*S. typhimurium*用には0.5 mM D-ビオチンおよび0.5 mM L-ヒスチジン水溶液，*E. coli*用には0.5 mM L-トリプトファン水溶液を1/10容加え，トップアガーとした．

6. S9 mix

エームステスト用凍結S9 mix(キッコーマン㈱)を購入し，製造後6ヶ月以内に使用した．S9は，誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawley系雄ラットの肝臓から調製されたものである．

7. 試験方法

試験は，プレインキュベーション法で行った．

試験管に使用溶媒，被験物質供試液あるいは陽性対照物質溶液を0.1 mL入れ，次いで直接法では0.1 Mリン酸ナトリウム緩衝液(pH 7.4)を0.5 mL，代謝活性化法ではS9 mixを0.5 mL加え，続いて試験菌液0.1 mLを分注し，37 ℃で20分間振盪培養した．培養終了後，45 ℃に保温したトップアガー2 mLを加えた混合液をプレート上に重層した．37 ℃で48時間培養後，復帰変異コロニーを計数し，同時に指標菌株の生育阻害の有無を実体顕微鏡を用いて観察した．プレートは，用量設定試験では各用量とも1枚，本試験では3枚を使用した．本試験は，同一用量を用いて2回行った．

8. 結果の判定

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)が溶媒対照値の2倍以上を示し，用量依存性および結果の再現性が認められる場合を陽性とした．

但し，明確な用量依存性が認められない場合においても，陽性値を示す試験結果に再現性が認められれば陽性と判定することとした．

結果および考察

50～5000 μg/plateの範囲で行った用量設定試験においては，代謝活性化の有無にかかわらず，いずれの菌株においても200 μg/plate以上の用量で菌の生育阻害が認められ，また，TA100，TA1535およびTA1537では直接法における100 μg/plateでも軽度な生育阻害が認められた．したがって，本試験における被験物質の用量は，最高用量を200 μg/plateとし，以下公比2で，100，50，25，12.5および6.25 μg/plateとした．

試験を2回行った結果(Tables 1～4)，直接法および代謝活性化法のいずれの場合も，供試した全ての菌株において復帰変異コロニー数は，溶媒対照値の2倍を越えることはなかった．菌の生育阻害については直接法の場合，*S. typhimurium*では100 μg/plate以上で，WP2 *uvrA*では200 μg/plateで認められ，代謝活性化法の場合は，TA100およびTA1535では100 μg/plate以上で，TA98，TA1537およびWP2 *uvrA*では200 μg/plateで認められた．

以上の成績から，本実験条件下では，2-*tert*-ブチルフェノールの遺伝子突然変異誘発性は陰性と判定した．

2-*tert*-ブチルフェノールの類縁化合物である4-*tert*-ブチルフェノール[3,4]，2-*sec*-ブチルフェノール[5,6]，4-*sec*-ブチルフェノール[7]，2,4-ジ-*tert*-ブチルフェノール[8]，2,6-ジ-*tert*-ブチルフェノール[3]，2,4,6-トリ-*tert*-ブチルフェノール[9]，6-*tert*-ブチル-*m*-クレゾール[10]，4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)[11]および2,2'-メチレンビス(6-*tert*-ブチル-*p*-クレゾール)[12]は，いずれも*S. typhimurium*および*E. coli*，または*S. typhimurium*を用いた復帰変異試験で陰性と報告されている．また，4-*tert*-ブチルフェノールおよび2,6-ジ-*tert*-ブチルフェノールにおいては酵母を用いた遺伝子突然変異試験でも陰性[3]と報告されている．

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Table 1 Results of reverse mutation test of 2-*tert*-butylphenol on bacteria (1st trial)
[direct method: -S9 mix]

Test substance dose (μg/plate)	Number of revertant colonies per plate [Mean±S.D.]															
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537			
0	97	103	125	8	15	9	21	13	12	15	18	18	10	6	6	
	[108±15]			[11±4]			[15±5]			[17±2]			[7±2]			
6.25	116	100	115	14	15	9	16	10	18	20	17	16	9	7	6	
	[110±9]			[13±3]			[15±4]			[18±2]			[7±2]			
12.5	116	114	107	11	14	5	14	12	14	14	28	18	15	6	13	
	[112±5]			[10±5]			[13±1]			[20±7]			[11±5]			
25	104	139	120	9	9	10	17	9	13	18	29	19	10	9	10	
	[121±18]			[9±1]			[13±4]			[22±6]			[10±1]			
50	126	110	98	11	11	15	17	8	13	22	25	27	12	9	14	
	[111±14]			[12±2]			[13±5]			[25±3]			[12±3]			
100	96*	90*	72*	7*	4*	9*	15	16	9	12*	16*	17*	3*	3*	2*	
	[86±12]			[7±3]			[13±4]			[15±3]			[3±1]			
200	0*	0*	0*	0*	1*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	
	[0±0]			[0±1]			[0±0]			[0±0]			[0±0]			
Positive control	1018	991	925[a]	426	454	434[b]	703	721	789[c]	369	402	347[d]	506	410	499[e]	
	[979±48]			[438±14]			[738±45]			[373±28]			[472±54]			

*: Growth inhibition was observed.
a) AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, 0.01 μg/plate
b) NaN_3: Sodium azide, 0.5 μg/plate
c) AF-2, 0.04 μg/plate
d) AF-2, 0.1 μg/plate
e) 9-AA: 9-Aminoacridine, 80 μg/plate

Table 2 Results of reverse mutation test of 2-*tert*-butylphenol on bacteria (1st trial)
[activation method: +S9 mix]

Test substance dose (μg/plate)	Number of revertant colonies per plate [Mean±S.D.]															
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537			
0	98	109	96	10	9	7	24	20	23	25	38	29	14	13	15	
	[101±7]			[9±2]			[22±2]			[31±7]			[14±1]			
6.25	142	139	124	14	8	8	16	17	10	23	27	33	10	12	13	
	[135±10]			[10±3]			[14±4]			[28±5]			[12±2]			
12.5	127	128	139	8	10	10	20	20	12	25	32	41	10	11	12	
	[131±7]			[9±1]			[17±5]			[33±8]			[11±1]			
25	152	139	133	8	11	11	4	22	14	38	36	30	7	14	10	
	[141±10]			[10±2]			[13±9]			[35±4]			[10±4]			
50	130	138	127	5	12	4	17	18	18	32	46	35	6	7	16	
	[132±6]			[7±4]			[18±1]			[38±7]			[10±6]			
100	104*	112*	127*	8*	4*	4*	28	18	16	31	26	34	5	5	8	
	[114±12]			[5±2]			[21±6]			[30±4]			[6±2]			
200	8*	0*	74*	4*	0*	0*	14*	10*	10*	22*	6*	28*	0*	0*	0*	
	[27±41]			[1±2]			[11±2]			[19±11]			[0±0]			
Positive control	536	554	498[a]	179	125	215[b]	708	697	796[c]	319	375	327[a]	87	83	100[b]	
	[529±29]			[150±27]			[734±54]			[340±30]			[90±9]			

