavin mononucleotide (FMN) and reducing conditions or riboflavin for benzidine-based or other azo dyes [Prival and Mitchell, 1982; Hartman et al, 1978; Sugimura et al, 1977; Robertson et al, 1982], or the need for testing volatile liquids in a sealed chamber [Simmon et al, 1977].

Received June 29, 1983; revised and accepted September 20, 1983.

Address correspondence to Dr. Errol Zeiger, Cellular and Genetic Toxicology Branch, N.I.E.H.S., P.O. Box 12233, Research Triangle Park, NC 27709.

Single copies of this Supplement will be available for purchase directly from the publisher: Alan R. Liss, Inc., Subscription Department, 150 Fifth Avenue, New York, NY 10011. Please do *not* address reprint requests to the authors.

The testing of large numbers of chemicals for mutagenicity in Salmonella and other test systems is being performed within the National Toxicology Program (NTP) [Zeiger and Drake, 1980]. All chemicals selected for genetic toxicology screening are initially tested in Salmonella using a preincubation procedure [Yahagi et al, 1975], which is a modification of the Salmonella/mammalian microsome test of Ames et al [1975]. Additional tests—Drosophila melanogaster sex-linked recessive lethal and heritable translocation tests, induction of chromosome aberrations and sister chromatid exchanges (SCEs) in Chinese Hamster ovary (CHO) cells, and induction of mutation in L5178Y mouse lymphoma cells—are performed on selected chemicals based upon the needs of the NTP and results obtained from other mutagenicity test systems and carcinogenesis tests. The results obtained in these short-term mutagenicity tests will be used by the NTP in the prioritization of chemicals for subchronic or chronic toxicity testing, to assist in the evaluation of rodent test data, and to alert the cognizant government agencies and industries about potentially hazardous chemicals. A number of the chemicals were already reported by other workers as having been tested in Salmonella. Some appeared in the literature after the NTP program had begun; others were tested to develop a NTP data base for these chemicals or because they were members of a larger class of chemicals of interest.

The Salmonella tests were performed by three laboratories: Case Western Reserve University (CWR), Dr. William Speck; Microbiological Associates (formerly EG&G Mason Research Institute [EGG]), Dr. Steve Haworth; and SRI International (SRI), Dr. Kristien Mortelmans. Salmonella strains TA98, TA100, TA1535, and TA1537 were used in a modification of the preincubation test of Yahagi et al [1975]. The preincubation procedure was selected because of reports that it was no less sensitive than the plate test, and was more effective than the plate test for various chemicals such as aliphatic nitrosamines [Prival et al, 1979; Yahagi et al, 1975], pyrrolizidine alkaloids [Yamanaka, et al, 1979], and volatile chemicals [Rosenkranz et al, 1980]. Liver S-9 was prepared from male Sprague-Dawley rats (RLI) and Syrian hamsters (HLI) that were induced with Aroclor 1254 [Ames et al, 1975]. Hamster liver was used because of indications from a prior study (unpublished) and reports that the use of hamster S-9 would detect a number of chemicals undetected with rat S-9 [Prival and Mitchell, 1981; Bartsch et al, 1975; Sugimura, personal communication]. The protocol was standardized among the three laboratories, as discussed below. Each chemical was coded and tested as an unknown; the primary purpose of each test was to determine whether or not the chemical was mutagenic. This is why, in the case of a positive result, only the strains and activation systems that gave the positive results were repeated, not the entire series. The protocol was designed to allow the individual investigators the flexibility to change doses based on their interpretations of the results of the initial experiment. To monitor the performance of each laboratory, a set of positive and negative control chemicals was chosen. These chemicals were included, on a random basis, in batches of coded test chemicals sent to the testing laboratories. In addition to these controls, a small number of test chemicals selected, at random, were resubmitted to the same laboratory or sent to a second laboratory to determine interlaboratory reproducibility.

This publication is a presentation of Salmonella testing results on 250 coded chemicals, encompassing 370 tests (see Table I). The majority of these results were previously summarized in issues of the National Toxicology Program Technical Bulletin [1980a,b, 1981a,b, 1983]. However, some interpretations were changed since

publication in the NTP Bulletin, based upon a reevaluation of the data. The presentation here is designed both to summarize the results in the text and to present the data (Appendix 2) so that the reader has the opportunity of performing an independent evaluation of the data. Results from additional chemicals will be presented in future publications.

MATERIALS AND METHODS

Chemicals

The chemicals tested, their source, and purity (where known) are listed in Table I and their structures are given in Appendix 1. They were supplied to the testing laboratories by a chemical repository (Radian Corporation, Austin, TX), which was responsible for purchase and inventory of the chemicals, collection of physical, toxicological and safety data for each chemical, coding each test sample, shipment to the testing laboratories, and chemical analyses (when requested). Each sample sent out by the repository carried a unique, six-digit code number (Aliquot number) so that it could be tested under code as an unknown. The laboratories were also supplied with available information on the volatility, density, solubility, flammability, and stability of each chemical; Radian only performed solubility tests. Also sent, but in a sealed envelope coded with the Aliquot number, was the chemical name(s) along with the available information on its toxicological effects and decontamination procedures. The laboratories were instructed to open this envelope only in the event of a spill or exposure to the chemical and to treat all coded chemicals as potential mutagens and carcinogens. After completion of the testing, the unopened envelopes were returned to the Radian Corporation.

All chemicals were stored at the testing laboratories as recommended by the chemical repository. Each chemical was dissolved and diluted immediately prior to testing. The solvent of choice was distilled water; dimethyl sulfoxide (DMSO) was used if the chemical was insoluble or not sufficiently soluble in water. Ethanol (95%) or acetone was used if the chemical was not soluble or stable in DMSO.

As a rule, if a chemical was mutagenic or gave a questionable response, it was analyzed by Radian for identity and purity. Analyses had been performed previously by Midwest Research Institute (MRI) on selected other chemicals and on chemicals that had been tested in the National Cancer Institute's Carcinogenesis Bioassay Program. The results of these analyses are in Table I.

Bacterial Strains

Salmonella typhimurium strains TA1535, TA1537, TA98, and TA100 were obtained by the individual laboratories from Dr. Bruce Ames, University of California, Berkeley. Cultures of each tester strain were prepared for storage essentially as described in the Supplement to the Methods Paper [Ames et al, 1975] supplied with the tester strains by Dr. Ames. Frozen cultures were stored in liquid nitrogen (EGG) or in a −70°C freezer (CWR, SRI) in 0.2-ml aliquots (EGG), or in 1-ml aliquots (SRI) in sterile, screw-cap vials. To inoculate overnight cultures, CWR transferred a loopful of cells that were maintained on Columbia agar slants kept at 4°C into Columbia broth. EGG transferred a loopful of the thawed cultures into Oxoid Nutrient Broth #2 (CM 67) and discarded the unused portion of the thawed culture. SRI used all of the thawed 1-ml culture to inoculate minimal glucose medium [Vogel and

TABLE I. Sources, Purities, and Mutagenicities of 250 Chemicals in Salmonella (Continued)

	Chemical name	CAS number	Chemical source	Lot number
194	Pentachloroethane	76-01-7	Aldrich	CE 072487
195	Pentachloronitrobenzene	82-68-8	Aldrich	AC 071177
196	Pentachlorophenol	87-86-5	Aldrich	CC 022487
197	Phenol	108-95-2	Fluka; Textile Chem.	187130280; 79380
198	o-Phenyl phenol	90-43-7	Dow Chemical	MM09157
199	Phosphamidon	13171-21-6	Chevron	—
200	β-Picoline	108-99-6	MC/B	IIJ28
201	Picric acid	88-89-1	MC/B	8H09A
202	Piperazine	110-85-0	Eastman	A7C
203	Piperonal	120-57-0	Eastman	A8X
204	Piperonyl butoxide	51-03-6	FNC	—
205	Piperonyl sulfoxide	120-62-7	CPC International	291-N100-12
206	Prednisone	53-03-2	Upjohn	52836
207	β-Propiolactone	57-57-8	Tridom	196050 1178
208	1,2-Propylene glycol	57-55-6	MC/B	C6J13
209	Pyridine	110-86-1	Fisher	732113
210	Pyrimethamine	58-14-0	Burroughs-Wellcome	51416
211	Quinoline	91-22-5	Eastman	D6H
212	Resorcinol (1,3-benzenediol)	108-46-3	MC/B	C3J23
213	Ricinoleic acid, Na salt	5323-95-5	MC/B	D6F04
214	Semicarbazide HCl	563-41-7	Aldrich	EC 050287
215	Sodium fluoride	7681-49-4	Aldrich	DC 030887
216	Sodium phosphate, dibasic	7558-79-4	Fisher	783607
217	cis-Stilbene	645-49-8	Pfaltz & Bauer	—
218	trans-Stilbene	103-30-0	Aldrich	JC 072087
219	Streptomycin sulphate (2:3)	3810-74-0	Sigma	107C-0168
220	Sulfallate	95-06-7	Monsanto	H5215
221	1,2,3,4-Tetrachlorobenzene	634-66-2	Pfaltz & Bauer	—
222	1,2,3,5-Tetrachlorobenzene	634-90-2	Aldrich	101777
223	1,2,4,5-Tetrachlorobenzene	95-94-3	Pfaltz & Bauer	—
224	1,1,1,2-Tetrachloroethane	630-20-6	Aldrich	KE 7912KE
225	1,1,2,2,-Tetrachloroethane	79-34-5	Aldrich	060207
226	Tetrachloroethylene	127-18-4	Fisher	772783
227	1,2,3,4-Tetrachloronaphthalene	20020-02-4	Aldrich	DC 052347
228	2,3,4,5-Tetrachloronitrobenzene	879-39-0	Aldrich	090777
229	2,3,5,6-Tetrachloronitrobenzene	117-18-0	Aldrich	BB 120557
230	Tetramethyl lead	75-74-1	Ethyl Corporation	—
231	Thiocarbanilide	102-08-9	MC/B	11E22
232	Titanocene dichloride	1271-19-8	Pfaltz & Bauer	—
233	Toluene	108-88-3	Fisher	782825
234	Tributoxyethyl phosphate	78-51-3	Pfaltz & Bauer	—
235	Tributyl borate	688-74-4	MC/B	5738
236	1,2,3-Trichlorobenzene	87-61-6	Aldrich	HC 060787
237	1,2,4-Trichlorobenzene	120-82-1	MC/B	B3J08
238	1,3,5-Trichlorobenzene	108-70-3	Aldrich	KC 081087
239	1,1,1-Trichloroethane (chlorothene)	71-55-6	Fluka	31086 680
240	2,4,5-Trichlorophenol	95-95-4	Aldrich	JD 02197
241	2,4,6-Trichlorophenol	88-06-2	Eastman	B7X
242	1,2,3-Trichloropropane	96-18-4	Shell Chemical	JG 32449

TABLE I. Sources, Purities, and Mutagenicities of 250 Chemicals in Salmonella (Continued)

	Chemical name	CAS number	Chemical source	Lot number
243	Tricresyl phosphate (tritolyl phosphate)	1330-78-5	MC/B	C3J16
244	Tri-m-cresyl phosphate (TMCP)	563-04-2	Pfaltz & Bauer	T20605
245	Tris(2-chloroethyl)phosphate	115-96-8	Stauffer	0101F-1-3
246	Tris(2-chloroethyl)phosphite	140-08-9	Aldrich	KC 073187
247	m-Xylene	108-38-3	Eastman	B7B
248	o-Xylene	95-47-6	Aldrich	HD 061297
249	p-Xylene	106-42-3	Aldrich	CD 030197
250	Ziram	137-30-4	Uniroyal	—

[a]CWR, Case Western Reserve University; EGG, EG&G Mason Research Institute; SRI, SRI International.
[b]+, Positive, −, negative; ?, equivocal.
[c]None available.
[d]Purity based on Radian analysis.
[e]Purity based on MRI analysis.
[f]Two different lots tested.

Bonner, 1956] supplemented with biotin and an excess of histidine. All overnight cultures (late log phase) were obtained by incubation at 37°C on a shaker for 12–15 hr and were routinely checked for genetic integrity as recommended by Ames et al [1975].

Preparation of S-9 Fraction

Liver S-9 fractions were routinely prepared from male Sprague-Dawley rats and male Syrian hamsters that were injected, ip, with Aroclor 1254 (200 mg/ml in corn oil) at 500 mg/kg. Five days after injection, the animals were sacrificed by decapitation (EGG, SRI) or cervical dislocation (CWR) and the livers were removed aseptically. The animals were fasted for 12–24 hr immediately preceding sacrifice.

Liver homogenates were prepared aseptically at 0–4°C. Excised livers were rinsed with 0.15 M KCl, then minced and homogenized (3 ml of 0.15 M KCl/g wet tissue) in a Potter-Elvehjem apparatus with a teflon pestle (EGG, SRI) or in a Waring blender (CWR). The homogenate was centrifuged for 10 min at 9,000g at 4°C. The supernatant (S-9) was decanted and distributed into freezing ampules and stored at −70°C.

The microsomal enzyme reaction mix (S-9 mix) was prepared immediately prior to each assay. Unused S-9 mix was discarded and not refrozen. One milliliter of S-9 mix has the following composition: S-9, 0.10 ml; 0.04 M $MgCl_2$, 0.02 ml; 1.65 M KCl, 0.02 ml; 0.04 M β-nicotinamide adenine dinucleotide phosphate (NADP), 0.10 ml; 0.05 M glucose-6-phosphate, 0.10 ml; 1.0 M NaH_2PO_4 (pH 7.4), 0.10 ml; and distilled water, 0.56 ml.

Preincubation Methodology

All chemicals were tested using the preincubation procedure of the Salmonella assay [Ames et al, 1975] as described by Yahagi et al [1975]. Briefly, 0.5 ml of S-9

Label purity	Analyzed purity	Testing lab	Test result	
Technical		CWR,SRI	−,−	243
—		SRI	−	244
99.5%	98.2%[c]	SRI	−	245
—	87.8%[d]	CWR,SRI	+,+	246
Practical		EGG	−	247
97%		EGG	−	248
99%		EGG	−	249
—	86.2%[c]	SRI	+	250

mix or 0.1 M PO_4 buffer was dispensed into an appropriate number of 13 × 100 mm culture tubes maintained at 37°C in a dry-bath. Then, 0.05 ml of cells and 0.05 ml of solvent or chemical dilution were added to each tube. The mixture was vortexed and allowed to incubate with shaking in the early tests (CWR, EGG), or standing (SRI) for 20 min at 37°C. The protocol was later changed to eliminate the shaking procedure, because the commercial shakers available would not fit in the Class II, Type B hoods and, for the purposes of laboratory safety, it was inadvisable to incubate the chemicals at 37°C in the open laboratory. Following the preincubation period, 2.5 ml (EGG) or 2.0 ml (CWR, SRI) of molten top agar (45°C) supplemented with 0.5 mM L-histidine and 0.5 mM d-biotin was pipetted into the tubes, which were immediately vortexed, and their contents poured onto 25 ml of minimal glucose bottom agar [Vogel and Bonner, 1956] in a 15 × 100-mm plastic petri dish (Falcon Muta-Assay, 1028 [EGG, SRI] or Fisher Scientific petri dishes [CWR]). After the overlay solidified, the plates were inverted and incubated at 37°C for 48 h.

At least five doses of test chemical, in addition to the concurrent solvent and positive controls, were tested on each strain in the presence of S-9 mix or buffer. Three plates were used, and the experiment was repeated no less than 1 week after completion of the initial test.

Dose-Setting Experiment

To select the dose range for the mutagenesis assay, the test chemicals were checked for toxicity to TA100 up to a concentration of 10 mg/plate or the limit of solubility, both in the presence and absence of S-9 mix. One or more parameters were used as an indication of toxicity: viability on complete medium (EGG) and reduced numbers of revertant colonies per plate and/or thinning or absence of the bacterial lawn (CWR, EGG, SRI). If toxicity was not apparent in the preliminary toxicity determination, the highest dose tested was 10 mg/plate; otherwise the upper limit of solubility was used. If toxicity was observed, the doses of test chemical were chosen so that the high dose exhibited some degree of toxicity. Occasionally, in the earlier tests, the high dose was greater than 10 mg/plate.

Positive Controls

The positive control chemicals were tested concurrently with each test chemical. 2-Aminoanthracene (2-AA) was tested on all strains in the presence of rat and hamster S-9. 4-Nitro-o-phenylenediamine (NOPD) was tested on TA98 without S-9. Also without S-9, sodium azide (SA) was tested on TA100 and TA1535, and 9-aminoacridine (9-AAD) was tested on TA1537. The actual concentration for each positive control chemical used for each strain and activation condition was selected by the individual laboratory based on dose-response curves generated at the beginning of the testing program. The doses of the positive controls used by each laboratory are given in Table II.

Data Evaluation

Although procedures for the statistical analysis of Salmonella plate test data have been developed [Margolin et al, 1981], they were not incorporated into the initial data evaluations. The data were evaluated in an ad hoc manner by each testing laboratory and by NTP personnel. Prior to statistical analysis no formal rules were used; however, a positive response was indicated by a reproducible, dose-related increase, whether it be twofold over background or not. The matrix of test strains and activation systems used allowed the investigators to detect trends or patterns that might not be as evident if only one strain and activation system were examined. In addition to the standard "positive" and "negative" categories, there is also "questionable" (or "inconclusive"). This applied to low-level responses that were not reproducible within the laboratory or to results that showed a definite trend but with which the investigator did not feel comfortable in making a "+" or "−" decision. It also included tests in which an elevated revertant colony yield occurred at only a single dose level. After a decision on the mutagenicity of a sample was made, a request to decode the sample was sent to the repository, and the code was broken. The data were subsequently evaluated using an analysis based on the models presented by Margolin et al [1981]; this analysis will be described elsewhere [Risko et al, manuscript in preparation]. As a result of these statistical analyses, a number of calls were changed from the original "negative" to "equivocal." The statistical analysis did not result in any "positive" or "equivocal" calls being called "negative."

Because the criteria for "positive" or "questionable" decisions have evolved during the course of this study and because of the recent use of statistical analysis,

TABLE II. Concentrations of Positive Control Chemicals (μg/plate)

	TA98		TA100		TA1535		TA1537	
	−S-9 (NOPD)[a]	+S-9 (2-AA)	−S-9 (SA)	+S-9 (2-AA)	−S-9 (SA)	+S-9 (2-AA)	−S-9 (9AAD)	+S-9 (2-AA)
CWR	3.3	1.0	3.2	1.0	3.3	2.0	33.0	2.0
EGG	12.0	RLI 1.5[b] HLI 0.75	2.5	RLI 1.5 HLI 0.75	2.5	RLI 1.5 HLI 0.75	80.0	RLI 1.5 HLI 0.75
SRI	5.0	1.0	1.0	1.0	1.0	2.5	50.0	2.5

[a]NOPD, 4-nitro-o-phenylenediamine; 2-AA, 2-aminoanthracene; SA, sodium azide; 9AAD, 9-aminoacridine.
[b]Different concentrations for each S-9 source.

some of the results assigned in Table I differ from the initial evaluations published in the NTP Technical Bulletins [1980a,b, 1981a,b, 1982a,b, 1983]. The chemicals whose interpretations differ from those in the Bulletin are o-anisidine, boric acid (EGG Aliquot), 3-chloronitrobenzene, cyclohexanol, 2.4-dichlorophenol, 3,5-dichlorophenol, gallic acid (SRI Aliquot), glycerol (CWR Aliquot), and thiocarbanilide (SRI Aliquot).

RESULTS AND DISCUSSION

Summary results are presented in Table I and data in Appendix 2, Tables 1–250. The 250 chemicals tested encompass 370 separate samples (aliquots) tested under code using a standardized protocol. Fourteen of the chemicals were tested as coded, positive controls (AF-2, 2-aminoanthracene, 4-aminobiphenyl, benzo(a)pyrene, calcium chromate, cyclophosphamide, dimethylcarbamyl chloride, 3-methylcholanthrene, 4,4′-methylene-bis-2-chloroaniline, nitrofurantoin, N-nitrosodimethylamine, N-nitrosopiperidine, picric acid, and β-propiolactone) and five as coded, negative controls (choline chloride, glycerol, glycine, mannitol, and sodium phosphate). Streptomycin sulphate was originally chosen as a negative control, but was mutagenic in TA98 in two of the three laboratories testing it (see Appendix 2, Table 219.1,2,3). Among the 230 chemicals that were not originally selected as controls, 143 were clearly negative, 70 were clearly positive, and 17 were either questionable or did not show agreement between laboratories. These last 17 chemicals included five that were equivocal in one or both laboratories (o-anisidine, 3-chloronitrobenzene, cyclohexanol, 2,4-dichlorophenol, and 3,5-dichlorophenol). Of the remaining 12 chemicals, five were equivocal in one laboratory and positive or negative in the other(s). The final seven chemicals showed a definite disagreement between laboratories (p-anisidine, bromoform, 2,6-dimethylmorpholine, ethyl acrylate, ferrocene, isoproterenol hydrochloride, and 2-aminobiphenyl, which was negative in one laboratory the first time it was tested but positive the next).

It can be seen that, for the most part, there was good reproducibility between laboratories, even for relatively weak mutagens (Figs. 1–14). Occasionally, relatively large variations in the degrees of the response were seen, but it was difficult to determine to what extent these were a function of the laboratory or of the chemical/activation/Salmonella strain combination.

