資料2－5

既存化学物質の人健康影響に関する情報（第二種監視化学物質審議関係）

（平成20年10月24日開催）

CASNo.	官報公示番号	物質名称	単回	２８日	Reproto	簡易生殖	Ames	染色体	小核	その他	頁
88-18-6	3-503	2-*tert*-ブチルフェノール	○	○			○	○		○	1
2243-62-1	4-323	1,５－ジアミノナフタレン								○	32

2-*tert*-ブチルフェノー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 2-*tert*-Butylphenol in Rats

要約

2-*tert*-ブチルフェノールは，農薬，香料，樹脂を製造する際の原料である[1]．今回，2-*tert*-ブチルフェノールを1群あたり雌雄各5匹のSD系ラットに1回経口投与し，その急性毒性を検討した．投与用量は雌雄ともに0，500，1000および2000 mg/kgとした．

その結果，1000 mg/kg群の雄1例および2000 mg/kg群の雄4例と雌全例が死亡した．死亡動物では，投与日に自発運動の低下，歩行失調，呼吸不整，腹臥位，側臥位および体温低下，第2日にはこの他にうずくまり，振戦，歩行異常，流涙がみられた．剖検では，腺胃のびらん／潰瘍が1000 mg/kg群の雄と2000 mg/kg群の雌雄，前胃の充血が2000 mg/kg群の雌雄，腹腔内の脂肪織の白色化および胸腔の脊椎周囲の出血巣が2000 mg/kg群の雄，空腸の黒色内容物が2000 mg/kg群の雌にみられた．病理組織学検査の結果，胃では前胃の重層扁平上皮の過形成と腺胃の胃腺上皮の変性・脱落が1000 mg/kg群の雄，前胃のびらん巣が2000 mg/kg群の雌雄，腺胃の壁細胞の空胞化が2000 mg/kg群の雄，胸部大動脈周囲脂肪組織の出血が2000 mg/kg群の雄に認められた．

生存動物では，投与日に500 mg/kg以上の投与群の雌雄で自発運動の低下，歩行失調，腹臥位，側臥位および体温低下がみられた．第2日以降には，1000 mg/kg群の雄で自発運動の低下および歩行異常が第4日まで，1000 mg/kg群の雌で歩行異常が第3日までみられた．体重の低値が1000および2000 mg/kg群の雄で第4日にみられた．剖検では，前胃の隆起巣が1000 mg/kg群の雄にみられ，病理組織学検査の結果，前胃の重層扁平上皮の過形成が認められた．

2-*tert*-ブチルフェノールを雌雄のラットに1回経口投与した結果，半数致死量(LD_{50}値)は，雄では1231 mg/kg(95 %信頼限界 838～1808 mg/kg)，雌では1414 mg/kgと結論した．

方法

1. 被験物質

大日本インキ化学工業㈱(東京)から提供された2-*tert*-ブチルフェノール(ロット番号C169，純度99.97 %)を室温，窒素封入条件下で保存し使用した．被験物質の安定性は，被験物質提供者より保証する資料を入手し，確認した．被験物質はオリブ油(丸石製薬㈱)に溶解調製し，窒素封入した．投与液の調製は投与4日前に行い，投与に供するまで冷蔵保存した．投与液中の被験物質の冷蔵保存条件下での8日間の安定性は，投与前に0.4から200 mg/mLの範囲で確認した．また，各用量群の投与液を分析し，被験物質の濃度が設定濃度±10 %以内であることを確認した．

2. 試験動物および動物飼育

日本チャールス・リバー㈱からCrj:CD(SD)IGSラット(SPF)を入手し，6日間検疫・馴化した．1群の動物数は雌雄各5匹とし，投与前日に，体重層別化無作為抽出法によって各群の体重がほぼ均一となるように群分けした．投与日の週齢は5週齢，体重範囲は雄が128～147 g，雌が107～121 gであった．

検疫・馴化期間を含む全飼育期間を通して，温度22±2 ℃(目標値)，相対湿度55±15 %(目標値)，換気約12回/時(オールフレッシュエアー供給)，照明12時間/日(7:00-19:00)に自動調節した飼育室を使用した．動物は，実験動物用床敷(ベータチップ，日本チャールス・リバー㈱)を敷いたポリカーボネート製ケージに，群分け前はケージあたり5匹以下(同性)，群分け後はケージあたり5匹(同性)収容し，飼育した．動物には，実験動物用固型飼料(MF,オリエンタル酵母工業㈱)と，5 μmのフィルター濾過後，紫外線照射した水道水を自由に摂取させた．

3. 投与量および投与方法

予備試験として，被験物質を0，500，1000および2000 mg/kgの用量で1群雌雄各3匹のSD系ラットに1回経口投与し，投与後7日間観察した．その結果，1000および2000 mg/kgの用量で雌全例が死亡した．1000および2000 mg/kgの用量の雄では投与日に重度の自発運動の低下がみられた．500 mg/kgの用量では重篤な変化はみられなかった．この結果から，本試験の用量は雌雄ともに2000 mg/kgを最高用量とし，以下公比2で1000および500 mg/kgの3用量を設定した．この他に溶媒のみを投与する対照群を設けた．投与前日より約18時間絶食させたラットに胃ゾンデを装着したシリンジを用いて1回強制経口投与し，投与後約3時間は飼料を与えなかった．投与液量は10 mL/kgとし，投与直前の体重に基づいて算出した．

4. 観察および検査方法

下記の項目を検査した．なお，日の表記は投与日を第1日とした．

1) 一般状態および体重

一般状態の観察は，投与日には15，30分，1，3および6時間の5回，以後は1日1回14日間にわたって行った．生存動物の体重は，投与直前，第4，8および15日に測定した．また，死亡動物については発見時に測定した．

2) 病理学検査

観察終了後(第15日)に生存動物をチオペンタール・ナトリウムによる麻酔下で腹大動脈を切断・放血し，安楽死させた後剖検した．また，死亡動物については発見後速やかに剖検した．剖検時に異常のみられた1000 mg/kg群の雄2例および2000 mg/kg群の雄1例，雌2例の胃，2000 mg/kg群の雌1例の十二指腸～直腸，2000 mg/kg群の雄1例の腎臓，副腎および周囲の脂肪織と小腸，腸間膜および周囲の脂肪織，胸部脊椎および周囲の組織を採取し，10 %中性リン酸緩衝ホルマリン液で固定し，保存した．また，採材した器官のうち，全例の胃，2000 mg/kg群の雄1例の脂肪織および胸部組織については，常法に従ってヘマトキシリン・エオジン染色標本を作製し，鏡検した．

5. 半数致死量(LD_{50}値)の算出

観察終了時の死亡率からVan der Waerden法で算出した．

結果

1. 死亡およびLD_{50}値(Table 1)

1000 mg/kg群の雄1例および2000 mg/kg群の雄4例と雌全例が投与後3時間から第2日までに死亡した．LD_{50}値は，雄では1231 mg/kg(95 %信頼限界 838～1808 mg/kg)，雌では1414 mg/kgであった．

2. 一般状態

死亡動物では，投与日に投与後15分から症状が発現し，自発運動の低下，歩行失調，呼吸不整，腹臥位，側臥位および体温低下，第2日にはこの他にうずくまり，振戦，歩行異常，流涙がみられた．

生存動物では，投与日に500 mg/kg以上の投与群の雌雄で自発運動の低下，歩行失調，腹臥位，側臥位および体温低下がみられた．第2日以降には，1000 mg/kg群の雄で自発運動の低下および歩行異常が第4日まで，1000 mg/kg群の雌で歩行異常が第3日までみられた．

3. 体重

生存動物では，体重の低値が1000および2000 mg/kg群の雄で第4日にみられたが，以降の体重増加は対照群と同様に推移した．

4. 剖検所見

死亡動物で，腺胃のびらん／潰瘍が1000 mg/kg群の雄1例，2000 mg/kg群の雄1例と雌2例，前胃の充血が2000 mg/kg群の雌雄各1例，空腸の異常内容物(黒色)が2000 mg/kg群の雌1例，腹腔内の脂肪織の白色化および胸腔の脊椎周囲の出血巣が2000 mg/kg群の雄1例にみられた．

生存動物では，1000 mg/kg群の雄1例に前胃の隆起巣がみられた．

5. 病理組織学所見

死亡動物では，剖検時にびらん／潰瘍および前胃の充血のみられた胃，腹腔内の白色化した脂肪織および胸腔の脊椎周囲の出血巣の検査を行った．その結果，胃では，前胃の重層扁平上皮の過形成と腺胃の胃腺上皮の変性・脱落が1000 mg/kg群の雄1例に，前胃のびらん巣が2000 mg/kg群の雌雄各1例に，腺胃の壁細胞の空胞化が2000 mg/kg群の雄1例に認められた．また，胸部大動脈周囲脂肪組織の出血が2000 mg/kg群の雄1例でみられた．なお，腹腔内脂肪織の白色化に対応する組織学的変化はみられなかった．

生存動物では，剖検時に前胃の隆起巣がみられた1000 mg/kg群の雄1例の胃を検査した結果，重層扁平上皮の過形成がみられた．

考察

2-*tert*-ブチルフェノールを雌雄のラットに0，500，1000および2000 mg/kgの用量を1回経口投与した結果，1000 mg/kg群の雄1例および2000 mg/kg群の雄4例と雌全例が死亡した．本被験物質のLD_{50}値は，雄では1231 mg/kg(95 %信頼限界 838～1808 mg/kg)，雌では1414 mg/kgと結論した．

文献

1) 化学工業日報社編，“1994年度版 新化学インデックス，”化学工業日報社，東京，1993, p.549.

連絡先

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Table 1 Mortality and LD_{50} values in rats after single oral administration of 2-*tert*-butylphenol

Sex	Dose (mg/kg)	Number of animals	Number of dead animals								Mortality[a]	LD_{50} values (95 % confidence limits)
			Day: 1	2	3	4	5	6	7	8-15		
Male	0	5	0	0	0	0	0	0	0	0	0/5	
	500	5	0	0	0	0	0	0	0	0	0/5	1231 mg/kg
	1000	5	0	1	0	0	0	0	0	0	1/5	(838 - 1808 mg/kg)
	2000	5	1	3	0	0	0	0	0	0	4/5	
Female	0	5	0	0	0	0	0	0	0	0	0/5	
	500	5	0	0	0	0	0	0	0	0	0/5	1414 mg/kg
	1000	5	0	0	0	0	0	0	0	0	0/5	
	2000	5	2	3							5/5	

a) number of dead animals/number of animals examined

2-*tert*-ブチルフェノールのラットを用いる28日間反復経口投与毒性試験

Twenty-eight-day Repeat Dose Oral Toxicity Test of 2-*tert*-Butylphenol in Rats

要約

2-*tert*-ブチルフェノールは，農薬，香料，樹脂を製造する際の原料である[1]．今回，2-*tert*-ブチルフェノールを0，4，20，100および500 mg/kgの用量で雌雄のSD系ラットに28日間反復経口投与し，その毒性と回復性を検討した．

一般状態において，投与期間中に歩行失調が500 mg/kg群の雌雄で，流涎が100および500 mg/kg群の雌雄で，器官重量において，投与期間終了時に肝臓相対重量の高値が500 mg/kg群の雌雄で，それぞれ認められた．これらの変化は，回復期間中あるいは終了時には認められなかった．

この他，体重測定，摂餌量測定，血液学検査，血液生化学検査，尿検査，剖検，病理組織学検査の結果には，被験物質投与に起因すると考えられる変化はみられなかった．

以上，雌雄いずれも100および500 mg/kg群で被験物質投与に起因すると考えられる変化が認められた．一般状態において100 mg/kg群の雌雄で流涎がみられたことから，本試験条件下における2-*tert*-ブチルフェノールの無影響量(NOEL)は，雌雄いずれも20 mg/kg/dayと判断した．

方法

1. 被験物質

大日本インキ化学工業㈱(東京)から提供された2-*tert*-ブチルフェノール(ロット番号C169，純度99.97 %)を室温，窒素封入条件下で保存し使用した．被験物質の安定性は，被験物質提供者より保証する資料を入手し，確認した．被験物質はオリブ油(丸石製薬㈱)に溶解調製し，窒素封入した．投与液の調製は週1回行い，投与に供するまで冷蔵保存した．投与液中の被験物質の冷蔵保存条件下での8日間の安定性は，投与開始前に0.4から200 mg/mLの範囲で確認した．また，初回調製時に各用量群の投与液を分析し，被験物質の濃度が設定濃度±10 %以内であることを確認した．

2. 試験動物および動物飼育

日本チャールス・リバー㈱からCrj:CD(SD)IGSラット(SPF)を入手し，7日間検疫・馴化した．投与開始前日に，体重層別化無作為抽出法によって各群の体重がほぼ均一となるように群分けした．1群の動物数は，雌雄各6匹とし，対照群，100および500 mg/kg群については雌雄各6匹の回復群(回復期間14日間)を設けた．投与開始時の週齢は5週齢，体重範囲は雄が154～185 g，雌が134～154 gであった．

検疫・馴化期間を含む全飼育期間を通して，温度22±2℃，相対湿度55±15 %，換気約12回/時(オールフレッシュエアー供給)，照明12時間/日(7:00～19:00)に自動調節した飼育室を使用した．動物を実験動物用床敷(ベータチップ，日本チャールス・リバー㈱)を敷いたポリカーボネート製ケージに群分け前はケージあたり5匹(同性)，群分け以降はケージあたり2匹(同性)収容し，飼育した．動物には，実験動物用固型飼料(MF，オリエンタル酵母工業㈱)と，5 μmのフィルター濾過後，紫外線照射した水道水を自由に摂取させた．

3. 投与量および投与方法

予備試験として被験物質を500，1000および2000 mg/kgの用量で単回経口投与した結果，1000および2000 mg/kgの雌全例が死亡し，両群の雄で重度の自発運動の低下が認められた．さらに0，100，300および500 mg/kgの用量をSD系ラットに12日間反復経口投与した結果，歩行失調が500 mg/kg群の雌雄および300 mg/kg群の雌，肝臓の絶対・相対重量の高値が500 mg/kg群の雄，肝臓および腎臓の相対重量の高値が500 mg/kg群の雌で認められた．これらの予備試験の結果から，本試験の高用量は500 mg/kgとし，以下公比5で100，20および4 mg/kgの計4用量群を設定した．さらに溶媒(オリブ油)のみを投与する対照群を設けた．

投与期間は28日間とし，胃ゾンデを装着したシリンジを用いて1日1回，午前中に強制経口投与した．投与液量は10 mL/kgとし，至近日に測定した体重に基づいて算出した．

4. 観察および検査方法

下記の項目を検査した．なお，日と週の表記は投与開始日を第1日，第1～7日を第1週とした．また，第29日以降を回復期間とした．

1) 一般状態，体重および摂餌量

全例について一般状態を毎日観察した．体重および摂餌量は投与開始日およびその後毎週1回測定した．摂餌量については各期間毎の1匹あたりの1日平均摂取量を算出した．

2) 血液学検査

第29日(最終投与日の翌日)および第43日(回復期間終了後)に全例を非絶食条件下で，チオペンタールナトリウムによる麻酔下で後大静脈より採血し，赤血球数(シースフローDCインピーダンス検出法)，ヘモグロビン濃度(SLSヘモグロビン法)，ヘマトクリット値(赤血球パルス波高値検出法)，血小板数(シースフローDCインピーダンス検出法)，白血球数(RF/DCインピーダンス検出法)を多項目自動血球分析装置(NE-4500，シスメックス㈱)で，網赤血球率(アルゴンレーザーを用いたフローサイトメトリー法)を自動網赤血球測定装置(R-2000，シスメックス㈱)で，プロトロンビン時間(PT;Quick一段法)および活性化部分トロンボプラスチン時間(APTT;活性化セファロプラスチン法)を血液凝固自動測定装置(KC 10A，アメルング社)で，白血球百分率(Wright染色塗抹標本)を血液細胞自動分析装置(MICROX HEG-70A，オムロン㈱)で，それぞれ測定した．また，検査の結果から平均赤血球容積(MCV)，平均赤血球血色素量(MCH)，平均赤血球血色素濃度(MCHC)を算出した．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の測定には凝固阻止剤として3.2 %クエン酸三ナトリウム水溶液を使用し，遠心分離して得られた血漿を用いた．その他の項目の測定には，凝固阻止剤EDTA-2Kで処理した血液を用いた．

3) 血液生化学検査

第29日(最終投与日の翌日)および第43日(回復期間終了後)に採取した血液の一部を室温で約30分間静置後3000 rpmで10分間遠心分離し，得られた血清を用いて，ASAT(GOT;JSCC改良法)，ALAT(GPT;JSCC改良法)，γGT(SSCC改良法)，ALP(JSCC改良法)，総ビリルビン(BOD法)，尿素窒素(Urease-GLDH法)，クレアチニン(Jaffé法)，グルコース(GlcK-G6PDH法)，総コレステロール(CES-CO-POD法)，トリグリセライド(LPL-GK-G3PO-POD法)，総蛋白(Biuret法)，アルブミン(BCG法)，カルシウム(OCPC法)，無機リン(PNP-XOD-POD法)，ナトリウム，カリウム，クロール(イオン選択電極法)を自動分析装置(日立736-10形;㈱日立製作所)により測定した．また，検査の結果からA/G比を算出した．

4) 尿検査

各群雌雄6匹の新鮮尿を第23日(投与期間最終週)に採取して，pH，蛋白，グルコース，ケトン体，ビリルビン，潜血，ウロビリノーゲン(試験紙法;マルティスティックス，バイエル・三共㈱)を尿分析装置(クリニテック100，バイエル・三共㈱)で測定した．