*: Growth inhibition was observed.
a) 2-AA: 2-Aminoanthracene, 1 μg/plate
b) 2-AA, 2 μg/plate
c) 2-AA, 10 μg/plate

Table 3 Results of reverse mutation test of 2-*tert*-butylphenol on bacteria (2nd trial)
[direct method: −S9 mix]

Test substance dose (μg/plate)	Number of revertant colonies per plate [Mean±S.D.]															
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537			
0	106	118	121	16	13	12	16	14	17	22	28	27	7	8	8	
	[115 ± 8]			[14 ± 2]			[16 ± 2]			[26 ± 3]			[8 ± 1]			
6.25	113	118	117	15	15	10	12	12	14	30	28	29	7	7	9	
	[116 ± 3]			[13 ± 3]			[13 ± 1]			[29 ± 1]			[8 ± 1]			
12.5	131	127	145	22	10	11	21	16	12	17	20	28	9	7	14	
	[134 ± 9]			[14 ± 7]			[16 ± 5]			[22 ± 6]			[10 ± 4]			
25	109	138	125	18	15	17	7	14	14	22	30	39	7	6	10	
	[124 ± 15]			[17 ± 2]			[12 ± 4]			[30 ± 9]			[8 ± 2]			
50	105	98	101	10	7	6	12	13	11	24	26	22	13	12	12	
	[101 ± 4]			[8 ± 2]			[12 ± 1]			[24 ± 2]			[12 ± 1]			
100	105*	80*	80*	14*	6	11*	10	16	13	15*	16*	15*	5*	4*	4*	
	[88 ± 14]			[10 ± 4]			[13 ± 3]			[15 ± 1]			[4 ± 1]			
200	0*	0*	0*	0*	0	0*	0*	0*	11*	0*	0*	0*	0*	0*	0*	
	[0 ± 0]			[0 ± 0]			[4 ± 6]			[0 ± 0]			[0 ± 0]			
Positive control	985	894	906[a]	409	521	442[b]	912	868	818[c]	405	372	383[d]	688	550	508[e]	
	[928 ± 49]			[457 ± 58]			[866 ± 47]			[387 ± 17]			[582 ± 94]			

*: Growth inhibition was observed.
a) AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide, 0.01 μg/plate
b) NaN_3: Sodium azide, 0.5 μg/plate
c) AF-2, 0.04 μg/plate
d) AF-2, 0.1 μg/plate
e) 9-AA: 9-Aminoacridine, 80 μg/plate

Table 4 Results of reverse mutation test of 2-*tert*-butylphenol on bacteria (2nd trial)
[activation method: +S9 mix]

Test substance dose (μg/plate)	Number of revertant colonies per plate [Mean±S.D.]															
	TA100			TA1535			WP2 *uvrA*			TA98			TA1537			
0	114	113	110	11	8	9	21	16	19	27	29	35	9	12	17	
	[112 ± 2]			[9 ± 2]			[19 ± 3]			[30 ± 4]			[13 ± 4]			
6.25	157	147	131	10	10	9	26	22	14	52	37	30	16	8	12	
	[145 ± 13]			[10 ± 1]			[21 ± 6]			[40 ± 11]			[12 ± 4]			
12.5	129	131	140	11	9	9	22	24	28	33	35	32	9	10	14	
	[133 ± 6]			[10 ± 1]			[25 ± 3]			[33 ± 2]			[11 ± 3]			
25	143	138	131	17	8	12	14	20	16	42	28	27	6	10	13	
	[137 ± 6]			[12 ± 5]			[17 ± 3]			[32 ± 8]			[10 ± 4]			
50	152	134	119	7	8	13	21	15	21	36	25	34	13	6	13	
	[135 ± 17]			[9 ± 3]			[19 ± 3]			[32 ± 6]			[11 ± 4]			
100	100*	87*	109*	5*	3*	5*	20	20	21	28	34	34	7	16	8	
	[99 ± 11]			[4 ± 1]			[20 ± 1]			[32 ± 3]			[10 ± 5]			
200	48*	1*	111*	0*	0*	0*	1*	9*	10*	13*	16*	6*	0*	0*	6*	
	[53 ± 55]			[0 ± 0]			[8 ± 3]			[12 ± 5]			[2 ± 3]			
Positive control	670	589	644[a]	162	164	162[b]	831	783	811[c]	318	330	367[a]	72	73	80[a]	
	[634 ± 41]			[163 ± 1]			[808 ± 24]			[338 ± 26]			[75 ± 4]			

*: Growth inhibition was observed.
a) 2-AA: 2-Aminoanthracene, 1 μg/plate
b) 2-AA, 2 μg/plate
c) 2-AA, 10 μg/plate

2-*tert*-ブチルフェノールのチャイニーズ・ハムスター培養細胞を用いる染色体異常試験

In Vitro Chromosomal Aberration Test of 2-*tert*-Butylphenol on Cultured Chinese Hamster Cells

要約

2-*tert*-ブチルフェノールの染色体異常誘発性の有無を検討するため，チャイニーズ・ハムスター肺由来の線維芽細胞株(CHL/IU)を用いて*in vitro*における短時間処理法による染色体異常試験を実施した．

染色体異常試験に用いる用量を決定するため，細胞増殖抑制試験を行った結果，S9 mix非存在下では120 μg/mL以上，S9 mix存在下では10 μg/mL以上の用量で50 %を上回る細胞増殖抑制が認められた．したがって，染色体異常試験における用量は，S9 mix非存在下では40，60，80，100，110および120 μg/mL，S9 mix存在下では1.25，2.5，5，7.5，10および20 μg/mLとした．

試験の結果，S9 mix非存在下では，染色体異常細胞の増加は認められなかった．S9 mix存在下では用量依存的な染色体構造異常細胞の増加が認められ，7.5および10 μg/mLでの増加(出現頻度5.5および11.0 %)は統計学的に有意なものであった．また，10 μg/mLで数的異常細胞(倍数性細胞)の有意な増加(出現頻度5.0 %)も認められた．S9 mix非存在下の110 μg/mL以上，S9 mix存在下の20 μg/mLでは細胞毒性のため観察可能な分裂中期像は認められなかった．