The negative control chemicals were all nonmutagenic in all tests with the exception of two positive responses from streptomycin sulphate and an equivocal response in one laboratory with glycerol. The positive controls were detected by all laboratories and were reproducible (Figs. 1–7).

The results presented in Appendix 2 are from the most definitive experiment conducted on each chemical. For the most part, the results from the confirmation (second) experiment are considered the most definitive. In a number of instances where the first or second experiment yielded weakly positive or questionably positive data, or the succeeding experiments were in disagreement with the first experiment, data from all of the experiments are presented. In all cases, however, if the reader wants data on a specific chemical in addition to that presented in Appendix 2, the specific testing laboratory should be contacted directly.

The majority of chemicals judged positive or questionable induced a response in TA100 with or without additional positive responses in one or more of the other

strains. A number of chemicals produced higher responses with hamster S-9 (for example, see Figs. 6, 7, 15–19), and vice versa (Figs. 12, 20–22), but a few were positve only with rat or hamster S-9, and some only in the absence of S-9. Substituted nitrobenzenes generally exhibited their strongest mutagenic responses in the presence of hamster S-9. The chlorinated nitrobenzenes were generally quite toxic to the tester strains, which limited the concentrations of chemical that could be tested. Two of these chemicals (2- and 4-chloronitrobenzene) were also tested (EGG) using the plate incorporation method (data not shown). Under these test conditions, higher concentrations of the chemicals could be tested. As might be expected, greater mutagenic activity was observed using the plate incorporation method. These data would indicate that when testing very toxic chemicals, negative or equivocal results with the preincubation method may require confirmation with the plate incorporation method. The similarities in the dose responses of azobenzene and hydrazobenzene (Fig. 21) suggest that both may be mutagenic via the same metabolic product. No information is available on the comparative metabolism of these substances.

The results from this testing can also be used to determine the extent of interlaboratory variability and, with some chemicals, intralaboratory variability as well. Representative examples of the degree of agreement between and within laboratories can be seen in the results on AF-2 (Fig. 1), 2-aminoanthracene (Fig. 2), 2- and 4-aminobiphenyl (Fig. 3), calcium chromate (Fig. 4), 2- and 4-chloronitrobenzene (Fig. 8), chloropicrin (Fig. 9), 2,3-dichloronitrobenzene (Fig. 10), dimethoate (Fig.11), 3,3'-dimethoxybenzidine (Fig. 12), ethylenediamine (Fig. 13), formaldehyde (Fig. 14), nitrofurantoin (Fig. 5), N-nitrosodimethylamine (Fig. 6), and N-nitrosopiperidine (Fig. 7).

Although we have not attempted to measure the extent of agreement between tests mathematically, it can be seen to vary with the chemical and is not necessarily related to the magnitude of the mutagenic response. Also, as seen with 2-aminoanthracene (cf TA100 and TA1535, Fig. 2C, D vs E, F), the variability can also be derived from the particular Salmonella strain examined.

In many instances, disagreements between laboratories occurred because the chemicals were coded. These disagreements may be relatively subtle and the result of low levels of activity in one laboratory versus questionable or no activity in the other. Many of these differences might have disappeared if the laboratory with the lower or negative response knew of the other data and adjusted the protocol accordingly. Other chemicals exist that are clearly positive in one laboratory and negative in another; the reasons for these differences are not obvious and would have to be investigated on an individual chemical basis.

A large number of chemicals were positive in TA100 but not in TA1535; a few were positive in TA1535 but not TA100. The chemicals that were positive in both strains usually showed two types of responses. The first type is best exemplified by 1-aziridine ethanol (Appendix 2, Table 22; Fig. 23). In this response, the mutagen induces approximately the same absolute numbers of revertants in both TA1535 and TA100; the only difference is that in TA100, the revertants appear against the higher background reversion frequency. Chemicals of this type which induce only low levels of revertants may be detected more readily using TA1535, and may be considered negative in TA100 if the number of induced revertants falls within or close to the range of spontaneous revertants; for example, ethylenediamine (Appendix 2, Table 123; Fig. 13) and N-nitrosopiperidine (Appendix 2, Table 185; Fig. 7). The other

type of response is the one in which mutagenicity is observed only in TA100, such as with chloropicrin (Appendix 2, Table 63; Fig. 9), dimethoate (Appendix 2, Table 107; Fig. 11), chloronitrobenzenes (Appendix 2, Tables 53–55; Figs. 8, 10) and others, or a higher number of revertants is induced in TA100 than in TA1535 (1,2,3,-trichloropropane [Appendix 2, Table 242; Fig. 17] and 2-aminoanthracene [Appendix 2, Table 10; Fig. 2]). These responses are not unexpected and reflect the mechanisms of action of the mutagens and the degree to which the error-prone repair system coded for by the pKM101 plasmid recognizes the DNA adduct produced.

Limitations

In any study using coded chemicals with fixed protocols, one would expect to obtain negative results for chemicals that have been reported elsewhere as mutagenic. This is because, with a coded chemical, the testing laboratory does not have any preconceptions about the "expected" responses based upon knowledge of the chemical's structure or responses in other biological systems. Having this knowledge affords the researcher the ability to test at varying dose ranges with different levels and types of S-9 and in varied protocols if the anticipated result is not obtained in the standardized protocol. In a study of the type reported here,the testing laboratory does not have the luxury of knowing the "expected" response; therefore, some of the chemicals that are reported here as nonmutagenic may have been reported as positive in the literature.

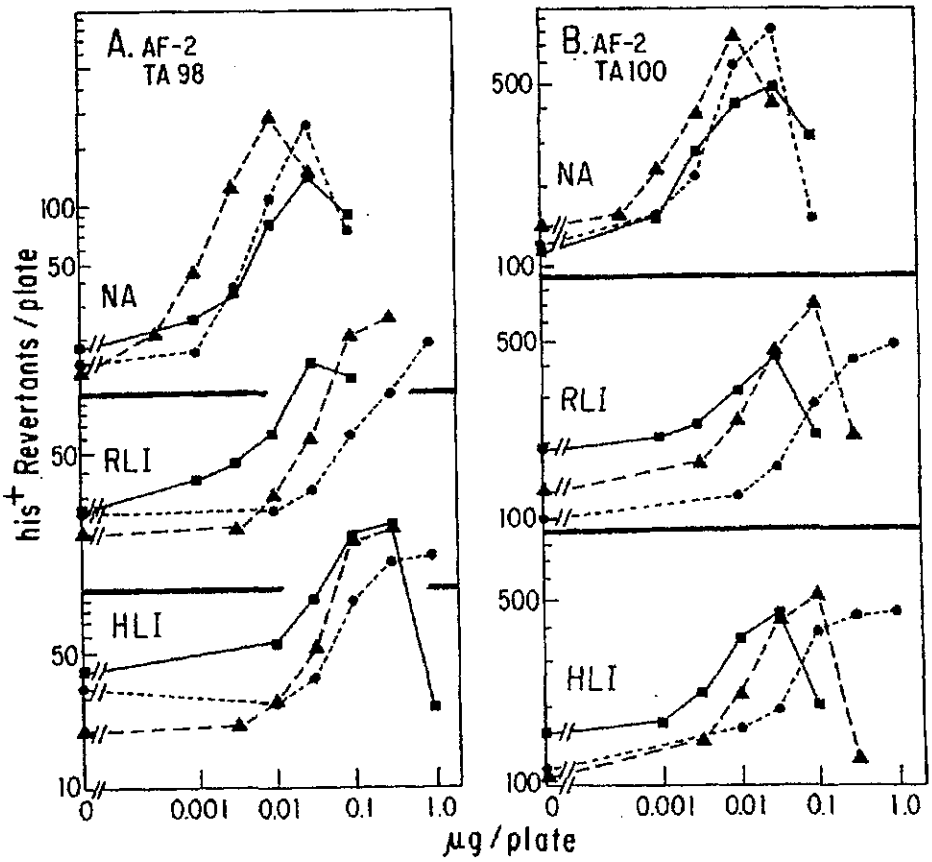


Fig. 1. Mutagenicity of AF-2 in S typhimurium TA98 (A) and TA100 (B) using no metabolic activation (NA), RLI, and HLI at CWR (■), EGG (▲), and SRI (●).

APPENDIX 2

Mutagenic responses of Salmonella tester strains TA100, TA1535, TA1537, and TA98 (mean ± SEM) to test chemicals. Chemicals are numbered as in Table I (pp. 6–17). Doses are in μg/plate; 0.0 dose is the solvent control.

Where only one test is reported for one chemical/laboratory combination (Aliquot), only the final test data are presented. If more than one Aliquot of the chemical was tested, the different aliquots are designated .1,.2, etc. Where more than one test from the same aliquot is reported, they are labeled with lower case letters, eg 1a . . . where a is the earliest data set.

Abbreviations are as follows: DMSO, Dimethyl sulfoxide (solvent); H_2O, Distilled water (solvent); 95% ETOH, 95% Ethanol (solvent); POS, Positive control (see Table II); CWR, Case Western Reserve University; EGG, EG&G Mason Research Corporation; SRI, SRI International; NA, not activated; RLI, rat liver S-9, Aroclor 1254 induced; HLI, hamster liver S-9, Aroclor 1254 induced; s, slight clearing of background lawn; t, complete clearing of background lawn; p, precipitate present in plates.

TABLE 1 ACETAMIDE [H2O] [EGG]

Dose	TA100 NA	TA100 RLI	TA100 HLI	TA1535 NA	TA1535 RLI	TA1535 HLI	TA1537 NA	TA1537 RLI	TA1537 HLI	TA98 NA	TA98 RLI	TA98 HLI
0.0	105± 1.8	123± 10.3	96± 5.7	17± 1.3	9± 2.1	11± 1.0	6± .9	8± 1.8	8± 2.5	20± 1.9	19± 3.5	18± 2.2
100.0	89± 5.0	135± 6.3	100± 4.3	17± 1.8	7± 1.5	12± 2.7	12± 3.8	4± .7	7± 2.0	19± 3.5	28± 1.3	25± 2.1
333.0	110± 16.1	100± 9.8	102± 2.7	17± 2.0	7± .6	8± 2.1	7± .9	8± 1.5	4± 1.2	22± .7	24± 2.2	20± 4.3
1000.0	96± 5.8	107± 8.2	105± 10.0	19± 1.7	10± 2.5	10± 1.5	6± 3.1	8± 2.8	8± 1.0	16± 2.3	27± 3.6	21± 2.0
3333.0	99± 8.9	98± 7.9	108± 8.5	21± 3.0	10± 1.3	10± 3.5	6± .3	9± 2.3	5± 1.9	23± 4.1	25± 4.3	19± 1.5
10000.0	102± 11.0	88± 5.4	93± 3.2	17± 2.6	9± 1.5	10± 1.5	9± 1.0	11± 2.7	5± 1.3	17± 1.7	27± 2.3	21± 3.1
POS	1432± 85.3	690± 82.6	1406± 64.9	1235± 75.6	50± 1.2	101± 2.3	174± 15.6	88± 4.1	154± 8.7	1605±193.3	402± 45.9	1043± 52.8

TABLE 2 ACETIN [H2O] [SRI]

Dose	TA100 NA	TA100 RLI	TA100 HLI	TA1535 NA	TA1535 RLI	TA1535 HLI	TA1537 NA	TA1537 RLI	TA1537 HLI	TA98 NA	TA98 RLI	TA98 HLI
0.0	97± 3.0	97± 3.5	108± 2.3	20± 3.1	10± 1.2	11± 3.1	5± .9	7± .7	6± .6	22± 3.2	35± 2.3	37± 3.4
100.0	82± 13.4	111± 1.8	116± 3.5				2± .3	6± .7	4± 1.0	31± 5.0	29± 5.3	48± 2.6
333.3	86± 7.2	98± 3.2	99± 7.8	17± 1.2	15± 3.2	22± 4.8	4± 1.2	6± 1.5	6± 1.3	24± 3.3	31± 1.2	35± 1.9
1000.0	89± 8.2	111± 10.3	119± 4.5	25± 2.0	20± 2.6	30± 1.5	3± .3	4± .6	7± 1.7	24± 2.7	31± 1.5	35± 3.3
3333.3	96± 5.3	97± 8.4	138± 10.5	42± .3	58± 3.5	64± 5.0	4± .6	5± .6	4± .9	25± 2.3	32± 5.7	25± 3.0
6666.7				79± 2.9	84± 6.0	117± 3.2						
10000.0	104± 6.1	133± 4.4	139± 9.8	106± 2.4	123± .3	149± 4.7	3± 1.2	4± 0.0	3± 0.0	20± 4.0	35± 2.1	31± 6.2
POS	268± 16.6	1492± 61.4	2368± 57.7	352± 17.7	211± 12.4	112± 14.0	278± 85.5	494± 38.2	522± 52.2	550± 97.1	1033± 57.8	1889±123.0

TABLE 3 P-ACETOPHENETIDIDE [DMSO] [CWR]

Dose	TA100 NA	TA100 RLI	TA100 HLI	TA1535 NA	TA1535 RLI	TA1535 HLI	TA1537 NA	TA1537 RLI	TA1537 HLI	TA98 NA	TA98 RLI	TA98 HLI
0.0	97± 6.8	121± 6.6	122± 11.6	6± .9	6± 2.1	6± 3.0	2± 1.2	6± 0.0	6± 1.2	15± 1.9	22± 1.2	19± .6
33.0	96± 3.8	121± 4.2	107± 6.9	3± .3	7± 1.2	8± 1.3	3± .7	5± .3	5± 1.2	9± 1.9	21± 3.3	20± 2.1
100.0	94± 6.8	103± 5.6	116± 9.3	3± 1.2	6± 1.2	7± .9	2± .9	5± 1.0	8± 1.5	14± 1.3	18± 2.4	21± 2.3
333.0	90± 2.6	110± 7.8	109± 4.8	4± .3	4± .9	7± .7	3± 1.2	5± 1.7	7± 1.5	18± 1.5	15± 4.3	17± 4.6
1000.0	87± 7.4	119± 2.7	124± 3.7	3± 1.3	4± .9	5± .9	3± .6	4± .3	7± 1.2	13± 4.3	18± 1.5	19± 4.7
2730.0	90± 5.9	105± 4.3	123± 11.6	3± .6	3± .9	7± .6	2± .6	8± 1.2	5± .6	15± 1.0	23± 2.4	25± 2.9
POS	859± 23.9	3057±178.4	1818±296.3	212± 33.8	38± 2.1	30± 3.7	196± 32.0	109± 2.3	107± 1.8	278± 25.4	626±139.2	984±100.2

TABLE 235.2 TRIBUTYL BORATE [ACETONE] [EGG]

Dose	TA100 NA	TA100 RLI	TA100 MLI	TA1535 NA	TA1535 RLI	TA1535 MLI	TA1537 NA	TA1537 RLI	TA1537 MLI	TA98 NA	TA98 RLI	TA98 MLI
0.0	111± 10.2	101± 3.7	99± 4.4	15± 2.3	9± 1.5	9± .9	7± 2.1	4± .7	6± 2.0	20± 5.2	17± .3	21± 1.5
77.0	118± 5.0	95± 4.2	102± .3	13± 1.7	9± 2.9	8± .6	6± 1.5	5± 1.3	6± 2.6	19± 2.4	15± 1.0	20± 2.9
256.7	106± 6.4	109± 7.5	97± 12.9	14± 2.1	7± .6	7± 1.2	5± .9	6± .3	4± 1.5	28± 3.3	18± 2.1	19± 2.4
770.0	112± 8.1	123± 4.4	114± 5.3	12± .9	7± .9	10± 1.5	4± .7	5± 1.8	5± .6	19± .6	19± 3.0	21± 1.2
2566.7	99± 9.8	106± 5.4	119± 1.8	10± 1.0	12± 2.6	7± .9	5± 2.0	7± 1.0	8± 1.5	15± 1.8	18± 3.3	23± 3.2
7700.0	81± 6.7	80± 5.9	103± 2.7	4± .9	4± .3	4± .3	3± .5	6± 1.3	t	7± 2.0	11± 2.0	11± 3.6a
POS	1338± 50.7	1862± 63.3	2536± 66.2	817± 19.0	149± 7.5	199± 6.9	393± 85.4	139± 10.2	81± 4.4	2515±139.9	1618± 62.3	2421± 74.8

TABLE 236 1,2,3-TRICHLOROBENZENE [DMSO] [SRI]

Dose	TA100 NA	TA100 RLI	TA100 MLI	TA1535 NA	TA1535 RLI	TA1535 MLI	TA1537 NA	TA1537 RLI	TA1537 MLI	TA98 NA	TA98 RLI	TA98 MLI
0.0	124± 9.9	138± 7.0	131± 7.3	25± 6.0	12± 2.9	14± .9	11± 2.7	20± .3	26± 3.5	25± 2.4	22± 5.0	35± 3.4
3.3	122± 13.2	122± 9.7	114± 4.1	17± 1.7	10± 2.2	16± 2.0	8± 2.1	15± 3.2	18± 3.0	20± 3.0	26± 2.2	32± 1.0
10.0	134± 14.0	131± 7.5	122± 7.2	20± 4.6	14± .3	13± .3	12± 1.3	14± 3.5	22± 3.0	16± 4.8	24± 1.7	31± 3.2
33.3	91± 3.8	131± 11.6	135± 10.6	17± 2.3	14± 2.9	16± 1.5	6± 1.3	13± 3.8	18± 1.5	20± .7	23± 3.7	32± 5.1
100.0	11± 6.9a	91± 6.4	124± 10.2	0± 0.0a	8± 1.5	14± 3.8	0± 0.0a	11± 1.8	21± 3.3	10± 2.9a	27± .6	31± 2.0
333.3	t	t	80± 2.8a	t	4± 2.2a	6± .7a	t	0± 0.0a	6± 1.6a	t	6± 3.2a	18± 6.2a
POS	550± 8.1	596± 29.5	556± 9.5	438± 5.3	334± 51.3	367± 6.2	163± 24.0	287± 3.6	467± 13.0	478± 16.3	388± 25.1	934± 19.9

TABLE 237 1,2,4-TRICHLOROBENZENE [DMSO] [SRI]

Dose	TA100 NA	TA100 RLI	TA100 MLI	TA1535 NA	TA1535 RLI	TA1535 MLI	TA1537 NA	TA1537 RLI	TA1537 MLI	TA98 NA	TA98 RLI	TA98 MLI
0.0	99± 5.2	113± 2.0	115± 8.7	20± 1.0	13± 3.0	13± .6	11± 1.5	14± 1.9	12± 2.7	37± 3.5	35± 2.3	37± 4.7
3.3	104± 4.0	107± 5.5	124± 2.0	16± 1.5	15± 2.5	13± 2.9	18± 3.8	10± 1.5	18± 5.2	38± 6.4	34± 2.3	31± .9
10.0	86± 6.7	115± 9.5	132± 5.8	18± 4.2	9± 0.0	10± 2.7	23± 2.7	11± 1.2	23± 3.7	28± 1.2	36± 4.8	35± 2.7
33.3	61± 10.1a	123± 2.2	124± 7.3	19± 1.2	13± 3.2	11± 1.7	11± 1.7	9± .3	17± .7	28± 1.7	29± .9	33± 2.3
100.0	t	112± 6.7	100± 6.9	6± 6.0a	9± 1.6	12± 2.7	9± 1.8	6± 1.7	17± 4.0	24± 1.3	34± 4.1	36± 6.4
333.3	t	114± 9.4	21± 21.5a	t	13± .7a	6± 1.5a	5± 2.3	7± 1.2	0± 0.0a	17± 2.5	34± 3.7	9± 8.5a
POS	625± 26.8	860± 15.3	963±104.3	448± 23.0	305± 3.2	268± 34.8	308± 8.0	158± 10.6	244± 5.8	850± 18.0	288± 3.0	1256±130.5

TABLE 238 1,3,5-TRICHLOROBENZENE [DMSO] [SRI]

Dose	TA100 NA	TA100 RLI	TA100 MLI	TA1535 NA	TA1535 RLI	TA1535 MLI	TA1537 NA	TA1537 RLI	TA1537 MLI	TA98 NA	TA98 RLI	TA98 MLI
0.0	124± 15.0	176± 1.2	108± 11.7	15± 2.4	18± 1.3	15± 1.7	24± 2.3	35± 5.2	26± 4.8	25± 4.8	43± 1.2	37± 5.8
33.3	99± 12.5	157± 1.8	126± 8.5	18± 3.2a	13± 2.0	8± .6	10± 1.2	30± 1.2	25± 3.5	18± .9	41± 1.2	36± 6.9
100.0	59± 1.8a	152± 2.8	117± 12.8	3± 1.3a	11± 2.0	9± 2.9	3± .6a	21± 3.3	25± 4.7	13± .6a	33± 4.8	39± 1.8
333.3	5± 5.3a	131± 2.3	101± 11.9	t	11± 2.4a	16± 1.5	0± 0.0a	10± 2.3a	13± 1.0	3± .3a	29± 2.0	31± 3.3
1000.0	28± 6.9a	115± 7.3a	81± 11.6a	0± 0.0a	7± 1.5a	13± 3.0	0± 0.0a	9± 1.7a	12± 1.2a	6± 1.3a	27± 4.5a	19± 1.5a
3333.3	62± 22.4a	121± 7.0a	70± 5.0a	6± 1.8a	6± 1.0a	11± 1.7	0± 0.0a	10± 1.3a	5± .6a	13± .3a	25± 4.3a	18± 1.0a
POS	617± 21.7	621± 5.1	442± 16.7	515± 8.9	197± 2.8	303± 4.8	136± 26.0	284± 7.2	322± 36.7	706± 14.6	534± 68.7	874± 31.9