5) 病理学検査

第29日(最終投与日の翌日)および第43日(回復期間終了後)に全例について，採血後，腹大動脈を切断・放血し，安楽死させた後剖検した．全例の脳，心臓，肺，肝臓，腎臓，副腎，胸腺，脾臓，精巣，卵巣，子宮，精巣上体，下垂体，甲状腺の重量を測定した．全例の脳，脊髄，下垂体，眼球およびハーダー腺，リンパ節(下顎・腸間膜)，胸腺，気管，肺および気管支，胃，十二指腸，空腸，回腸，盲腸，結腸，直腸，膵臓，甲状腺および上皮小体，心臓，肝臓，脾臓，腎臓，副腎，膀胱，精巣，精巣上体，精嚢，前立腺腹葉，卵巣，子宮，大腿骨および骨髄，大腿筋および坐骨神経を採取し，眼球とハーダー腺はダビドソン液で，精巣および精巣上体はブアン液で，それ以外の器官・組織は10 %中性リン酸緩衝ホルマリン液で固定し，保存した．

投与期間終了時に採取した対照群と500 mg/kg群の雌雄全例の胸腺，心臓，肝臓，脾臓，腎臓，副腎，精巣，精巣上体，精嚢，卵巣，脳，脊髄(頚部，胸部，腰部)，坐骨神経，大腿筋ならびに対照群を含む全動物の肉眼的異常部位は常法に従ってヘマトキシリン・エオジン(H.E)染色標本を作製し，鏡検した．

5. 統計解析

計量データは，Bartlett法で等分散の検定を行い，分散が等しい場合は一元配置分散分析，分散が等しくない場合はKruskal-Wallisの検定を行った．群間に有意な差が認められた場合はDunnett法またはDunnett型の多重比較検定を行った．尿検査データおよび病理組織所見は，a × bのχ^2検定を行い，有意差が認められた場合はArmitageのχ^2検定で対照群と各用量群を比較した．有意水準は5 %とした．

結果

1. 一般状態

被験物質投与に起因する変化として，歩行失調，自発運動の低下，流涎が認められた．歩行失調は投与期間中に500 mg/kg群で雄9例，雌全例に散発的に認められた．本変化は第1日からみられ，投与後に発現し，投与後5時間以内に消失した．自発運動の低下は500 mg/kg群の雌2例に1度または散発的に認められた．本変化は投与後に発現し，発現翌日の投与前までに消失した．流涎が100 mg/kg群の雄6例，雌2例，500 mg/kg群の雄全例，雌11例にみられた．本変化は100 mg/kg群の雄で第13日，雌で第14日，500 mg/kg群の雌雄で第7日以降の投与後30分以内にみられる一過性の変化であった．また，500 mg/kg群の雌1例では投与前の流涎がみられた．これらの変化は回復期間中にはみられなかった．

2. 体重(Fig. 1)

いずれの被験物質投与群においても，対照群と同様に推移した．

3. 摂餌量(Fig. 2)

被験物質投与に起因すると思われる変化は認められなかった．

回復期間中の第36日に100および500 mg/kg群の雄で低値がみられたが，投与期間中および回復終了時にみられない軽微な変化であり，被験物質投与とは関連のない

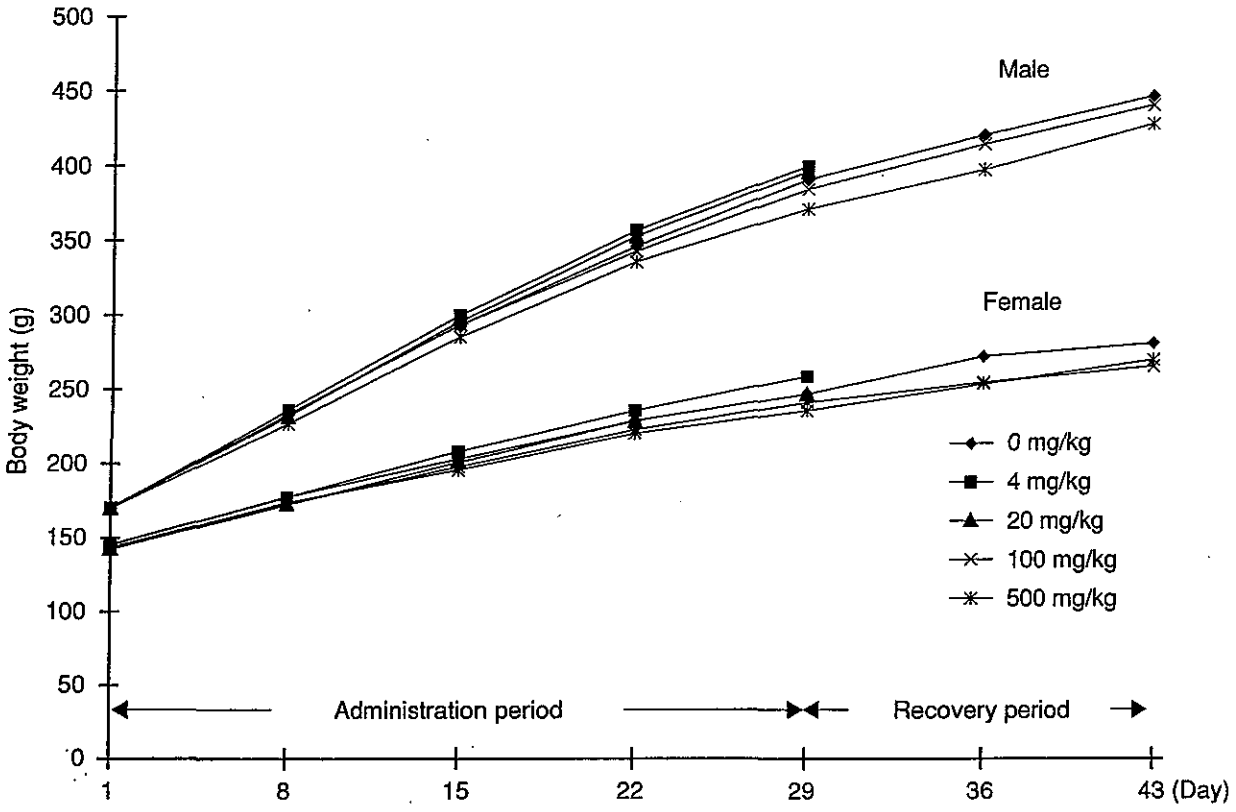

Fig. 1 Body weight changes of rats treated orally with 2-*tert*-butylphenol in twenty-eight-day repeat dose toxicity test

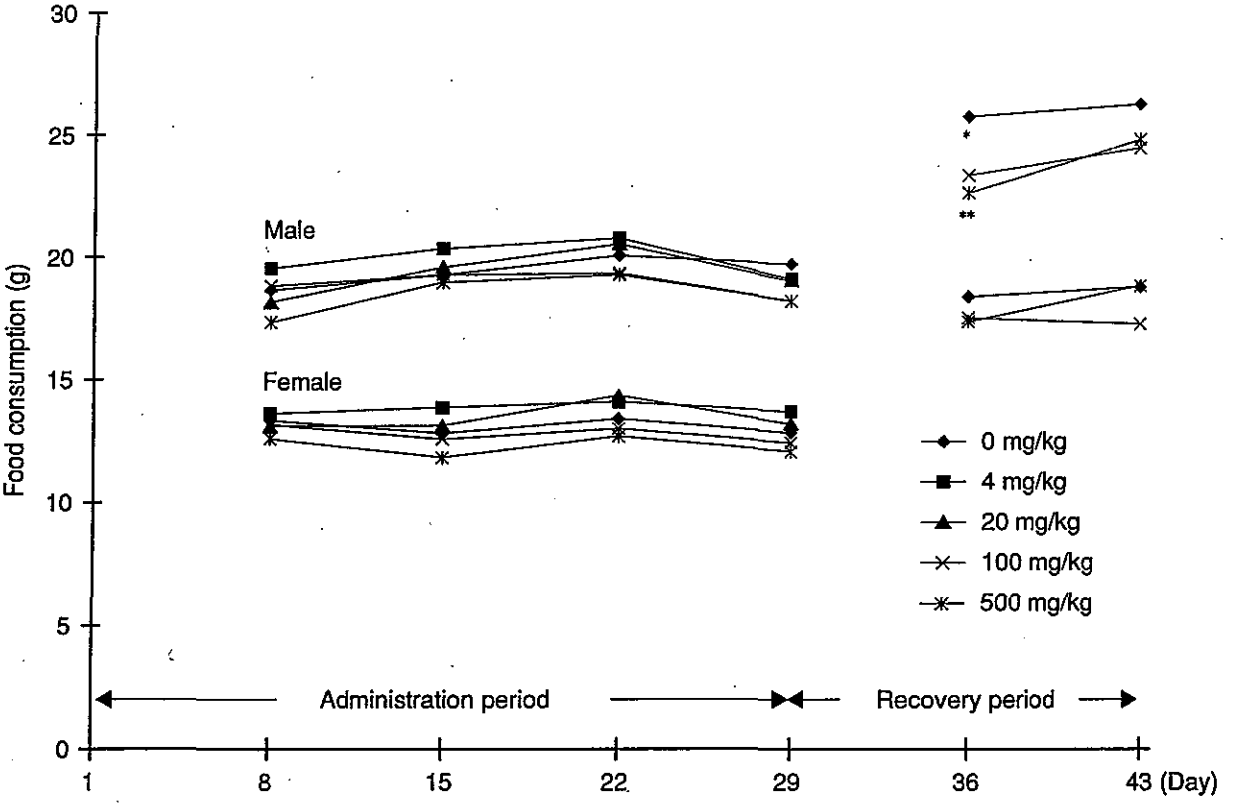

Fig. 2 Food consumption of rats treated orally with 2-*tert*-butylphenol in twenty-eight-day repeat dose toxicity test

Significantly different from 0 mg/kg group; $*p<0.05$, $**p<0.01$

変化と判断した.

4. 血液学検査(Table 1)

投与期間終了時および回復期間終了時の検査で，被験物質投与に起因すると思われる変化は認められなかった.

回復期間終了時に500 mg/kg群の雄で白血球数の高値がみられたが，軽微な変化であり，投与期間終了時にはみられないことから，被験物質投与とは関連のない変化と判断した.

5. 血液生化学検査(Table 2)

投与期間終了時および回復期間終了時の検査で，被験物質投与に起因すると思われる変化は認められなかった.

ALPの低値が投与期間終了時に4，100，500 mg/kg群の雄で，回復期間終了時に500 mg/kg群の雄で認められた．しかし，いずれも軽微な変化であり，正常範囲内の変動であることから，被験物質投与と関連のない変化と判断した.

6. 尿検査(Table 3)

投与期間最終週の検査で，変化は認められなかった.

7. 器官重量(Table 4)

投与期間終了時に肝臓相対重量の高値が500 mg/kg群の雌雄で認められた．回復期間終了時には，本変化はみられなかった.

投与期間終了時に胸腺相対重量の低値が4および500 mg/kg群の雌でみられたが，いずれも軽微な変化であり，絶対重量には有意な差はみられないこと，正常範囲内の変動であることから，被験物質投与と関連のない変化と判断した.

8. 剖検所見

投与期間終了時の検査で被験物質投与に起因すると思われる変化は認められなかった.

投与期間終了時および回復期間終了時に被験物質投与群で胸腺の頚部残留，腎臓ののう胞，肺の褐色斑，子宮の膨満，甲状腺の小型が散発的に認められた．これらはいずれもラットで認められる偶発病変であり，その発現に用量との関連がないことから被験物質投与に起因した変化ではないと判断した.

9. 病理組織学所見(Table 5)

投与期間終了時の解剖動物の500 mg/kg群の雌雄に被験物質投与に起因すると思われる変化は認められなかった.

被験物質投与群で心臓の炎症性細胞浸潤巣，肝臓の門脈周囲性の肝細胞脂肪化，小肉芽腫および壊死巣，腎臓の好塩基性尿細管，のう胞，線維化巣，近位尿細管上皮の硝子滴および乳頭部の鉱質沈着，精巣上体の炎症性細胞浸潤巣，肺の炎症性細胞浸潤巣，子宮腔の拡張が認められたが，いずれもラットを用いた毒性試験でしばしば自然発生性に認められる変化であり，その発現に用量との関連がないことから，被験物質投与に起因した変化ではないと判断した.

考察

2-tert-ブチルフェノールを0，4，20，100および500 mg/kgの用量で雌雄のSD系ラットに28日間反復経口投与し，その毒性と回復性を検討した.

一般状態において，投与期間中に歩行失調が500 mg/kg群の雌雄，流涎が100および500 mg/kg群の雌雄に認められた．歩行失調は投与後5時間以内にみられる一過性の変化であった．類似化合物であるフェノールでは，吸入暴露によりラットに一過性の協調運動失調を起こすことが報告されている[2]．また，有機溶剤の大量投与では，一過性の中枢神経障害により協調運動失調が起こることが知られている[3]．病理組織学的検査では，歩行失調に関連すると思われる変化は認められなかった．流涎は，主に投与後短時間に発現する一過性の変化であり，被験物質の直接的な刺激による可能性が考えられるが，500 mg/kg群では歩行失調と併せてみられることから，中枢神経障害に起因する可能性も否定できない．この他に投与期間中に500 mg/kg群の雌で自発運動の低下が散見された．これらの症状は回復期間中にはみられなかった.

器官重量測定において，投与期間終了時に肝臓相対重量の高値が500 mg/kg群の雌雄で認められた．しかし，肝臓の絶対重量には有意な変化はなく，病理組織学的にも相対重量の変動に関連する変化は認められなかった．回復期間終了時には，本変化はみられなかった.

体重測定，摂餌量測定，血液学検査，血液生化学検査，尿検査，剖検，病理組織学検査の結果には，被験物質投与に起因すると考えられる変化はみられなかった.

以上，雌雄いずれも100および500 mg/kg群で被験物質投与に起因すると考えられる変化が認められた．一般状態において100 mg/kg群の雌雄で流涎がみられたことから，本試験条件下における2-tert-ブチルフェノールの無影響量(NOEL)は，雌雄いずれも20 mg/kg/dayと判断した.

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Table 1 Hematology of rats treated orally with 2-*tert*-butylphenol in twenty-eight-day repeat dose toxicity test

Sex	Administration period					Recovery period		
Dose level (mg/kg)	0	4	20	100	500	0	100	500
Male								
Number of animals	6	6	6	6	6	6	6	6
RBC ($\times10^4/\mu L$)	749.8±34.4	727.5±26.7	735.3±30.3	732.2±15.0	755.0±27.0	789.5±27.5	784.7±19.1	801.2±41.7
Hemoglobin (g/dL)	14.50±0.40	14.70±0.51	14.38±0.64	14.72±0.33	14.85±0.35	14.85±0.52	14.95±0.38	15.05±0.23
Hematocrit (%)	41.97±0.98	41.95±1.24	41.77±1.24	42.52±1.39	43.32±1.65	41.97±1.63	42.30±0.82	42.23±0.80
MCV (fL)	56.02±1.72	57.70±2.02	56.85±2.15	58.05±1.07	57.38±1.85	53.18±1.28	53.93±1.14	52.78±2.17
MCH (pg)	19.38±0.61	20.23±0.74	19.57±0.86	20.10±0.11	19.67±0.57	18.82±0.55	19.05±0.54	18.82±0.81
MCHC (%)	34.55±0.45	35.03±0.73	34.42±0.52	34.63±0.52	34.32±0.68	35.40±0.54	35.35±0.48	35.62±0.28
Reticulocyte (‰)	30.92±4.15	30.97±3.46	30.17±4.28	28.70±3.41	31.23±2.08	24.42±2.39	25.10±1.81	25.07±4.61
Platelet ($\times10^4/\mu L$)	94.87±8.62	88.67±6.55	94.57±10.00	90.17±18.19	88.75±5.19	92.92±7.71	93.08±11.59	91.80±11.00
PT (sec)	13.58±0.21	13.65±0.45	13.13±0.26	13.27±0.30	13.28±0.29	14.32±0.42	14.62±0.43	14.32±0.22
APTT (sec)	16.10±1.04	15.85±1.18	16.23±0.58	15.30±1.98	16.32±1.77	17.00±0.89	16.87±1.26	16.93±0.99
WBC ($\times10^2/\mu L$)	108.18±21.35	119.88±34.28	97.73±20.91	99.72±19.94	116.25±20.30	102.17±12.82	101.43±8.46	124.02±13.87*
Differential leukocyte counts (%)								
Lymphocytes	84.8±6.0	86.3±6.3	87.8±4.0	88.7±4.5	86.2±4.2	85.3±4.6	85.2±4.4	87.5±3.7
Neutrophils								
Segmented	9.7±5.3	8.7±5.1	7.5±3.0	6.7±3.4	8.5±3.2	8.0±3.2	9.5±4.0	7.8±3.4
Band	0.0±0.0	0.3±0.5	0.3±0.5	0.2±0.4	0.3±0.5	0.5±0.5	0.3±0.5	0.2±0.4
Eosinophils	0.3±0.5	0.5±1.2	0.3±0.5	0.5±0.5	0.3±0.5	1.3±1.0	1.2±0.8	0.7±0.8
Basophils	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Monocytes	5.2±2.3	4.2±4.1	4.0±1.8	4.0±1.9	4.7±2.9	4.8±3.0	3.8±1.9	3.8±2.2
Female								
Number of animals	6	6	6	6	6	6	6	6
RBC ($\times10^4/\mu L$)	710.0±24.7	687.3±17.7	737.0±30.6	719.7±22.6	720.8±8.8	727.5±56.5	751.8±39.6	760.7 ± 12.8
Hemoglobin (g/dL)	14.30±0.68	14.08±0.45	14.83±0.48	14.55±0.16	14.20±0.31	14.23±1.14	14.80±0.96	14.83 ± 0.30
Hematocrit (%)	40.40±1.42	40.02±1.06	41.70±1.59	40.92±0.75	40.42±1.34	39.15±2.96	41.27±2.55	41.77 ± 0.78
MCV (fL)	56.93±1.45	58.23±1.45	56.58±1.01	56.90±1.00	56.07±2.15	53.83±1.26	54.90±2.37	54.90 ± 0.74
MCH (pg)	20.13±0.94	20.48±0.57	20.12±0.50	20.23±0.50	19.68±0.53	19.57±0.56	19.72±0.97	19.52 ± 0.35
MCHC (%)	35.40±0.85	35.20±0.32	35.60±0.64	35.60±0.44	35.15±0.48	36.33±0.43	35.87±0.46	35.52 ± 0.70
Reticulocyte (‰)	24.10±2.10	26.37±3.70	21.40±1.65	24.98±1.81	24.65±4.78	24.02±8.95	22.20±3.54	29.78 ± 6.62
Platelet ($\times10^4/\mu L$)	87.27±10.11	84.45±9.46	82.60±4.73	88.33±9.14	91.28±13.28	80.62±17.41	77.03±8.13	84.95 ± 7.41
PT (sec)	14.18±0.34	14.12±0.48	13.85±0.65	13.93±0.69	13.77±0.61	15.18±0.72	14.68±1.05	14.83 ± 0.39
APTT (sec)	13.95±1.03	13.87±1.64	12.62±0.56	13.77±1.05	14.28±0.52	14.87±1.10	14.43±2.09	13.32 ± 1.46
WBC ($\times10^2/\mu L$)	84.30±22.91	77.23±13.45	75.02±4.33	72.82±9.93	78.90±5.55	83.28±25.86	104.20±11.67	91.72 ± 15.45
Differential leukocyte counts (%)								
Lymphocytes	88.0±3.3	85.8±4.3	89.3±3.9	83.8±7.3	86.5±3.1	84.3±6.3	87.0±4.7	85.2 ± 3.9
Neutrophils								
Segmented	7.7±3.2	6.3±2.3	6.0±3.0	10.5±7.3	8.5±2.2	8.5±6.4	5.5±3.4	8.3 ± 3.4
Band	0.2±0.4	0.5±0.8	0.2±0.4	0.0±0.0	0.8±1.0	0.0±0.0	0.2±0.4	0.2 ± 0.4
Eosinophils	0.8±1.2	1.8±1.8	0.5±0.8	0.7±0.5	1.2±1.5	0.7±1.2	1.5±1.0	1.0 ± 0.6
Basophils	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0 ± 0.0
Monocytes	3.3±1.8	5.5±2.7	4.0±3.2	5.0±1.8	3.0±2.4	6.5±2.9	5.8±2.3	5.3 ± 3.7