以上の成績から，2-*tert*-ブチルフェノールは，CHL/IU細胞に対し染色体異常を誘発する(陽性)と結論した．

方法

1. 試験細胞株

国立医薬品食品衛生研究所変異遺伝部(元:国立衛生試験所変異原性部)から昭和60年1月13日に分与を受けたチャイニーズ・ハムスター肺由来の線維芽細胞株(CHL/IU)を使用した．供試細胞は，浮遊細胞液に10 vol%の割合でジメチルスルホキシド(DMSO，和光純薬工業㈱)を添加し，液体窒素条件下で保存したものを培養液に戻し，解凍後の継代数が3回までのものを使用した．

2. 培養液

Eagle-MEM粉末培地(Gibco Laboratories)を常法に従い調製し，これに非働化仔牛血清(Gibco Laboratories)を10 vol%の割合で添加したものを用いた．

3. 培養条件

4×10^5個/mLの細胞を含む培養液5 mLをディッシュ(径6 cm, Becton Dickinson Co.)に加え，37 ℃のCO_2インキュベーター(5 % CO_2)内で培養した．

培養開始3日後にS9 mix非存在および存在下で被験物質を6時間処理し，処理終了後，新鮮培養液でさらに18時間培養した．

4. S9 mix

染色体異常試験用凍結S9 mix(キッコーマン㈱)を購入し，製造後6ヶ月以内に使用した．S9は，誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawley系雄ラットの肝臓から調製されたものである．

5. 被験物質

2-*tert*-ブチルフェノール(ロット番号C169，大日本インキ化学工業㈱(東京)提供)は，無色透明の液体で，水に難溶，ジメチルスルホキシド(DMSO)，アルコールおよびアセトンに易溶であり，純度99.97 %(不純物として，フェノール0.03 %を含む)の物質である．被験物質は，冷暗所(4 ℃)で密栓(窒素充填)保管した．

実験終了後，残余被験物質を分析した結果，安定性に問題はなかった．

6. 被験物質供試液の調製

溶媒にDMSO(和光純薬工業㈱)を用い，被験物質を溶解して最高用量の供試液(原液)を調製した．この原液の一部を溶媒で順次希釈して所定用量の供試液を調製した．供試液は，用時調製し，そのディッシュ内への添加量は培養液量の0.5 vol%とした．

7. 細胞増殖抑制試験

染色体異常試験に用いる被験物質の用量を決定するため，被験物質の細胞増殖に及ぼす影響を調べた．0.1 w/v%クリスタルバイオレット水溶液で染色した細胞の密度を単層培養細胞密度計(モノセレーターII，MI-60，オリンパス光学工業㈱)を用いて測定し，溶媒対照群の細胞増殖率を100 %とした時の各用量群の細胞増殖率を求めた．

その結果(Fig. 1)，S9 mix非存在下では120 μg/mL以上，S9 mix存在下では10 μg/mL以上の用量で50 %を上回る細胞増殖抑制が認められ，50 %細胞増殖抑制用量は，それぞれ100～120 μg/mLおよび5～10 μg/mLの用

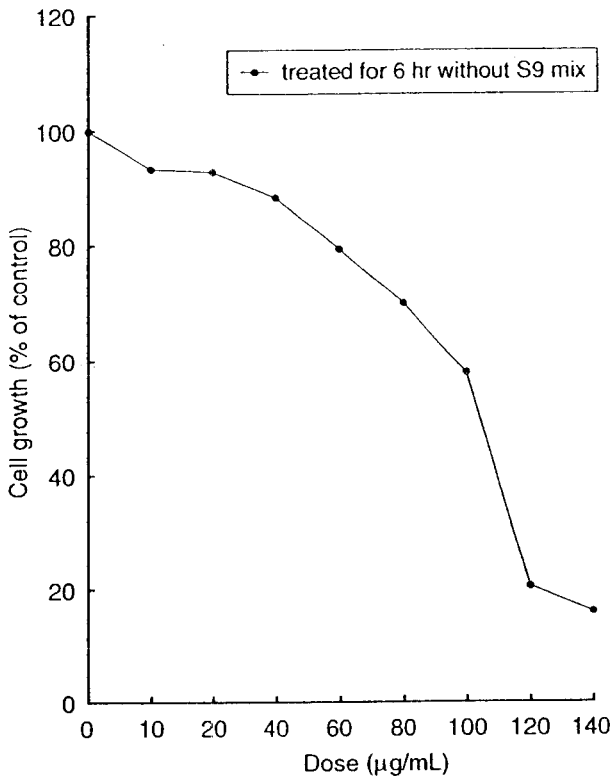

Fig. 1 Growth inhibition of CHL/IU cells treated with 2-*tert*-butylphenol

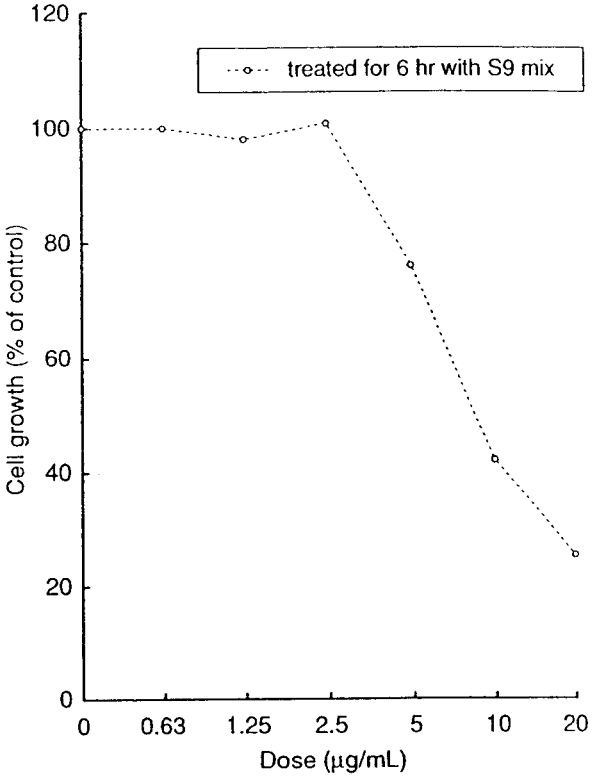

Fig. 2 Growth inhibition of CHL/IU cells treated with 2-*tert*-butylphenol

量域にあるものと判断された.