TABLE 239.1 1,1,1-TRICHLOROETHANE (CHLOROTHENE) [DMSO] [CWR]

Dose	TA100 NA	TA100 RLI	TA100 MLI	TA1535 NA	TA1535 RLI	TA1535 MLI	TA1537 NA	TA1537 RLI	TA1537 MLI	TA98 NA	TA98 RLI	TA98 MLI
0.0	199± 8.8	240± 16.3	271± 5.9	6± .3	6± 1.3	7± 2.0	5± 1.2	13± 1.5	8± .9	22± 1.5	29± 1.2	23± 3.2
33.0												27± 2.2
100.0	176± 13.4	284± 6.6	323± 9.1	4± .7	7± 1.3	7± 1.9	8± .7	11± 2.7	7± 1.9	21± 1.8	28± .7	26± 4.7
333.0	193± 6.1	292± 15.6	335± 23.0	5± .3	7± 0.0	7± 1.2	6± .7	12± 2.7	9± 1.5	24± 3.6	31± 1.9	29± .9
1000.0	204± 15.2	290± 11.1	300± 12.9	4± .7	8± 1.3	7± 1.5	4± .9	11± 4.2	9± .6	20± 1.0	35± 2.4	28± 3.3
3333.0	203± 33.3	287± 15.9	292± 5.9	3± 0.0	5± 1.2	5± 1.6	5± .6	3± .3	4± .9	24± 1.3	26± 4.9	30± 1.8
10000.0	180± 13.4	293± 18.9	165± 30.4	1± 0.0	3± .6	4± 2.5	4± 0.0	4± 2.0	4± .9	22± 2.4	6± 3.3	
POS	867± 63.7	1126± 47.1	1905± 56.4	936± 79.2	99± 12.2	116± 16.2	63± 9.3	120± 1.2	163± 9.5	464± 49.0	645± 12.6	1946±183.3

1,2,4-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 1,2,4-Trimethylbenzene in Rats

要約

1,2,4-トリメチルベンゼンは，トリメリット酸，ビタミンEなどの合成用，染料，顔料，医薬品の中間体，メチル化してデュレンを経てピロメリット酸の合成原料として広く使用されている．当物質をラットに5 ml/kg経口投与した場合には3/10例死亡するとの報告[1]があるが，反復投与毒性に関する報告は皆無に等しい．今回，既存化学物質の毒性を評価するために，当物質を雌雄ラットに1日1回，28日間反復経口投与し，その毒性について検討した．一部の動物については，14日間の回復期間を設けた．投与量は，1000 mg/kgを最高用量とし，以下公比約3により300，100および30 mg/kgとした．なお，対照として媒体(コーンオイル)投与群を設けた．

死亡は，いずれの群にも発現しなかった．一般状態観察において，300 mg/kg以上の群の雌雄で投与直後に流涎がみられた．

体重は，1000 mg/kg群の雌雄で増加抑制がみられた．回復期間中には，1000 mg/kg群の雌雄とも体重は低値で推移した．

摂餌量は，各投与群の雌雄とも対照群とほぼ同様に推移した．

摂水量は，300 mg/kg群の雄と1000 mg/kg群の雌雄で増加がみられた．回復期間中には，1000 mg/kg群の雌雄とも摂水量は高値で推移した．

尿検査において，1000 mg/kg群の雌雄で尿量の高値がみられた．この変動は，回復期間終了前には消失した．

血液学検査において，各投与群の雌雄とも各検査項目に投与による変動はみられなかった．

血液生化学検査において，各投与群の雌雄とも各検査項目に投与による変動はみられなかった．

剖検では，いずれの群の雌雄とも異常はみられなかった．

器官重量において，雄では300 mg/kg以上の群で腎臓の相対重量の高値，1000 mg/kg群で肝臓の相対重量の高値および腎臓の絶対重量の高値がみられた．雌では，300 mg/kg以上の群で肝臓の絶対・相対重量の高値，1000 mg/kg群で腎臓の相対重量の高値がみられた．これらの変動は，回復期間終了時には消失した．

病理組織学的検査において，雄では300 mg/kg以上の群で腎臓に尿細管の硝子滴変性がみられた．この変化は，回復期間終了時には消失した．雌では，投与による変化はみられなかった．

以上のことから，当試験条件下における1,2,4-トリメチルベンゼンの28日間反復経口投与による毒性学的無影響量は，雌雄とも100 mg/kg/dayと考えられる．

方法

1. 被験物質，媒体および投与検体

被験物質の1,2,4-トリメチルベンゼン(CAS No.95-63-6)は，分子量：120.20，融点：-43.9℃，沸点：169.4℃，比重：0.88で水に難溶，有機溶媒に易溶の無色透明の液体である(Lot No.H5-CH-11，製造元：東洋合成工業㈱，純度：98.75％)．入手後は，室温・遮光下で気密容器に入れて保管した．投与期間終了後に被験物質の一部を製造元に送付して分析した結果，純度は98.64％であり，使用期間中の安定性が確認された．

被験物質は秤取し，コーンオイルに溶解して必要濃度の投与検体を調製した．なお，0.6，2および20％濃度の調製液は，室温・遮光・気密条件下で7日間保存しても安定性に問題のないことが確認されていたため，各濃度の調製液は調製後，室温・遮光・気密条件下で保管し，調製後7日以内に使用した．また，被験物質は純度換算しないで，投与量は原体重量で表示した．

2. 使用動物および飼育条件

Sprague-Dawley系ラット［Crj:CD(SD)，(SPF)］を雄は4週齢，雌は3週齢で日本チャールス・リバー㈱から購入した．入手した動物は，5日間の検疫期間およびその後，雄は7日間，雌は14日間の馴化期間を設け，一般状態および体重推移に異常の認められない6週齢の動物を群分けして試験に用いた．群分けは，コンピュータを用いて体重を層別に分けた後に，無作為抽出法により各群の平均体重および分散がほぼ等しくなるように投与開始の前日に行った．1群の動物数は，雌雄各10あるいは15匹とした．

動物は，室温20～24℃，湿度40～70％，明暗各12時間(照明：午前6時～午後6時)，換気回数12回／時に設定した飼育室で飼育した．検疫・馴化期間中はステンレス製懸垂式ケージを用いて1ケージあたり5匹までの群飼育とし，群分け後はステンレス製五連ケージを用いて個別飼育した．

飼料は固型飼料(CRF-1，オリエンタル酵母工業㈱)を，飲料水は水道水をいずれも自由に摂取させた．なお，剖検前日の午後4時からは絶食とした．

3. 投与経路，投与方法，群構成および投与量

1,2,4-トリメチルベンゼンは，継続して経口的に人に摂取される可能性が考えられるため，投与経路として経口投与を選択した．投与に際しては，金属製経口胃ゾンデを取り付けたプラスチック製ディスポーザブル注射筒を用いて，強制経口投与した．投与液量は，投与日あるいは投与日に最も近い測定日の体重を基準とし，5 ml/kgで算出した．

投与期間は，1日1回で28日間反復投与とした．また，28日間の投与後に一部の動物について14日間の回復期間を設けた．なお，投与開始日を投与1日とし，最終投与日の翌日を回復1日とした．投与開始日の週齢は6週齢であり，体重範囲は雄が154～180 g，雌が133～165 gであった．

投与量は，先に実施した雄ラットを用いた2週間投与による予備試験(投与段階：0，62.5，125，250，500および1000 mg/kg，各群5例)の結果により決定した．すなわち，125 mg/kg以上の群で投与直後に流涎がみられたのみで，各群とも死亡発現はなく，体重推移および剖検でも異常はみられなかった．そこで，当試験の投与量は，1000 mg/kgを最高用量とし，以下公比約3で300，100および30 mg/kgとした．また，対照として被験物質と同一液量の媒体(コーンオイル)を投与する群を設けた．

1群の動物数は，対照群および1000 mg/kg群では投与期間終了時剖検例雌雄各10匹と回復期間終了時剖検例雌雄各5匹の雌雄各15匹とした．また，30，100および300 mg/kg群では，投与期間終了時剖検例雌雄各10匹とした．

4. 観察および検査項目

1) 一般状態

一般状態および死亡の有無は，投与期間中には投与前・後の1日2回ならびに回復期間中には毎日1回観察した．

2) 体重測定

体重は，投与期間中および回復期間中とも1週間に2回測定した．

3) 摂餌量測定

摂餌量は，投与期間中および回復期間中ともに1週間に1回測定した．

4) 摂水量測定

摂餌量測定と同様にして摂水量を測定した．

5) 尿検査

投与期間終了前に投与期間終了時の剖検用動物を，回復期間終了前に回復期間終了時の剖検用動物について実施した．すなわち，採尿ケージを用いて絶食・給水下で3時間で採取した尿(3時間尿)と引き続いて給餌・給水下で21時間で採取した尿(21時間尿)ならびにそれらを合計した尿(24時間尿)について，以下の検査を実施した．

3時間尿：色調は，外観判定とした．pH，蛋白，糖，ケトン体，ビリルビン，潜血，ウロビリノーゲンは，エームスクリニテック用検査紙(マイルス・三共㈱)に尿を滴下後にエームス尿分析器(クリニテック200，マイルス・三共㈱)を用いて検査した．尿沈渣は，沈渣を尿沈渣染色液で染色後に顕微鏡下で観察した．なお，投与期間中の採尿は，当日の検体投与後に行った．

21時間尿：比重を屈折率により屈折型尿比重計(ユリペット・ⅡD，㈱ニコン)を用いて測定した．

24時間尿：尿量を比重と重量から算出した．

6) 血液学検査

最終投与の翌日および回復期間終了後に，ペントバルビタールナトリウムの腹腔内投与による麻酔下で腹大動脈から血液を採取し，以下の検査を実施した．

赤血球数(RBC)，ヘモグロビン量，ヘマトクリット値，血小板数および白血球数(WBC)は，EDTA-2Kコーティングしたシスメックスサンプルカップに採取した血液について，多項目自動血球計数装置(Sysmex E-2000，東亜医用電子㈱)を用いて測定した．さらに，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．

網状赤血球数は，EDTA-2K処理した血液をBrecher法により超生体染色してスライドグラスに塗抹後，Giemsa染色した標本を作製して顕微鏡下で赤血球1000個中の数を計数した．

白血球百分率は，EDTA-2K処理した血液をスライドグラスに塗抹し，May-Giemsa染色した標本を作製して顕微鏡下で白血球100個を分類計数した．

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)は，3.13％クエン酸ナトリウムで処理した血漿について，散乱光検出方式により血液凝固分析装置(コアグマスターⅡ，三共㈱)を用いて測定した．

7) 血液生化学検査

血液学的検査用の血液と同時期に腹大動脈から採取した血液を遠心分離し，得られた血清について，以下の検査を実施した．

GOTおよびGPTはHenry変法，ALPは*p*-NPP基質法，γ-GTPはγ-G-P-NA基質法，総蛋白はBiuret法，総ビリルビンはAzobilirubin法，尿素窒素(BUN)はUrease・GlDH法，クレアチニンはJaffé法，ブドウ糖はGlucose dehydrogenase法，総コレステロールはCOD・DAOS法，トリグリセライドはGPO・DAOS法，Caは*o*-CPC法，無機リンはMolybdenum blue法により，自動分析装置(AU 500，オリンパス光学工業㈱)を用いて測定した．

NaおよびKはイオン選択電極法により，Clは電量滴定法により，いずれも全自動電解質分析装置(EA04，㈱A＆T)を用いて測定した．

蛋白分画は，電気泳動法により自動電気泳動装置(AES 600，オリンパス光学工業㈱)を用いて測定した．

アルブミン量は総蛋白量および蛋白分画値から，A/G

比は蛋白分画値から算出した.

8) 剖検

上記の6)および7)の項で採血した動物をさらに放血致死させた後に器官・組織の肉眼的観察を行った.

9) 器官重量の測定

剖検時に以下の器官重量を測定した.さらに,剖検前に測定した体重を基準として器官重量の体重比(相対重量)を算出した.

脳(大脳,小脳,延髄),胸腺,心臓,肝臓,脾臓,腎臓,副腎,精巣および精巣上体または卵巣.

10) 病理組織学的検査

以下の器官または組織を摘出して10%中性緩衝ホルマリン液(ただし,眼球はグルタールアルデヒド・ホルマリン液)で固定し,全例について常法に従ってパラフィン包埋標本を作製した.

心臓,肺,肝臓,胃,脾臓,胸腺,腎臓,膀胱,精巣,精巣上体,卵巣,下垂体,副腎,甲状腺(上皮小体を含む),脳(大脳,小脳,延髄),眼球,骨髄(大腿骨).

投与期間終了時剖検例の対照群および1000 mg/kg群の心臓,肝臓,脾臓,腎臓および副腎についてH-E染色組織標本を作製し,病理組織学的に検査した.さらに,投与期間終了時の1000 mg/kg群の検査で対照群と比べて異常を示す動物数に差があると考えられた肝臓は30,100および300 mg/kg群ならびに回復期間終了時の対照群および1000 mg/kg群の雌雄について,腎臓は30,100および300 mg/kg群ならびに回復期間終了時の対照群および1000 mg/kg群の雄について同様に検査した.

5. 統計解析

体重,摂餌量,摂水量,尿量,尿比重,血液学検査,血液生化学検査,器官重量(相対重量を含む)については,各群で平均値および標準偏差を算出した.有意差検定は対照群と各投与群との間で多重比較検定を用いて行い,危険率5%未満を有意とした.すなわち,Bartlett法による等分散性の検定を行い,等分散ならば一元配置法による分散分析[2]を行い,有意ならばDunnett法[3]またはScheffé法[4]を用いて行った.一方,等分散と認められなかった場合は,順位を利用した一元配置法による分析(Kruskal-Wallisの検定[5])を行い,有意ならば順位を利用したDunnett法またはScheffé法を用いて行った.なお,病理組織学的検査において,1000 mg/kg群で毒性学的影響が示唆された器官・組織については,対照群との群間比較を上記の順位を利用したDunnett法またはScheffé法を用いて行った.さらに対照群との間に有意差が認められた所見については,Cochran・Armitageの傾向検定を用いて用量反応性を確認した.

結果

1. 一般状態

投与期間中には,対照群および100 mg/kg以下の群の雌雄とも異常症状はみられなかった.300 mg/kg以上の群の雌雄では,投与後に流涎がみられた.流涎は,300 mg/kg群の雄で投与7日,雌で投与20日,1000 mg/kg群の雄で投与2日,雌で投与3日からみられ,300 mg/kg群の雌雄では少数例～約半数例,1000 mg/kg群の雌雄では全例に認められた.

回復期間中には,対照群および1000 mg/kg群の雌雄とも異常症状はみられなかった.

2.体重推移(Fig.1)

投与期間中には,300 mg/kg以下の群の雌雄は対照群とほぼ同様の体重推移であり,いずれの測定日とも有意差はみられなかった.1000 mg/kg群の雄では,対照群と比べて投与4日頃から体重増加抑制傾向がみられ,投

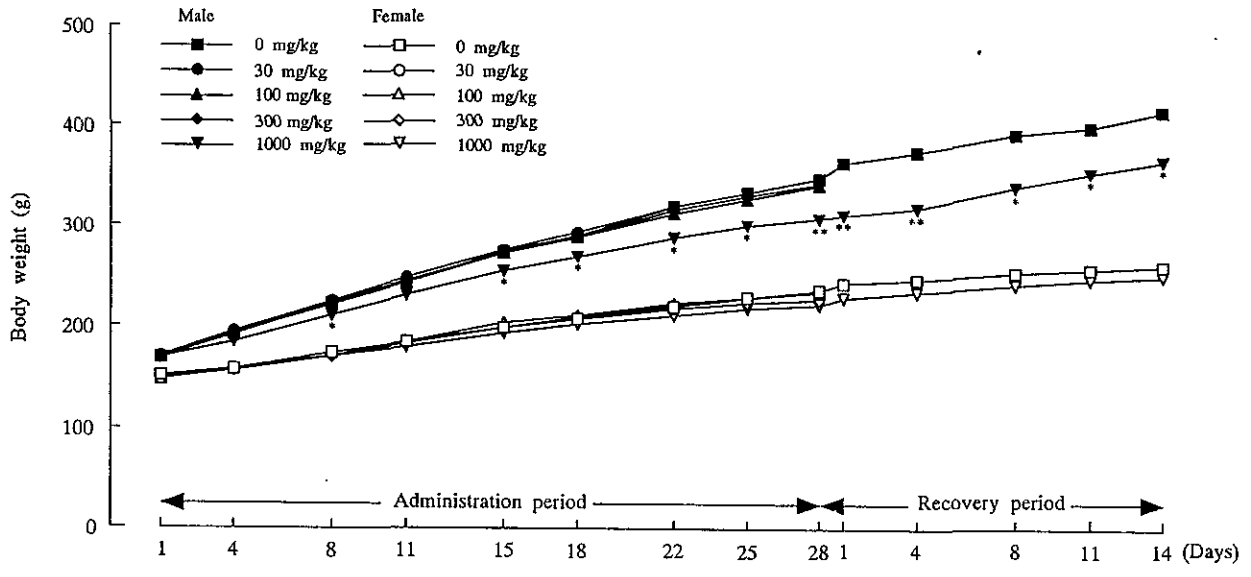


Fig. 1 Body weight of male and female rats in 28-day repeat dose oral toxicity test of 1,2,4-trimethylbenzene
Significantly different from control (*: $p<0.05$, **: $p<0.01$).

与8日と投与15日から最終投与日まで体重の有意な低値が認められた．1000 mg/kg群の雌では，対照群と比べて有意な差ではないが，投与25日頃から最終投与日まで体重増加抑制傾向がみられた．

回復期間中には，1000 mg/kg群の雄では対照群と比べて回復1日から回復14日まで体重の有意な低値がみられた．1000 mg/kg群の雌では，対照群と比べて有意な差ではないが，回復期間を通して体重の低値傾向がみられた．

3. 摂餌量

投与期間中には，各投与群の雌雄とも対照群とほぼ同様の摂餌量であり，いずれの測定日とも有意差はみられなかった．

回復期間中には，1000 mg/kg群の雌雄では対照群とほぼ同様の摂餌量であり，いずれの測定日とも有意差はみられなかった．

4. 摂水量

投与期間中には，100 mg/kg以下の群の雄および300 mg/kg以下の群の雌では対照群とほぼ同様の摂水量であり，いずれの測定日とも有意差はみられなかった．300 mg/kg群の雄では，対照群と比べて投与17および24日に摂水量の有意な高値がみられた．1000 mg/kg群の雄では，対照群と比べて投与10日から投与24日まで摂水量の有意な高値がみられた．1000 mg/kg群の雌では，対照群と比べて投与3，17および24日に摂水量の有意な高値が認められた．

回復期間中には，1000 mg/kg群の雄では回復3日に摂水量の高値傾向がみられた．1000 mg/kg群の雌では，対照群と比べて回復3および10日に摂水量の有意な高値が認められた．

5. 尿検査

投与期間終了前には，300 mg/kg以下の群の雌雄では対照群と比べて尿量および比重に有意差はみられなかった．1000 mg/kg群の雌雄では，対照群と比べて尿量の有意な高値がみられた．なお，色調，pH，蛋白，糖，ケトン体，ビリルビン，潜血，ウロビリノーゲンおよび沈渣は，各投与群の雌雄とも対照群とほぼ同程度であった．

回復期間終了前には，尿量および尿比重は1000 mg/kg群の雌雄とも対照群と比べて有意差はみられなかった．また，色調，pH，蛋白，糖，ケトン体，ビリルビン，潜血，ウロビリノーゲンおよび沈渣は，1000 mg/kg群の雌雄とも対照群とほぼ同程度であった．

6. 血液学検査(Table 1，2)

投与期間終了時には，100 mg/kg群の雄で対照群と比べて好酸球率の有意な高値がみられたが，投与量に依存した変化ではなかった．なお，300 mg/kg以下の群の雌雄では，対照群と比べてその他の測定項目に有意差はみられなかった．1000 mg/kg群の雄では，対照群と比べてヘモグロビン量の有意な高値がみられた．1000 mg/kg群の雌では，対照群と比べて血小板数の有意な低値がみられた．

回復期間終了時には，1000 mg/kg群の雄では対照群と比べていずれの測定項目とも有意差はみられなかった．1000 mg/kg群の雌では，対照群と比べて活性化部分トロンボプラスチン時間の短縮がみられた．

7. 血液生化学検査(Table 3，4)

投与期間終了時には，30 mg/kg群の雄および100 mg/kg以下の群の雌では対照群と比べていずれの測定項目とも有意差はみられなかった．300 mg/kg以上の群の雄では対照群と比べてアルブミン率，A/G比およびKの有意な高値，α_1-グロブリン率の有意な低値，1000 mg/kg群の雄では総ビリルビンおよびNaの有意な低値がみられた．その他に100 mg/kg群の雄では，対照群と比べてGOTの有意な低値がみられたが，投与量に依存した変化ではなかった．また，300 mg/kg群の雄では，対照群と比べて総蛋白およびブドウ糖の有意な低値がみられたが，投与量に依存した変化ではなかった．300 mg/kg以上の群の雌では対照群と比べて総コレステロールの有意な高値，1000 mg/kg群の雌ではGPTおよびα_2-グロブリン率の有意な高値，α_1-グロブリン率，α_3-グロブリン率および総ビリルビンの有意な低値がみられた．