Values are expressed as Mean±S.D.
Significantly different from 0 mg/kg group; *$p<0.05$

Table 2 Blood chemistry of rats treated orally with 2-*tert*-butylphenol in twenty-eight-day repeat dose toxicity test

Sex	Administration period					Recovery period		
Dose level (mg/kg)	0	4	20	100	500	0	100	500
Male								
Number of animals	6	6	6	6	6	6	6	6
GOT (U/L)	83.3±12.6	71.2±7.9	74.5±5.0	67.0±15.3	68.8±8.3	91.5±13.1	91.0±8.9	80.0±11.1
GPT (U/L)	42.3±10.1	40.7±5.4	42.0±6.6	33.5±1.5	38.7±4.3	35.2±4.6	32.5±4.4	31.0±4.2
γ-GT (U/L)	0.2±0.4	0.3±0.5	0.2±0.4	0.3±0.8	0.3±0.5	0.2±0.4	0.0±0.0	0.2±0.4
ALP (U/L)	1151.7±273.9	887.2±150.4*	936.8±116.2	763.5±157.0**	813.7±111.4**	704.8±54.9	738.8±85.8	560.7±75.6**
Total bilirubin (mg/dL)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.04	0.00±0.00	0.00±0.00
Urea nitrogen (mg/dL)	12.13±1.66	12.35±2.15	12.97±2.14	13.23±2.63	12.65±1.36	16.40±2.37	16.33±3.07	17.27±1.80
Creatinine (mg/dL)	0.40±0.00	0.40±0.00	0.40±0.00	0.38±0.08	0.38±0.04	0.45±0.05	0.45±0.05	0.45±0.05
Glucose (mg/dL)	151.3±13.7	151.8±7.4	145.3±8.1	150.2±13.2	152.5±8.7	146.3±11.0	141.0±3.5	139.3±9.7
Total chol. (mg/dL)	66.0±8.3	65.7±9.0	62.7±5.9	71.8±8.5	73.0±6.0	57.3±5.7	62.8±6.8	66.3±9.1
Triglyceride (mg/dL)	104.0±76.8	81.2±34.7	60.3±18.5	89.7±36.9	71.2±37.5	114.8±43.3	98.8±33.5	104.8±48.4
Total protein (g/dL)	6.42±0.21	6.53±0.20	6.53±0.20	6.58±0.32	6.67±0.23	6.75±0.45	6.78±0.26	6.87±0.42
Albumin (g/dL)	3.33±0.08	3.38±0.04	3.35±0.08	3.35±0.10	3.42±0.12	3.38±0.08	3.38±0.12	3.38±0.15
A/G ratio	1.083±0.038	1.075±0.060	1.055±0.045	1.040±0.057	1.052±0.052	1.015±0.103	0.995±0.067	0.975±0.059
Calcium (mg/dL)	9.45±0.19	9.55±0.33	9.48±0.31	9.53±0.29	9.57±0.34	9.95±0.49	9.97±0.21	9.98±0.46
Inorganic phos. (mg/dL)	8.52±0.17	8.33±0.71	8.67±0.62	8.23±0.49	8.22±0.39	8.75±0.33	8.37±0.27	8.50±0.37
Na (mmol/L)	143.7±1.4	143.0±1.3	143.8±1.0	143.8±0.4	143.3±0.5	143.5±0.5	143.2±0.8	143.8±1.2
K (mmol/L)	4.48±0.19	4.43±0.22	4.45±0.24	4.32±0.17	4.43±0.12	4.47±0.19	4.57±0.21	4.47±0.25
Cl (mmol/L)	99.5±1.0	98.8±1.0	99.0±0.9	99.2±1.5	98.2±1.2	98.8±1.5	99.2±1.0	100.0±1.3
Female								
Number of animals	6	6	6	6	6	6	6	6
GOT (U/L)	66.8±7.4	67.8±7.7	74.7±19.4	67.5±10.3	74.8±19.1	72.2±12.9	70.0±8.9	70.5±11.4
GPT (U/L)	24.8±3.9	26.3±5.3	25.7±5.0	23.8±3.3	22.5±3.2	28.3±2.3	27.2±4.8	27.7±4.4
γ-GT (U/L)	0.0±0.0	0.2±0.4	0.2±0.4	0.2±0.4	0.2±0.4	0.0±0.0	0.2±0.4	0.3±0.5
ALP (U/L)	512.3±180.8	551.5±170.5	487.8±134.2	530.5±110.9	396.0±100.0	316.3±97.6	325.3±37.3	394.0±170.3
Total bilirubin (mg/dL)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.04	0.00±0.00	0.00±0.00
Urea nitrogen (mg/dL)	13.85±1.39	14.02±1.80	12.65±2.65	14.23±3.34	12.83±1.75	17.20±2.29	17.73±2.28	17.55±1.31
Creatinine (mg/dL)	0.48±0.04	0.47±0.05	0.47±0.05	0.45±0.05	0.45±0.08	0.53±0.05	0.53±0.05	0.55±0.05
Glucose (mg/dL)	151.2±6.0	162.5±11.9	162.0±13.5	160.7±6.6	155.5±11.9	148.0±5.9	147.8±10.6	149.5±9.4
Total chol. (mg/dL)	64.2±6.5	61.0±8.1	62.3±10.8	70.0±7.5	76.0±8.3	83.2±12.9	75.5±9.6	78.5±8.9
Triglyceride (mg/dL)	16.0±5.8	15.0±7.3	32.0±25.3	21.3±11.2	21.7±10.2	51.0±50.2	31.2±14.2	52.3±48.1
Total protein (g/dL)	6.67±0.25	6.53±0.16	6.53±0.30	6.72±0.22	6.68±0.42	7.07±0.58	7.15±0.28	7.02±0.26
Albumin (g/dL)	3.48±0.15	3.42±0.10	3.47±0.12	3.52±0.08	3.58±0.19	3.53±0.29	3.58±0.10	3.52±0.08
A/G ratio	1.093±0.043	1.098±0.050	1.133±0.066	1.102±0.048	1.158±0.056	1.000±0.027	1.007±0.053	1.008±0.066
Calcium (mg/dL)	9.12±0.19	9.07±0.12	9.27±0.16	9.12±0.16	9.12±0.29	9.88±0.17	9.85±0.30	9.82±0.38
Inorganic phos. (mg/dL)	7.63±0.29	7.52±0.26	7.63±0.41	7.45±0.39	7.17±0.22	7.43±0.55	7.88±0.38	7.37±0.32
Na (mmol/L)	142.8±0.8	142.3±1.0	142.5±1.0	142.5±1.0	141.7±0.8	143.0±1.3	143.0±1.3	142.0±1.1
K (mmol/L)	4.05±0.31	4.05±0.29	4.17±0.16	3.92±0.21	4.05±0.22	4.07±0.33	4.03±0.26	4.07±0.10
Cl (mmol/L)	101.2±1.0	101.0±1.3	100.2±1.3	99.7±1.2	100.0±1.8	100.5±2.0	100.8±1.5	99.8±2.6

Values are expressed as Mean±S.D.
Significantly different from 0 mg/kg group; *$p<0.05$, **$p<0.01$

Table 3 Urinalysis of rats treated orally with 2-*tert*-butylphenol in twenty-eight-day repeat dose toxicity test

		Administration period									
	Sex	Male					Female				
Dose level (mg/kg)		0	4	20	100	500	0	4	20	100	500
Number of animals		6	6	6	6	6	6	6	6	6	6
pH	6.0	0	0	0	0	0	0	1	0	0	0
	6.5	0	0	0	0	0	0	1	0	0	0
	7.0	0	0	0	0	0	1	0	0	0	0
	7.5	0	0	0	0	0	0	0	0	1	2
	8.0	2	2	2	2	2	3	2	2	2	1
	8.5	4	4	4	4	4	2	2	4	3	2
	>=9	0	0	0	0	0	0	0	0	0	1
Protein	-	0	0	0	0	0	2	2	3	2	1
(mg/dL)	+/-	1	0	1	0	0	1	1	0	1	3
	30	2	0	2	0	1	2	2	2	2	1
	100	3	6	3	6	5	1	1	1	1	1
	>=300	0	0	0	0	0	0	0	0	0	0
Glucose	-	6	6	6	5	6	6	5	5	6	6
(g/dL)	0.1	0	0	0	1	0	0	1	1	0	0
	0.25	0	0	0	0	0	0	0	0	0	0
	0.5	0	0	0	0	0	0	0	0	0	0
	>=1.	0	0	0	0	0	0	0	0	0	0
Ketones	-	0	0	1	0	0	3	2	3	3	1
(mg/dL)	5	3	1	3	3	5	3	3	3	2	4
	15	2	5	2	3	1	0	1	0	1	1
	40	1	0	0	0	0	0	0	0	0	0
	>=80	0	0	0	0	0	0	0	0	0	0
Bilirubin	-	5	3	6	5	4	6	6	6	6	6
	1+	1	3	0	1	2	0	0	0	0	0
	2+	0	0	0	0	0	0	0	0	0	0
	3+	0	0	0	0	0	0	0	0	0	0
Occult blood	-	6	6	6	6	6	6	6	6	6	6
	+/-	0	0	0	0	0	0	0	0	0	0
	1+	0	0	0	0	0	0	0	0	0	0
	2+	0	0	0	0	0	0	0	0	0	0
	3+	0	0	0	0	0	0	0	0	0	0
Urobilinogen	0.1	4	5	6	6	6	6	6	6	5	4
(EU/dL)	1	2	1	0	0	0	0	0	0	1	2

Grade; -:negative, +/-:trace, 1+:slight, 2+:moderate, 3+:severe

Table 4 Absolute and relative organ weights of rats treated orally with 2-*tert*-butylphenol in twenty-eight-day repeat dose toxicity test

Sex	Administration period					Recovery period		
Dose level (mg/kg)	0	4	20	100	500	0	100	500
Male								
Number of animals	6	6	6	6	6	6	6	6
Final body weight (g)	395.2±39.1	397.7±30.8	393.7±23.5	386.2±22.8	373.0±33.1	444.0±32.1	437.7±22.1	425.2±17.6
Absolute organ weight								
Brain (g)	2.088±0.060	2.048±0.095	2.105±0.062	2.070±0.085	2.042±0.062	2.138±0.072	2.147±0.064	2.097±0.053
Pituitary (mg)	16.15±1.69	15.82±1.19	16.23±1.63	14.58±1.08	14.50±2.03	15.82±1.48	16.27±1.32	15.95±0.81
Thyroids (mg)	34.40±7.93	31.70±6.86	30.80±4.69	32.57±5.12	28.98±2.42	33.42±3.21	37.08±5.69	31.07±4.11
Thymus (mg)	558.3±82.2	607.0±129.2	609.2±93.6	624.2±69.7	543.7±96.0	509.5±131.6	517.0±122.7	529.5±77.5
Lungs (g)	1.428±0.091	1.497±0.127	1.468±0.083	1.467±0.091	1.420±0.119	1.462±0.063	1.507±0.065	1.427±0.037
Heart (g)	1.300±0.092	1.315±0.150	1.363±0.073	1.390±0.157	1.330±0.100	1.447±0.182	1.432±0.084	1.313±0.082
Liver (g)	16.315±2.977	16.197±2.243	16.137±1.318	16.565±2.089	18.173±1.797	16.235±1.200	15.877±1.076	14.945±1.073
Spleen (g)	0.810±0.114	0.832±0.081	0.845±0.125	0.727±0.107	0.750±0.096	0.855±0.076	0.912±0.083	0.783±0.103
Kidneys (g)	2.735±0.308	2.760±0.352	2.852±0.220	2.850±0.329	2.835±0.155	3.122±0.271	2.965±0.169	2.898±0.165
Adrenals (mg)	47.45±6.67	53.15±8.21	50.38±5.83	51.75±5.64	45.30±8.26	50.60±8.59	53.93±10.89	49.77±1.60
Testes (g)	3.048±0.293	3.058±0.220	3.192±0.247	3.063±0.194	3.177±0.123	3.212±0.180	3.285±0.141	3.123±0.130
Epididymides (g)	0.803±0.050	0.807±0.040	0.783±0.085	0.792±0.065	0.753±0.042	1.118±0.071	1.137±0.054	1.083±0.040
Relative organ weight								
Brain (%)	0.533±0.044	0.517±0.037	0.537±0.031	0.538±0.039	0.550±0.039	0.480±0.023	0.493±0.024	0.495±0.022
Pituitary ($\times10^{-3}$ %)	4.10±0.45	3.98±0.32	4.12±0.39	3.78±0.21	3.88±0.44	3.55±0.21	3.73±0.38	3.78±0.31
Thyroids ($\times10^{-3}$ %)	8.72±1.98	7.97±1.47	7.82±0.93	8.50±1.68	7.83±1.14	7.60±1.18	8.47±0.96	7.35±1.18
Thymus ($\times10^{-3}$ %)	142.20±23.38	151.78±22.87	154.67±21.33	161.42±12.13	145.07±16.57	114.25±24.82	117.90±25.85	124.77±20.02
Lungs (%)	0.365±0.026	0.375±0.020	0.373±0.023	0.382±0.013	0.380±0.014	0.332±0.016	0.343±0.015	0.337±0.015
Heart (%)	0.328±0.023	0.330±0.020	0.345±0.010	0.360±0.022	0.362±0.044	0.327±0.031	0.328±0.031	0.310±0.018
Liver (%)	4.103±0.349	4.058±0.278	4.100±0.227	4.280±0.365	4.870±0.172**	3.660±0.139	3.628±0.118	3.512±0.156
Spleen (%)	0.205±0.015	0.210±0.011	0.215±0.033	0.187±0.023	0.202±0.028	0.193±0.016	0.208±0.020	0.183±0.026
Kidneys (%)	0.692±0.035	0.692±0.043	0.725±0.062	0.740±0.064	0.765±0.071	0.705±0.052	0.678±0.024	0.682±0.034
Adrenals ($\times10^{-3}$ %)	12.03±1.27	13.40±2.26	12.80±1.24	13.43±1.80	12.13±1.84	11.38±1.63	12.33±2.52	11.72±0.35
Testes (%)	0.777±0.109	0.773±0.092	0.810±0.026	0.793±0.044	0.858±0.085	0.725±0.055	0.753±0.059	0.735±0.052
Epididymides (g)	0.207±0.031	0.203±0.020	0.198±0.013	0.207±0.023	0.203±0.027	0.253±0.019	0.258±0.015	0.255±0.019

Values are expressed as Mean±S.D.
Significantly different from 0 mg/kg group; *$p<0.05$, **$p<0.01$

Table 4 (continued)