8. 実験群の設定

細胞増殖抑制試験の結果から，染色体異常試験における被験物質の用量は，50 %細胞増殖抑制用量の前後が含まれ，かつ3用量以上のデータが得られることを考慮して，S9 mix非存在下では40，60，80，100，110および120 μg/mL，S9 mix存在下では1.25，2.5，5，7.5，10および20 μg/mLのそれぞれ6用量を設定した．対照として，溶媒対照群と陽性対照群を設けた.

陽性対照として，短時間処理法S9 mix存在下では3,4-benzo[*a*]pyrene(B[*a*]P, Sigma Chemical Co.)を10 μg/mL，S9 mix非存在下では1-methyl-3-nitro-1-nitrosoguanidine(MNNG, Aldrich Chemical Co.)を2.5 μg/mLの用量で用いた．陽性対照物質の溶媒には，いずれもDMSO(和光純薬工業㈱)を使用した.

9. 染色体標本の作製

培養終了2時間前にコルセミド(Gibco Laboratories)を最終濃度として0.2 μg/mLとなるように添加した．トリプシン処理で細胞を剥離し，遠心分離により細胞を回収した．75 mM塩化カリウム水溶液で低張処理後，用時調製した冷却メタノール・酢酸(3:1)混合液で細胞を固定した．空気乾燥法で染色体標本を作製した後，1.4 vol%ギムザ液で約15分間染色した.

10. 染色体の観察

各ディッシュあたり100個，すなわち，1用量当たり2ディッシュ，200個の分裂中期像を，総合倍率600倍の顕微鏡下で観察した．標本は全てコード化し，盲検法で観察を行った．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)による分類法[1]に基づいて行い，染色体型あるいは染色分体型の切断，交換などの構造異常と倍数性細胞(Polyploid)の有無について観察した.

11. 記録と判定

観察した細胞数，構造異常の種類と数および倍数性細胞の数について集計し，記録した.

染色体構造異常細胞および倍数性細胞の出現頻度について，多試料χ^2検定を行い有意差(有意水準5 %以下)が認められた場合は，フィッシャーの直接確率法を用いて溶媒対照群と各用量群との間の有意差検定(有意水準は多重性を考慮して，5 %または1 %を処理群の数で割ったものを用いた)を行った.

その結果，溶媒対照群と比較して，被験物質による染色体異常細胞の出現頻度が2用量以上で有意に増加し，かつ用量依存性あるいは再現性が認められた場合，陽性と判定した.

結果および考察

短時間処理法による結果をTable 1に示す．S9 mix非存在下では，染色体の構造異常および倍数性細胞の誘発作用は認められなかった．一方，S9 mix存在下においては，用量依存的な染色体の構造異常を有する細胞の増

加が認められ，7.5および10 μg/mLでの増加(出現頻度5.5および11.0 %)は溶媒対照群と比較して統計学的に有意なものであった．また，10 μg/mLで倍数性細胞の有意な増加(出現頻度5.0 %)も認められた．S9 mix非存在下の110 μg/mL以上およびS9 mix存在下の20 μg/mLにおいては，被験物質の細胞に対する毒性のため，観察可能な分裂中期像が認められなかった．

以上の成績から，2-*tert*-ブチルフェノールは，その代謝物に染色体構造異常を誘発する作用があると考えられた．したがって，本実験条件下では，2-*tert*-ブチルフェノールのCHL/IU細胞に対する染色体異常誘発性は陽性と判定した．陽性結果が得られたため，D_{20}値[2](分裂中期像の20 %に異常を誘発させる被験物質の推定用量)を算出したところ，本被験物質のD_{20}値は，構造異常に関して0.022 mg/mL，数的異常に関しては，0.043 mg/mLであった．本試験結果は，CHL/IU細胞において，染色体異常を有する細胞の出現頻度が5 %以上10 %未満を疑陽性，10 %以上を陽性とする石館らの判定基準[3]からみても，陽性と判断されるものであった．

2-*tert*-ブチルフェノールの類縁化合物については，4-*tert*-ブチルフェノールでは，CHL/IU細胞を用いた染色体異常試験において連続処理法24時間および48時間処理で倍数性細胞の誘発作用が，また，短時間処理法S9 mix存在下では構造異常および倍数性細胞の誘発作用が報告され[4]，2,4-ジ-*tert*-ブチルフェノール[5]および6-*tert*-ブチル-*m*-クレゾール[6]では同様の試験において，短時間処理法S9 mix存在下で構造異常細胞の誘発作用が報告されている．また，2-*sec*-ブチルフェノールでもCHL/IU細胞を用いた染色体異常試験において連続処理法48時間処理および短時間処理法S9 mix存在下で構造異常細胞の誘発作用が認められ[7]，さらに，4-*sec*-ブチルフェノールでは同様の試験において，連続処理法48時間処理および短時間処理法S9 mix存在下での構造異常細胞の誘発に対し，疑陽性の判定が示されている[8]．一方，2,6-*tert*-ブチルフェノールおよび4-*tert*-ブチルフェノールでは，ラット肝細胞を用いた染色体異常試験において陰性[9]，また，2,2'-メチレンビス(6-*tert*-ブチル-*p*-クレゾール)[10]および4,4'-チオビス(6-*tert*-ブチル-*m*-クレゾール)[11]ではCHL/IU細胞を用いた染色体異常試験において陰性と報告されている．

このように，類縁化合物にも染色体異常誘発性を示す物質が多いことから，2-*tert*-ブチルフェノールの染色体異常誘発性はこれらに共通した化学構造との関連性が考えられる．

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Table 1 Chromosome analysis of Chinese hamster cells (CHL/IU) treated with 2-*tert*-butylphenol with and without S9 mix