回復期間終了時には，1000 mg/kg群の雄では対照群と比べていずれの測定項目とも有意差はみられなかった．1000 mg/kg群の雌では，対照群と比べて尿素窒素の有意な高値がみられた．

8. 剖検

投与期間終了時および回復期間終了時には，いずれの群の雌雄とも異常はみられなかった．

9. 器官重量(Table 5，6)

投与期間終了時には，100 mg/kg以下の群の雌雄では対照群と比べていずれの器官重量とも有意差はみられなかった．300 mg/kg以上の群の雄では対照群に比べて腎臓の相対重量の有意な高値，1000 mg/kg群の雄では肝臓の相対重量の有意な高値，腎臓の絶対重量の有意な高値，有意差はないが肝臓の絶対重量の高値傾向がみられた．その他には，1000 mg/kg群の雄で対照群と比べて脾臓の絶対重量の有意な低値，脳，精巣および精巣上体の相対重量の有意な高値がみられたが，絶対重量と相対重量で一定方向の変動ではないもの，もしくは軽度の差であるものであった．300 mg/kg以上の群の雌では，対照群に比べて肝臓の絶対・相対重量の有意な高値，1000 mg/kg群の雌では腎臓の相対重量の有意な高値，有意差はないが腎臓の絶対重量の高値傾向がみられた．その他には，1000 mg/kg群の雌で対照群と比べ脳の相対重量の有意な高値がみられたが，軽度な差であった．

回復期間終了時には，1000 mg/kg群の雄では対照群と比べて脳，脾臓および精巣の相対重量の有意な高値が

Table 1 Hematological examination of male rats in 28-day repeat dose oral toxicity test of 1, 2, 4-trimethylbenzene

Test period	Termination of administration period					Termination of recovery period	
Dose (mg/kg)	0	30	100	300	1000	0	1000
Number of males	10	10	10	10	10	5	5
RBC (10^4/mm^3)	784.5 ± 36.6	784.2 ± 33.6	778.5 ± 24.3	777.4 ± 29.3	799.4 ± 23.1	840.8 ± 30.2	845.8 ± 13.1
Hemoglobin (g/dl)	14.84 ± 0.54	14.90 ± 0.72	14.91 ± 0.30	14.85 ± 0.44	15.59 ± 0.46**	15.22 ± 0.28	15.20 ± 0.33
Hematocrit (%)	45.26 ± 2.14	45.17 ± 1.91	45.01 ± 0.83	45.30 ± 1.09	47.18 ± 1.44	45.24 ± 0.82	45.22 ± 1.27
MCV (μm^3)	57.72 ± 2.01	57.62 ± 1.23	57.85 ± 1.49	58.31 ± 1.83	59.04 ± 1.33	53.84 ± 1.14	53.48 ± 1.96
MCH (pg)	18.93 ± 0.58	19.02 ± 0.37	19.18 ± 0.36	19.12 ± 0.60	19.52 ± 0.58	18.14 ± 0.39	17.98 ± 0.36
MCHC (g/dl)	32.80 ± 0.65	32.99 ± 0.50	33.12 ± 0.42	32.79 ± 0.78	33.06 ± 0.48	33.64 ± 0.44	33.62 ± 0.79
Platelet (10^4/mm^3)	133.39 ± 14.75	132.32 ± 14.00	126.61 ± 15.08	120.83 ± 14.28	121.29 ± 8.96	120.44 ± 13.75	108.08 ± 4.51
Reticulocyte (‰)	27.3 ± 3.7	27.9 ± 5.4	26.0 ± 5.1	28.9 ± 6.7	26.5 ± 5.7	28.6 ± 1.5	27.4 ± 6.9
PT (sec.)	14.65 ± 1.54	16.20 ± 2.57	16.89 ± 1.91	14.80 ± 2.59	14.86 ± 2.08	14.40 ± 1.80	16.46 ± 1.63
APTT (sec.)	27.23 ± 2.24	28.58 ± 2.77	29.27 ± 2.46	27.60 ± 3.67	29.61 ± 2.72	24.88 ± 2.65	26.12 ± 1.17
WBC (10^2/mm^3)	56.0 ± 8.8	56.0 ± 14.7	50.6 ± 13.2	52.9 ± 10.0	60.4 ± 18.4	69.4 ± 14.7	84.4 ± 16.6
Differential leukocyte (%)							
Lymphocyte	92.3 ± 3.2	93.2 ± 2.8	92.6 ± 3.3	93.4 ± 2.8	92.7 ± 3.2	95.8 ± 1.1	94.6 ± 3.0
Neutrophil	7.3 ± 3.4	6.6 ± 2.7	6.3 ± 3.1	5.9 ± 2.9	6.6 ± 3.1	3.6 ± 1.1	5.0 ± 2.8
Eosinophil	0.1 ± 0.3	0.1 ± 0.3	0.6 ± 0.5*	0.1 ± 0.3	0.3 ± 0.5	0.4 ± 0.5	0.0 ± 0.0
Basophil	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
Monocyte	0.3 ± 0.5	0.1 ± 0.3	0.5 ± 0.7	0.6 ± 0.7	0.4 ± 0.5	0.2 ± 0.4	0.4 ± 0.5

Each value shows mean ± S.D.
Significantly different from control (*: $p<0.05$, **: $p<0.01$).

Table 2 Hematological examination of female rats in 28-day repeat dose oral toxicity test of 1, 2, 4-trimethylbenzene

Test period	Termination of administration period					Termination of recovery period	
Dose (mg/kg)	0	30	100	300	1000	0	1000
Number of females	10	10	10	10	10	5	5
RBC (10^4/mm^3)	751.9 ± 32.3	743.4 ± 35.3	757.5 ± 22.9	743.7 ± 20.4	749.9 ± 34.6	798.2 ± 14.3	797.8 ± 21.1
Hemoglobin (g/dl)	14.38 ± 0.52	14.32 ± 0.53	14.23 ± 0.49	14.35 ± 0.33	14.48 ± 0.68	14.90 ± 0.16	14.80 ± 0.60
Hematocrit (%)	42.9 ± 1.29	42.69 ± 1.35	42.42 ± 1.32	42.85 ± 0.68	43.36 ± 1.66	43.70 ± 0.53	43.12 ± 1.50
MCV (μm^3)	57.13 ± 1.55	57.47 ± 1.76	56.00 ± 0.85	57.66 ± 1.49	57.86 ± 1.46	54.76 ± 1.57	54.04 ± 0.92
MCH (pg)	19.15 ± 0.50	19.27 ± 0.62	18.79 ± 0.29	19.29 ± 0.56	19.32 ± 0.50	18.64 ± 0.44	18.58 ± 0.41
MCHC (g/dl)	33.51 ± 0.62	33.55 ± 0.73	33.54 ± 0.18	33.49 ± 0.72	33.39 ± 0.46	34.06 ± 0.28	34.32 ± 0.26
Platelet (10^4/mm^3)	108.49 ± 34.49	110.96 ± 6.58	116.72 ± 14.92	102.56 ± 11.95	91.62 ± 11.58**	121.82 ± 8.76	123.66 ± 20.92
Reticulocyte (‰)	25.9 ± 4.9	24.3 ± 4.4	25.4 ± 6.4	26.6 ± 5.9	24.4 ± 3.2	24.8 ± 2.8	31.2 ± 8.7
PT (sec.)	12.61 ± 0.46	12.62 ± 0.32	12.17 ± 0.36	12.52 ± 0.23	12.36 ± 0.54	12.60 ± 0.23	12.48 ± 0.50
APTT (sec.)	19.84 ± 1.46	20.27 ± 1.65	20.65 ± 1.79	20.39 ± 0.89	20.78 ± 1.63	21.50 ± 0.80	19.78 ± 1.22*
WBC (10^2/mm^3)	45.6 ± 14.4	41.4 ± 10.5	47.7 ± 11.3	42.2 ± 9.7	52.3 ± 20.0	66.0 ± 15.3	58.6 ± 18.4
Differential leukocyte (%)							
Lymphocyte	94.0 ± 3.4	93.2 ± 4.7	95.0 ± 2.0	93.5 ± 2.8	93.5 ± 3.3	95.8 ± 1.6	95.2 ± 1.9
Neutrophil	5.4 ± 2.8	6.1 ± 4.1	4.5 ± 2.0	5.9 ± 2.3	5.9 ± 3.1	3.8 ± 1.3	3.6 ± 1.1
Eosinophil	0.3 ± 0.5	0.4 ± 0.5	0.2 ± 0.4	0.4 ± 0.5	0.3 ± 0.5	0.2 ± 0.4	0.8 ± 0.8
Basophil	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
Monocyte	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.5	0.2 ± 0.4	0.3 ± 0.5	0.2 ± 0.4	0.4 ± 0.5

Each value shows mean ± S.D.
Significantly different from control (*: $p<0.05$, **: $p<0.01$).

Table 3 Blood chemical examination of male rats in 28-day repeat dose oral toxicity test of 1, 2, 4-trimethylbenzene

Test period	Termination of administration period					Termination of recovery period	
Dose (mg/kg)	0	30	100	300	1000	0	1000
Number of males	10	10	10	10	10	5	5
GOT (IU/l)	84.13 ± 23.46	72.69 ± 6.90	64.47 ± 7.23**	73.87 ± 12.77	69.07 ± 10.12	67.92 ± 5.06	66.38 ± 7.41
GPT (IU/l)	27.21 ± 11.18	21.63 ± 2.88	22.27 ± 4.39	23.24 ± 3.58	24.23 ± 3.29	24.44 ± 1.40	25.46 ± 3.34
ALP (IU/l)	171.29 ± 16.15	181.03 ± 28.69	187.27 ± 24.46	191.57 ± 17.38	191.19 ± 31.87	133.68 ± 18.20	143.60 ± 14.97
γ-GTP (IU/l)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T-protein (g/dl)	5.33 ± 0.16	5.29 ± 0.23	5.17 ± 0.25	5.04 ± 0.22**	5.20 ± 0.14	5.36 ± 0.09	5.26 ± 0.27
Albumin (g/dl)	3.013 ± 0.152	3.028 ± 0.071	2.965 ± 0.148	2.945 ± 0.095	3.090 ± 0.083	2.944 ± 0.045	2.926 ± 0.170
Protein fraction (%)							
Albumin	56.51 ± 1.54	57.30 ± 1.93	57.35 ± 1.81	58.45 ± 1.87*	59.45 ± 1.29**	54.94 ± 0.86	55.62 ± 2.25
α_1-globulin	20.11 ± 1.20	19.04 ± 1.99	18.88 ± 1.66	17.63 ± 1.57**	16.86 ± 1.50**	20.50 ± 1.20	19.64 ± 2.23
α_2-globulin	5.88 ± 0.48	5.97 ± 0.54	6.02 ± 0.36	6.02 ± 0.47	6.34 ± 0.69	5.76 ± 0.27	5.74 ± 0.63
α_3-globulin	5.32 ± 0.83	5.34 ± 0.35	5.36 ± 0.38	5.16 ± 0.40	4.97 ± 0.45	5.74 ± 0.61	5.96 ± 0.36
β-globulin	10.18 ± 0.53	10.35 ± 0.51	10.27 ± 0.77	10.57 ± 0.57	10.25 ± 0.70	10.60 ± 0.41	10.84 ± 0.21
γ-globulin	2.00 ± 0.37	2.00 ± 0.48	2.12 ± 0.53	2.17 ± 0.54	2.13 ± 0.68	2.46 ± 0.38	2.20 ± 0.63
A/G ratio	1.301 ± 0.085	1.346 ± 0.111	1.350 ± 0.098	1.411 ± 0.108*	1.469 ± 0.079**	1.220 ± 0.041	1.258 ± 0.116
T-bilirubin (mg/dl)	0.055 ± 0.010	0.054 ± 0.011	0.047 ± 0.008	0.053 ± 0.011	0.042 ± 0.009*	0.068 ± 0.015	0.064 ± 0.011
BUN (mg/dl)	12.51 ± 1.99	13.49 ± 2.40	12.01 ± 2.60	13.01 ± 2.53	12.95 ± 2.80	18.02 ± 2.14	15.04 ± 2.21
Creatinine (mg/dl)	0.458 ± 0.033	0.451 ± 0.032	0.442 ± 0.037	0.439 ± 0.021	0.423 ± 0.037	0.566 ± 0.046	0.518 ± 0.018
Glucose (mg/dl)	119.85 ± 7.98	118.76 ± 12.23	108.53 ± 12.36	107.45 ± 9.11*	112.35 ± 10.37	126.44 ± 13.79	123.48 ± 8.37
T-cholesterol (mg/dl)	48.11 ± 9.53	46.61 ± 6.85	50.87 ± 8.90	47.65 ± 6.01	49.88 ± 8.47	49.72 ± 5.67	43.38 ± 9.14
Triglyceride (mg/dl)	35.41 ± 15.64	29.05 ± 7.92	34.55 ± 14.45	27.72 ± 7.94	23.42 ± 5.59	38.36 ± 6.56	44.06 ± 21.19
Na (mEq/l)	146.16 ± 1.08	146.03 ± 1.55	146.29 ± 0.76	145.26 ± 1.37	144.75 ± 0.96*	144.12 ± 1.21	144.68 ± 0.88
K (mEq/l)	4.365 ± 0.286	4.432 ± 0.256	4.433 ± 0.160	4.668 ± 0.251*	4.772 ± 0.285**	4.604 ± 0.183	4.432 ± 0.167
Cl (mEq/l)	105.82 ± 1.35	106.16 ± 1.53	106.00 ± 1.10	105.86 ± 0.70	104.69 ± 1.02	104.48 ± 1.14	105.10 ± 0.73
Ca (mg/dl)	10.43 ± 0.23	10.34 ± 0.23	10.28 ± 0.20	10.16 ± 0.27	10.26 ± 0.22	9.66 ± 0.11	9.72 ± 0.33
I-phosphate (mg/dl)	8.21 ± 0.85	8.54 ± 0.47	8.43 ± 0.65	8.54 ± 0.69	8.21 ± 0.44	7.46 ± 0.54	7.68 ± 0.43

Each value shows mean ± S.D.
Significantly different from control (*: $p<0.05$, **: $p<0.01$).

Table 4 Blood chemical examination of female rats in 28-day repeat dose oral toxicity test of 1, 2, 4-trimethylbenzene

Test period	Termination of administration period					Termination of recovery period	
Dose (mg/kg)	0	30	100	300	1000	0	1000
Number of males	10	10	10	10	10	5	5
GOT (IU/l)	66.22 ± 14.48	67.93 ± 9.11	61.33 ± 6.43	60.08 ± 9.32	61.33 ± 8.27	52.74 ± 4.26	47.70 ± 4.77
GPT (IU/l)	17.71 ± 2.87	18.15 ± 1.76	17.44 ± 2.19	17.87 ± 2.82	21.91 ± 1.98**	19.78 ± 3.03	16.96 ± 1.04
ALP (IU/l)	101.37 ± 12.49	97.41 ± 12.26	98.22 ± 15.08	94.68 ± 20.76	114.67 ± 19.38	71.48 ± 12.21	71.78 ± 11.18
γ-GTP (IU/l)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T-protein (g/dl)	5.55 ± 0.23	5.44 ± 0.38	5.52 ± 0.40	5.25 ± 0.29	5.48 ± 0.43	5.84 ± 0.27	5.44 ± 0.34
Albumin (g/dl)	3.349 ± 0.197	3.236 ± 0.296	3.358 ± 0.327	3.181 ± 0.260	3.395 ± 0.311	3.380 ± 0.257	3.176 ± 0.125
Protein fraction (%)							
Albumin	60.31 ± 1.94	59.45 ± 2.31	60.76 ± 2.36	60.55 ± 2.24	61.94 ± 1.79	57.82 ± 1.88	58.48 ± 1.59
α_1-globulin	16.36 ± 1.43	16.47 ± 1.60	16.86 ± 1.43	15.61 ± 1.56	13.91 ± 1.85**	19.66 ± 1.09	19.76 ± 1.39
α_2-globulin	4.63 ± 0.54	5.22 ± 0.79	4.66 ± 0.58	5.40 ± 0.79	5.80 ± 1.13**	4.06 ± 0.26	3.86 ± 0.57
α_3-globulin	5.24 ± 0.63	4.95 ± 0.62	4.80 ± 0.82	4.80 ± 0.54	4.21 ± 0.43**	5.40 ± 0.47	5.12 ± 0.57
β-globulin	10.05 ± 0.76	10.73 ± 0.83	9.61 ± 0.82	10.53 ± 0.97	10.80 ± 0.66	9.78 ± 0.93	9.80 ± 0.61
γ-globulin	3.41 ± 0.87	3.18 ± 0.71	3.31 ± 0.47	3.11 ± 0.95	3.34 ± 0.59	3.28 ± 0.53	2.98 ± 0.54
A/G ratio	1.524 ± 0.115	1.474 ± 0.148	1.557 ± 0.153	1.540 ± 0.143	1.633 ± 0.123	1.372 ± 0.111	1.410 ± 0.093
T-bilirubin (mg/dl)	0.075 ± 0.017	0.069 ± 0.016	0.064 ± 0.014	0.058 ± 0.008	0.049 ± 0.007**	0.080 ± 0.020	0.076 ± 0.005
BUN (mg/dl)	14.83 ± 2.05	14.21 ± 1.24	13.71 ± 2.53	13.85 ± 2.82	13.15 ± 2.48	16.42 ± 1.05	18.32 ± 0.95*
Creatinine (mg/dl)	0.488 ± 0.034	0.487 ± 0.030	0.487 ± 0.016	0.492 ± 0.029	0.475 ± 0.033	0.532 ± 0.020	0.512 ± 0.022
Glucose (mg/dl)	130.27 ± 15.68	125.77 ± 10.84	125.21 ± 9.79	120.96 ± 11.34	136.80 ± 13.35	130.76 ± 8.50	130.50 ± 9.64
T-cholesterol (mg/dl)	51.90 ± 9.00	58.59 ± 12.47	54.34 ± 10.88	66.84 ± 12.67*	75.94 ± 9.59**	55.82 ± 12.86	64.24 ± 7.93
Triglyceride (mg/dl)	24.25 ± 8.90	20.99 ± 6.18	23.57 ± 11.94	19.43 ± 7.80	21.53 ± 4.00	25.46 ± 5.96	26.12 ± 5.02
Na (mEq/l)	144.01 ± 1.56	144.11 ± 0.39	144.15 ± 1.36	143.31 ± 1.32	143.04 ± 1.41	143.56 ± 0.42	143.82 ± 1.19
K (mEq/l)	4.338 ± 0.403	4.349 ± 0.217	4.241 ± 0.087	4.242 ± 0.273	4.328 ± 0.239	3.616 ± 0.092	3.570 ± 0.223
Cl (mEq/l)	106.67 ± 1.68	107.04 ± 1.16	105.74 ± 1.23	105.80 ± 0.86	105.62 ± 1.48	106.50 ± 0.92	105.62 ± 1.52
Ca (mg/dl)	9.84 ± 0.33	9.81 ± 0.19	10.00 ± 0.29	9.90 ± 0.24	9.90 ± 0.31	10.46 ± 0.18	10.34 ± 0.21
I-phosphate (mg/dl)	6.40 ± 0.95	6.55 ± 0.75	6.81 ± 0.80	6.67 ± 0.51	6.61 ± 0.56	5.20 ± 0.64	5.86 ± 0.29

Each value shows mean ± S.D.
Significantly different from control (*: $p<0.05$, **: $p<0.01$).