Sex	Administration period					Recovery period		
Dose level (mg/kg)	0	4	20	100	500	0	100	500
Female								
Number of animals	6	6	6	6	6	6	6	6
Final body weight (g)	242.0±10.3	257.8±21.4	246.0±24.1	243.0±22.7	231.7±9.2	279.8±27.0	264.5±17.2	268.7±31.4
Absolute organ weight								
Brain (g)	1.913±0.059	1.882±0.079	1.918±0.064	1.907±0.059	1.943±0.074	1.970±0.106	1.937±0.094	1.930±0.026
Pituitary (mg)	16.35±1.56	18.13±1.58	16.72±2.48	17.13±2.77	16.37±0.85	18.47±3.15	18.92±2.45	20.27±1.66
Thyroids (mg)	23.67±3.71	23.35±5.20	26.15±3.24	24.98±3.67	25.03±3.97	26.30±3.25	26.13±2.91	24.50±4.26
Thymus (mg)	586.3±161.7	464.8±39.8	517.3±65.9	534.7±95.7	423.0±59.6	427.0±93.9	481.3±100.3	449.2±55.9
Lungs (g)	1.078±0.081	1.078±0.121	1.090±0.094	1.083±0.099	1.097±0.072	1.188±0.072	1.145±0.075	1.135±0.077
Heart (g)	0.860±0.127	0.892±0.100	0.888±0.040	0.838±0.073	0.813±0.072	0.958±0.097	0.898±0.066	0.943±0.056
Liver (g)	8.863±0.633	9.450±1.121	9.080±1.176	9.062±0.818	9.993±0.726	9.980±1.217	8.830±0.835	9.658±1.347
Spleen (g)	0.525±0.076	0.543±0.083	0.528±0.055	0.502±0.065	0.548±0.057	0.812±0.531	0.623±0.108	0.605±0.080
Kidneys (g)	1.648±0.092	1.733±0.062	1.698±0.121	1.722±0.173	1.738±0.099	1.822±0.131	1.768±0.087	1.867±0.117
Adrenals (mg)	64.10±8.21	66.93±9.72	63.77±6.33	59.32±7.86	54.83±5.97	62.93±8.35	66.10±6.61	65.40±10.75
Ovaries (mg)	86.03±8.66	90.02±11.12	88.68±23.24	85.88±5.81	81.20±8.98	94.00±13.50	82.98±6.36	97.25±13.88
Uterus (g)	0.662±0.244	0.550±0.299	0.393±0.067	0.480±0.222	0.580±0.198	0.527±0.291	0.423±0.052	0.485±0.144
Relative organ weight								
Brain (%)	0.790±0.032	0.733±0.059	0.787±0.089	0.790±0.078	0.840±0.042	0.707±0.042	0.735±0.060	0.728±0.081
Pituitary ($\times10^{-3}$ %)	6.75±0.72	7.05±0.77	6.82±0.86	7.08±1.06	7.07±0.61	6.65±1.27	7.20±1.14	7.68±1.39
Thyroids ($\times10^{-3}$ %)	9.77±1.32	9.03±1.72	10.63±0.55	10.30±1.20	10.83±1.82	9.48±1.57	9.85±0.80	9.22±1.88
Thymus ($\times10^{-3}$ %)	240.97±60.69	180.73±15.00*	211.80±32.15	221.07±40.03	182.67±25.18*	153.03±30.49	181.80±35.74	168.22±22.00
Lungs (%)	0.443±0.025	0.418±0.019	0.443±0.019	0.445±0.018	0.472±0.021	0.427±0.025	0.433±0.036	0.427±0.037
Heart (%)	0.357±0.048	0.347±0.026	0.362±0.025	0.347±0.023	0.355±0.027	0.345±0.048	0.340±0.017	0.357±0.056
Liver (%)	3.663±0.217	3.660±0.204	3.683±0.165	3.737±0.227	4.315±0.304**	3.572±0.361	3.337±0.224	3.588±0.103
Spleen (%)	0.218±0.026	0.212±0.019	0.215±0.023	0.208±0.024	0.238±0.029	0.295±0.209	0.235±0.033	0.225±0.015
Kidneys (%)	0.682±0.037	0.675±0.034	0.697±0.078	0.708±0.030	0.752±0.049	0.652±0.036	0.670±0.046	0.703±0.094
Adrenals ($\times10^{-3}$ %)	26.50±3.09	26.15±4.52	26.02±2.57	24.40±1.82	23.65±1.88	22.53±2.67	25.00±2.11	24.73±5.58
Ovaries ($\times10^{-3}$ %)	35.57±3.44	34.97±3.89	35.80±7.23	35.52±3.09	35.02±3.34	33.55±3.21	31.52±3.37	36.42±5.33
Uterus (%)	0.273±0.096	0.210±0.101	0.160±0.028	0.198±0.095	0.250±0.091	0.192±0.113	0.160±0.021	0.185±0.061

Values are expressed as Mean±S.D.
Significantly different from 0 mg/kg group; **$p<0.01$

Table 5 Summary of histopathological findings of rats treated orally with 2-*tert*-butylphenol in twenty-eight-day repeat dose toxicity test

Sex / Organ / Finding	Dose level (mg/kg) / Number of animals	Administration period					Recovery period		
		0	4	20	100	500	0	100	500
		6	6	6	6	6	6	6	6
Male	(Grade)								
Heart		<6>	<0>	<0>	<0>	<6>	<0>	<0>	<0>
Inflammatory cell infiltration, focal	1+	2				2			
Spleen		<6>	<0>	<0>	<0>	<6>	<0>	<0>	<0>
Increase in hematopoietic cell, erythrocytic	1+	1				0			
Lung		<0>	<0>	<0>	<0>	<0>	<1>	<0>	<0>
Inflammatory cell infiltration, focal	1+						1		
Liver		<6>	<0>	<0>	<0>	<6>	<0>	<0>	<0>
Microgranuloma	1+	1				0			
Necrosis, focal	1+	0				1			
Kidney		<6>	<0>	<1>	<1>	<6>	<0>	<0>	<0>
Basophilic tubule	1+	2		1	0	2			
Cyst	1+	3		1	1	1			
Fibrosis, focal	1+	0		1	0	0			
Hyaline droplet, tubular epithelium, proximal	1+	5		1	1	5			
Mineralization, papilla	1+	1		0	0	1			
Epididymis		<6>	<0>	<0>	<0>	<6>	<0>	<0>	<0>
Inflammatory cell infiltration, lymphocyte, focal	1+	1				1			
Female	(Grade)								
Spleen		<6>	<0>	<0>	<0>	<6>	<1>	<0>	<0>
Congestion	1+	0				0	1		
Lung		<1>	<0>	<0>	<0>	<1>	<0>	<0>	<0>
Foreign body granuloma	1+	1				0			
Inflammatory cell infiltration, focal	1+	0				1			
Liver		<6>	<0>	<0>	<0>	<6>	<0>	<0>	<0>
Fatty change, hepatocyte, periportal	1+	1				1			
Microgranuloma	1+	1				1			
Kidney		<6>	<0>	<0>	<0>	<6>	<1>	<0>	<0>
Basophilic tubule	1+	2				3	0		
Cyst	1+	2				1	0		
Mineralization, papilla	1+	0				1	0		
Dilatation, pelvis	1+	0				0	1		
Uterus		<3>	<2>	<0>	<1>	<2>	<1>	<0>	<1>
Dilatation, lumen	1+	3	2		1	2	1		1

<>:Number of animals examined.
Grade; 1+:slight

2-*tert*-ブチルフェノールの細菌を用いる復帰変異試験

Reverse Mutation Test of 2-*tert*-Butylphenol on Bacteria

要約

2-*tert*-ブチルフェノールの遺伝子突然変異誘発性の有無を検討するため，細菌を用いる復帰変異試験を実施した．

試験は，指標菌株として*Salmonella typhimurium* TA100，TA1535，TA98，TA1537および*Escherichia coli* WP2 *uvrA*を用い，S9 mix非存在(直接法)および存在(代謝活性化法)下でプレインキュベーション法により行った．

用量は，用量設定試験の結果から菌の生育阻害が認められる用量を最高用量とし，直接法および代謝活性化法ともに6.25～200 μg/plateの範囲(公比2)で設定した．

試験を2回行った結果，代謝活性化の有無にかかわらず，全ての菌株において復帰変異コロニー数の増加は認められなかった．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以上で認められ，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以上で認められた．

以上の成績から，2-*tert*-ブチルフェノールの細菌に対する遺伝子突然変異誘発性は陰性と判定した．

方法

1. 指標菌株

国立公衆衛生院地域環境衛生学部から1994年12月19日に分与を受けた*S. typhimurium* TA98，TA100，TA1535，TA1537[1]および*E. coli* WP2 *uvrA*[2]の5菌株を用いた．各菌株は，超低温槽で-80℃以下に凍結保存した．

試験に際して，各凍結菌株を解凍後，その25 μLをニュートリエントブロス(Bacto nutrient broth dehydrated, Difco Laboratories)液体培地15 mLに接種し，37 ℃で12時間振盪培養した．培養後の懸濁菌液は濁度を測定し，濁度と生菌数の換算式より1 mLあたり1 × 10^9以上の生菌数が得られていることを確認し，試験菌液とした．

各菌株の遺伝的特性検査は，凍結保存菌の調製時並びに各実験ごとに行い，本試験に用いた菌株が規定の特性を保持していることを確認した．

2. 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．

3. 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，用時調製した．

4. 陽性対照物質

陽性対照物質として下記のものを使用した．

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

2-AA：2-アミノアントラセン(和光純薬工業㈱)

NaN_3：アジ化ナトリウム(和光純薬工業㈱)

9-AA：9-アミノアクリジン(Aldrich Chemical Co.)

AF-2および2-AAはDMSO(和光純薬工業㈱)に，NaN_3および9-AAは蒸留水(㈱大塚製薬工場)に溶解した．

5. 培地

1) 最少グルコース寒天平板培地(プレート)

テスメディアAN培地(オリエンタル酵母工業㈱)を購入し，使用した．培地1 Lあたりの組成は下記のとおりであり，径90 mmのシャーレ1枚あたりに30 mLを分注したものである．

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	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
寒天(OXOID Agar No.1)	15 g

2) アミノ酸添加軟寒天培地(トップアガー)

0.6 w/v%寒天粉末(Difco Laboratories)および0.5 w/v%塩化ナトリウムの組成の軟寒天を調製し，これに，

*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	994	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	245[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*	4*	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[b]
	[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

4×10^3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

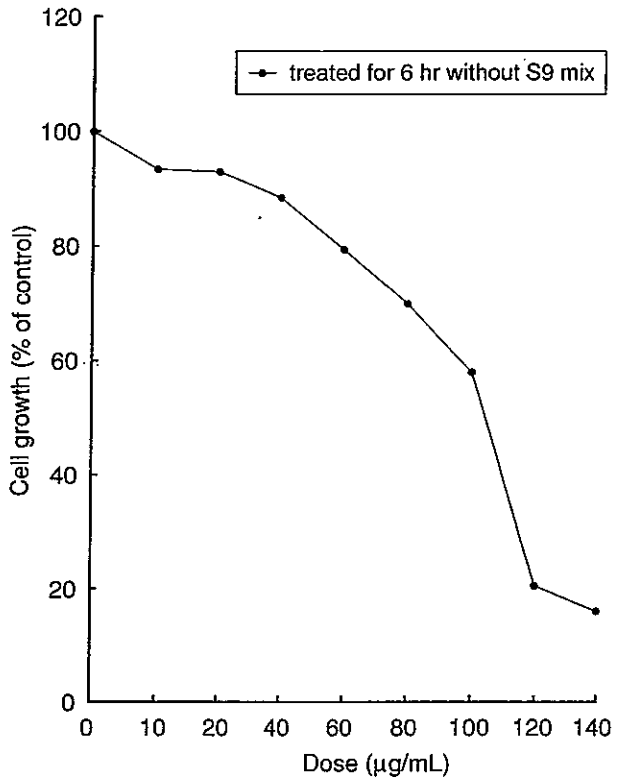

Fig. 1 Growth inhibition of CHL/IU cells treated with 2-*tert*-butylphenol

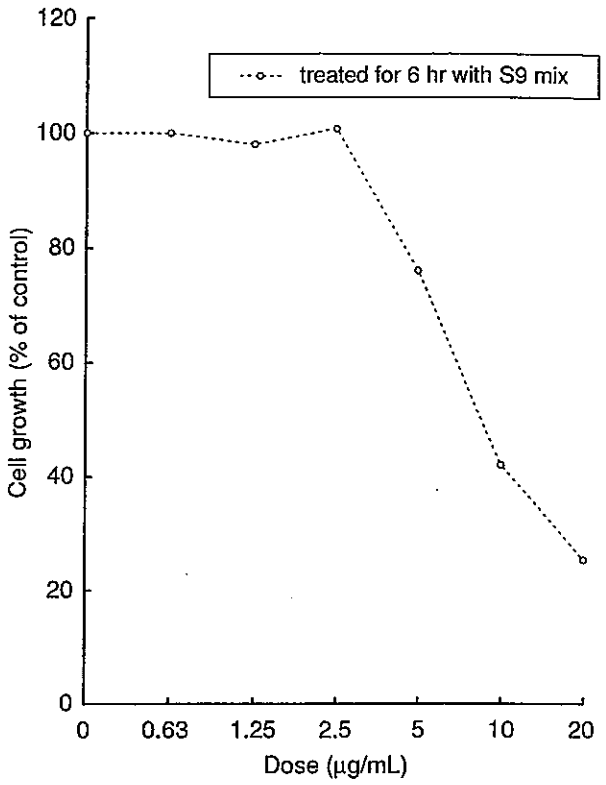

Fig. 2 Growth inhibition of CHL/IU cells treated with 2-*tert*-butylphenol

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.

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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Received June 17, 2005; revised and accepted September 2, 2005.

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,

Table 4 Summary of the results of the newborn and young rat study of 2,4-di-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	5	40	300	
Death	–	–	M: 2/12, F: 1/12	
Clinical signs	–	–	Decrease in locomotor activity bradypnea, hypothermia	
Body weight changes	–	–	9–25%↓	
Urinalysis	n.d.	n.d.	n.d.	
Hematological changes	–	–	–	
Blood biochemical changes	–	–	Various†	
Changes in relative organ weights	–	F: Liver 14%↑	Liver 39–51%↑, Kidney 37–41%↑ M: Spleen 24%↓	
Histopathological findings	–	F: Fatty degeneration in liver	Various changes in liver and kidney‡	
Developmental parameters	–	–	*Slight delay in preputial separation*	
Young rat study				
Dose (mg/kg/day)	5	20	75	300
Death	–	–	–	–
Clinical signs	–	–	–	–
Body weight changes	–	–	–	–
Urinalysis	–	–	–	UV↑ SG↓ OP↓
Hematological changes	–	–	–	Various§
Blood biochemical changes	–	–	F: Tcho↑ Pho↑	M: TB↑ F: Tcho↑ Pho↑
Changes in relative organ weights	–	–	F: Liver 13%↑	Liver 40–43%↑
Histopathological findings	n.d.	n.d.	–	Various changes in liver and kidney¶

Data on death are shown as no. of dead animals/no. of animals examined, according to sex. Statistically significant increases ($P < 0.05$) in body weights, urinalysis and blood biochemical parameters, and relative organ weights are shown as ↑, while decreases are shown as ↓. 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. †Increase in total bilirubin and decrease in the A/G ratio in both sexes, increase in γ-GTP in males, and increase in total protein and BUN in females were noted. ‡Various changes were observed as shown in Table 3. §Various hematological changes were noted such as decrease in hemoglobin and hematocrit and increase in segmented neutrophils in females and prolongation of PT and APTT in males. ¶Various changes were observed as shown in Table 5. OP: osmotic pressure; Pho: phospholipid; SG: specific gravity; TB: total bilirubin; Tcho: total cholesterol; UV: urine volume; –: no change; n.d.: not determined.

or critical histopathological damage (Koizumi *et al.* 2001). We here tried to apply this UETL approach to the present study. For 2TBP, clinical signs such as decrease in locomotor activity and ataxic gait were noted in most of the animals given 200 mg/kg (newborn rats) and 500 mg/kg (young rats) (Table 2). Furthermore, a 8–17% lowering of body weight was observed at 200 mg/kg in newborn rats, but not in the young rat study. Therefore, equivalent toxic effects to these observed at 500 mg/kg in young rats might be expected to appear at 100–150 mg/kg in newborn animals. The UETLs were concluded to be 100–150 and 500 mg/kg/day in newborn and young rats, respectively. In the case of DTBP, clear toxicity was observed at the top dose of 300 mg/kg in both newborn and young rat studies (Table 4), but the level of severity was very different, for example, deaths were only noted in the newborn cases. It was considered difficult to estimate the UETLs from the results of main studies only. However, the most critical endpoint for toxicity, mortality, was also noted at 100 mg/kg and more, and 500 mg/kg, in the dose-finding studies of newborn and young rats, respectively. Therefore, it would be possible to estimate the appropriate UETLs as the minimum lethal dose by taking the results of the dose-finding studies into consideration. The UETLs were concluded to be 100 mg/kg/day for the newborn, and 500 mg/kg/day for young rats, at which one out of eight rats was found dead in both cases. These analyzes of UETLs, considering equivalence in toxic degree, showed 3.3–5.0 times higher susceptibility of newborn rats to 2TBP and DTBP than young rats, consistent with our analytical results for NOAELs.