Group	Dose (μg/mL)	S9 mix	Time of exposure (hr)	No. of cells analysed	No. of cells with structural aberrations						gap (%)	No. of cells with numerical aberrations			Cell Growth rate (%)
					ctb	cte	csb	cse	oth	total (%)[2]		Polyploid	Polyploid[3]	total (%)[2]	
Solvent[1]	0	-	6-(18)	200	2	1	0	0	0	3(15)	0(0)	0	0	0(0)	100.0
2TBP	40	-	6-(18)	200	0	0	0	0	0	0(0)	1(0.5)	0	0	0(0)	105.5
	60	-	6-(18)	200	0	1	0	1	0	2(1.0)	0(0)	0	0	0(0)	97.0
	80	-	6-(18)	200	0	2	0	2	0	4(2.0)	0(0)	1	0	1(0.5)	95.0
	100	-	6-(18)	200	3	6	0	0	0	8(4.0)	0(0)	0	0	0(0)	50.5
	110	-	6-(18)	Toxic	—	—	—	—	—	—	—	—	—	—	13.5
	120	-	6-(18)	Toxic	—	—	—	—	—	—	—	—	—	—	4.0
MNNG	2.5	-	6-(18)	200	64	188	3	1	0	190(95.0)**	3(1.5)	0	0	0(0)	—
Solvent	0	+	6-(18)	200	0	0	0	1	0	1(0.5)	0(0)	1	0	1(0.5)	100.0
2TBP	1.25	+	6-(18)	200	1	1	0	0	0	2(1.0)	0(0)	1	0	1(0.5)	90.0
	2.5	+	6-(18)	200	1	2	0	2	0	4(2.0)	1(0.5)	0	0	0(0)	81.5
	5	+	6-(18)	200	1	2	0	0	0	2(1.0)	0(0)	0	0	0(0)	70.0
	7.5	+	6-(18)	200	2	10	0	0	0	11(5.5)*	1(0.5)	7	0	7(3.5)	43.0
	10	+	6-(18)	200	8	18	0	0	0	22(11.0)**	0(0)	7	3	10(5.0)*	26.5
	20	+	6-(18)	Toxic	—	—	—	—	—	—	—	—	—	—	16.5
BP	10	+	6-(18)	200	13	68	1	0	0	75(37.5)**	0(0)	0	0	0(0)	—

Abbreviations; gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring), oth: others, SA: structural aberration, NA: numerical aberration, 2TBP: 2-*tert*-butylphenol, MNNG: 1-methyl-3-nitro-1-nitrosoguanidine, BP: 3,4-benzo[*a*]pyrene

1) Dimethyl sulfoxide was used as solvent.

2) Multi-sample χ^2 test was done at $p<0.05$ and then Fisher's exact test was done at $p<0.05$ or $p<0.01$.

3) endoreduplication

*: Significantly different from solvent group data at $p<0.05$ by Fisher's exact test.

**: Significantly different from solvent group data at $p<0.01$ by Fisher's exact test.

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ORIGINAL ARTICLE

Elevated susceptibility of newborn as compared with young rats to 2-*tert*-butylphenol and 2,4-di-*tert*-butylphenol toxicity

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ABSTRACT **In order to determine the susceptibility of newborn rats to 2-*tert*-butylphenol (2TBP) and 2,4-di-*tert*-butylphenol (DTBP) toxicity, studies were conducted with oral administration from postnatal days (PND) 4 to 21 and the findings were compared with results for young rats exposed from 5 or 6 weeks of age for 28 days. In the newborn rats, specific effects on physical and sexual development and reflex ontogeny were not observed. While there were no clear differences in toxicological profiles between newborn and young rats, the no-observed-adverse-effect levels (NOAELs) differed markedly. For 2TBP, clinical signs such as ataxic gait, decrease in locomotor activity and effects on liver, such as increase in organ weight, were observed and the NOAELs were concluded to be 20 and 100 mg/kg/day in newborn and young rats, respectively. Based on hepatic and renal toxicity (histopathological changes and increase in organ weight with blood biochemical changes), the respective NOAELs for DTBP were concluded to be 5 and 20 mg/kg/day. Therefore, the susceptibility of newborn rats to 2TBP and DTBP was found to be 4–5 times higher than that of young rats.**

Key Words: 2, 4-di-*tert*-butylphenol, 2-*tert*-butylphenol, susceptibility of newborn rats

INTRODUCTION

Protection of humans against disease and injury caused by chemicals in the environment is the ultimate goal of risk assessment and risk management (Landrigan *et al.* 2004). However, the focus has long been solely on adult exposure and toxicity and the fetus via maternal transfer, with little consideration given to early childhood. In the past decade, stimulated especially by the 1993 US National Research Council (NRC) report *Pesticides in the Diets of Infants and Children* (NAS 1993), recognition that special consideration is required for children in risk assessment has grown. The NRC report noted that 'children are not little adults', because of their unique patterns of exposures to environmental hazards and their particular vulnerability.

For the susceptibility of children to environmental chemicals, the early postnatal period (the suckling period) is of particular note. During this period, the infant could be exposed to various chemicals not only through mothers' milk, but also directly, by having chemical-contaminated baby food, mouthing toys or household materials, and so on; however, current risk assessment gives no consideration to toxic effects resulting from direct exposure to chemicals. An approach that adequately takes into account the susceptibility of infancy is urgently required. However, because there is no standard testing protocol intended for direct exposure of preweaning animals (newborn animals) to chemicals, and toxicity studies using newborn animals are complicated by practical difficulties regarding grouping, direct dosing, and general and functional observation, there is only limited information on susceptibility of the newborn at the present.

We therefore have established a new protocol for repeated dose toxicity studies using newborn rats (newborn rat studies) (Koizumi *et al.* 2001) for systematic application. Results have been compared with those of 28-day repeated dose toxicity studies using young rats (young rat studies) to provide a basis of analyzing susceptibility. Since young rat studies are routinely conducted as one of a battery of minimum toxicity tests and data are stored for many chemicals, comparative analyzes should provide important information for considering effects of direct exposure to chemicals during the suckling period.

We have already reported analytical results for eight chemicals (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, 1,3-dibromopropane, 1,1,2,2-tetrabromoethane, 2,4,6-trinitrophenol, and tetrabromobisphenol A) (Koizumi *et al.* 2001, 2002, 2003; Fukuda *et al.* 2004; Takahashi *et al.* 2004; Hirata-Koizumi *et al.* 2005). The susceptibility of newborn rats to the toxicity of the first four agents was four times higher than that of their young counterparts at a maximum. For 1,3-dibromopropane and 1,1,2,2-tetrabromoethane, while the doses causing clear toxicity were lower in newborn rats, doses at which toxic signs began to appear were paradoxically higher in the newborn case. These six chemicals had no impact on development in the newborn period and showed similar toxicity profiles in both age groups. For the other two chemicals, there were marked differences in toxicity profile between the newborn and young rats. Especially, in the case of tetrabromobisphenol A, a specific rather than enhanced renal toxicity was observed in newborn case.

In the present investigation, two *tert*-butylphenols, 2-*tert*-butylphenol (2TBP), and 2,4-di-*tert*-butylphenol (DTBP), were chosen for comparative toxicity analysis. 2TBP has been used in the production of agricultural chemicals, aroma chemicals, and resins (New Chemical Index 2001), and DTBP in the production of antioxidants and ultraviolet absorbers (Chemical Products' Handbook 2004). For either chemical, there is no available toxicity information on human. Regarding toxicity to experimental animals, results from young rat studies of both chemicals are available in

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Toxicity Testing Reports of Environmental Chemicals of the Japanese government (MHLW 2001a, 2001b), but no other data have been reported regarding repeated dose toxicity. Since the young rats were only evaluated for toxicity profiles and no-observed-effect levels, we re-evaluated the results for a more practical evaluation index, the no-observed-adverse-effect level (NOAEL), which could serve as the basis for determining tolerable daily intake (TDI) or acceptable daily intake (ADI) for risk assessment, and conducted comparative analyzes with newborn rats.