Table 5 Absolute and relative organ weights of male rats in 28-day repeat dose oral toxicity test of 1, 2, 4-trimethylbenzene

Test period	Termination of administration period					Termination of recovery period	
Dose (mg/kg)	0	30	100	300	1000	0	1000
Number of males	10	10	10	10	10	5	5
Body weight (g)	323.0 ± 25.5	324.9 ± 40.5	320.5 ± 16.2	320.8 ± 18.5	289.2 ± 22.2*	385.8 ± 34.0	337.0 ± 14.6*
Brain (g)	1.916 ± 0.069	1.915 ± 0.051	1.881 ± 0.056	1.912 ± 0.074	1.891 ± 0.065	1.932 ± 0.025	1.946 ± 0.050
(g%)	0.595 ± 0.036	0.597 ± 0.074	0.588 ± 0.023	0.598 ± 0.043	0.656 ± 0.044*	0.504 ± 0.046	0.580 ± 0.040*
Thymus (g)	0.546 ± 0.084	0.486 ± 0.058	0.476 ± 0.098	0.516 ± 0.093	0.484 ± 0.125	0.550 ± 0.107	0.418 ± 0.088
(g%)	0.169 ± 0.023	0.153 ± 0.023	0.147 ± 0.028	0.162 ± 0.025	0.167 ± 0.037	0.142 ± 0.023	0.126 ± 0.027
Heart (g)	1.121 ± 0.124	1.186 ± 0.122	1.172 ± 0.083	1.150 ± 0.089	1.056 ± 0.146	1.226 ± 0.049	1.196 ± 0.087
(g%)	0.348 ± 0.032	0.366 ± 0.025	0.365 ± 0.018	0.357 ± 0.027	0.364 ± 0.027	0.318 ± 0.024	0.358 ± 0.038
Liver (g)	9.440 ± 1.025	9.567 ± 1.529	9.578 ± 0.777	9.842 ± 0.681	10.571 ± 0.803	10.370 ± 1.251	9.808 ± 1.120
(g%)	2.918 ± 0.144	2.937 ± 0.141	2.986 ± 0.126	3.066 ± 0.048	3.658 ± 0.132**	2.686 ± 0.175	2.902 ± 0.235
Spleen (g)	0.614 ± 0.078	0.592 ± 0.099	0.571 ± 0.049	0.626 ± 0.110	0.506 ± 0.091*	0.650 ± 0.066	0.644 ± 0.030
(g%)	0.189 ± 0.020	0.184 ± 0.026	0.179 ± 0.014	0.196 ± 0.037	0.172 ± 0.021	0.170 ± 0.010	0.190 ± 0.012*
Kidneys (g)	2.391 ± 0.181	2.374 ± 0.264	2.414 ± 0.175	2.590 ± 0.137	2.638 ± 0.255*	2.646 ± 0.091	2.594 ± 0.228
(g%)	0.742 ± 0.047	0.732 ± 0.057	0.753 ± 0.041	0.809 ± 0.040*	0.915 ± 0.064**	0.690 ± 0.060	0.772 ± 0.075
Adrenals (mg)	51.96 ± 9.95	56.20 ± 4.11	49.81 ± 5.52	50.30 ± 5.97	50.81 ± 8.33	54.44 ± 5.89	47.58 ± 4.96
(mg%)	16.09 ± 2.83	17.50 ± 2.34	15.54 ± 1.47	15.80 ± 2.51	17.65 ± 3.19	14.18 ± 1.68	14.14 ± 1.71
Testes (g)	3.051 ± 0.144	3.054 ± 0.217	3.037 ± 0.146	3.006 ± 0.195	3.036 ± 0.193	3.074 ± 0.170	3.198 ± 0.156
(g%)	0.948 ± 0.065	0.953 ± 0.138	0.948 ± 0.038	0.938 ± 0.071	1.055 ± 0.085*	0.802 ± 0.089	0.952 ± 0.090*
Epididymides (g)	0.736 ± 0.050	0.743 ± 0.043	0.757 ± 0.070	0.756 ± 0.038	0.745 ± 0.041	1.014 ± 0.061	0.974 ± 0.047
(g%)	0.231 ± 0.025	0.231 ± 0.028	0.237 ± 0.018	0.236 ± 0.013	0.259 ± 0.017*	0.264 ± 0.030	0.290 ± 0.020

Each value shows mean ± S.D.
Significantly different from control (*: $p<0.05$, **: $p<0.01$).

Table 6 Absolute and relative organ weights of female rats in 28-day repeat dose oral toxicity test of 1, 2, 4-trimethylbenzene

Test period	Termination of administration period					Termination of recovery period	
Dose (mg/kg)	0	30	100	300	1000	0	1000
Number of females	10	10	10	10	10	5	5
Body weight (g)	216.2 ± 18.9	217.7 ± 13.4	217.8 ± 14.0	211.5 ± 15.9	202.4 ± 12.2	239.2 ± 11.9	226.2 ± 8.8
Brain (g)	1.792 ± 0.068	1.814 ± 0.086	1.786 ± 0.060	1.790 ± 0.049	1.818 ± 0.051	1.834 ± 0.029	1.826 ± 0.039
(g%)	0.834 ± 0.072	0.835 ± 0.045	0.823 ± 0.052	0.851 ± 0.057	0.901 ± 0.058*	0.768 ± 0.051	0.806 ± 0.041
Thymus (g)	0.526 ± 0.097	0.539 ± 0.107	0.547 ± 0.074	0.510 ± 0.086	0.475 ± 0.066	0.476 ± 0.021	0.456 ± 0.028
(g%)	0.243 ± 0.037	0.246 ± 0.042	0.252 ± 0.039	0.240 ± 0.031	0.236 ± 0.038	0.200 ± 0.010	0.202 ± 0.015
Heart (g)	0.823 ± 0.101	0.794 ± 0.053	0.826 ± 0.089	0.772 ± 0.063	0.782 ± 0.082	0.870 ± 0.034	0.876 ± 0.113
(g%)	0.383 ± 0.068	0.365 ± 0.029	0.380 ± 0.024	0.366 ± 0.020	0.387 ± 0.031	0.364 ± 0.021	0.388 ± 0.052
Liver (g)	6.487 ± 0.726	6.539 ± 0.452	6.763 ± 0.571	7.367 ± 0.463*	9.019 ± 0.931**	6.908 ± 0.221	6.608 ± 0.315
(g%)	2.997 ± 0.178	3.004 ± 0.110	3.105 ± 0.161	3.491 ± 0.222**	4.448 ± 0.259**	2.892 ± 0.114	2.918 ± 0.042
Spleen (g)	0.480 ± 0.049	0.508 ± 0.073	0.470 ± 0.063	0.489 ± 0.067	0.416 ± 0.035	0.522 ± 0.101	0.476 ± 0.038
(g%)	0.224 ± 0.023	0.233 ± 0.032	0.217 ± 0.027	0.232 ± 0.027	0.207 ± 0.015	0.220 ± 0.049	0.212 ± 0.013
Kidneys (g)	1.667 ± 0.122	1.621 ± 0.114	1.669 ± 0.168	1.606 ± 0.090	1.722 ± 0.188	1.712 ± 0.090	1.682 ± 0.120
(g%)	0.774 ± 0.055	0.745 ± 0.052	0.767 ± 0.050	0.763 ± 0.043	0.849 ± 0.055**	0.716 ± 0.048	0.742 ± 0.030
Adrenals (mg)	64.41 ± 4.74	59.57 ± 3.46	63.28 ± 5.85	58.56 ± 7.72	66.97 ± 8.01	68.42 ± 9.86	69.40 ± 8.87
(mg%)	29.91 ± 2.47	27.43 ± 1.84	29.15 ± 3.25	27.91 ± 4.72	33.10 ± 3.31	28.50 ± 3.09	30.62 ± 3.10
Ovaries (mg)	85.84 ± 10.71	86.46 ± 9.81	87.67 ± 11.41	83.65 ± 13.93	78.76 ± 13.51	90.98 ± 11.48	87.88 ± 10.85
(mg%)	39.84 ± 4.74	39.69 ± 3.45	40.32 ± 5.06	39.61 ± 6.46	38.89 ± 6.07	38.14 ± 5.65	38.84 ± 4.33

Each value shows mean ± S.D.
Significantly different from control (*: $p<0.05$, **: $p<0.01$).

Table 7 Histopathological examination of male rats in 28-day repeat dose oral toxicity test of 1, 2, 4-trimethylbenzene

Test period	Termination of administration period																				Termination of recovery period									
Dose (mg/kg)	0					30					100					300					1000					0					1000				
Number of males	10					10					10					10					10					5					5				
Finding Grade	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+
Liver																																			
Vacuolar degeneration of periportal hepatocytes	1	0	4	4	1	0	3	6	1	0	1	8	1	0	0*	4	5	1	0	0**	9	1	0	0	0**	4	1	0	0	0	2	3	0	0	0
Cell infiltration	10	0	0	0	0	10	0	0	0	0	9	1	0	0	0	10	0	0	0	0	10	0	0	0	0	5	0	0	0	0	5	0	0	0	0
Degeneration and necrosis of hepatocytes	10	0	0	0	0	10	0	0	0	0	9	1	0	0	0	10	0	0	0	0	10	0	0	0	0	5	0	0	0	0	5	0	0	0	0
Focal necrosis	10	0	0	0	0	10	0	0	0	0	10	0	0	0	0	10	0	0	0	0	10	0	0	0	0	5	0	0	0	0	4	1	0	0	0
Kidney																																			
Hyaline droplet degeneration	8	2	0	0	0	3	7	0	0	0	3	7	0	0	0	2	5	3	0	0*	0	2	7	1	0**	3	2	0	0	0	5	0	0	0	0
Basophilic change of urinary tubules	10	0	0	0	0	10	0	0	0	0	10	0	0	0	0	10	0	0	0	0	10	0	0	0	0	5	0	0	0	0	4	1	0	0	0

Grade of histopathological finding; -: No abnormality detected, ±: Slight, +: Mild, 2+: Moderate, 3+: Marked.
No remarkable changes were recognized in heart, spleen and adrenal of control group and 1000 mg/kg group.
Significantly different from control (*: $p<0.05$, **: $p<0.01$).

Table 8 Histopathological examination of female rats in 28-day repeat dose oral toxicity test of 1,2,4-trimethylbenzene

Test period	Termination of administration period																				Termination of recovery period									
Dose (mg/kg)	0					30					100					300					1000					0					1000				
Number of males	10					10					10					10					10					5					5				
Finding Grade	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+	-	±	+	2+	3+
Liver																																			
Vacuolar degeneration of periportal hepatocytes	0	6	3	1	0	1	4	5	0	0	1	8	1	0	0	0	10	0	0	0	3	7	0	0	0*	1	4	0	0	0	3	2	0	0	0
Cell infiltration	10	0	0	0	0	10	0	0	0	0	10	0	0	0	0	9	1	0	0	0	10	0	0	0	0	5	0	0	0	0	5	0	0	0	0
Kidney																																			
Basophilic change of urinary tubules	10	0	0	0	0	/					/					/					9	1	0	0	0	5	0	0	0	0	5	0	0	0	0

Grade of histopathological finding; -: No abnormality detected, ±: Slight, +: Mild, 2+: Moderate, 3+: Marked.
No remarkable changes were recognized in heart, spleen and adrenal of control group and 1000 mg/kg group.
/: Not examined.
Significantly different from control (*: $p<0.05$).

みられたが，絶対重量と相対重量で一定方向の変動ではないもの，もしくは軽度の差であるものであった．1000 mg/kg群の雌では，対照群と比べていずれの器官重量とも有意差はみられなかった．

10. 病理組織学的検査(Table 7, 8)

投与期間終了時には，肝臓において門脈周囲の肝細胞の空胞変性が雄では対照群で9例，30 mg/kg群で10例，100 mg/kg群で9例，300 mg/kg群で6例，1000 mg/kg群で1例にみられ，投与量が増えるに従い出現例数は減少し，その程度も弱かった．この変化は，対照群と比べて100 mg/kg以上の群で有意差が認められ，また，用量相関性も確認された．この変化は，雌では対照群で10例，30 mg/kg群で9例，100 mg/kg群で9例，300 mg/kg群で10例，1000 mg/kg群で7例にみられ，1000 mg/kg群で有意差が認められた．なお，その他に，肝臓に認められた所見は，いずれも程度はごく軽度であり，かつ投与量に依存した変化ではなかった．腎臓において，尿細管の硝子滴変性が雄では対照群で2例，30 mg/kg群で7例，100 mg/kg群で7例，300 mg/kg群で8例，1000 mg/kg群で10例にみられた．また，300 mg/kg以上の群では有意差が認められ，用量相関性も確認された．尿細管の好塩基性変化は，雌では1000 mg/kg群で1例にみられた．その他には，対照群および1000 mg/kg群の雌雄とも心臓，脾臓および副腎に異常はみられなかった．

回復期間終了時には，肝臓において門脈周囲の肝細胞の空胞変性が雄では対照群で1例，1000 mg/kg群で3例にみられた．この変化は，雌では対照群で4例，1000 mg/kg群で2例にみられた．なお，その他に，巣状壊死

が1000 mg/kg群の雄で1例にみられたのみであった．腎臓において，尿細管の硝子滴変性が対照群の雄で2例にみられた．尿細管の好塩基性変化は，1000 mg/kg群の雄で1例にみられた．

考察

1,2,4-トリメチルベンゼンを雌雄ラットに30，100，300および1000 mg/kgの投与量で1日1回，28日間反復経口投与し，その毒性について検討した．一部の動物については，14日間の回復期間を設けた．

死亡は，いずれの群にも発現しなかった．一般状態では，雌雄とも300 mg/kg以上の投与で投与直後に流涎がみられた．この流涎は，高濃度の検体投与でより顕著に認められていることから，1,2,4-トリメチルベンゼンの刺激により生じた可能性が考えられる．

体重は，雌雄とも1000 mg/kgの投与で増加抑制がみられ，この変化は回復期間中も継続して認められた．しかし，1000 mg/kgの投与でも摂餌量に影響はみられなかった．摂水量は，雄では300 mg/kg以上の投与で，雌では1000 mg/kgの投与で増加がみられ，この変化は回復期間中も継続して認められた．

尿検査では，雌雄とも1000 mg/kgの投与で摂水量の増加に伴う尿量の高値がみられたが，これらの変化は回復期間終了前には消失した．

血液学検査では，投与期間終了時に1000 mg/kg群の雄でヘモグロビン量の高値，雌で血小板数の低値，回復期間終了時には1000 mg/kg群の雌で活性化部分トロンボプラスチン時間の短縮がみられた．しかし，これらの変化は，いずれも軽微なものであり，生理的変動の範囲内の変化であると考えられる．したがって，1,2,4-トリメチルベンゼンの1000 mg/kgを投与しても，血液学的検査の各項目に影響は及ぼさないと判断した．

血液生化学検査において，雄では投与期間終了時に300 mg/kg以上の群でアルブミン率，A/G比およびKの高値，α_1-グロブリン率の低値，1000 mg/kg群で総ビリルビンおよびNaの有意な低値がみられた．雌では，投与期間終了時に300 mg/kg以上の群で総コレステロールの高値，1000 mg/kg群でGPTおよびα_2-グロブリン率の高値，α_1-グロブリン率，α_3-グロブリン率および総ビリルビンの低値がみられた．しかし，これらの変化はいずれも軽微であり，当社のほぼ同週齢ラットの集積データの範囲内の数値であることから，毒性学的意義はないと判断した．

剖検では，雌雄とも1000 mg/kgを投与しても1,2,4-トリメチルベンゼンによる影響はみられなかった．

器官重量については，雄では300 mg/kg以上の群で腎臓の相対重量の高値，1000 mg/kg群で肝臓の相対重量の高値と絶対重量の高値傾向，腎臓の絶対重量の高値がみられた．雌では，300 mg/kg以上の群で肝臓の絶対・相対重量の高値，1000 mg/kg群で腎臓の相対重量の高値と絶対重量の高値傾向がみられた．なお，類似化学物質である1,4-ジエチルベンゼンの反復投与においても，肝臓および腎臓重量の増加が報告されている[6]．したがって，1,2,4-トリメチルベンゼン投与により肝臓および腎臓重量に影響が生じると考えられる．なお，雌雄で認められたこれら器官の重量増加は回復期間終了時には消失した．

病理組織学的検査では，投与期間終了時に雄で腎臓に尿細管の硝子滴変性がみられた．この変化は，ラットの雄でしばしば観察される変化であるが，本試験では軽度以上の変化の出現例数が300 mg/kg以上の群で増加し，程度も増強されていることから，変化を増強する何らかの影響を受けたものと考えられる．しかし，この変化は可逆性のものであった．

肝臓では，投与期間終了時に雌雄で門脈周囲の肝細胞の空胞変性がみられ，特に対照群および30 mg/kg群で多く観察された．この変化は，コーンオイルを反復投与した場合によくみられている所見であり，100 mg/kg以上の群の雄と1000 mg/kg群の雌で軽微であった機序は不明であるが，毒性学的には問題のない変化と考えられる．なお，病理組織学的検査において，肝臓重量の増加と関連した所見が得られなかったこと，ならびに類似化学物質での結果から，重量増加は薬物代謝亢進に適応した変化と考えられる．

以上のことから，1,2,4-トリメチルベンゼンは肝臓および腎臓に影響を及ぼすことが示唆された．なお，雄では300 mg/kg以上の投与で流涎，摂水量の増加，腎臓重量の増加および腎臓に尿細管の硝子滴変性，1000 mg/kgの投与で体重増加の抑制，尿量の増加および肝臓重量の増加，雌では300 mg/kg以上の投与で流涎および肝臓重量の増加，1000 mg/kgの投与で体重増加の抑制，摂水量の増加，尿量の増加および腎臓重量の増加がみられたことから，当試験条件下における1,2,4-トリメチルベンゼンの28日間反復経口投与による毒性学的無影響量は雌雄とも100 mg/kg/dayと考えられる．

文献

1) 丸善石油㈱社内資料
2) 佐久間昭，“薬効評価－計画と解析Ⅰ，Ⅱ，” 東大出版会，東京，1977，1981.
3) C. W. Dunnett, *Biometrics*, **20**, 482(1964).
4) H. Scheffé , *Biometrika*, **40**, 87(1953).
5) W. H. Kruskal, W. A. Wallis, *J. Amer. Statist. Assoc.*, **47**, 583(1952).
6) 萩田孝一ら，化学物質毒性試験報告，**2**，37(1995).

連絡先

試験責任者：古橋忠和

試験担当者：長瀬孝彦，藤村高志，虫賀勇樹，外山勝己，牧野浩平，木村　均

㈱日本バイオリサーチセンター　羽島研究所

〒501-62　岐阜県羽島市福寿町間島6-104

Tel 058-392-6222　Fax 058-392-1284

Correspondence

Authors: Tadakazu Furuhashi (Study director) Takahiko Nagase, Takashi Fujimura, Yuuki Mushiga, Katsumi Toyama, Kohei Makino and Hitoshi Kimura

Nihon Bioresearch Inc. Hashima Laboratory

6-104, Majima, Fukuju-cho, Hashima, Gifu, 501-62, Japan

Tel +81-58-392-6222　Fax +81-58-392-1284

1,2,4-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 1,2,4-Trimethylbenzene on Bacteria

要約

既存化学物質安全性調査事業の一環として，1,2,4-トリ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無添加試験およびS9 mix添加試験において，すべての検定菌で強い抗菌性が認められた．また，TA1535のS9 mix無添加試験とTA1537のS9 mix添加試験では，本試験Ⅰで抗菌性のない用量が4用量に達しなかったため，本試験の用量を下げた．したがって，本試験ではS9 mix無添加試験をTA100, TA1535, TA98およびTA1537は7.81～250 μg/プレート，WP2 *uvrA*は15.6～500 μg/プレートの範囲で，S9 mix添加試験をTA1537は7.81～250 μg/プレート，TA100, TA1535およびWP2 *uvrA*は15.6～500 μg/プレート，TA98は31.3～1000 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, TA1535, TA98およびTA1537では125 μg/プレート以上，WP2 *uvrA*では，250 μg/プレート以上，S9 mix添加試験では，TA1537では125 μg/プレート以上，TA100およびTA1535では250 μg/プレート以上，TA98およびWP2 *uvrA*では，500 μg/プレート以上の用量で抗菌性が認められた．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，1,2,4-トリメチルベンゼンは，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，試験に際して，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〕

1,2,4-トリメチルベンゼン(CAS No. 95-63-6)は，分子量120.20の無色透明液体である．試験には，東洋合成工業㈱製〔ロット番号：H5-CH-11，純度98.75％(不純物：不明)〕を，㈳日本化学工業協会から供与され，使用時まで室温遮光保管して，使用した．

1,2,4-トリメチルベンゼンは，ジメチルスルホキシド(DMSO)に溶解性がよいことから，DMSOに50，10または2.5 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，1,2,4-トリメチルベンゼンの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(78.1 μg/ml)溶液は，本試験Ⅱで調製したものについて，高濃度(60.0 mg/ml)溶液は，染色体異常試験で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，104および99.2％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，低濃度(78.1 μg/ml)溶液は91.6％，高濃度(10.0 mg/ml溶液)は99.8％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．

小試験管中にトップアガー2 m*l*，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*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無添加試験では，TA100, TA98およびTA1537は150 μg/プレート以上，TA1535およびWP2 *uvrA*は500 μg/プレート以上の用量で抗菌性が認められた．また，S9 mix添加試験ではTA100, TA1535, WP2 *uvrA*およびTA1537は500 μg/プレート以上，TA98は1500 μg/プレート以上の用量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．1,2,4-トリメチルベンゼンの用量は，TA1535のS9 mix無添加試験とTA1537のS9 mix添加試験では，本試験Ⅰにおいて抗菌性のない用量が4用量に達しなかったため，本試験における最高用量をともに250 μg/プレートに下げることとした．したがって，本試験での用量は，S9 mix無添加試験ではTA100, TA1535, TA98およびTA1537は7.81～250 μg/プレート，WP2 *uvrA*は15.6～500 μg/プレートの範囲で，S9 mix添加試験ではTA1537は7.81～250 μg/プレート，TA100, TA1535およびWP2 *uvrA*は15.6～500 μg/プレート，TA98は31.3～1000 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，1,2,4-トリメチルベンゼンは，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 1,2,4-trimethylbenzene** 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	136 138 152 (142± 8.7)	5 14 14 (11± 5.2)	19 32 26 (26± 6.5)	18 34 24 (25± 8.1)	5 5 9 (6± 2.3)
	7.81	133 121 127 (127± 6.0)	6 12 12 (10± 3.5)	ND	15 26 29 (23± 7.4)	7 11 4 (7± 3.5)
	15.6	132 126 123 (127± 4.6)	12 7 8 (9± 2.6)	12 30 15 (19± 9.6)	23 27 19 (23± 4.0)	13 6 3 (7± 5.1)
	31.3	131 142 134 (136± 5.7)	20 15 9 (15± 5.5)	21 16 16 (18± 2.9)	25 25 31 (27± 3.5)	6 3 6 (5± 1.7)
	62.5	121 97 115 (111±12.5)	9 14 8 (10± 3.2)	19 18 22 (20± 2.1)	25 23 24 (24± 1.0)	16 5 9 (10± 5.6)
	125	102* 91* 106* (100± 7.8)	7* 9* 18* (11± 5.9)	23 17 24 (21± 3.8)	22* 20* 17* (20± 2.5)	5* 6* 12* (8± 3.8)
	250	86* 86* 79* (84± 4.0)	12* 6* 14* (11± 4.2)	14* 20* 24* (19± 5.0)	30* 20* 11* (20± 9.5)	6* 6* 5* (6± 0.6)
	500			24* 20* 15* (20± 4.5)		
S9mix (+)	0	117 130 139 (129±11.1)	14 14 16 (15± 1.2)	25 21 26 (24± 2.6)	36 29 41 (35± 6.0)	19 18 32 (23± 7.8)
	7.81	ND	ND	ND	ND	14 21 17 (17± 3.5)
	15.6	136 155 147 (146± 9.5)	19 23 22 (21± 2.1)	15 26 33 (25± 9.1)	ND	22 18 14 (18± 4.0)
	31.3	136 154 145 (145± 9.0)	15 12 9 (12± 3.0)	25 23 26 (25± 1.5)	45 43 40 (43± 2.5)	17 15 13 (15± 2.0)
	62.5	130 146 168 (148±19.1)	12 18 20 (17± 4.2)	23 24 23 (23± 0.6)	39 23 43 (35±10.6)	15 20 18 (18± 2.5)
	125	141 112 109 (121±17.7)	10 13 9 (11± 2.1)	25 29 25 (26± 2.3)	33 26 37 (32± 5.6)	14* 10* 17* (14± 3.5)
	250	91* 116* 121* (109±16.1)	13* 11* 11* (12± 1.2)	24 26 19 (23± 3.6)	42 24 31 (32± 9.1)	12* 11* 9* (11± 1.5)
	500	105* 91* 104* (100± 7.8)	9* 7* 9* (8± 1.2)	25* 15* 12* (17± 6.8)	25* 33* 20* (26± 6.6)	
	1000				20* 19* 15* (18± 2.6)	
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	770 678 696 (715±48.8)	142 171 162 (158±14.8)	135 112 123 (123±11.5)	756 803 831 (797±37.9)	742 764 624 (710±75.3)
Positive control S9 mix (+)	Chemical	2AA	2AA	2AA	2AA	2AA
	Dose (μg/plate)	1	2	10	0.5	2
	Number of colonies/plate	1417 1332 1306 (1352±58.1)	333 332 325 (330± 4.4)	1435 1465 1409 (1436±28.0)	389 369 355 (371± 17.1)	222 198 195 (205±14.8)

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 98.75% and impurity was unknown.