Higher susceptibility of newborn rats was also demonstrated in our previous analyzes of five phenols (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol and 2,4,6-trinitrophenol) (Koizumi *et al.* 2001, 2002, 2003; Takahashi *et al.* 2004), considered mainly due to their poor metabolic and excretory capacity (Horster 1977; Cresteil *et al.* 1986). It has actually been reported that UDP-glucuronyltransferase and sulfotransferase activities, when 4-nitrophenol is used as the substrate, are lower in microsomes prepared from livers of newborn rats, and that the elimination rate of 2,4-dinitrophenol from serum of newborn rabbits is markedly slower than in young adults (Gehring & Buerge 1969; Matsui & Watanabe 1982). Unfortunately, there is no information on the toxicity mechanism and toxicokinetics of both 2TBP

Table 5 Histopathological findings for the young rat study of 2,4-di-*tert*-butylphenol

		Scheduled-sacrifice group†			Recovery group	
Dose (mg/kg/day)	Grade	0	75	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	6/6	6/6
Liver						
– Centrilobular hypertrophy of hepatocytes	+	0/0	0/0	4/4	0/0	0/0
Kidneys						
– Basophilic tubules	+	0/0	0/0	1/4	0/0	3/1
	++	0/0	0/0	4/0	0/0	2/0
	+++	0/0	0/0	1/1	0/0	1/0
– Granular casts	+	0/0	0/0	5/2	0/0	4/0
	++	0/0	0/0	1/1	0/0	0/0
– Proteinaceous casts	+	0/0	0/0	5/1	0/0	2/0
	++	0/0	0/0	1/0	0/0	0/0

†No histopathological examination was conducted for the 5 and 20 mg/kg scheduled-sacrifice groups. +, mild; ++, moderate; +++, marked.

and DTBP; however, the immature functions involved in the toxicokinetics in newborn rats would be implicated in the higher susceptibility, as in the case of five phenols previously analyzed. While there are very little data on toxicokinetics of environmental chemicals in the newborn, relatively plentiful information has been reported in humans for pharmaceuticals which are clinically applied during the early postnatal period. Recently, Ginsberg *et al.* (2002) conducted comparative analysis of pharmacokinetic parameters for 45 drugs in both children and adults, and showed half-lives in children aged two months or under to generally be two-fold longer than in adults.

As for the susceptibility of the newborn to toxicity of chemicals, although it is generally important to take the sensitivity of target organs and tissues themselves (toxicodynamics) into consideration besides toxicokinetics, there are insufficient data on differences between newborn and young/adult animals. For appearance of toxicity, which is the outcome of toxicokinetics and toxicodynamics, some comparative studies have relied on LD_{50} values (Goldenthal 1971; Sheehan & Gaylor 1990). However, it is not considered that information on acute toxicity at lethal dosage is appropriate when considering the susceptibility of newborn in risk assessment, because dose–response curves could differ, as mentioned above. With prolonged, subtoxic doses, which are basis for TDI or ADI, our series of comparative studies constitute the first systematic assessment, providing an important base for development of new methods of risk assessment of susceptibility of the newborn.

In conclusion, clinical signs and effects on the liver were observed for 2TBP, and hepatic and renal toxicity for DTBP. Although there were no clear differences in toxicity profiles between the newborn and young rats for both chemicals, the toxicity levels differed markedly. The susceptibility of the newborn to these chemicals appears to be 4–5 times higher than that of young animals.

ACKNOWLEDGMENT

The authors gratefully acknowledge the financial support of the Office of Chemical Safety, Pharmaceutical and Medical Safety Bureau, Ministry of Health, Labor and Welfare, Japan.

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BIOASSAY OF

1,5-NAPHTHALENEDIAMINE

FOR POSSIBLE CARCINOGENICITY

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Public Health Service
National Institutes of Health

DHEW Publication No. (NIH) 78-1398

REPORT ON THE BIOASSAY OF 1,5-NAPHTHALENEDIAMINE
FOR POSSIBLE CARCINOGENICITY

CARCINOGENESIS TESTING PROGRAM
DIVISION OF CANCER CAUSE AND PREVENTION
NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH

FOREWORD: This report presents the results of the bioassay of 1,5-naphthalenediamine conducted for the Carcinogenesis Testing Program, Division of Cancer Cause and Prevention, National Cancer Institute (NCI), National Institutes of Health, Bethesda, Maryland. This is one of a series of experiments designed to determine whether selected chemicals have the capacity to produce cancer in animals. Negative results, in which the test animals do not have a significantly greater incidence of cancer than control animals, do not necessarily mean the test chemical is not a carcinogen because the experiments are conducted under a limited set of circumstances. Positive results demonstrate that the test chemical is carcinogenic for animals under the conditions of the test and indicate a potential risk to man. The actual determination of the risk to man from animal carcinogens requires a wider analysis.

CONTRIBUTORS: This bioassay of 1,5-naphthalenediamine was conducted by Mason Research Institute, Worcester, Massachusetts, initially under direct contract to the NCI and currently under a subcontract to Tracor Jitco, Inc., prime contractor for the NCI Carcinogenesis Testing Program.

The experimental design was determined by the NCI Project Officers, Dr. J. H. Weisburger (1,2) and Dr. E. K. Weisburger (1). The principal investigators for the contract were Dr. E. Smith (3) and Dr. A. Handler (3). Animal treatment and observation were supervised by Mr. G. Wade (3) and Ms. E. Zepp (3). Chemical analysis was performed by Midwest Research Institute (4) and the analytical results were reviewed by Dr. N. Zimmerman (5).

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Compilation of individual animal survival, pathology, and summary tables was performed by EG&G Mason Research Institute (7); the

statistical analysis was performed by Mr. W. W. Belew (5,8) and Mr. R. M. Helfand (5), using methods selected for the Carcinogenesis Testing Program by Dr. J. J. Gart (9).

This report was prepared at METREK, a Division of The MITRE Corporation (5) under the direction of the NCI. Those responsible for this report at METREK are the project coordinator, Dr. L. W. Thomas (5), task leader Dr. M. R. Kornreich (5,10), senior biologist Ms. P. Walker (5), biochemist Mr. S. C. Drill (5), and technical editor Ms. P. A. Miller (5). The final report was reviewed by members of the participating organizations.

The following other scientists at the National Cancer Institute were responsible for evaluating the bioassay experiment, interpreting the results, and reporting the findings: Dr. K. C. Chu (1), Dr. C. Cueto, Jr. (1), Dr. J. F. Douglas (1), Dr. D. G. Goodman (1,10), Dr. R. A. Griesemer (1), Dr. M. H. Levitt (1), Dr. H. A. Milman (1), Dr. T. W. Orme (1), Dr. R. A. Squire (1,11), Dr. S. F. Stinson (1), Dr. J. M. Ward (1), and Dr. C. E. Whitmire (1).

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SUMMARY

A bioassay of 1,5-naphthalenediamine for possible carcinogenicity was conducted using Fischer 344 rats and B6C3F1 mice. 1,5-Naphthalenediamine was administered in the feed, at either of two concentrations, to groups of 50 male and 50 female animals of each species. The high and low dietary concentrations utilized in the chronic bioassay were, respectively, 0.1 and 0.05 percent for rats and 0.2 and 0.1 percent for mice. The compound was administered in the diet for 103 weeks, followed by up to 4 weeks of observation. Fifty mice of each sex and 25 rats of each sex were placed on test as controls. These animals were observed for up to 110 weeks.

There were no significant positive associations between the administered concentrations of 1,5-naphthalenediamine and mortality in either sex of rats or mice. In all groups adequate numbers of animals survived sufficiently long to be at risk from late-developing tumors.

Among dosed female rats, a statistically significant increase in endometrial stromal polyps was observed. Several of these tumors underwent malignant transformation to endometrial stromal sarcomas. The incidence of female rats having either adenoma or carcinoma of the clitoral gland was statistically significant. No neoplasms were observed at significantly increased incidences in dosed male rats. Based on lack of clinical signs or weight loss, the male rats may have been able to withstand a higher dose.

In mice, dose-related increases in thyroid neoplasms were observed in both sexes. The incidence of thyroid C-cell carcinomas was significant for high dose female mice. The combined incidences of papillary adenomas, follicular-cell adenomas and papillary cystadenomas of the thyroid were significant for mice of both sexes. The incidence of hepatocellular carcinomas and the incidence of alveolar/bronchiolar adenomas were each significant for dosed female mice.

Under the conditions of this bioassay, 1,5-naphthalenediamine was carcinogenic in female Fischer 344 rats, causing clitoral and uterine neoplasms. 1,5-Naphthalenediamine was also carcinogenic for B6C3F1 mice, producing thyroid neoplasms in males and neoplasms of the thyroid, liver, and lung in females. Insufficient evidence was provided for the carcinogenicity of the compound in male Fischer 344 rats.

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I. INTRODUCTION

1,5-Naphthalenediamine (Figure 1) (NCI No. C03021), a bicyclic aromatic amine used in the dye industry, was selected for bioassay by the National Cancer Institute because of the high incidence of bladder cancer reported among dye manufacturing industry workers (Anthony and Thomas, 1970; Wynder et al., 1963). Aromatic amines are one class of chemicals believed to contribute to the increased cancer risk in this industry (Wynder et al., 1963). The structural similarity of 1,5-naphthalenediamine to both the human bladder carcinogen 2-naphthylamine (International Agency for Research on Cancer [IARC], 1974) and the suspected carcinogen 1-naphthylamine (IARC, 1974) was an additional factor in its selection for testing.

The Chemical Abstracts Service (CAS) Ninth Collective Index (1977) name for this compound is 1,5-naphthalenediamine.* It is also known as 1,5-diaminonaphthalene.

1,5-Naphthalenediamine can be used as an oxidation base (Colour Index [C.I.] 76595), an intermediate in the synthesis of the dye Naphthylene Red (C.I. 21650) (Society of Dyers and Colourists, 1956), and in the production of a black trisazo dye for cotton (Taube, 1973). 1,5-Naphthalenediamine has also been used as a precursor for 1,5-naphthalenediisocyanate (Hirai and Yamamoto, 1975); as an intermediate in the synthesis of drugs for the symptomatic treatment of asthma or

*The CAS registry number is 2243-62-1

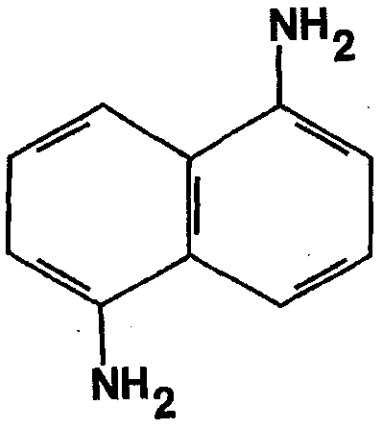


FIGURE 1
CHEMICAL STRUCTURE OF 1,5-NAPHTHALENEDIAMINE

2

rhinitis (Hall, 1976); as a component of piperazine-modified aromatic polyamides (Fujiwara et al., 1974); and as a modifier for phenolic resins used in rapid curing compounds (Freeman et al., 1974); however, these uses appear to be purely experimental.

Specific production data for 1,5-naphthalenediamine are not available; however, the exclusion of this compound from the 1977 Directory of Chemical Producers, U.S.A. (Stanford Research Institute, 1977) implies that it is not produced in commercial quantities (in excess of 1000 pounds or $1000 in value annually).

The potential for exposure to 1,5-naphthalenediamine may be greatest for workers in the dye industry and persons engaged in chemical research with this compound.

II. MATERIALS AND METHODS

A. Chemicals

1,5-Naphthalenediamine was purchased from Carroll Products, Wood River Junction, Rhode Island by the NCI for Mason Research Institute, Worcester, Massachusetts, and chemical analysis was performed by Midwest Research Institute, Kansas City, Missouri. The experimentally determined melting point of 190° to 191°C suggested a compound of high purity based on its narrow range and its close proximity to the value (190°C) reported in the literature (Pollock and Stevens, 1965). Elemental analysis was consistent with $C_{10}H_{10}N_2$, the molecular formula for 1,5-naphthalenediamine. However, nonaqueous amine group titration was approximately 89 to 90 percent of that expected on a theoretical basis. Vapor-phase chromatography revealed one homogeneous peak, but thin-layer chromatography utilizing two solvent systems (acetone:ammonium hydroxide and methylethylketone:formic acid), each visualized with 254 nm and 367 nm light, indicated the presence of one nonmotile impurity. Nuclear magnetic and infrared analyses were consistent with the structure of the compound. Ultraviolet analysis showed λ_{max} at 232, 328 and 498 nm with ϵ values of 62,800, 10,640 and 9, respectively. The literature (Sadtler Standard Spectra) indicates a λ_{max} at 328.5 nm with ϵ = 10,000 for 1,5-naphthalenediamine. The observed ϵ at 328 nm was 10,640 (6 percent greater than expected).

Throughout this report the term 1,5-naphthalenediamine is used to represent this compound.

B. Dietary Preparation

The basal laboratory diet for both dosed and control animals consisted of Wayne Lab-Blox® (Allied Mills, Inc., Chicago, Illinois). 1,5-Naphthalenediamine was administered to the dosed animals as a component of the diet. Under an exhaust hood, proper amounts of the chemical were removed from the stock bottle. The compound was blended in an aluminum bowl with an aliquot of the ground feed. Once visual homogeneity was attained, the mixture was placed into a 6 kg capacity Patterson-Kelley twin-shell stainless steel V-blender, along with the remainder of the meal and blended for 20 minutes. Prepared diets were placed in double plastic bags and stored in the dark at 4°C. The mixture was used for 1 week only.

C. Animals

Two animal species, rats and mice, were used in the carcinogenicity bioassay. Fischer 344 rats and B6C3F1 mice were obtained through contracts of the Division of Cancer Treatment, National Cancer Institute. All animals were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts. Dosed and control animals were received in separate shipments. Upon arrival, a sample of animals was examined for parasites and other signs of disease. All animals appeared to have parasites. They were treated with 3.0 gm of piperazine adipate per liter of drinking water, ad libitum, for 3 days, followed by 3 days of plain tapwater and 3 subsequent days of piperazine adipate administration. During this period, new cages

with fresh bedding were provided daily. Animals were held in quarantine by species for 2 weeks prior to initiation of test. Animals were assigned to groups and distributed among cages so that average body weight per cage was approximately equal for a given sex and species.

D. Animal Maintenance

All animals were housed by species in rooms having a temperature range of 23° to 34°C. Incoming air was filtered through Tri-Dek® 15/40 denier Dacron® filters (Tri-Dim Filter Corp., Hawthorne, New Jersey) providing six changes of room air per hour. Fluorescent lighting was provided on a 12-hour-daily cycle.

Rats were housed five per cage by sex. During quarantine and for the first 14 months of study rats were housed in galvanized-steel wire-mesh cages suspended over newspapers. Newspapers under cages were replaced daily and cages and racks washed weekly. For the remainder of the study, rats were held in suspended polycarbonate cages equipped with disposable nonwoven fiber filter sheets. Clean bedding and cages were provided twice weekly. SAN-I-CEL® corncob bedding (Paxton Processing Company, Paxton, Illinois) was used for the first 2 months that rats were housed in polycarbonate cages. For the remainder of the study, Aspen hardwood chip bedding (American Excelsior Company, Baltimore, Maryland) was used. Stainless steel cage racks were cleaned once every 2 weeks, and disposable filters were replaced at that time.

Mice were housed by sex in polycarbonate shoe box type cages. Cages were fitted with perforated stainless steel lids (Lab Products, Inc., Garfield, New Jersey). Nonwoven fiber filter bonnets were used over cage lids. Control mice were housed ten per cage for the first month of study and five per cage thereafter. Dosed mice were held five per cage throughout the study. Clean cages, lids, and bedding were provided twice per week. SAN-I-CEL® was used during the first 9 months of study. A second corncob bedding (Bed-o-Cobs®, The Andersons Cob Division, Maumee, Ohio) was used for the next 8 months. Aspen bedding was used for the remainder of the study. Reusable filter bonnets and pipe racks were sanitized every 2 weeks throughout the study.

Water was available from 250 ml polycarbonate water bottles equipped with rubber stoppers and stainless steel sipper tubes. Bottles were replaced twice weekly and, for rats only, water was supplied as needed between changes. Food and water were available ad libitum.

Wayne Lab-Blox® meal was supplied to rats for 12 months and mice for 11 months from Alpine® aluminum feed cups (Curtin Matheson Scientific, Inc., Woburn, Massachusetts) containing stainless steel baffles. After that period, meal was supplied from stainless steel gangstyle food hoppers (Scientific Cages, Inc., Bryan, Texas). During the 2-year period of chemical administration, dosed animals were supplied

with meal containing the appropriate concentrations of 1,5-naphthalenediamine. Control animals had untreated meal available. Food hoppers were changed on the same schedule as were cages. Food was replenished daily in Alpine® feed cups.

All rats utilized in the 1,5-naphthalenediamine bioassay were housed in a room with other rats receiving diets containing* acetylaminofluorene (53-96-3); sodium nitrite (76-32-00-0); L-arginine glutamate (4320-30-3); N-butylurea (592-31-4); N,N-dimethyl-p-nitrosoaniline (138-89-6); 2,5-toluenediamine sulfate (6369-59-1); 2,4-dinitrotoluene (121-14-2); 4-nitroanthranilic acid (619-17-0); N-(1-naphthyl)ethylenediamine dihydrochloride (1465-25-4); 2-chloro-p-phenylenediamine sulfate (61702-44-1); aniline hydrochloride (142-04-1); and p-anisidine hydrochloride (20265-97-8).

Dosed mice were in a room with mice intubated with m-cresidine (102-50-1); and with other mice receiving diets containing N-(1-naphthyl)ethylenediamine dihydrochloride (1465-25-4) and 1H-benzotriazole (95-14-7). Control mice were in a room with other mice receiving diets containing hydrazobenzene (530-50-7); 2,3,5,6-tetrachloro-4-nitroanisole (2438-88-2); tris(2,3-dibromopropyl)phosphate (126-72-7); N-(1-naphthyl)ethylenediamine dihydrochloride (1465-25-4); aniline hydrochloride (142-04-1); and 2-chloro-o-phenylenediamine sulfate.

*CAS registry numbers are given in parentheses.

E. Selection of Initial Concentrations

In order to establish the maximum tolerated concentrations of 1,5-naphthalenediamine for administration to dosed animals in the chronic studies, subchronic toxicity studies were conducted with both rats and mice. Animals of each species were distributed among six groups, each consisting of five males and five females. 1,5-Naphthalenediamine was incorporated into the basal laboratory diet and supplied ad libitum to five of the six rat groups and five of the six mouse groups in concentrations of 0.03, 0.1, 0.3, 1.0, and 3.0 percent. The sixth group of each species served as a control group, receiving only the basal laboratory diet. The dosed dietary preparations were administered for 8 weeks.