MATERIALS

2-*tert*-Butylphenol (2TBP, CAS no. 88-18-6, purity: 99.97%) and 2,4-di-*tert*-butylphenol (DTBP, CAS no. 96-76-4, purity: 99.67%), obtained from Dainippon Ink and Chemicals, Incorporated (Tokyo, Japan), were dissolved in olive oil and corn oil, respectively. The test solutions were prepared once a week as stability for eight days had been confirmed. All other reagents used in this study were specific purity grade.

METHODS

All studies were performed under Good Laboratory Practice conditions and in accordance with 'Guidance for Animal Care and Use' of Panapharm Laboratories Co., Ltd, Research Institute for Animal Science in Biochemistry and Toxicology, or Mitsubishi Chemical Safety Institute Ltd.

Animals

In the newborn rat studies of 2TBP and DTBP, pregnant SPF Sprague-Dawley rats [Crj:CD(SD)IGS] were purchased at gestation days 13–15 from Charles River Japan Inc. (Yokohama, Japan), and allowed to deliver spontaneously. All newborn were separated from dams at postnatal day (PND) 3 (the date of birth was defined as PND 0), and pooled according to sex. At the same time, 12 foster mothers were selected among dams, based on the nursing condition. Each foster mother suckled four male and four female newborn, assigned to each of the four dose groups, including the controls, up to weaning on PND 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group (see Study Design) were individually maintained for nine weeks.

In the young rat studies, 4–5 week-old males and females of the same strain were obtained from the same supplier as for the newborn rat studies, and used at ages of 5–6 weeks after acclimation.

All animals were maintained in an environmentally controlled room at 20–26°C with a relative humidity of 40–70%, a ventilation rate of more than ten times per hour, and a 12:12 h light/dark cycle. They were allowed free access to a basal diet (MF: Oriental Yeast Co. Ltd, Tokyo, Japan, or LABO MR Stock: Nihon Nosan Kogyo Inc., Yokohama, Japan) and water (sterile tap water or well water treated with sodium hypochlorite) throughout.

Study design

1. 18-day repeated dose toxicity study in newborn rats (newborn rat study)

Newborn rats (12/sex/dose) were administered the test substances by gastric intubation on PNDs 4–21. On PND 22, six males and six females in each treated group were sacrificed for autopsy (the scheduled-sacrifice group). The remaining animals in all groups (6 rats/sex/dose) were maintained for nine weeks without chemical treatment and then sacrificed at 12 weeks of age (the recovery-maintenance group).

Based on the results of dose-finding studies conducted prior to the main study, the dose, which would show clear toxicity, was selected as the top dose, that without potentially toxic effects as the lowest dose, and the medium dose was set between them. In the dose-finding study for 2TBP (oral administration from PNDs 4–21), some clinical signs and suppressed body weight gain were observed at 200 mg/kg and an increase in relative liver weight at 60 mg/kg and more. For DBTP (oral administration from PNDs 4–17), all of the four males and four females died at 500 mg/kg, and the death of one of the four males, an increase in serum total cholesterol and phospholipid, and increase in relative liver weight were noted in the 100 mg/kg group. Therefore, the doses were set at 0, 20, 60, or 200 mg/kg/day for 2TBP and at 0, 5, 40, or 300 mg/kg/day for DTBP.

During the study, the rats' general condition was observed at least once a day (details of clinical signs noted in this study are described in 'Glossary of terms for toxicity testing' [NIHS 1994]). Body weight and food consumption (only the recovery-maintenance period) was examined once or more a week. As developmental parameters, fur appearance, incisor eruption, pinna detachment and eye opening were assessed for physical development, and testes descent or preputial separation and vaginal opening for sexual development (OECD 2004). In addition, reflex ontogeny, such as visual placing reflex, and surface and mid-air righting reflexes, were also examined (Adams 1986; Jensh & Brent 1988). Urinalysis (color, occult blood, pH, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, specific gravity, and volume of the urine) was conducted in the last week of the recovery-maintenance period.

At PNDs 22 and 85, blood was collected from the abdominal aorta under ether anesthesia (for 2TBP) or from the postcaval vein under pentobarbital sodium anesthesia (for DTBP) after overnight starvation for the scheduled-sacrifice and recovery-maintenance groups, respectively. One portion was treated with EDTA-2K and examined for hematological parameters, such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count. In the recovery-maintenance group, part of the blood was treated with 3.8% sodium citrate, and blood clotting parameters such as prothrombin time (PT) and activated partial thromboplastin time (APTT) were examined. Serum from the remaining portions of blood for both the scheduled-sacrifice and recovery-maintenance groups were analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio [A/G ratio], glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen [BUN], creatinine, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, γ-glutamyl transpeptidase [γ-GTP], calcium, inorganic phosphorus, sodium, potassium, and chlorine). Following collection of blood, all animals were sacrificed by exsanguination, and all organs and tissues were macroscopically examined. Then, the brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, and ovaries were removed and weighed. Histopathological examination was conducted for the control and the highest dose groups. The above-listed organs were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides), and paraffin sections were routinely prepared and stained with Hematoxylin-Eosin for microscopy. For other groups, organs with macroscopically abnormal findings or in which chemical-related effects were evident on microscopic examination for the highest dose group, were similarly investigated.

2. 28-day repeated dose toxicity study in young rats (young rat study)

Five to six week old rats were given the test substances by gastric intubation daily for 28 days and sacrificed following the last treatment (the scheduled-sacrifice group). Recovery groups were maintained for two weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. The number of animals was six for each sex/dose for both scheduled-sacrificed and recovery cases.

The doses were selected in the same way as the newborn rat studies. In the 12-day dose-finding study for 2TBP, ataxic gait was observed at 300 mg/kg and more, and increase in relative liver and kidney weight at 500 mg/kg. For DTBP, with 14-day administration, the death of one of the four females, various changes in some blood biochemical parameters, increase in relative liver weights and light gray macules on kidneys were found at 500 mg/kg. Increase in serum phospholipid and relative liver weights were also demonstrated in the 100 mg/kg group. Based on the results, the doses were determined at 0, 4, 20, 100, or 500 mg/kg/day for 2TBP and at 0, 5, 20, 75, or 300 mg/kg/day for DTBP. Recovery groups were set at 0, 100, 500 mg/kg/day for 2TBP and 0, 300 mg/kg/day for DTBP.