ND: Not done

Table 2. Mutagenicity of 1,2,4-trimethylbenzene** in reverse mutation test (II) 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	115 106 120 (114± 7.1)	16 15 12 (14± 2.1)	25 26 36 (29± 6.1)	32 23 21 (25± 5.9)	7 8 6 (7± 1.0)	
	7.81	105 128 105 (113±13.3)	13 8 11 (11± 2.5)	ND	22 25 21 (23± 2.1)	5 6 7 (6± 1.0)	
	15.6	93 113 117 (108±12.9)	13 14 11 (13± 1.5)	25 17 17 (20± 4.6)	19 23 29 (24± 5.0)	9 3 13 (8± 5.0)	
	31.3	101 107 97 (102± 5.0)	16 22 15 (18± 3.8)	26 27 27 (27± 0.6)	22 23 26 (24± 2.1)	3 9 7 (6± 3.1)	
	62.5	125 106 109 (113±10.2)	12 10 12 (11± 1.2)	20 14 20 (18± 3.5)	26 26 22 (25± 2.3)	7 7 6 (7± 0.6)	
	125	95* 108* 84* (96±12.0)	5* 13* 12* (10± 4.4)	17 22 25 (21± 4.0)	16* 16* 16* (16± 0.0)	5* 4* 6* (5± 1.0)	
	250	55* 82* 62* (66±14.0)	6* 6* 8* (7± 1.2)	17* 16* 19* (17± 1.5)	17* 7* 14* (13± 5.1)	0* 6* 3* (3± 3.0)	
	500			13* 14* 14* (14± 0.6)			
S9mix (+)	0	129 117 119 (122± 6.4)	16 11 10 (12± 3.2)	39 26 27 (31± 7.2)	37 31 35 (34± 3.1)	15 7 15 (12± 4.6)	
	7.81	ND	ND	ND	ND	18 20 18 (19± 1.2)	
	15.6	111 89 111 (104±12.7)	10 7 11 (9± 2.1)	31 30 29 (30± 1.0)	ND	18 19 19 (19± 0.6)	
	31.3	109 118 105 (111± 6.7)	6 9 11 (9± 2.5)	32 25 54 (37±15.1)	23 34 31 (29± 5.7)	16 15 18 (16± 1.5)	
	62.5	102 106 120 (109± 9.5)	13 10 16 (13± 3.0)	23 30 30 (28± 4.0)	27 25 29 (27± 2.0)	21 14 18 (18± 3.5)	
	125	117 96 107 (107±10.5)	8 14 10 (11± 3.1)	22 30 21 (24± 4.9)	35 31 25 (30± 5.0)	9 20 24 (18± 7.8)	
	250	88* 103* 81* (91±11.2)	10 10 16 (12± 3.5)	23 26 18 (22± 4.0)	32 33 25 (30± 4.4)	13* 21* 21* (18± 4.6)	
	500	95* 108* 89* (97± 9.7)	12* 15* 16* (14± 2.1)	22* 19* 22* (21± 1.7)	21* 18* 32* (24± 7.4)		
	1000				20* 20* 14* (18± 3.5)		
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	754 769 582 (702±103.9)	158 173 156 (162± 9.3)	188 185 105 (159±47.1)	825 866 908 (866±41.5)	1052 1228 1133 (1138±88.1)	
Positive control S9 mix (+)	Chemical	2AA	2AA	2AA	2AA	2AA	
	Dose (μg/plate)	1	2	10	0.5	2	
	Number of colonies/plate	1100 1146 1128 (1125±23.2)	315 292 311 (306±12.3)	1364 1438 1356 (1386±45.2)	298 300 295 (298± 2.5)	299 266 221 (262±39.2)	

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 98.75% and impurity was unknown.

ND: Not done

文献

1) D. M. Maron, B. N. Ames, *Mutat. Res.*, **113**, 173 (1983).
2) M. H. L. Green, "Handbook of Mutagenicity Test Procedures," eds. by B. J. Kilbey, M. Legator, W. Nichols, C. Ramel, Elsevier, Amsterdam, New York, Oxford, 1984, pp. 161-187.

連絡先

試験責任者：澁谷　徹
試験担当者：原　　巧，坂本京子，川上久美子，清水ゆり，松木容彦，中込まどか，飯田さやか
(財)食品薬品安全センター　秦野研究所
〒257　秦野市落合 729-5
Tel 0463-82-4751　Fax 0463-82-9627

Correspondence

Authors: Tohru Shibuya (Study Director)
Takumi Hara, Kyoko Sakamoto, Kumiko Kawakami, Yuri Shimizu, Yasuhiko Matsuki, Madoka Nakagomi and Sayaka Iida
Hatano Research Institute, Food and Drug Safety Center
729-5 Ochiai, Hadano-shi, Kanagawa 257 Japan
Tel +81-463-82-4751　Fax +81-463-82-9627

1,2,4-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
1,2,4-Trimethylbenzene 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，1,2,4-トリ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0.08 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%を明らかに越える増殖抑制濃度，すなわち0.1 mg/mlおよび0.3 mg/mlの濃度を最高処理濃度とした．連続処理および短時間処理のS9 mix存在下では，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．短時間処理のS9 mix非存在下では，増殖抑制試験でのデータのバラツキが大きかったことから，最高処理濃度とその1/2，1/4および1/8の4処理群を設定した．最高処理濃度の1/2濃度ですでに強い細胞毒性が認められたことから，染色体分析では，最高処理濃度の1/2(0.15 mg/ml)，1/4および1/8の3処理濃度を観察対照とした．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．一方，48時間連続処理した高濃度群(0.08 mg/ml)では，細胞毒性のため十分な細胞数を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は誘発されなかった．短時間処理では，非存在下で6時間処理した高濃度群(0.15 mg/ml)において，細胞毒性のため十分な細胞数を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，S9 mix存在下では，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，1,2,4-トリメチルベンゼンは，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

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

1,2,4-トリメチルベンゼン(略号：TMB，CAS No.：95-63-6，ロット番号：H5-CH-11，東洋合成工業㈱製造，㈳日本化学工業協会提供)は，無色透明液体で，水に対して難溶，融点-43.9℃，沸点169.4℃，蒸気圧0.5 KPa(20℃)，分子式C_9H_{12}，分子量120.20，純度98.75%(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，78.1 μg/ml～60.0 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，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，0.08 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，それぞれ0.1 mg/mlおよび0.3 mg/mlであった(Fig. 1)．

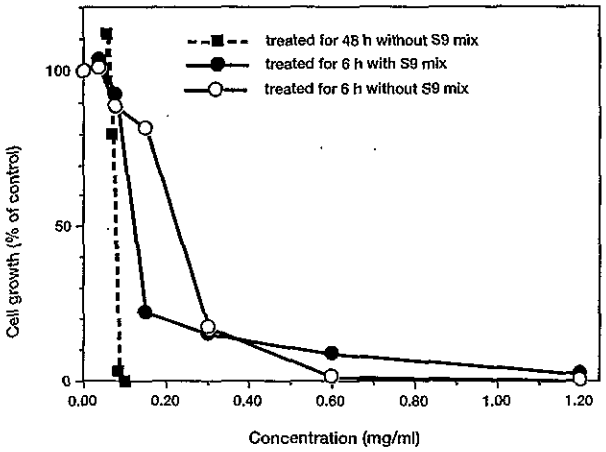


Fig. 1 Growth inhibition of CHL/IU cells treated with 1,2,4-trimethylbenzene

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0.08 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0.1 mg/mlおよび0.3 mg/mlとした．連続処理および短時間処理のS9 mix存在下では，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．短時間処理のS9 mix非存在下では，増殖抑制試験でのデータのバラツキが大きかったことから，最高処理濃度とその1/2，1/4および1/8の4処理群を設定した．最高処理濃度の1/2濃度で，すでに強い細胞毒性が認められたことから，染色体分析では，最高処理濃度の1/2(0.15 mg/ml)，1/4および1/8の3処理濃度を観察対照とした．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．1,2,4-トリメチルベンゼンを加えて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．一方，48時間連続処理した高濃度群(0.08 mg/ml)では，細胞毒性のため十分な細胞数を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．

短時間処理による染色体分析の結果をTable 2に示した．1,2,4-トリメチルベンゼンを加えてS9 mix非存在下で6時間処理した高濃度群(0.15 mg/ml)においては，細胞毒性のため十分な細胞数を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，S9 mix存在下では，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．

従って，1,2,4-トリメチルベンゼンは，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 1,2,4-trimethylbenzene (TMB)* 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	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.25		
Solvent[1]	0	24	200	1	0	0	0	0	0	1	0	1	(0.5)	0	(0.0)	0.50		
TMB	0.020	24	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.13		
TMB	0.040	24	200	0	1	0	0	0	0	1	0	1	(0.5)	1	(0.5)	0.00	NT	NT
TMB	0.080	24	187	0	1	1	0	0	0	2	0	2	(1.1)	2	(1.1)	0.00[6]		
MC	0.00005	24	200	6	34	76	6	1	0	123	1	74	(37.0)	71	(35.5)	0.00		
Solvent[1]	0	48	200	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00		
TMB	0.020	48	200	5	1	0	0	0	0	6	0	6	(3.0)	1	(0.5)	0.13		
TMB	0.040	48	200	2	1	0	0	0	0	3	0	2	(1.0)	1	(0.5)	0.13	NT	NT
TMB	0.080	48	68	0	0	0	0	0	0	0	0	0	(0.0)	0	(0.0)	0.00[7]T		
MC	0.00005	48	200	7	25	76	1	8	0	117	4	76	(38.0)	72	(36.0)	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, 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 different from historical solvent control at $p<0.05$ by Fisher's exact test. 6) Seven hundred and eleven cells were analysed. 7) One hundred and twelve cells were analysed. *: Purity was 98.75%.

Table 2 Chromosome analysis of Chinese hamster cells (CHL/IU) treated with 1,2,4-trimethylbenzene (TMB)* 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	1	0	0	0	0	1	0	1	(0.5)	1 (0.5)	0.00		
Solvent[1]	0	-	6-(18)	200	0	1	0	0	2	0	3	0	3	(1.5)	3 (1.5)	0.13		
TMB	0.038	-	6-(18)	200	1	1	0	0	0	0	2	0	2	(1.0)	1 (0.5)	0.38		
TMB	0.075	-	6-(18)	200	0	0	0	0	1	0	1	0	1	(0.5)	1 (0.5)	0.13	NT	NT
TMB	0.15	-	6-(18)	58T	0	0	0	0	0	0	0	0	0	(0.0)	0 (0.0)	0.00[6]T		
CPA	0.005	-	6-(18)	200	1	1	0	0	1	0	3	0	3	(1.5)	2 (1.0)	0.13		
Solvent[1]	0	+	6-(18)	200	3	2	0	0	1	0	6	0	6	(3.0)	3 (1.5)	0.00		
TMB	0.025	+	6-(18)	200	3	0	0	0	0	0	3	0	3	(1.5)	0 (0.0)	0.50		
TMB	0.050	+	6-(18)	200	5	1	1	0	0	0	7	0	6	(3.0)	2 (1.0)	0.13	NT	NT
TMB	0.10	+	6-(18)	196	2	3	0	0	0	0	5	2	4	(2.0)	2 (1.0)	0.38[7]		
CPA	0.005	+	6-(18)	200	9	40	127	7	4	10	197	1	96	(48.0)	91 (45.5)	0.25		

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, 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 different from historical solvent control at $p<0.05$ by Fisher's exac test 7) Seven hundred and eighty eight cells were analysed. *: Purity was 98.75%.

文献

1) 日本環境変異原学会・哺乳動物試験分科会編，“化学物質による染色体異常アトラス，” 朝倉書店，東京，1988.
2) 林　真，変異原性試験，1，255(1992).
3) 吉村　功　編著，“毒性・薬効データの統計解析，事例研究によるアプローチ，” サイエンティスト社，東京，1987.
4) 吉村　功，大橋靖夫　編，“毒性試験講座14，毒性試験データの統計解析，” 地人書館，東京，1992.

連絡先

試験責任者：田中憲穂

試験担当者：山影康次，中川ゆづき，日下部博一，橋本恵子，長尾哲二，太田　亮

(財)食品薬品安全センター秦野研究所

〒257　神奈川県秦野市落合729-5

Tel 0463-82-4751　Fax 0463-82-9627

Correspondence

Authors: Noriho Tanaka (Study director)
Kohji Yamakage, Yuzuki Nakagawa, Hirokazu Kusakabe, Keiko Hashimoto, Tetsuji Nagao, Ryo Ohta

Hatano Research Institute, Food and Drug Safety Center

729-5 Ochiai, Hadano, Kanagawa, 257, Japan

Tel +81-463-82-4751　Fax +81-463-82-9627

4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)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 4,4'-Thiobis(6-*tert*-butyl-*m*-cresol) in Rats

要約

既存化学物質の毒性評価の一環として，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)の0(5%アラビアゴム水溶液)，15，60，および250 mg/kgをSD系ラットに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群については，別に14日間の回復群を設けた．

被験物質投与に起因する一般状態の変化はみられず，体重への影響もみられなかった．

摂餌量では，投与開始初期に一時的な減少が250 mg/kg群の雌雄でみられたが，雄ではその後対照群をやや上回って推移した．60 mg/kg群の雄の摂餌量も対照群をやや上回って推移した．尿検査では，pHの低下が60 mg/kg群の雌と250 mg/kg群の雌雄に，尿蛋白およびケトン体の増加が60および250 mg/kg群の雌に，血液学検査では，血小板数の増加が250 mg/kg群の雌雄に，分葉核好中球比率の増加およびリンパ球比率の減少が250 mg/kg群の雌に，血液生化学検査では，無機リンの増加が60 mg/kg以上の投与群の雌に，総コレステロールの増加が250 mg/kg群の雌雄に，リン脂質および尿素窒素の増加と血糖値の低下が250 mg/kg群の雌にみられた．

病理学検査では，250 mg/kg群の雌雄に肝臓重量の増加，肉眼的な小腸壁の肥厚と盲腸の拡張がみられた．組織学的には，肝臓で小葉中心帯肝細胞の肥大と回腸における腸絨毛の過形成が250 mg/kg群の雌雄に，盲腸と結腸で吸収上皮細胞の空胞化が60 mg/kg以上の投与群の雌雄に，また，盲腸粘膜の細胞浸潤が60 mg/kg以上の投与群の雌雄に，結腸粘膜の細胞浸潤が60 mg/kg以上の投与群の雌にみられた．さらに，腸間膜リンパ節では，傍皮質領域における“tingible body macrophage”が250 mg/kg群の雌に多くみられた．回復群においては，回腸以外のほとんどの変化は消失した．

以上の結果から，本試験条件下における4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)の無影響量は雌雄とも15 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4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)は，分子量358.54，融点160～165℃，水に不溶，アセトンおよびメタノールに可溶の白色結晶性粉末である．本試験にはロット番号40701(住友化学工業㈱製)，純度98%以上のものを用いた．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5 ml/kg体重となるよう，5%アラビアゴム水溶液に懸濁して0.3，1.2および5%(w/v)懸濁液を調製した．0.1～5%(w/v)懸濁液は，室温で1日間および冷蔵(約4℃)・暗所(褐色ガラス瓶)で8日間まで安定であったことから，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て冷蔵庫(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測定した結果，いずれも濃度は適正でかつ均一であった．

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42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193～222 g(平均値：208.0 g)，雌で148～171 g(平均値：157.4 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群で多数例が死亡し，250 mg/kg群ではGOTおよびGPTの上昇，血糖値の低下，盲腸の拡張などがみられた．一方，60 mg/kg以下の投与群では変化はみられなかった．これらの成績から，本試験では250，60および15 mg/kgの3用量を設定し，これに対照群を加えて計4群を使用した．さらに，対照群，60および250 mg/kg群では回復群を設けた．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(5%アラビアゴム水溶液)を同様に投与した．投

与液量は最新の体重を基準に算出した．回復期間は14日間とした．

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AIP(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(DTNB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，このうち対照群と高用量群は包埋した全ての器官・組織について，また，中および低用量群は被験物質投与による変化が疑われた小腸(十二指腸～回腸)，大腸(盲腸～直腸)，肝臓および腸間膜リンパ節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腸管，肝臓および腸間膜リンパ節について全動物を検査した．

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[17]．

結果

1. 一般状態

死亡はみられず，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

雌雄ともに，各投与群の体重は対照群と同様に推移した．

2) 回復期間

雌雄ともに，各投与群の体重は対照群と同様に推移した．

3. 摂餌量

1) 投与期間

雄では，250 mg/kg群で投与4日に対照群を有意に下回った．しかし，その後は対照群をやや上回って推移し，有意差もみられた．また，60 mg/kg群でも対照群をやや上回って推移し，有意差もみられた．雌では，250 mg/kg群で投与4日に対照群を有意に下回った．しかし，その後は対照群と同様に推移した．

2) 回復期間

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．

4. 血液学検査(Table 1)

1) 投与終了時

雄では，250 mg/kg群で血小板数の有意な増加がみられた．他に，プロトロンビン時間の有意な短縮が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，250 mg/kg群で血小板数および分葉核好中球比率の有意な増加とリンパ球比率の有意な減少がみられた．

2) 回復終了時

雌雄ともに，250 mg/kg群で血小板数の有意な増加がみられた．

5. 血液生化学検査(Table 2)

1) 投与終了時

雄では，250 mg/kg群で総コレステロールの有意な増加がみられた．雌では，60 mg/kg以上の投与群で無機

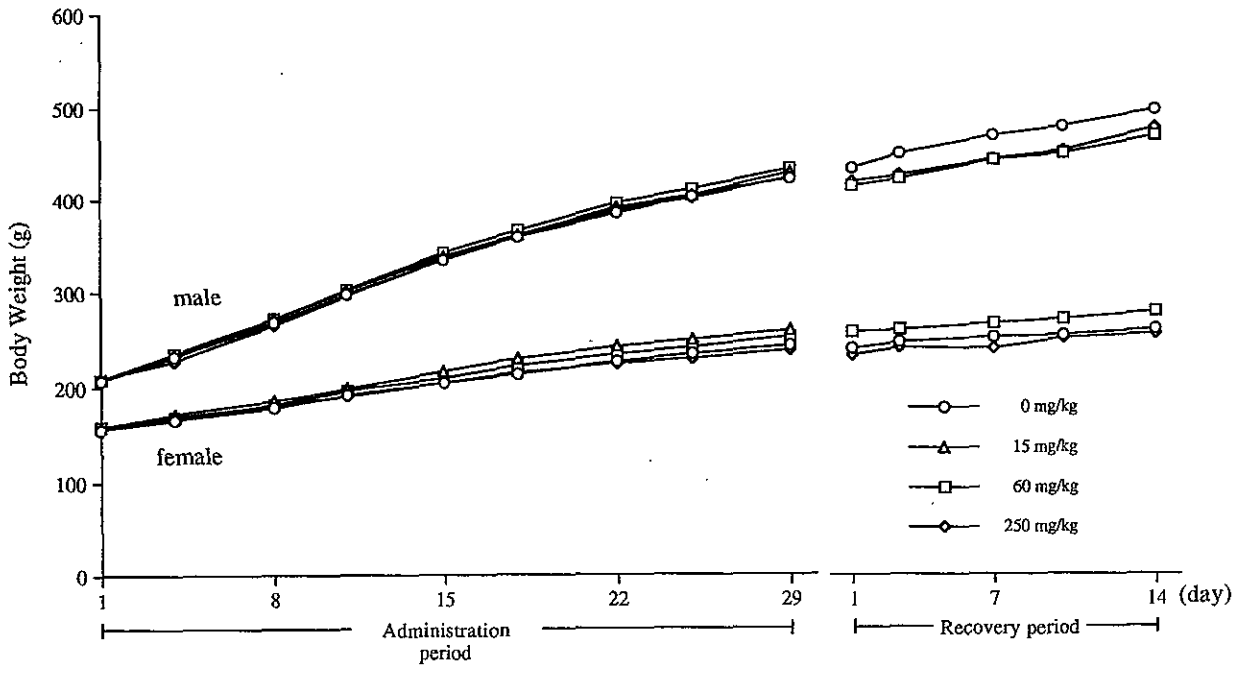


Fig. 1 Body weight changes of rats treated orally with 4,4'-thiobis(6-*tert*-butyl-*m*-cresol) in the twenty-eight-day repeated dose toxicity test