The highest concentration causing no deaths, no compound-related gross abnormalities, and no mean body weight depression in excess of 20 percent relative to controls was selected as the high concentration for the chronic bioassay.

Deaths were recorded for all groups of rats receiving concentrations of 0.3 percent or more. Mean body weight depression was approximately 19 and 9 percent, respectively, in males and females dosed with 0.1 percent 1,5-naphthalenediamine. The concentration of 1,5-naphthalenediamine selected for administration as the high dose in the rat chronic bioassay was 0.1 percent.

Deaths were recorded for all groups of mice receiving concentrations of 0.3 percent or more and in the group of female mice

receiving 0.03 percent. Mean body weight depression was approximately 22 and 3 percent, respectively, in males and females dosed with 0.3 percent. Males receiving 0.1 percent experienced mean body weight depression of approximately 3 percent, while females receiving the same concentration had a greater mean body weight than the controls. The concentration of 1,5-naphthalenediamine selected for administration as the high dose in the mouse chronic bioassay was 0.2 percent.

F. Experimental Design

The experimental design parameters for the chronic study (species, sex, group size, concentrations administered, and duration of treated and untreated observation periods) are summarized in Tables 1 and 2.

Rats were all approximately 7 weeks old at the time they were placed on test. Dosed rats were born approximately 1 month earlier than controls and were started on test 1 month earlier than controls. The dietary concentrations of 1,5-naphthalenediamine administered were 0.10 and 0.05 percent. Throughout this report those rats receiving the former concentration are referred to as the high dose groups and those receiving the latter concentration are referred to as the low dose groups. The dosed rats were supplied with feed containing 1,5-naphthalenediamine for a total of 103 weeks, followed by a 3- to 4-week observation period.

All mice were approximately 7 weeks old at the time they were placed on test. Dosed mice were born approximately 1 month earlier

TABLE 1

DESIGN SUMMARY FOR FISCHER 344 RATS
1,5-NAPHTHALENEDIAMINE FEEDING EXPERIMENT

	INITIAL GROUP SIZE	1,5-NAPHTHALENE-DIAMINE CONCENTRATION (PERCENT)	OBSERVATION PERIOD TREATED (WEEKS)	OBSERVATION PERIOD UNTREATED (WEEKS)
MALE				
CONTROL	25	0	0	109
LOW DOSE	50	0.05 0	103	3
HIGH DOSE	50	0.10 0	103	3
FEMALE				
CONTROL	25	0	0	110
LOW DOSE	50	0.05 0	103	3
HIGH DOSE	50	0.10 0	103	4

TABLE 2

DESIGN SUMMARY FOR B6C3F1 MICE
1,5-NAPHTHALENEDIAMINE FEEDING EXPERIMENT

	INITIAL GROUP SIZE	1,5-NAPHTHALENE-DIAMINE CONCENTRATION (PERCENT)	OBSERVATION PERIOD	
			TREATED (WEEKS)	UNTREATED (WEEKS)
MALE				
CONTROL	50	0	0	109
LOW DOSE	50	0.1 0	103	2
HIGH DOSE	50	0.2 0	103	2
FEMALE				
CONTROL	50	0	0	109
LOW DOSE	50	0.1 0	103	2
HIGH DOSE	50	0.2 0	103	3

than controls and were started on test 1 month earlier than controls. The dietary concentrations of 1,5-naphthalenediamine administered were 0.2 and 0.1 percent. Throughout this report those mice receiving the former concentration are referred to as the high dose groups and those receiving the latter concentration are referred to as the low dose groups. The dosed mice were supplied with feed containing 1,5-naphthalenediamine for a total of 103 weeks, followed by a 2- to 3-week observation period.

G. Clinical and Histopathologic Examinations

Animals were weighed immediately prior to initiation of the experiment. Body weights were recorded twice weekly for the first 12 weeks of the study and at monthly intervals thereafter. From the first day, all animals were inspected twice daily for mortality. Food consumption, for two cages from each group, was monitored for seven consecutive days once a month for the first nine months of the bioassay and for three consecutive days each month thereafter. The presence of tissue masses and lesions was determined by monthly observation and palpation of each animal.

A necropsy was performed on each animal regardless of whether it died, was killed when moribund, or was sacrificed at the end of the bioassay. The animals were euthanized by carbon dioxide inhalation, and were immediately necropsied. The histopathologic examination consisted of gross and microscopic examination of major tissues, organs,

and gross lesions taken from sacrificed animals and, whenever possible, from animals found dead.

Tissues were preserved in 10 percent buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin prior to microscopic examination. An occasional section was subjected to special staining techniques for more definitive diagnosis.

Slides were prepared from the following tissues: skin, subcutaneous tissue, larynx, lungs and bronchi, trachea, bone marrow, spleen, lymph nodes, thymus, heart, salivary gland, liver, gallbladder (mice), pancreas, esophagus, stomach, small intestine, large intestine, kidney, urinary bladder, pituitary, adrenal, thyroid, parathyroid, ear, brain, testis, prostate, uterus, mammary gland, and ovary.

A few tissues were not examined for some animals, particularly for those that died early. Also, some animals were missing, cannibalized, or judged to be in such an advanced state of autolysis as to preclude histopathologic interpretation. Thus, the number of animals for which particular organs, tissues, or lesions were examined microscopically varies and does not necessarily represent the number of animals that were placed on experiment in each group.

H. Data Recording and Statistical Analyses

Pertinent data on this experiment have been recorded in an automatic data processing system, the Carcinogenesis Bioassay Data System (Linhart et al., 1974). The data elements include descriptive information on the chemicals, animals, experimental design, clinical

observations, survival, body weight, and individual pathologic results, as recommended by the International Union Against Cancer (Berenblum, 1969). Data tables were generated for verification of data transcription and for statistical review.

These data were analyzed using the statistical techniques described in this section. Those analyses of the experimental results that bear on the possibility of carcinogenicity are discussed in the statistical narrative sections.

Probabilities of survival were estimated by the product-limit procedure of Kaplan and Meier (1958) and are presented in this report in the form of graphs. Animals were statistically censored as of the time that they died of other than natural causes or were found to be missing; animals dying from natural causes were not statistically censored. Statistical analyses for a possible dose-related effect on survival used the method of Cox (1972) when testing two groups for equality and used Tarone's (1975) extensions of Cox's methods when testing a dose-related trend. One-tailed P-values have been reported for all tests except the departure from linearity test, which is only reported when its two-tailed P-value is less than 0.05.

The incidence of neoplastic or nonneoplastic lesions has been given as the ratio of the number of animals bearing such lesions at a specific anatomic site (numerator) to the number of animals in which that site was examined (denominator). In most instances, the denominators included only those animals for which that site was examined

histologically. However, when macroscopic examination was required to detect lesions prior to histologic sampling (e.g., skin or mammary tumors), or when lesions could have appeared at multiple sites (e.g., lymphomas), the denominators consist of the numbers of animals necropsied.

The purpose of the statistical analyses of tumor incidence is to determine whether animals receiving the test chemical developed a significantly higher proportion of tumors than did the control animals. As a part of these analyses, the one-tailed Fisher exact test (Cox, 1970, pp. 48-52) was used to compare the tumor incidence of a control group to that of a group of treated animals at each dose level. When results for a number of treated groups, k, are compared simultaneously with those for a control group, a correction to ensure an overall significance level of 0.05 may be made. The Bonferroni inequality (Miller, 1966, pp. 6-10) requires that the P-value for any comparison be less than or equal to 0.05/k. In cases where this correction was used, it is discussed in the narrative section. It is not, however, presented in the tables, where the Fisher exact P-values are shown.

The Cochran-Armitage test for linear trend in proportions, with continuity correction (Armitage, 1971, pp. 362-365), was also used when appropriate. Under the assumption of a linear trend, this test determined if the slope of the dose-response curve is different from zero at the one-tailed 0.05 level of significance. Unless otherwise

noted, the direction of the significant trend was a positive dose relationship. This method also provides a two-tailed test of departure from linear trend.

A time-adjusted analysis was applied when numerous early deaths resulted from causes that were not associated with the formation of tumors. In this analysis, deaths that occurred before the first tumor was observed were excluded by basing the statistical tests on animals that survived at least 52 weeks, unless a tumor was found at the anatomic site of interest before week 52. When such an early tumor was found, comparisons were based exclusively on animals that survived at least as long as the animal in which the first tumor was found. Once this reduced set of data was obtained, the standard procedures for analyses of the incidence of tumors (Fisher exact tests, Cochran-Armitage tests, etc.) were followed.

When appropriate, life-table methods were used to analyze the incidence of tumors. Curves of the proportions surviving without an observed tumor were computed as in Saffiotti et al. (1972). The week during which animals died naturally or were sacrificed was entered as the time point of tumor observation. Cox's methods of comparing these curves were used for two groups; Tarone's extension to testing for linear trend was used for three groups. The statistical tests for the incidence of tumors which used life-table methods were one-tailed and, unless otherwise noted, in the direction of a positive dose

relationship. Significant departures from linearity ($P < 0.05$, two-tailed test) were also noted.

The approximate 95 percent confidence interval for the relative risk of each dosed group compared to its control was calculated from the exact interval on the odds ratio (Gart, 1971). The relative risk is defined as p_t/p_c where p_t is the true binomial probability of the incidence of a specific type of tumor in a treated group of animals and p_c is the true probability of the spontaneous incidence of the same type of tumor in a control group. The hypothesis of equality between the true proportion of a specific tumor in a treated group and the proportion in a control group corresponds to a relative risk of unity. Values in excess of unity represent the condition of a larger proportion in the treated group than in the control.

The lower and upper limits of the confidence interval of the relative risk have been included in the tables of statistical analyses. The interpretation of the limits is that in approximately 95 percent of a large number of identical experiments, the true ratio of the risk in a treated group of animals to that in a control group would be within the interval calculated from the experiment. When the lower limit of the confidence interval is greater than one, it can be inferred that a statistically significant result (a $P < 0.025$ one-tailed test when the control incidence is not zero, $P < 0.050$ when the control incidence is zero) has occurred. When the lower limit is less than unity but the upper limit is greater than unity,

the lower limit indicates the absence of a significant result while the upper limit indicates that there is a theoretical possibility of the induction of tumors by the test chemical which could not be detected under the conditions of this test.

III. CHRONIC TESTING RESULTS: RATS

A. Body Weights and Clinical Observations

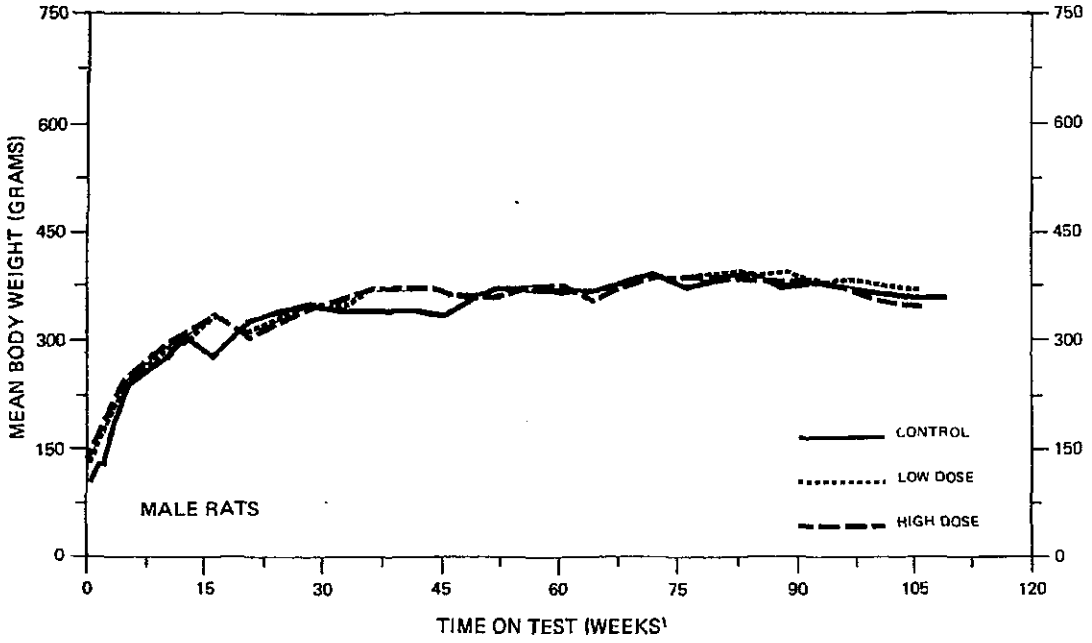
There was no appreciable depression in mean body weight when dosed rats were compared with their respective controls (Figure 2).

Subcutaneous masses were observed in 2 high dose, 3 low dose, and 1 control males, and in 12 high dose, 3 low dose, and 2 control females. Crusted cutaneous masses occurred in 4 high dose males, 1 low dose male, 2 low dose females, and 1 control female, while firm nodular growths were detected in 1 high dose, 2 low dose, and 2 control males, and in 1 low dose female. Swelling of the eyes was exhibited by 2 high dose males, 2 high dose females, and 2 low dose females and swelling of the nose by 1 low dose male. Only 1 control female experienced crusted lesions in the vaginal area while 4 low dose and 9 high dose females were so effected. Alopecia was recorded for 1 low dose female, emaciation was observed in 1 male and 1 female control, and 1 female control exhibited abdominal distention.

B. Survival

The estimated probabilities of survival for male and female rats in the control and 1,5-naphthalenediamine-dosed groups are shown in Figure 3. There was no significant positive association between dosage and mortality for either male or female rats.

Adequate numbers of male rats were at risk from late-developing tumors with 74 percent (37/50) of the high dose, 80 percent (40/50) of the low dose and 68 percent (17/25) of the control surviving on



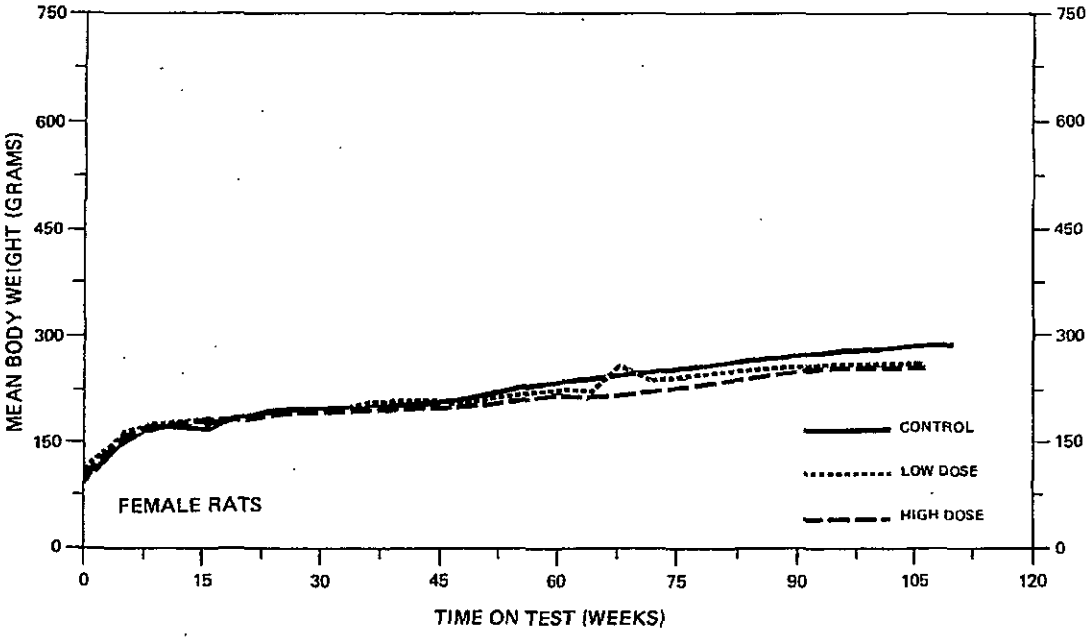
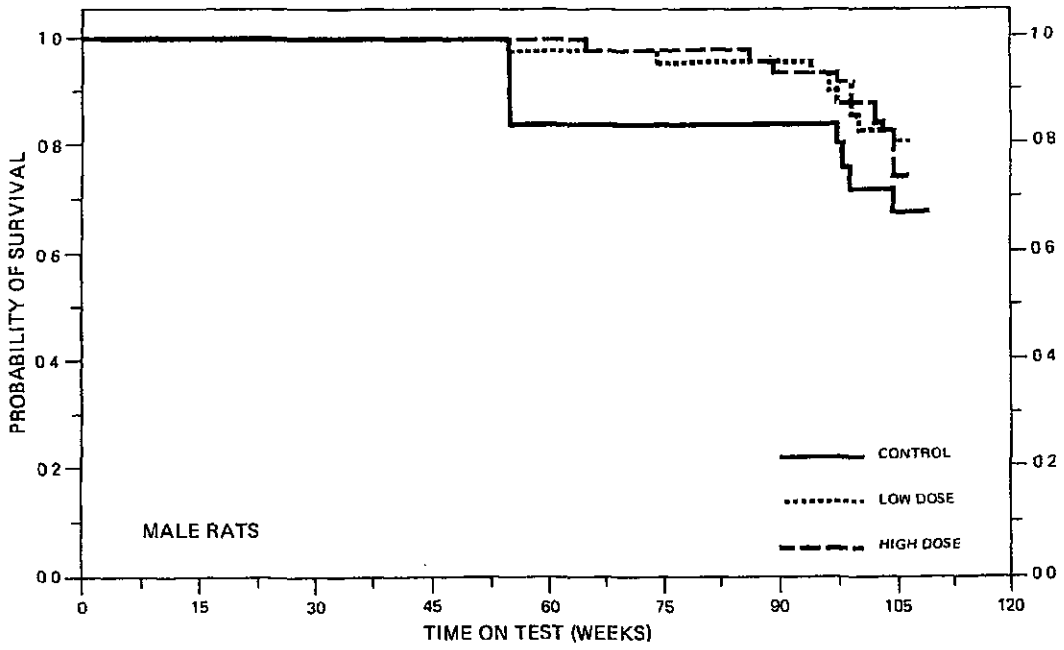