During the study, rats were examined for general condition, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights, and histopathological findings in compliance with the Test Guideline in the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances).

Statistical analysis

Data for body weights, food consumption, urinalysis findings (except for the results of qualitative analysis), hematological, blood biochemical findings (except for differential leukocyte count), and organ weights were analyzed by the Bartlett's test (Bartlett 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett 1964) was conducted for comparison between control and individual treatment groups ($P < 0.01$ or 0.05). If not homogeneous or for qualitative urinalysis data and differential leukocyte count, the data were analyzed using Steel's multiple comparison tests (Steel 1959), or tests of the Dunnett type (Hollander & Wolfe 1973) ($P < 0.01$ or 0.05). For reflex ontogeny, and physical and sexual development parameters in the newborn rat studies, the χ^2-test (Fisher 1922) was conducted ($P < 0.01$ or 0.05).

RESULTS

2-*tert*-butylphenol (2TBP)

Newborn rat study

Various clinical signs such as decrease in locomotor activity, ataxic gait, deep respiration, and muscle weakness were observed throughout the dosing period in the 200 mg/kg group, as shown in Table 1. With 60 mg/kg, transient decrease in locomotor activity was noted on the first dosing day limited to only one of 12 males. Body weights were lowered by 8–17% from dosing day 7 through to the end of the dosing period in males and to recovery-maintenance day 14 in females given 200 mg/kg. At the scheduled sacrifice, there were no hematological changes at any dose, but blood biochemical examination of the 200 mg/kg group showed increases in γ-GTP in both sexes and total protein in males. In addition, significant increase in relative liver weights was noted in 9% of the females in the 60 mg/kg group and in 21–23% of both males and females in the 200 mg/kg group. On histopathological examination, slight hypertrophy of centrilobular hepatocytes was found in one female of the 60 mg/kg group, and in four males and three females from the 200 mg/kg group. During the recovery-maintenance period, no clinical signs were observed and the lowered body weights showed a tendency for recovery. In parameters for physical and sexual development and reflex ontogeny, no definitive changes were detected. At the end of the recovery-maintenance period, no chemical-related changes, also in urinalysis data, were found in any dose group.

The results of the newborn rat study of 2TBP are summarized in Table 2. Since clinical signs and histopathological changes in the liver were observed in the 60 mg/kg group, the NOAEL was concluded to be 20 mg/kg/day.

Young rat study

Ataxic gait were observed sporadically during the dosing period in nine males and 12 females, and decrease in locomotor activity in two females from the 500 mg/kg group. During the dosing period, there were no changes in body weight, food consumption, and urinalysis data. At the scheduled sacrifice, hematological and blood biochemical examination also showed no changes. Eighteen to 19% increases were found in relative liver weights of both sexes receiving 500 mg/kg, but no histopathological changes in liver were observed at any dose. No chemical-related changes were noted during and at the end of the recovery period.

Table 1 Clinical signs observed during the dosing period in the newborn rat study of 2-*tert*-butylphenol

	Dose (mg/kg/day)			
	0	20	60	200
No. animals (Male/Female)	12/12	12/12	12/12	12/12
No. animals with clinical signs				
Decrease in locomotor activity	0/0	0/0	1†/0	12/12
Ataxic gait	0/0	0/0	0/0	4/6
Deep respiration	0/0	0/0	0/0	12/12
Tremors	0/0	0/0	0/0	2/4
Muscle weakness	0/0	0/0	0/0	12/12
Emaciation	0/0	0/0	0/0	2/2
Pale skin	0/0	0/0	0/0	4/2

†Observed only on the first dosing day.

Table 2 Summary of the results of the newborn and young rat study of 2-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	20	60	200	
Clinical signs	–	M: Decrease in locomotor activity	Various†	
Body weight changes	–	–	8–17%↓	
Blood biochemical changes	–	–	GTP↑, M: TP↑	
Changes in relative organ weights	–	F: Liver 9%↑	Liver 21–23%↑	
Histopathological findings in liver				
– Slight centrilobular hypertrophy of hepatocytes	–	M: 0/6, F: 1/6	M: 4/6, F: 3/6	
Young rat study				
Dose (mg/kg/day)	4	20	100	500
Clinical signs	–	–	–	Ataxic gait F: Decrease in locomotor activity
Body weight changes	–	–	–	–
Blood biochemical changes	–	–	–	–
Changes in relative organ weights	–	–	–	Liver 18–19%↑
Histopathological findings	n.d.	n.d.	n.d.	–

Statistically significant increases ($P < 0.05$) in body weights, blood biochemical parameters and relative organ weights are shown as ↑, while decreases are shown as ↓. Data on histopathological findings are given as no. of animals with the findings/no. of animals examined, according to sex. Changes observed only in males or females are shown as 'M' or 'F', respectively, while neither 'M' nor 'F' is mentioned in the case of changes noted in both sexes. No chemical-related changes were observed in developmental parameters (conducted only in newborn rat study), urinalysis (only in young rat study), and hematological parameters. †Decrease in locomotor activity, ataxic gait, deep respiration, tremors, muscle weakness, emaciation, and pale skin were observed, as shown in Table 1. GTP, γ-GTP; TP, total protein; –, no change; n.d., not determined.

A summary of the results of the young rat study of 2TBP is given in Table 2. The NOAEL was concluded to be 100 mg/kg/day, at which no changes were observed.

2,4-di-*tert*-butylphenol (DTBP)