Table 1 Hematology of rats treated orally with 4,4'-thiobis(6-*tert*-butyl-*m*-cresol) in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)		
	0	15	60	250	0	60	250
Male							
No. of animals	6	6	6	6	6	6	6
RBC ($\times 10^4$/mm^3)	800 ± 38	791 ± 28	790 ± 38	793 ± 27	806 ± 21	812 ± 28	801 ± 15
Hb (g/l)	16.0 ± 0.4	15.9 ± 0.4	15.6 ± 0.4	15.9 ± 0.7	15.8 ± 0.4	15.9 ± 0.3	15.4 ± 0.4
Ht (%)	48 ± 2	48 ± 1	47 ± 2	48 ± 2	47 ± 1	47 ± 1	46 ± 1
MCV (μ^3)	59.8 ± 1.9	60.4 ± 0.6	59.8 ± 2.1	60.0 ± 0.9	58.1 ± 1.0	58.1 ± 2.4	57.6 ± 2.5
MCH (pg)	20.0 ± 0.7	20.1 ± 0.5	19.8 ± 0.7	20.0 ± 0.3	19.6 ± 0.2	19.6 ± 0.8	19.3 ± 0.8
MCHC (%)	33.5 ± 0.4	33.2 ± 0.6	33.2 ± 0.4	33.3 ± 0.4	33.8 ± 0.4	33.8 ± 0.4	33.4 ± 0.3
Reticulocyte (‰)	20 ± 3	22 ± 3	21 ± 3	22 ± 6	21 ± 5	20 ± 4	23 ± 4
Platelet ($\times 10^4$/mm^3)	118.0 ± 7.3	122.2 ± 8.1	116.5 ± 11.9	135.0 ± 9.2*	107.4 ± 3.6	109.3 ± 7.4	118.8 ± 9.2*
WBC ($\times 10^2$/mm^3)	95 ± 34	105 ± 39	96 ± 28	97 ± 37	92 ± 18	99 ± 24	97 ± 8
Differential leukocyte counts (%)							
Lymph	84.5 ± 5.5	88.3 ± 4.1	81.1 ± 2.2	87.8 ± 3.6	79.9 ± 9.2	84.0 ± 4.8	80.3 ± 8.9
Stab	0.1 ± 0.2	0.1 ± 0.2	0.2 ± 0.3	0.3 ± 0.3	0.2 ± 0.3	0.1 ± 0.2	0.0 ± 0.0
Seg	14.8 ± 5.6	10.7 ± 4.7	17.8 ± 1.9	11.5 ± 3.4	18.7 ± 8.6	14.9 ± 4.3	18.7 ± 9.0
Eosino	0.4 ± 0.6	0.9 ± 0.8	0.8 ± 1.0	0.3 ± 0.4	0.9 ± 0.4	0.6 ± 0.5	0.7 ± 0.6
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
Mono	0.2 ± 0.3	0.1 ± 0.2	0.1 ± 0.2	0.3 ± 0.3	0.3 ± 0.4	0.4 ± 0.4	0.3 ± 0.4
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
PT (Sec.)	12.8 ± 1.1	11.8 ± 0.8	11.4 ± 0.5*	13.6 ± 1.3	12.3 ± 1.5	12.5 ± 1.1	12.1 ± 0.8
APTT (Sec.)	19.4 ± 1.8	18.0 ± 1.2	17.3 ± 1.4	21.3 ± 1.8	19.8 ± 2.4	19.0 ± 1.4	18.9 ± 1.8
Female							
No. of animals	6	6	6	6	6	6	6
RBC ($\times 10^4$/mm^3)	753 ± 35	760 ± 36	724 ± 26	758 ± 49	812 ± 46	810 ± 27	808 ± 19
Hb (g/l)	15.5 ± 0.5	16.1 ± 0.6	15.3 ± 0.4	15.8 ± 0.8	15.6 ± 0.3	15.8 ± 0.5	15.5 ± 0.2
Ht (%)	45 ± 2	45 ± 2	43 ± 1	44 ± 2	46 ± 1	47 ± 2	46 ± 1
MCV (μ^3)	59.2 ± 1.4	59.8 ± 1.2	59.5 ± 1.2	58.5 ± 1.0	56.8 ± 2.3	57.3 ± 0.5	57.1 ± 0.8
MCH (pg)	20.6 ± 0.6	21.2 ± 0.5	21.2 ± 0.5	20.8 ± 0.4	19.2 ± 0.9	19.4 ± 0.2	19.2 ± 0.4
MCHC (%)	34.8 ± 0.9	35.4 ± 0.4	35.6 ± 0.4	35.5 ± 0.2	33.9 ± 0.4	33.9 ± 0.4	33.6 ± 0.3
Reticulocyte (‰)	22 ± 4	17 ± 5	19 ± 3	19 ± 4	20 ± 2	23 ± 5	22 ± 5
Platelet ($\times 10^4$/mm^3)	114.6 ± 8.7	120.6 ± 11.6	116.2 ± 6.3	136.6 ± 9.2**	113.3 ± 10.8	112.4 ± 8.3	128.1 ± 7.2*
WBC ($\times 10^2$/mm^3)	66 ± 10	76 ± 18	86 ± 23	93 ± 37	84 ± 32	100 ± 23	80 ± 26
Differential leukocyte counts (%)							
Lymph	86.0 ± 3.1	84.3 ± 3.2	83.2 ± 6.8	73.0 ± 9.0**	79.2 ± 9.0	88.6 ± 2.2	85.4 ± 8.0
Stab	0.0 ± 0.0	0.2 ± 0.3	0.2 ± 0.3	0.3 ± 0.4	0.2 ± 0.3	0.3 ± 0.4	0.3 ± 0.3
Seg	13.2 ± 3.2	14.1 ± 2.4	15.4 ± 6.3	24.4 ± 7.7**	19.6 ± 8.8	9.9 ± 1.7	13.3 ± 7.9
Eosino	0.7 ± 0.3	1.2 ± 0.9	1.2 ± 0.7	1.9 ± 1.3	0.8 ± 0.6	1.0 ± 0.8	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
Mono	0.2 ± 0.3	0.3 ± 0.3	0.1 ± 0.2	0.3 ± 0.4	0.3 ± 0.3	0.2 ± 0.3	0.3 ± 0.3
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
PT (Sec.)	10.6 ± 0.5	10.9 ± 0.3	10.5 ± 0.7	10.0 ± 0.3	10.7 ± 0.5	10.5 ± 0.6	10.8 ± 0.3
APTT (Sec.)	14.0 ± 0.9	13.7 ± 0.5	14.1 ± 1.4	14.3 ± 1.6	16.6 ± 1.4	16.3 ± 1.9	16.4 ± 1.2

Values are expressed as Mean ± S.D.
Significant difference from control group; *: $P<0.05$ **: $P<0.01$

Table 2 Blood chemistry of rats treated orally with 4,4'-thiobis(6-*tert*-butyl-*m*-cresol) in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)		
	0	15	60	250	0	60	250
Male							
No. of animals	6	6	6	6	6	6	6
GOT (IU/*l*)	54 ± 9	57 ± 10	53 ± 5	60 ± 5	56 ± 11	57 ± 16	61 ± 17
GPT (IU/*l*)	36 ± 6	34 ± 4	35 ± 5	36 ± 9	41 ± 4	36 ± 8	33 ± 5
LDH (IU/*l*)	26 ± 5	25 ± 3	24 ± 6	27 ± 8	33 ± 9	25 ± 6	28 ± 12
AlP (IU/*l*)	289 ± 61	297 ± 38	312 ± 65	326 ± 69	232 ± 67	247 ± 56	244 ± 25
γ-GTP (IU/*l*)	1.5 ± 0.7	1.4 ± 0.5	1.5 ± 0.3	1.5 ± 0.4	1.7 ± 0.2	1.7 ± 0.3	1.8 ± 0.2
ChE (IU/*l*)	672 ± 39	707 ± 92	690 ± 74	787 ± 95	693 ± 89	802 ± 229	650 ± 52
TP (g/d*l*)	6.0 ± 0.2	6.0 ± 0.2	5.8 ± 0.2	5.7 ± 0.3	6.3 ± 0.1	6.2 ± 0.2	6.2 ± 0.2
Albumin (g/d*l*)	3.5 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.4 ± 0.2	3.4 ± 0.1
A/G (%)	1.39 ± 0.12	1.32 ± 0.09	1.41 ± 0.09	1.41 ± 0.13	1.19 ± 0.08	1.24 ± 0.13	1.22 ± 0.10
T. cho (mg/d*l*)	59 ± 13	62 ± 12	62 ± 15	81 ± 9*	66 ± 15	65 ± 14	67 ± 14
TG (mg/d*l*)	74 ± 23	94 ± 20	97 ± 22	77 ± 33	113 ± 36	110 ± 54	88 ± 38
PL (mg/d*l*)	113 ± 22	114 ± 15	122 ± 22	132 ± 11	125 ± 21	125 ± 26	123 ± 18
T. bilirubin (mg/d*l*)	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.02	0.16 ± 0.07	0.13 ± 0.02	0.13 ± 0.02	0.12 ± 0.01
Glucose (mg/d*l*)	119 ± 22	118 ± 8	124 ± 10	106 ± 11	139 ± 15	127 ± 11	122 ± 4*
BUN (mg/d*l*)	12 ± 1	12 ± 1	13 ± 2	13 ± 1	14 ± 2	13 ± 1	16 ± 2
Creatinine (mg/d*l*)	0.57 ± 0.03	0.58 ± 0.03	0.58 ± 0.02	0.56 ± 0.03	0.56 ± 0.05	0.60 ± 0.05	0.62 ± 0.06
Na (mEq/*l*)	142 ± 1	142 ± 1	142 ± 1	142 ± 1	145 ± 1	145 ± 1	145 ± 1
K (mEq/*l*)	4.8 ± 0.4	4.6 ± 0.3	4.4 ± 0.3	4.6 ± 0.2	4.8 ± 0.3	4.7 ± 0.3	4.7 ± 0.3
Cl (mEq/*l*)	110 ± 1	110 ± 1	110 ± 1	110 ± 1	111 ± 1	112 ± 1	111 ± 2
Ca (mg/d*l*)	9.3 ± 0.2	9.2 ± 0.2	9.2 ± 0.3	9.3 ± 0.3	9.0 ± 0.2	8.9 ± 0.1	9.0 ± 0.2
P (mg/d*l*)	9.2 ± 0.5	9.2 ± 0.5	9.2 ± 0.5	9.8 ± 0.2	7.8 ± 0.5	7.8 ± 0.4	8.4 ± 0.4
Female							
No. of animals	6	6	6	6	6	6	6
GOT (IU/*l*)	63 ± 10	59 ± 8	64 ± 4	57 ± 8	64 ± 5	69 ± 7	66 ± 11
GPT (IU/*l*)	36 ± 7	33 ± 3	35 ± 3	37 ± 9	34 ± 5	35 ± 8	31 ± 4
LDH (IU/*l*)	24 ± 17	20 ± 6	22 ± 7	26 ± 6	23 ± 6	24 ± 5	27 ± 10
AlP (IU/*l*)	192 ± 34	185 ± 26	198 ± 71	186 ± 25	142 ± 36	147 ± 32	131 ± 30
γ-GTP (IU/*l*)	1.7 ± 0.7	1.9 ± 0.7	1.5 ± 0.4	1.4 ± 0.8	1.8 ± 0.4	1.8 ± 0.6	1.3 ± 0.6
ChE (IU/*l*)	2610 ± 663	2268 ± 695	1857 ± 508	1832 ± 592	2410 ± 703	2665 ± 927	2275 ± 473
TP (g/d*l*)	6.4 ± 0.5	6.2 ± 0.3	6.1 ± 0.3	6.5 ± 0.4	6.7 ± 0.2	7.1 ± 0.4	6.9 ± 0.3
Albumin (g/d*l*)	3.7 ± 0.2	3.7 ± 0.2	3.5 ± 0.2	3.7 ± 0.3	3.6 ± 0.2	3.7 ± 0.1	3.7 ± 0.2
A/G (%)	1.36 ± 0.10	1.48 ± 0.10	1.35 ± 0.10	1.30 ± 0.08	1.18 ± 0.09	1.12 ± 0.10	1.12 ± 0.08
T. cho (mg/d*l*)	70 ± 14	68 ± 8	84 ± 16	101 ± 15**	62 ± 12	88 ± 18*	63 ± 15
TG (mg/d*l*)	32 ± 4	37 ± 9	36 ± 5	54 ± 28	39 ± 10	42 ± 7	36 ± 14
PL (mg/d*l*)	133 ± 24	134 ± 15	161 ± 30	192 ± 39**	138 ± 23	178 ± 20*	136 ± 30
T. bilirubin (mg/d*l*)	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.48 ± 0.38	0.13 ± 0.02	0.13 ± 0.02	0.14 ± 0.02
Glucose (mg/d*l*)	107 ± 12	104 ± 9	107 ± 9	83 ± 16**	108 ± 14	120 ± 18	105 ± 8
BUN (mg/d*l*)	14 ± 2	15 ± 1	13 ± 1	17 ± 3*	15 ± 2	14 ± 2	15 ± 2
Creatinine (mg/d*l*)	0.60 ± 0.06	0.60 ± 0.06	0.58 ± 0.04	0.63 ± 0.05	0.59 ± 0.04	0.61 ± 0.08	0.60 ± 0.05
Na (mEq/*l*)	141 ± 1	141 ± 1	140 ± 1	140 ± 2	142 ± 1	142 ± 0	142 ± 1
K (mEq/*l*)	4.8 ± 0.4	5.1 ± 0.2	5.0 ± 0.2	5.2 ± 0.6	5.1 ± 0.3	5.1 ± 0.3	5.2 ± 0.3
Cl (mEq/*l*)	112 ± 2	111 ± 1	112 ± 2	114 ± 2	114 ± 1	114 ± 1	114 ± 1
Ca (mg/d*l*)	9.0 ± 0.1	9.1 ± 0.2	8.9 ± 0.2	9.2 ± 0.4	9.2 ± 0.3	9.2 ± 0.2	9.1 ± 0.2
P (mg/d*l*)	8.2 ± 0.7	8.6 ± 0.6	9.2 ± 0.5*	9.2 ± 0.8*	8.2 ± 0.3	7.9 ± 0.7	8.3 ± 1.0

Values are expressed as Mean ± S.D.
Significant difference from control group; *: $P<0.05$ **: $P<0.01$

リンの有意な増加がみられたほか，250 mg/kg群で総コレステロール，リン脂質および尿素窒素の有意な増加と血糖値の有意な低下がみられた．

2) 回復終了時

雄では，血糖の有意な低下が250 mg/kg群でみられた．雌では，総コレステロールとリン脂質の有意な増加が60 mg/kg群でみられたが，用量に関連した変化ではなかった．

6. 尿検査(Table 3)

1) 投与第4週

雄では，250 mg/kg群に尿pHの低下傾向と尿沈渣中のリン酸塩の増加傾向がみられた．雌では，60 mg/kg以上の投与群で尿pHの低下傾向，尿蛋白，ケトン体および尿沈渣中のリン酸塩の増加傾向がみられた．

2) 回復第2週

雄では，250 mg/kg群に尿蛋白の増加傾向がみられた．雌では，250 mg/kg群で尿pHの低下傾向がみられた．

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250 mg/kg群の雌雄にみられた．

2) 回復終了時剖検例

心臓で，相対重量の有意な増加が雄の各投与群にみられた．

他に，脳の絶対重量の有意な増加が雌の60 mg/kg群にみられたが，用量と関連した変化ではなかった．

8. 剖検所見(Table 5)

1) 投与終了時剖検例

盲腸の拡張が250 mg/kg群の雄5例と雌4例に，小腸壁の肥厚が250 mg/kg群の雌雄各4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小腸，大腸，肝臓および腸間膜リンパ節にみられた．

回腸：腸絨毛のごく軽度な過形成が250 mg/kg群の雄1例と雌3例にみられた．本所見は，腸絨毛の丈の高さの増大として観察され，組織構築の変化あるいは炎症反応は認められなかった．

盲腸：吸収上皮細胞のごく軽度から軽度な空胞化が60 mg/kg群の雄3例と雌1例，250 mg/kg群の雌雄各5例にみられた．また，粘膜におけるごく軽度から軽度な細胞浸潤が60 mg/kg群の雄5例と雌1例，250 mg/kg群の雌雄各3例にみられたが，吸収上皮細胞の空胞化を示す個体と細胞浸潤を示す個体とは必ずしも一致していなかった．

結腸：吸収上皮細胞のごく軽度な空胞化が60 mg/kg群の雌雄各1例，250 mg/kg群の雄1例と雌2例にみられた．また，粘膜におけるごく軽度な細胞浸潤が雌の60および250 mg/kg群でそれぞれ1および2例にみられた．

直腸：粘膜におけるごく軽度な細胞浸潤が250 mg/kg群の雌雄各1例にみられた．

肝臓：小葉中心帯肝細胞のごく軽度な肥大が250 mg/kg群の雌雄各5例にみられた．

腸間膜リンパ節：傍皮質領域において，核崩壊物を容れたマクロファージ“tingible body macrophage”の出現が，対照群で雄1例(ごく軽度)，15 mg/kg群で雌雄各1例(いずれもごく軽度)，60 mg/kg群で雄1例と雌2例(いずれもごく軽度)にみられたのに対し，250 mg/kg群では雄で2例(1例がごく軽度，残る1例が軽度)と雌で5例(4例がごく軽度，残る1例が軽度)にみられ，250 mg/kg群の雌で例数がやや多かった．

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

回腸：腸絨毛のごく軽度から軽度な過形成が，250 mg/kg群の雌雄各2例にみられた．

盲腸：吸収上皮細胞の変化は認められなかった．なお，粘膜におけるごく軽度から軽度な細胞浸潤が対照群で雌雄各2例，60 mg/kg群で雄2例と雌1例，250 mg/kg群で雄3例と雌4例にみられた．

直腸：粘膜におけるごく軽度な細胞浸潤が60 mg/kg群の雌1例，250 mg/kg群の雄1例にみられた．

肝臓：肝細胞の肥大は認められなかった．なお，ごく軽度な微小肉芽腫が対照群の雌雄各1例，250 mg/kg群の雄1例にみられた．

十二指腸，空腸，結腸および腸間膜リンパ節には異常所見は認められなかった．

考察

試験期間を通じて死亡はみられず，また，一般状態および体重にも被験物質投与による変化は認められなかった．

摂餌量では，250 mg/kg群の雌雄で投与開始4日に減少を示したが，雄ではその後対照群をやや上回った．60 mg/kg群でも雄の摂餌量は対照群をやや上回った．これらの投与群の体重は，対照群とほぼ同様であったことから，食餌効率は低下しているものと考えられ，後述の腸管に対する障害がその要因と推察される．

尿検査では，60 mg/kg以上の投与群で尿pHの低下，尿蛋白およびケトン体の増加が主として雌にみられた．同時に，血液生化学検査において，雌で尿素窒素や無機

Table 3 Urinalysis of rats treated orally with 4,4'-thiobis(6-*tert*-butyl-*m*-cresol) in the twenty-eight-day repeated dose toxicity test