FIGURE 2
GROWTH CURVES FOR 1,5-NAPHTHALENEDIAMINE CHRONIC STUDY RATS



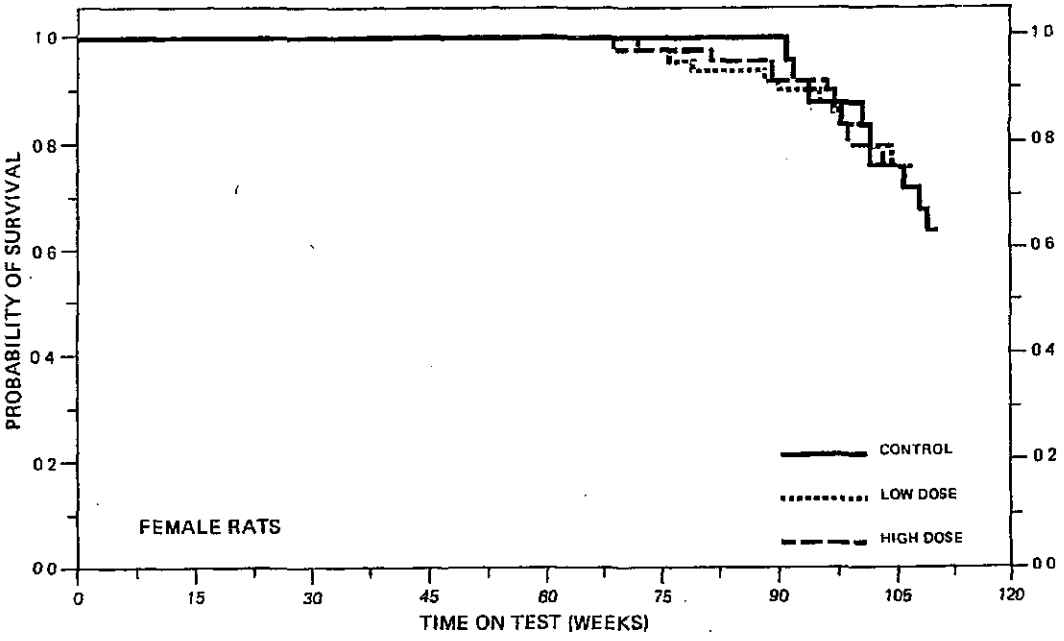


FIGURE 3
SURVIVAL COMPARISONS OF 1,5-NAPHTHALENEDIAMINE CHRONIC STUDY RATS

test until the termination of the study. No lesions were reported for the 4 control rats that died in week 55.

With 76 percent (38/50) of the high dose, 76 percent (38/50) of the low dose and 64 percent (16/25) of the control rats surviving on test until the termination of the study, adequate numbers of females were at risk from late-developing tumors.

C. Pathology

Histopathologic findings on neoplasms in rats are summarized in Appendix A (Tables A1 and A2); findings on nonneoplastic lesions are summarized in Appendix C (Tables C1 and C2).

The incidence of liver neoplasms in male and female rats administered 1,5-naphthalenediamine in the diet appeared to be increased relative to controls. In female rats, tumors of the clitoral gland, uterus, and C-cell neoplasms of the thyroid appeared to be related to compound administration. The incidences of these tumors are as follows:

	MALES			FEMALES		
	Control	Low Dose	High Dose	Control	Low Dose	High Dose
LIVER						
(Number of animals with tissues examined histopathologically)	(25)	(49)	(49)	(24)	(50)	(49)
Neoplastic Nodule	1	3	2	0	3	4
Hepatocellular Carcinoma	0	4	2	0	1	0
PREPUTIAL/CLITORAL GLAND						
(Number of animals necropsied)	(25)	(49)	(50)	(24)	(50)	(50)
Carcinoma	0	0	1	1	3	8
Adenoma	0	0	1	0	0	5

	MALES			FEMALES		
	Control	Low Dose	High Dose	Control	Low Dose	High Dose
UTERUS AND ENDOMETRIUM						
(Number of animals with tissues examined histopathologically)	-	-	-	(24)	(49)	(48)
Adenocarcinoma				1	2	4
Endometrial Stromal Polyp				2	14	20
Endometrial Stromal Sarcoma				1	2	2
THYROID						
(Number of animals with tissues examined histopathologically)	(21)	(47)	(47)	(21)	(49)	(48)
C-Cell Adenoma	0	2	5	0	7	3
C-Cell Carcinoma	2	3	3	1	5	1

Neoplasms of the clitoral (preputial) gland were presented grossly as round, fluctuant cystic subcutaneous lesions in the genital area, which on section were filled with pasty green material. On microscopic examination, the cyst contents consisted of desquamated epithelial cells, frequently mixed with leukocytes from secondary inflammation. The inner portion of the cyst wall was lined by hyperkeratinized squamous epithelium often thrown into papillary folds. Peripheral to this was a zone of large, round glandular cells at least a few of which had coarse, brightly eosinophilic cytoplasmic granules. If the peripheral border appeared smooth and intact, the lesion was classified as an adenoma. If there was disorganization of the glandular structure and invasion into the surrounding stroma, the tumor was called a carcinoma.

Thyroid C-cell tumors were observed in dosed female rats at incidences increased relative to controls (4/48 [8 percent] high dose, 12/49 [24 percent] low dose, 1/21 [5 percent] controls). C-cell adenomas were discrete masses of these cells, often containing small cysts lined by flat epithelium and containing colloid-like material. In C-cell carcinomas, the tumor cells often assumed a spindle shape and tended to invade surrounding tissue.

Uterine horns containing neoplasms were usually grossly enlarged. The neoplasms themselves were varicolored, polypoid, frequently gelatinous masses projecting into the uterine cavity. Endometrial stromal polyps had a fibrous connective tissue core richly supplied with large vessels. The surface of the polyps was covered with well-differentiated endometrium which often formed glands in the superficial portion of the polyps. These tumors frequently became necrotic at the tip and exhibited hemorrhage and secondary inflammation. In a few rats, the connective tissue stroma of these lesions underwent malignant transformation characterized by increased cellularity, mitoses, and formation of plump, pleomorphic nuclei. Such tumors were classified as stromal sarcomas. A uterine adenocarcinoma was a collection of fairly well-differentiated glands arranged back-to-back with no obvious intervening stroma. Nuclei of the glands were markedly pleomorphic with frequent mitoses. There was invasion into the myometrium and sometimes into extra uterine structures.

There were instances in this study, as noted in the summary tables, where neoplastic lesions occurred only in dosed animals, or with increased frequency when compared to the control group. No pulmonary neoplasms were found in the controls; alveolar/bronchiolar tumors were seen in dosed rats of both sexes. There was only one urinary tract neoplasm in a female control; a few more occurred in dosed rats, both male and female. No gliomas of the brain were seen in controls; a few gliomas were found in dosed rats of both sexes. These neoplasms occurred in such small numbers that a conclusive interpretation as to their significance is not possible.

Rats in all groups exhibited a variety of nonneoplastic inflammatory and degenerative changes, and none were associated with administration of the compound.

Based upon the results of this pathologic examination, 1,5-naphthalenediamine was carcinogenic to female Fischer 344 rats since feeding of the compound was associated with adenomas and carcinomas of the clitoral gland. In addition, 1,5-naphthalenediamine feeding appeared to be associated with increased incidences of thyroid, liver and uterine neoplasms in female rats and liver neoplasms in male rats.

D. Statistical Analyses of Results

The results of the statistical analyses of tumor incidence in rats are summarized in Tables 3 and 4. The analysis is included for

TABLE 3

ANALYSES OF THE INCIDENCE OF PRIMARY TUMORS AT
SPECIFIC SITES IN MALE RATS TREATED WITH 1,5-NAPHTHALENEDIAMINE[a]

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Subcutaneous Tissue: Fibroma[b]	1/25(0.04)	3/49(0.06)	2/50(0.04)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.531	1.000
Lower Limit	---	0.133	0.056
Upper Limit	---	78.493	56.712
Weeks to First Observed Tumor	99	106	102
Skin: Squamous-Cell Papilloma[b]	2/25(0.08)	1/49(0.02)	1/50(0.02)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.255	0.250
Lower Limit	---	0.005	0.004
Upper Limit	---	4.707	4.616
Weeks to First Observed Tumor	109	106	106
Lung: Alveolar/Bronchiolar Adenoma or Alveolar/Bronchiolar Carcinoma[b]	0/25(0.00)	3/49(0.06)	4/47(0.09)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	0.315	0.508
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	104	106

TABLE 3 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Hematopoietic System: Leukemia or Malignant Lymphoma[b]	1/25(0.04)	10/49(0.20)	10/50(0.20)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	5.102	5.000
Lower Limit	---	0.801	0.787
Upper Limit	---	212.137	213.351
Weeks to First Observed Tumor	109	100	97
Liver: Hepatocellular Carcinoma or Neoplastic Nodule[b]	1/25(0.04)	7/49(0.14)	4/49(0.08)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	3.571	2.041
Lower Limit	---	0.503	0.218
Upper Limit	---	156.046	96.949
Weeks to First Observed Tumor	109	106	104
Pituitary: Adenoma NOS, Chromophobe Adenoma, Acidophil Adenoma, or Basophil Adenoma[b]	2/22(0.09)	7/44(0.16)	11/44(0.25)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.750	2.750
Lower Limit	---	0.376	0.683
Upper Limit	---	16.365	24.081
Weeks to First Observed Tumor	98	96	65

TABLE 3 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Adrenal: Pheochromocytoma or Malignant Pheochromocytoma[b]	2/24(0.08)	4/48(0.08)	5/48(0.10)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.000	1.250
Lower Limit	---	0.157	0.226
Upper Limit	---	10.563	12.529
Weeks to First Observed Tumor	109	106	102
Thyroid: C-Cell Carcinoma[b]	2/21(0.10)	3/47(0.06)	3/47(0.06)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.670	0.670
Lower Limit	---	0.084	0.084
Upper Limit	---	7.650	7.650
Weeks to First Observed Tumor	97	100	106
Thyroid: C-Cell Adenoma or C-Cell Carcinoma[b]	2/21(0.10)	5/47(0.11)	8/47(0.17)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.117	1.787
Lower Limit	---	0.205	0.405
Upper Limit	---	11.249	16.445
Weeks to First Observed Tumor	97	100	104

TABLE 3 (CONCLUDED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Pancreatic Islets: Islet-Cell Adenoma or Islet-Cell Carcinoma[b]	1/25(0.04)	2/48(0.04)	5/45(0.11)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.042	2.778
Lower Limit	---	0.058	0.340
Upper Limit	---	60.184	128.213
Weeks to First Observed Tumor	98	106	104
Testis: Interstitial-Cell Tumor[b]	21/25(0.84)	44/49(0.90)	45/49(0.92)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.069	1.093
Lower Limit	---	0.890	0.912
Upper Limit	---	1.325	1.324
Weeks to First Observed Tumor	97	94	65

[a]Treated groups received doses of 0.05 or 0.10 percent in feed.

[b]Number of tumor-bearing animals/number of animals examined at site (proportion).

[c]The probability level for the Cochran-Armitage test is given beneath the incidence of tumors in the control group when $P < 0.05$; otherwise, not significant (N.S.) is indicated. The probability level for the Fisher exact test for the comparison of a treated group with the control group is given beneath the incidence of tumors in the treated group when $P < 0.05$; otherwise, not significant (N.S.) is indicated. For both Cochran-Armitage and Fisher exact tests a negative designation (N) indicates a lower incidence in the treated group(s) than in the control group.

[d]The 95% confidence interval on the relative risk of the treated group to the control group.

TABLE 4

ANALYSES OF THE INCIDENCE OF PRIMARY TUMORS AT
SPECIFIC SITES IN FEMALE RATS TREATED WITH 1,5-NAPHTHALENEDIAMINE[a]

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Hematopoietic System: Leukemia or Malignant Lymphoma[b]	3/24(0.13)	7/50(0.14)	1/50(0.02)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.120	0.160
Lower Limit	---	0.287	0.003
Upper Limit	---	6.292	1.890
Weeks to First Observed Tumor	94	76	103
Liver: Hepatocellular Carcinoma or Neoplastic Nodule[b]	0/24(0.00)	4/50(0.08)	4/49(0.08)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	0.458	0.467
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	102	106
Pituitary: Adenoma NOS, Chromophobe Adenoma, Acidophil Adenoma, or Basophil Adenoma[b]	6/21(0.29)	10/50(0.20)	17/47(0.36)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.700	1.266
Lower Limit	---	0.275	0.577
Upper Limit	---	2.090	3.426
Weeks to First Observed Tumor	91	98	98

TABLE 4 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Pituitary: Carcinoma NOS, Adenoma NOS, Chromophobe Adenoma, Chromophobe Carcinoma, Acidophil Adenoma, or Basophil Adenoma[b]	6/21(0.29)	11/50(0.22)	18/47(0.38)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.770	1.340
Lower Limit	---	0.312	0.618
Upper Limit	---	2.262	3.606
Weeks to First Observed Tumor	91	88	98
Adrenal: Cortical Adenoma or Cortical Carcinoma[b]	0/24(0.00)	3/50(0.06)	1/49(0.02)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	0.297	0.027
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	106	106
Adrenal: Pheochromocytoma[b]	1/24(0.04)	0/50(0.00)	3/49(0.06)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.000	1.469
Lower Limit	---	0.000	0.127
Upper Limit	---	8.966	75.534
Weeks to First Observed Tumor	110	---	106

TABLE 4 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Thyroid: C-Cell Carcinoma[b]	1/21(0.05)	5/49(0.10)	1/48(0.02)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	2.143	0.438
Lower Limit	---	0.266	0.006
Upper Limit	---	99.147	33.659
Weeks to First Observed Tumor	109	106	106
Thyroid: C-Cell Adenoma or C-Cell Carcinoma[b]	1/21(0.05)	12/49(0.24)	4/48(0.08)
P Values[c]	N.S.	P = 0.046	N.S.
Departure from Linear Trend[e]	P = 0.009	---	---
Relative Risk (Control)[d]	---	5.143	1.750
Lower Limit	---	0.855	0.192
Upper Limit	---	215.370	83.548
Weeks to First Observed Tumor	109	104	103
Thyroid: Papillary Carcinoma, Follicular-Cell Carcinoma, or Papillary Cystadenocarcinoma NOS[b]	1/21(0.05)	1/49(0.02)	3/48(0.06)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.429	1.313
Lower Limit	---	0.006	0.115
Upper Limit	---	32.983	67.452
Weeks to First Observed Tumor	110	106	99

TABLE 4 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Thyroid: Papillary Carcinoma, Follicular-Cell Carcinoma, Papillary Cystadenocarcinoma NOS, or Papillary Cystadenoma[b]	1/21(0.05)	2/49(0.04)	4/48(0.08)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.857	1.750
Lower Limit	---	0.648	0.191
Upper Limit	---	49.555	84.310
Weeks to First Observed Tumor	110	106	81
Mammary Gland: Fibroadenoma[b]	4/24(0.17)	5/50(0.10)	13/50(0.26)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.600	1.560
Lower Limit	---	0.145	0.556
Upper Limit	---	2.812	6.019
Weeks to First Observed Tumor	109	102	98
Mammary Gland: Fibroadenoma, Adenocarcinoma NOS, or Papillary Adenocarcinoma[b]	4/24(0.17)	5/50(0.10)	14/50(0.28)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.600	1.680
Lower Limit	---	0.145	0.609
Upper Limit	---	2.807	6.412
Weeks to First Observed Tumor	109	102	98

TABLE 4 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Clitoral Gland: Carcinoma NOS[b]	1/24(0.04)	3/50(0.06)	8/50(0.16)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.440	3.840
Lower Limit	---	0.125	0.566
Upper Limit	---	75.487	168.221
Weeks to First Observed Tumor	110	106	69
Clitoral Gland: Adenoma NOS or Carcinoma NOS[b]	1/24(0.04)	3/50(0.06)	13/50(0.26)
P Values[c]	P = 0.003	N.S.	P = 0.021
Relative Risk (Control)[d]	---	1.440	6.240
Lower Limit	---	0.125	1.043
Upper Limit	---	74.077	258.268
Weeks to First Observed Tumor	110	106	69
Uterus: Endometrial Stromal Polyp[b]	2/24(0.08)	14/49(0.29)	20/48(0.42)
P Values[c]	P = 0.003	P = 0.043	P = 0.003
Relative Risk (Control)[d]	---	3.429	5.000
Lower Limit	---	0.892	1.385
Upper Limit	---	29.588	41.202
Weeks to First Observed Tumor	102	88	96

TABLE 4 (CONCLUDED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Uterus and Endometrium: Adenocarcinoma NOS[b]	1/24(0.04)	2/49(0.04)	4/48(0.08)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.980	2.000
Lower Limit	---	0.054	0.216
Upper Limit	---	56.627	96.367
Weeks to First Observed Tumor	110	104	106
Zymbal's Gland: Sebaceous Adenocarcinoma[b]	0/24(0.00)	0/50(0.00)	3/50(0.06)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	---	Infinite
Lower Limit	---	---	0.296
Upper Limit	---	---	Infinite
Weeks to First Observed Tumor	---	---	89

[a]Treated groups received doses of 0.05 or 0.10 percent in feed.

[b]Number of tumor-bearing animals/number of animals examined at site (proportion).