Newborn rat study

Two males and one female of the 300 mg/kg group were found dead on dosing days 3, 4, and 7. In this group, decrease in locomotor activity (12 males and 12 females), bradypnea (10 males and 10 females), and hypothermia (one male) were observed from the first dosing day, but then the incidence decreased, with disappearance after dosing day 7. Body weights of the 300 mg/kg group were lowered by 15–25% in males and by 9–20% in females during the dosing period, compared with the control values. There were no definitive changes in parameters for physical development and reflex ontogeny in any dose group. At the scheduled sacrifice, blood biochemical examination showed an increase in total bilirubin and a decrease in the A/G ratio in both sexes, an increase in γ-GTP in males, and an increase in total protein and BUN in females of the 300 mg/kg group. In the 300 mg/kg group, there was a 39–51% increase in relative liver weights, a 37–41% increase in relative kidney weights in both sexes, and a 24% decrease in relative spleen weights in males. In the 40 mg/kg group, 14% increases in relative weight of liver were found in females. On histopathological examination, various changes were observed in livers and kidneys in the 300 mg/kg group, as shown in Table 3. Furthermore, periportal fatty degeneration of hepatocytes was evident in one female given 40 mg/kg, and basophilic tubules in kidneys in one animal of each sex receiving 40 mg/kg and one control group male. Regarding parameters of sexual development, a slight delay in preputial separation was noted in the 300 mg/kg group (the incidences were 0/5, compared with 2/6 in the control group at PND 42 [recovery-maintenance day 21]; 0/5, 3/6 at PND 43; 2/5, 5/6 at PND 44; 2/5, 6/6 at PND 46; 4/5, 6/6 at PND 47; and 5/5, 6/6 at PND 48). During this observation period, body weights were lowered by approximately 10% in males given 300 mg/kg than control levels, which was not statistically significant. In the last week of the recovery-maintenance period, there were no chemical-related changes on urinalysis in any dose group. At the end of the recovery period, changes noted in the scheduled-sacrifice group were not observed except for histopathological changes in the kidneys, significant in the 300 mg/kg group (Table 3).

A summary of the results of the newborn rat study of DTBP is shown in Table 4. Since fatty degeneration of hepatocytes and increase in liver weight were demonstrated at 40 mg/kg, the NOAEL was concluded to be 5 mg/kg/day.

Young rat study

No chemical-related changes were found in general condition, body weight, and food consumption at any dose. On urinalysis at the fourth week of dosing, an increase in urine volume, and a decrease in specific gravity and osmotic pressure were noted in both sexes of the 300 mg/kg group. At the scheduled sacrifice, hematological examination showed a decrease in hemoglobin and hematocrit, an increase in segmented neutrophils in females, and prolongation of PT and APTT in males at 300 mg/kg. On blood biochemical examination, there was an increase in total bilirubin in males given 300 mg/kg, and an increase in total cholesterol and phospholipid in females given 75 mg/kg and above. For organ weights, there were

Table 3 Histopathological findings for the newborn rat study of 2,4-di-*tert*-butylphenol

		Scheduled-sacrifice group				Recovery-maintenance group†	
Dose (mg/kg/day)	Grade	0	5	40	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	5/6	6/6	5/5
Liver							
– Fatty degeneration of periportal hepatocytes	+	0/0	0/0	0/1	0/0	0/0	0/0
	++	0/0	0/0	0/0	3/4	0/0	0/0
	+++	0/0	0/0	0/0	2/2	0/0	0/0
Kidneys							
– Basophilic tubules	+	1/0	n.d.	1/1	4/4	0/0	3/0
– Granular casts	+	0/0	n.d.	0/0	4/2	0/0	0/0
– Cystic dilatation of collecting tubules	+	0/0	n.d.	0/0	0/0	0/0	5/4
	++	0/0	n.d.	0/0	3/4	0/0	0/0
	+++	0/0	n.d.	0/0	2/2	0/0	0/0
– Cellular infiltration of neutrophils	+	0/0	n.d.	0/0	2/1	0/0	1/0
	++	0/0	n.d.	0/0	1/1	0/0	1/0
	+++	0/0	n.d.	0/0	1/1	0/0	0/0

†No histopathological examination was conducted at 5 and 40 mg/kg in the recovery-maintenance group. +, mild; ++, moderate; +++, marked; n.d., not determined.

increases in relative liver weights by 40–43% in both sexes given 300 mg/kg, and by 13% in females receiving 75 mg/kg. On histopathological examination, mild to marked changes in livers and kidneys were observed in both sexes from the 300 mg/kg group, as shown in Table 5. At the end of the recovery period, the increase in total cholesterol and phospholipid and renal histopathological changes observed in the scheduled-sacrifice group remained significant in the highest-dose group (Table 5).

The results of the young rat study are summarized in Table 4. Based on increase in the relative liver weights with some changes in blood biochemical parameters in females given 75 mg/kg, the NOAEL was concluded to be 20 mg/kg/day.

DISCUSSION

During development, many rapid and complex biological changes occur, which can have profound consequences on sensitivity to the effects of exogenous chemicals (Scheuplein *et al.* 2002). Although the neonatal body at birth is reasonably well prepared for the abrupt changes associated with parturition, and most functional systems possess a significant portion of their adult capacity (Dourson *et al.* 2002), it is known that the various functions remain immature in early postnatal period and that some organs and tissues, especially in the nervous, immune and reproductive systems, continue to develop after birth (NAS 1993). Therefore, it is important to evaluate toxic effects by exposure to chemicals during the early postnatal period as well as the fetal period for comprehensive risk assessment. However, economic issues and lack of human resources, arising from practical difficulties regarding protocols, have hindered routine implementation of toxicity studies using newborn animals. Our series of comparative analyzes on susceptibility of the newborn are therefore of particular importance for risk assessment.

In the present study on 2TBP and DTBP, there were no clear differences in toxicity profiles between the newborn and young rats in either case. For 2TBP, clinical signs such as a decrease in locomotor activity and ataxic gait, and effects on liver such as an increase in organ weight were observed. In the DTBP case, hepatic and renal toxicity (histopathological changes, increase in organ weight, etc.) were noted. As a characteristic effect of DTBP on male sexual development, slight delay in preputial separation was also observed in the newborn rat study. Preputial separation, an androgen-dependent process which is an early marker of puberty, represents a reliable non-invasive indicator of chemical-induced perturbation of male pubertal development in the rat (Gaytan *et al.* 1988). However, it is known that decreased body weights can result in non-specific delay in puberty (Ashby & Lefevre 2000). Since DTBP lowered body weights in the period of observation of preputial separation and there were no DTBP-related changes in weights or histopathology of the testes and epididymides, well known to be essentially androgen-dependent, no specific effect on male sexual development could be concluded in the present study. As for NOAELs of both chemicals, clear differences were observed between newborn and young rats, with values of 20 and 5 mg/kg/day in newborn rats, and 100 and 20 mg/kg/day in young rats for 2TBP and DTBP, respectively. Therefore, the susceptibility was four- to five-fold higher in newborn than in young rats.

Our previous analysis of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane (Hirata-Koizumi *et al.* 2005) showed dose–response curves to be very different between newborn and young rats. The same was recently reported for the widely used organophosphorus insecticide, chlorpyrifos (Zheng *et al.* 2000), as well as pyrethroid insecticides (Shafer *et al.* 2005). These data showed the importance of estimating unequivocally toxic levels (UETLs), defined for our comparative toxicity analysis as equivalent toxic doses inducing clear toxicity, including death, clinical toxic signs,