Item		28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)		
		0	15	60	250	0	60	250
Male								
No. of animals		6	6	6	6	6	6	6
Volume (ml)		9.9 ± 3.6	9.3 ± 5.3	12.9 ± 5.4	13.5 ± 5.0	10.4 ± 3.3	9.2 ± 6.5	8.9 ± 6.0
Specific gravity		1.072 ± 0.012	1.073 ± 0.018	1.063 ± 0.015	1.058 ± 0.011	1.079 ± 0.019	1.074 ± 0.017	1.078 ± 0.019
Water intake (ml)		40 ± 8	41 ± 6	45 ± 4	51 ± 12	44 ± 5	41 ± 8	42 ± 4
pH	7.5	0	0	0	1	0	0	0
	8	0	0	0	2	0	0	0
	8.5	1	4	2	1	0	0	1
	9	5	2	4	2	6	6	5
Protein	−	1	1	0	1	0	1	0
	−/+	4	3	3	1	3	0	2
	1+	1	1	2	3	3	5	1
	2+	0	1	1	1	0	0	3
Ketons	−	6	5	5	6	6	5	4
	−/+	0	1	1	0	0	1	1
	1+	0	0	0	0	0	0	1
Glucose	−	6	6	6	6	6	6	6
Occult blood	−	4	5	5	5	3	4	6
	−/+	1	1	0	0	1	1	0
	1+	1	0	0	0	1	1	0
	2+	0	0	1	0	1	0	0
	3+	0	0	0	1	0	0	0
Bilirubin	−	6	6	6	6	6	6	6
Urobilinogen	−/+	6	6	6	6	6	6	4
	1+	0	0	0	0	0	0	2
Color	Yellow	6	6	6	5	6	6	6
	Dark yellow	0	0	0	1	0	0	0
RBC	−	6	6	6	5	6	6	6
	−/+	0	0	0	0	0	0	0
	1+	0	0	0	0	0	0	0
	2+	0	0	0	1	0	0	0
WBC	−	6	6	6	6	6	6	6
SEC	−	0	0	0	0	0	0	0
	−/+	6	6	6	6	6	6	6
	3+	0	0	0	0	0	0	0
SREC	−	6	6	6	6	6	6	6
Cast	−	6	6	6	6	6	6	6
PS	−	6	4	5	3	3	4	4
	−/+	0	2	1	2	3	2	2
	1+	0	0	0	1	0	0	0
Co	−	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

Table 3 (Continued)

Item		28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)		
		0	15	60	250	0	60	250
Female								
No. of animals		6	6	6	6	6	6	6
Volume (ml)		8.4±3.0	10.2 ± 7.1	4.8 ± 2.4	5.6 ± 3.7	6.1 ± 3.2	7.2 ± 3.6	6.6 ± 4.1
Specific gravity		1.070 ± 0.018	1.060 ± 0.016	1.079 ± 0.018	1.066 ± 0.026	1.068 ± 0.013	1.066 ± 0.018	1.072 ± 0.023
Water intake (ml)		35 ± 7	41 ± 8	39 ± 25	41 ± 12	30 ± 7	31 ± 8	42 ± 12
pH	5.5	0	0	0	1	0	0	0
	6	0	1	0	1	0	0	0
	6.5	0	0	3	1	0	0	1
	7	0	1	1	0	0	0	1
	7.5	1	0	0	2	1	0	1
	8	2	2	1	0	0	1	1
	8.5	2	2	0	1	3	2	2
	9	1	0	1	0	2	3	0
Protein	−	5	4	2	1	3	0	1
	−/+	1	1	0	0	1	4	3
	1+	0	1	2	4	1	0	2
	2+	0	0	2	1	1	2	0
Ketons	−	6	5	3	2	5	6	5
	−/+	0	1	2	3	1	0	1
	1+	0	0	1	1	0	0	0
Glucose	−	6	6	6	6	6	6	6
Occult blood	−	5	5	6	4	6	6	6
	−/+	0	0	0	1	0	0	0
	1+	1	1	0	0	0	0	0
	2+	0	0	0	0	0	0	0
	3+	0	0	0	1	0	0	0
Bilirubin	−	6	6	6	6	6	6	6
Urobilinogen	−/+	6	6	6	6	5	6	6
	1+	0	0	0	0	1	0	0
Color	Yellow	6	6	6	4	6	6	6
	Dark yellow	0	0	0	2	0	0	0
RBC	−	6	6	6	6	6	6	6
WBC	−	6	6	6	6	6	6	6
SEC	−	0	0	0	0	0	0	0
	−/+	6	6	6	6	6	6	6
SREC	−	6	6	5	6	6	6	6
	−/+	0	0	1	0	0	0	0
Cast	−	6	6	6	6	6	6	6
PS	−	6	5	4	3	1	3	1
	−/+	0	1	2	3	5	3	4
	1+	0	0	0	0	0	0	1
Co	−	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

Table 4 Absolute and relative organ weights of rats treated orally with 4,4'-thiobis(6-*tert*-butyl-*m*-cresol) in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)		
	0	15	60	250	0	60	250
Male							
No. of animals	6	6	6	6	6	6	6
Body weight (g)	375 ± 19	401 ± 26	415 ± 42	378 ± 18	475 ± 35	447 ± 40	455 ± 17
Absolute organ weight							
Brain (g)	2.01 ± 0.07	2.04 ± 0.05	2.03 ± 0.05	2.00 ± 0.03	2.10 ± 0.04	2.11 ± 0.12	2.12 ± 0.05
Thymus (mg)	535 ± 74	647 ± 105	553 ± 77	464 ± 76	468 ± 78	475 ± 136	489 ± 52
Heart (g)	1.27 ± 0.10	1.32 ± 0.11	1.32 ± 0.09	1.31 ± 0.20	1.43 ± 0.09	1.41 ± 0.11	1.44 ± 0.07
Lung (g)	1.29 ± 0.03	1.33 ± 0.12	1.39 ± 0.11	1.35 ± 0.07	1.47 ± 0.09	1.46 ± 0.11	1.50 ± 0.09
Liver (g)	11.65 ± 1.08	12.63 ± 1.30	13.07 ± 1.94	13.78 ± 1.64	14.63 ± 1.59	13.43 ± 1.26	14.67 ± 0.85
Spleen (g)	0.69 ± 0.07	0.72 ± 0.12	0.70 ± 0.11	0.73 ± 0.04	0.80 ± 0.13	0.73 ± 0.15	0.74 ± 0.12
Kidneys (g)	2.87 ± 0.22	2.96 ± 0.25	3.02 ± 0.27	2.98 ± 0.27	3.24 ± 0.20	3.09 ± 0.26	3.25 ± 0.18
Adrenals (mg)	60 ± 8	67 ± 11	63 ± 6	61 ± 9	64 ± 7	66 ± 5	65 ± 10
Testes (g)	3.15 ± 0.19	3.35 ± 0.18	3.27 ± 0.29	3.29 ± 0.28	3.36 ± 0.24	3.47 ± 0.61	3.22 ± 0.11
Relative organ weight							
Brain (%)	0.54 ± 0.03	0.51 ± 0.03	0.49 ± 0.05	0.53 ± 0.02	0.44 ± 0.03	0.47 ± 0.05	0.47 ± 0.02
Thymus (%)	143 ± 19	162 ± 26	134 ± 19	123 ± 20	99 ± 19	107 ± 29	108 ± 11
Heart (%)	0.34 ± 0.02	0.33 ± 0.02	0.32 ± 0.03	0.35 ± 0.05	0.30 ± 0.01	0.32 ± 0.01*	0.32 ± 0.01*
Lung (%)	0.35 ± 0.02	0.33 ± 0.01	0.33 ± 0.02	0.36 ± 0.01	0.31 ± 0.01	0.33 ± 0.02	0.33 ± 0.01
Liver (%)	3.10 ± 0.14	3.15 ± 0.13	3.14 ± 0.22	3.64 ± 0.33**	3.07 ± 0.16	3.02 ± 0.31	3.23 ± 0.15
Spleen (%)	0.18 ± 0.02	0.18 ± 0.02	0.17 ± 0.03	0.19 ± 0.02	0.17 ± 0.02	0.16 ± 0.02	0.16 ± 0.02
Kidneys (%)	0.77 ± 0.03	0.74 ± 0.05	0.73 ± 0.03	0.79 ± 0.07	0.69 ± 0.04	0.69 ± 0.06	0.72 ± 0.04
Adrenals (%)	16 ± 2	17 ± 3	15 ± 2	16 ± 2	14 ± 2	15 ± 2	14 ± 3
Testes (%)	0.84 ± 0.05	0.84 ± 0.06	0.80 ± 0.13	0.87 ± 0.09	0.71 ± 0.08	0.78 ± 0.14	0.71 ± 0.05
Female							
No. of animals	6	6	6	6	6	6	6
Body weight (g)	228 ± 12	243 ± 15	227 ± 8	220 ± 12	249 ± 29	264 ± 29	238 ± 17
Absolute organ weight							
Brain (g)	1.84 ± 0.06	1.86 ± 0.05	1.84 ± 0.07	1.86 ± 0.06	1.86 ± 0.04	1.99 ± 0.15*	1.88 ± 0.08
Thymus (mg)	420 ± 108	447 ± 68	434 ± 87	460 ± 97	341 ± 50	384 ± 57	339 ± 85
Heart (g)	0.85 ± 0.08	0.84 ± 0.05	0.83 ± 0.04	0.78 ± 0.04	0.86 ± 0.09	0.94 ± 0.11	0.84 ± 0.04
Lung (g)	1.09 ± 0.05	1.07 ± 0.10	1.06 ± 0.08	1.04 ± 0.07	1.08 ± 0.08	1.11 ± 0.09	1.05 ± 0.10
Liver (g)	6.75 ± 0.76	6.86 ± 0.34	6.87 ± 0.58	7.65 ± 0.86	6.84 ± 0.93	7.49 ± 1.08	7.00 ± 0.65
Spleen (g)	0.51 ± 0.06	0.49 ± 0.03	0.47 ± 0.04	0.50 ± 0.05	0.51 ± 0.07	0.52 ± 0.08	0.50 ± 0.04
Kidneys (g)	1.72 ± 0.21	1.86 ± 0.11	1.84 ± 0.13	1.74 ± 0.14	1.81 ± 0.14	1.87 ± 0.22	1.74 ± 0.16
Adrenals (mg)	72 ± 8	75 ± 8	76 ± 10	74 ± 5	70 ± 6	76 ± 5	78 ± 10
Ovaries (mg)	81.3 ± 9.8	96.6 ± 14.9	98.0 ± 15.5	97.4 ± 10.2	88.3 ± 13.0	92.4 ± 9.9	96.0 ± 12.5
Relative organ weight							
Brain (%)	0.81 ± 0.04	0.77 ± 0.04	0.81 ± 0.04	0.85 ± 0.04	0.75 ± 0.08	0.76 ± 0.09	0.80 ± 0.07
Thymus (%)	184 ± 42	185 ± 33	192 ± 41	210 ± 49	138 ± 18	147 ± 22	142 ± 34
Heart (%)	0.37 ± 0.03	0.35 ± 0.02	0.37 ± 0.02	0.36 ± 0.01	0.35 ± 0.03	0.36 ± 0.02	0.36 ± 0.01
Lung (%)	0.48 ± 0.02	0.44 ± 0.03	0.47 ± 0.04	0.47 ± 0.04	0.43 ± 0.03	0.42 ± 0.02	0.44 ± 0.02
Liver (%)	2.96 ± 0.26	2.83 ± 0.12	3.02 ± 0.19	3.46 ± 0.20**	2.75 ± 0.13	2.83 ± 0.13	2.94 ± 0.12
Spleen (%)	0.23 ± 0.04	0.20 ± 0.02	0.21 ± 0.01	0.23 ± 0.03	0.21 ± 0.03	0.20 ± 0.03	0.21 ± 0.01
Kidneys (%)	0.76 ± 0.07	0.77 ± 0.05	0.81 ± 0.04	0.79 ± 0.05	0.73 ± 0.05	0.71 ± 0.01	0.74 ± 0.06
Adrenals (%)	32 ± 3	31 ± 4	33 ± 5	34 ± 3	28 ± 3	29 ± 3	33 ± 5
Ovaries (%)	35.7 ± 4.3	40.0 ± 6.2	43.3 ± 7.6	44.3 ± 4.7	36.0 ± 6.8	35.1 ± 1.4	40.4 ± 4.6

Values are expressed as Mean ± S.D.

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

Table 5 Summary of gross findings of rats treated orally with 4,4'-thiobis(6-*tert*-butyl-*m*-cresol) 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	15	60	250	0	60	250
Male							
No. of animals necropsied	6	6	6	6	6	6	6
Small intestine							
Thickening of wall	0	0	0	4	0	0	0
Large intestine							
Dilatation of cecum	0	0	0	5	0	0	0
Testes							
Enlargement (unilateral)	0	0	0	0	0	1	0
Epididymis							
Yellow nodule (unilateral)	0	0	0	0	0	0	1
Female							
No. of animals necropsied	6	6	6	6	6	6	6
Thyroid							
Enlargement (bilateral)	0	1	0	0	0	0	0
Small intestine							
Thickening of wall	0	0	0	4	0	0	0
Large intestine							
Dilatation of cecum	0	0	0	4	0	0	0

リンの増加がみられたことから，尿蛋白の増加は本被験物質の腎臓に対する影響と考えられるが，腎臓では組織学的変化は認められなかった．また，250 mg/kg群の雌の血糖値は低下していたことから，尿中ケトン体の増加は，血糖値の低下に伴い，エネルギー源としての脂質要求が増大したためと推察される．

血液学検査では，血小板数の増加，リンパ球比率の減少と分葉核好中球比率の増加が250 mg/kg群の主として雌にみられた．250 mg/kg群の雌におけるリンパ球および分葉核好中球の実数を，白血球数とその分画比率から求めると，リンパ球数は対照群平均値の約20%増，分葉核好中球数は対照群平均値の約160%増であったことから，本試験における白血球分画比率の変化は，分葉核好中球の実質的な増加に起因し，大腸粘膜での細胞浸潤との関連性が示唆される．

血液生化学検査では，既に述べた血糖，尿素窒素および無機リンの変動のほか，総コレステロールとリン脂質の増加が250 mg/kg群にみられ，脂質代謝への影響も示唆された．

病理学検査では，小腸，大腸，腸間膜リンパ節および肝臓に変化がみられた．小腸では，肉眼的な壁の肥厚が250 mg/kg群にみられ，組織学的には，回腸で腸絨毛の過形成がみられた．既に述べた如く，60 mg/kg以上の投与群では食餌効率の低下が示唆されたが，小腸における形態学的変化は，消化・吸収能の低下に対する適応的な生体反応と考えられる．また，大腸では，肉眼的な盲腸の拡張が250 mg/kg群にみられ，組織学的には盲腸および結腸で吸収上皮細胞の空胞化および粘膜の細胞浸潤が60 mg/kg以上の投与群にみられ，大腸に対する障害性が示唆された．ただし，吸収上皮細胞の変化を示す個体と細胞浸潤を示す個体とは必ずしも一致せず，両者の関連性は明らかでなかった．腸間膜リンパ節では，傍皮質領域における“tingible body macrophage”が250 mg/kg群にやや多くみられた．この変化は，頸部リンパ節や胸腺，脾臓など他のリンパ器官では認められなかったことから，腸管障害と関連した所見であり，リンパ系器官に対する直接的な作用ではないと考えられる．なお，投与終了時剖検例のうち，250 mg/kg群の雌雄各1例に，直腸粘膜の細胞浸潤がみられたが発現例数が少なく，また，この種の動物では背景的に観察されることから，被験物質投与との関連性はないと判断された．

肝臓では，250 mg/kg群で重量が増加し，組織学的には小葉中心帯肝細胞の肥大が認められた．血液生化学検査では，GOTやGPTの上昇など肝機能障害を示す所見はみられなかったことから，肝臓の組織所見は，薬物代謝酵素の誘導を示唆するもの[21]と推察される．

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群の尿pHの低下，尿蛋白の増加，血小板数の増加および回腸における腸絨毛の過形成を除く所見は認められず，概ね可逆性の変化と考えられた．

以上の如く，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)をラットに28日間反復投与した結果，主な変化が60 mg/kg以上の投与群の大腸に，さらに250 mg/kg群では小腸および肝臓にみられ，本被験物質の主な標的器官は

Table 6 Summary of histopathological findings of rats treated orally with 4,4'-thiobis (6-*tert*-butyl-*m*-cresol) in the twenty-eight-day repeated dose toxicity test

Item	28 days dosing groups (mg/kg)				14 days recovery groups (mg/kg)		
	0	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
Male							
No. of animals necropsied	6	6	6	6	6	6	6
Ileum	(6)	(6)	(6)	(6)	(6)	(6)	(6)
hyperplasia/ villus	0 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0	0 0 0 0	0 0 0 0	1 1 0 0
Cecum	(6)	(6)	(6)	(6)	(6)	(6)	(6)
vacuolization/cytoplasmic/ absorptive epithelial cell	0 0 0 0	0 0 0 0	3 0 0 0	4 1 0 0	0 0 0 0	0 0 0 0	0 0 0 0
cellular infiltration/mucosa	0 0 0 0	0 0 0 0	4 1 0 0	2 1 0 0	1 1 0 0	2 0 0 0	2 1 0 0
Colon	(6)	(6)	(6)	(6)	(0)	(0)	(0)
vacuolization/cytoplasmic/ absorptive epithelial cell	0 0 0 0	0 0 0 0	1 0 0 0	1 0 0 0	- - - -	- - - -	- - - -
Rectum	(6)	(6)	(6)	(6)	(6)	(6)	(6)
cellular infiltration/mucosa	0 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0
Liver	(6)	(6)	(6)	(6)	(6)	(0)	(6)
hypertrophy/hepatocyte/ centrilobular	0 0 0 0	0 0 0 0	0 0 0 0	5 0 0 0	0 0 0 0	- - - -	0 0 0 0
altered hepatocellular foci	0 0 0 1	0 0 0 0	0 0 0 0	0 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	- - - -	1 0 0 0
Mesenteric lymph node	(6)	(6)	(6)	(6)	(0)	(0)	(0)
tingible body macrophage/ paracortical/increased	1 0 0 0	1 0 0 0	1 0 0 0	1 1 0 0	- - - -	- - - -	- - - -
Kidney	(6)	(0)	(0)	(6)	(0)	(0)	(0)
basophilic change/proximal tubule/ focal	0 0 0 0	- - - -	- - - -	1 0 0 0	- - - -	- - - -	- - - -
Prostate	(6)	(0)	(0)	(6)	(0)	(0)	(0)
inflammation	1 0 0 0	- - - -	- - - -	0 0 0 0	- - - -	- - - -	- - - -
Femxale							
No. of animals necropsied	6	6	6	6	6	6	6
Ileum	(6)	(6)	(6)	(6)	(6)	(6)	(6)
hyperplasia/villus	0 0 0 0	0 0 0 0	0 0 0 0	3 0 0 0	0 0 0 0	0 0 0 0	2 0 0 0
Cecum	(6)	(6)	(6)	(6)	(6)	(6)	(6)
vacuolization/cytoplasmic/ absorptive epithelial cell	0 0 0 0	0 0 0 0	1 0 0 0	3 2 0 0	0 0 0 0	0 0 0 0	0 0 0 0
cellular infiltration/mucosa	0 0 0 0	0 0 0 0	1 0 0 0	2 1 0 0	2 0 0 0	1 0 0 0	2 2 0 0
Colon	(6)	(6)	(6)	(6)	(0)	(0)	(0)
vacuolization/cytoplasmic/ absorptive epithelial cell	0 0 0 0	0 0 0 0	1 0 0 0	2 0 0 0	- - - -	- - - -	- - - -
cellular infiltration/mucosa	0 0 0 0	0 0 0 0	1 0 0 0	2 0 0 0	- - - -	- - - -	- - - -
Rectum	(6)	(6)	(6)	(6)	(6)	(6)	(6)
cellular infiltration/mucosa	0 0 0 0	0 0 0 0	0 0 0 0	1 0 0 0	0 0 0 0	1 0 0 0	0 0 0 0
Liver	(6)	(6)	(6)	(6)	(6)	(0)	(6)
hypertrophy/hepatocyte/ centrilobular	0 0 0 0	0 0 0 0	0 0 0 0	5 0 0 0	0 0 0 0	- - - -	0 0 0 0
microgranuloma	0 0 0 0	0 0 0 0	1 0 0 0	0 0 0 0	1 0 0 0	- - - -	0 0 0 0
Thyroid (Parathyroid)	(6)	(1)	(0)	(6)	(0)	(0)	(0)
hyperplasia/follicle cell/diffuse	0 0 0 0	0 1 0 0	- - - -	0 0 0 0	- - - -	- - - -	- - - -
Mesenteric lymph node	(6)	(6)	(6)	(6)	(0)	(0)	(0)
tingible body macrophage/ paracortical/increased	0 0 0 0	1 0 0 0	2 0 0 0	4 1 0 0	- - - -	- - - -	- - - -

1:Slight 2:Mild 3:Moderate P:Present (grading of severity was not done, such as case in the neoplastic lesion)
Numbers in parenthesis indicate No. of animals examined microscopically at this site.

腸管と肝臓と考えられた．一方，15 mg/kg群では変化は認められなかった．これらの結果から，本試験条件下における4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)の無影響量は雌雄とも15 mg/kg/dayと考えられた．

文献

1) S. C. Gad and C. S. Weil, "Principles and Methods of Toxicology," 2, ed. by A. Wallace Hayes, Raven Press, Ltd., New York, 1989, pp. 435-483.
2) J. R. Glaister. "毒性病理学の基礎," 高橋道人監訳, ソフトサイエンス社, 東京, 1992, pp.85-98.

連絡先
試験責任者：岡崎修三
試験担当者：榎並倫宣，中村英明，畠山和久，田村一利，沼田弘明，勝亦倶慶
㈱ボゾリサーチセンター　御殿場研究所
〒412　静岡県御殿場市かまど1284
Tel. 0550-82-2000　Fax. 0550-82-2379

Correspondence
Authors: Shuzo Okazaki (Study director)
Tomonori Enami, Hideaki Nakamura, Kazuhisa Hatayama, Kazutoshi Tamura, Hiroaki Numata, Tomoyoshi Katsumata
Gotemba Laboratory, Bozo Research Center Inc.
1284, Kamado, Gotemba-shi, Shizuoka, 412, Japan
Tel.+81-550-82-2000　Fax.+81-550-82-2379