[c]The probability level for the Cochran-Armitage test is given beneath the incidence of tumors in the control group when $P < 0.05$; otherwise, not significant (N.S.) is indicated. The probability level for the Fisher exact test for the comparison of a treated group with the control group is given beneath the incidence of tumors in the treated group when $P < 0.05$; otherwise, not significant (N.S.) is indicated. For both Cochran-Armitage and Fisher exact tests a negative designation (N) indicates a lower incidence in the treated group(s) than in the control group.

[d]The 95% confidence interval on the relative risk of the treated group to the control group.

[e]The probability level of the test for departure from linear trend is given beneath the control group when $P < 0.05$.

every type of tumor in either sex where at least two such tumors were observed in at least one of the control or 1,5-naphthalenediamine-dosed groups and where such tumors were observed in at least 5 percent of the group.

For female rats an increased incidence of endometrial stromal polyps was observed in both the high and low dose groups compared to the control group. The Cochran-Armitage test indicated a significant ($P = 0.003$) positive association between compound administration and tumor incidence. The Fisher exact tests supported this result with a significant ($P = 0.003$) comparison of the high dose group to the control; for the low dose comparison the probability level was $P = 0.043$, a marginal result which was not significant under the Bonferroni criterion. Based on these results, the administration of 1,5-naphthalenediamine was associated with an elevated incidence of endometrial stromal polyps in female rats.

A number of adenomas NOS and carcinomas NOS of the clitoral gland were observed in female rats. The Cochran-Armitage test indicated a significant ($P = 0.003$) positive association between dose and the combined incidence of adenomas NOS or carcinomas NOS of the clitoral gland. The Fisher exact test comparing high dose to control was also significant ($P = 0.021$). In historical data collected by this laboratory for the NCI Carcinogenesis Testing Program, 4/249 (2 percent) of the untreated female Fischer 344 rats had one of these tumors, compared to the 13/50 (26 percent) observed in the high dose group in

this bioassay. Based upon these statistical results, the administration of 1,5-naphthalenediamine was associated with an elevated incidence of clitoral gland neoplasms in female rats.

For females the Fisher exact test comparing control to low dose for the combined incidence of C-cell adenomas or C-cell carcinomas of the thyroid had a probability level of $P = 0.046$, a marginal result which was not significant under the Bonferroni criterion.

Based on these statistical tests, it is concluded that 1,5-naphthalenediamine was carcinogenic for female rats, producing tumors of the clitoral gland and uterus.

IV. CHRONIC TESTING RESULTS: MICE

A. Body Weights and Clinical Observations

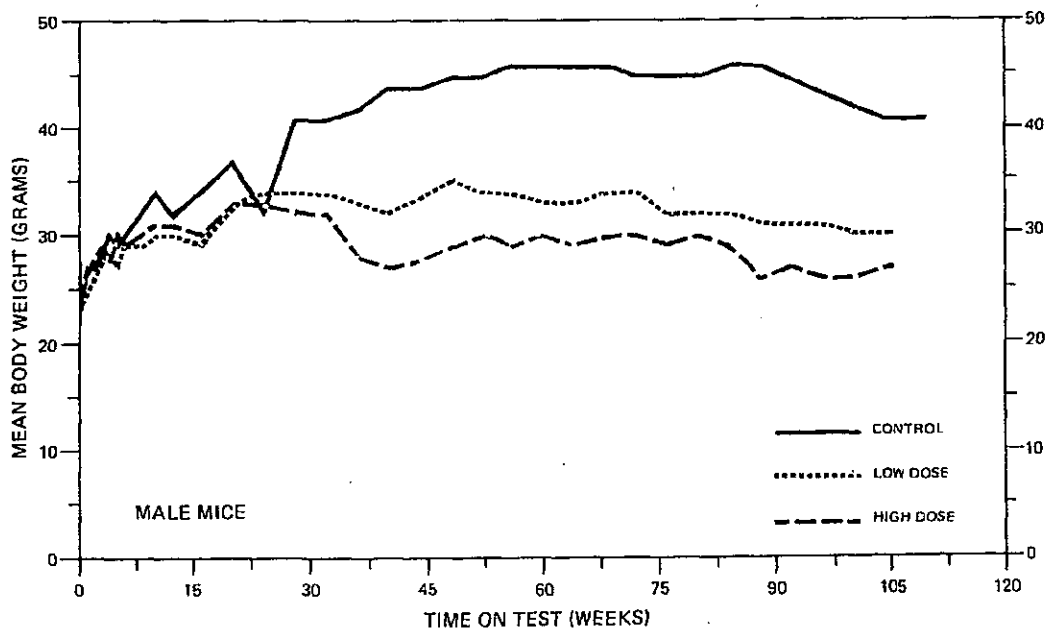
Mean body weight depression was readily apparent in dosed male mice when compared to controls. A similar but less pronounced trend was evident in dosed females (Figure 4).

One low dose male had a soft subcutaneous mass on the leg and two males in this group had palpable abdominal masses. Firm nodular growths developed in one low dose male and two high dose females. Alopecia was observed in 27 control males, 16 low dose males, 4 high dose males, 25 control females, and 3 low dose females. Two low dose and two high dose males experienced noticeable swelling of the eyes. Abdominal distention was observed in one control male and one control female mouse.

B. Survival

The estimated probabilities of survival for male and female mice in the control and 1,5-naphthalenediamine-dosed groups are shown in Figure 5. There was no significant positive association between dosage and mortality for either male or female mice.

Adequate numbers of male mice were at risk from late-developing tumors with 58 percent (29/50) of the high dose, 78 percent (39/50) of the low dose and 66 percent (33/50) of the controls surviving on test until the termination of the study. The 6 control male mice that died in week 11 were autolyzed, as were 2 of the 4 high dose male mice that died in week 41.



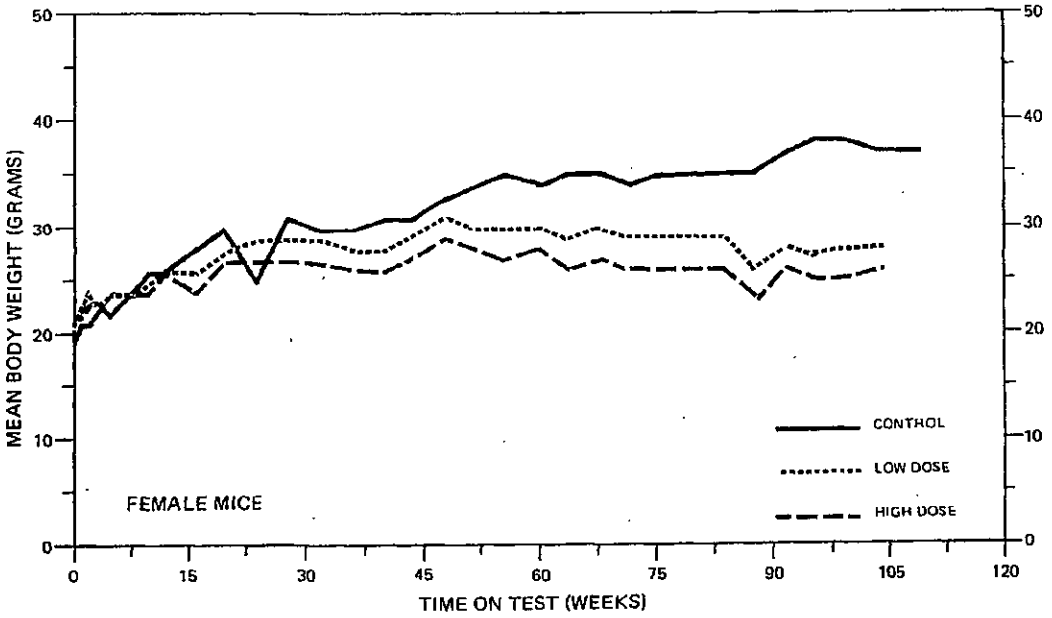
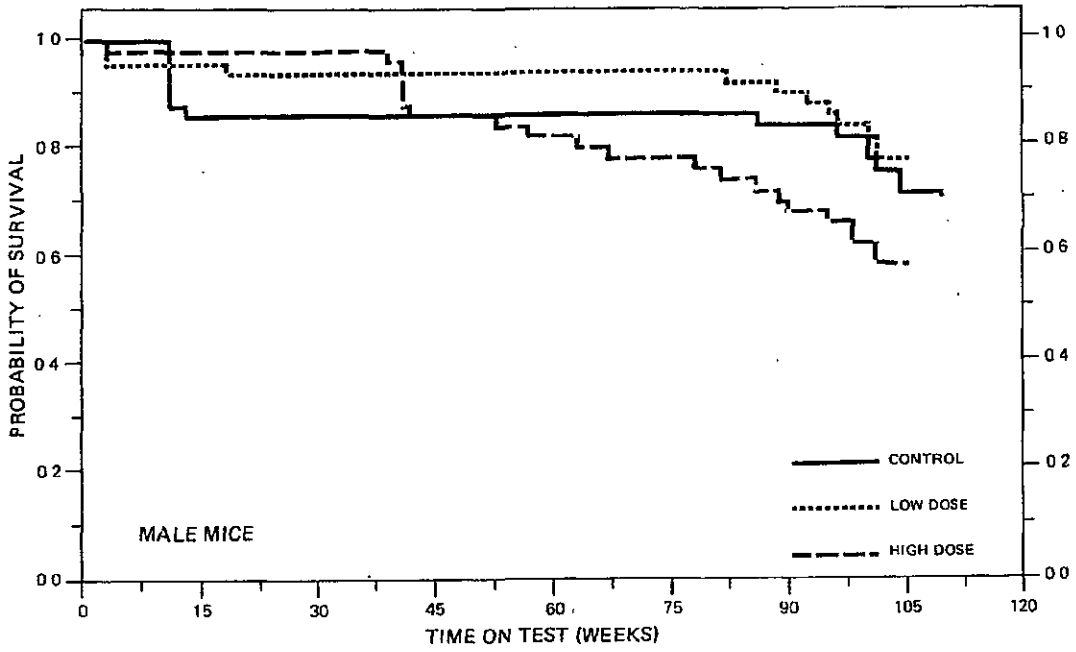


FIGURE 4
GROWTH CURVES FOR 1,5-NAPHTHALENEDIAMINE CHRONIC STUDY MICE



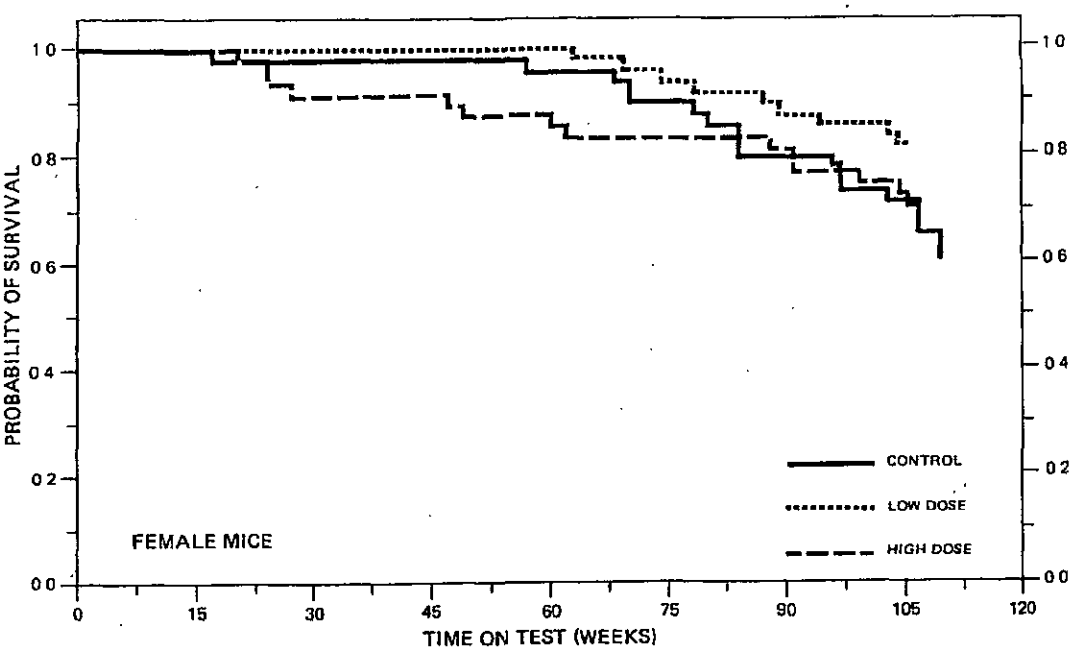


FIGURE 5
SURVIVAL COMPARISONS OF 1,5-NAPHTHALENEDIAMINE CHRONIC STUDY MICE

For female mice, with 68 percent (34/50) of the high dose, 82 percent (41/50) of the low dose and 60 percent (30/50) of the control mice surviving on test until the termination of the study, adequate numbers were at risk from late-developing tumors.

C. Pathology

Histopathologic findings on neoplasms in mice are summarized in Appendix B (Tables B1 and B2); findings on nonneoplastic lesions are summarized in Appendix D (Tables D1 and D2).

Dietary administration of 1,5-naphthalenediamine produced an increase in hepatocellular neoplasms in female mice, and it produced a dose-related increase in thyroid neoplasms and compound-related nonneoplastic thyroid lesions in both sexes. The compound-related lesions are summarized below:

	MALES			FEMALES		
	Control	Low Dose	High Dose	Control	Low Dose	High Dose
LIVER						
(Number of animals with tissues examined histopathologically)	(39)	(45)	(43)	(46)	(49)	(46)
Hepatocellular Carcinoma	12	10	7	1	25	16
Hepatocellular Adenoma	0	3	6	0	3	11
THYROID						
(Number of animals with tissues examined histopathologically)	(38)	(46)	(43)	(44)	(49)	(45)
Follicular-Cell Adenoma (Papillary or Follicular-Cell Adenoma, Papillary Cystadenoma)	0	8	16	2	17	14
Follicular-Cell Carcinoma	0	1	1	2	0	1
Follicular-Cell Hyperplasia	2	12	9	2	1	4
C-Cell Adenoma	0	2	0	0	1	2
C-Cell Carcinoma	0	0	4	0	1	6

In male mice, dietary administration of the compound did not increase the incidence of hepatocellular neoplasms, whereas dosed females showed a striking increase in hepatocellular carcinomas and hepatocellular adenomas.

Grossly, hepatocellular neoplasms appeared as smooth, nodular, rounded masses distorting the normal shape of the liver. Color varied, many neoplasms appearing pale tan or dark red. Microscopically, hepatocellular carcinomas were expansive masses of hepatocytes exhibiting loss of normal architectural pattern, the cells being arranged in sheets or trabeculae instead of the normal lobules. Nuclei were frequently uniform, although variable amounts of pleomorphism did occur. The cytoplasm was either basophilic or acidophilic, sometimes varying from one region of the tumor to another, and was frequently pale. Lesions classified as hepatocellular adenomas were smaller, usually better differentiated, and were less pleomorphic than the hepatocellular carcinomas.

The criteria for classification of thyroid neoplasms in mice were the same as those used to classify thyroid neoplasms in rats. The nonneoplastic thyroid lesions found in dosed mice were similar to those in the rats but occurred in higher incidences. Hyperplasia of follicular cells (focal, papillary or adenomatous) were found in 2/38 (5 percent) control, 12/46 (26 percent) low dose, and 9/43 (21 percent) high dose male mice. Abundant golden brown pigment was seen in follicular epithelium, colloid, and macrophages. In the mice,

there were frequent foci of lymphocytes in the thyroid parenchyma and occasional cystic areas filled with amorphous material containing long clefts suggesting cholesterol crystals.

Three transitional-cell papillomas occurred in the bladder or urethra of dosed mice (two high dose males and one high dose female), but none occurred in controls.

Based upon the results of this pathologic examination, 1,5-naphthalenediamine was carcinogenic to B6C3F1 mice, producing hepatocellular neoplasms in females and thyroid neoplasms in both sexes.

D. Statistical Analyses of Results

The results of the statistical analyses of tumor incidence in mice are summarized in Tables 5 and 6. The analysis is included for every type of tumor in either sex where at least two such tumors were observed in at least one of the control or 1,5-naphthalenediamine-dosed groups and where such tumors were observed in at least 5 percent of the group.

For both male and female mice elevated incidences of thyroid tumors were observed in the dosed groups. In female mice the Cochran-Armitage test indicated a significant ($P = 0.005$) positive association between dietary concentration and the incidence of C-cell carcinomas. This was supported by a significant ($P = 0.014$) Fisher exact test for the high dose group. For males the Cochran-Armitage test result was also significant ($P = 0.017$), but the Fisher exact tests were

TABLE 5

ANALYSES OF THE INCIDENCE OF PRIMARY TUMORS AT
SPECIFIC SITES IN MALE MICE TREATED WITH 1,5-NAPHTHALENEDIAMINE[a]

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Lung: Alveolar/Bronchiolar Carcinoma[b]	2/39(0.05)	3/46(0.07)	0/45(0.00)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.272	0.000
Lower Limit	---	0.153	0.000
Upper Limit	---	14.686	4.478
Weeks to First Observed Tumor	109	82	---
Lung: Alveolar/Bronchiolar Adenoma or Alveolar/Bronchiolar Carcinoma[b]	4/39(0.10)	9/46(0.20)	2/45(0.04)
P Values[c]	N.S.	N.S.	N.S.
Departure from Linear Trend[e]	P = 0.037	---	---
Relative Risk (Control)[d]	---	1.908	0.433
Lower Limit	---	0.582	0.041
Upper Limit	---	7.882	2.871
Weeks to First Observed Tumor	109	82	105
Hematopoietic System: Malignant Lymphoma[b]	13/39(0.33)	14/47(0.30)	5/49(0.10)
P Values[c]	P = 0.007(N)	N.S.	P = 0.008(N)
Relative Risk (Control)[d]	---	0.894	0.306
Lower Limit	---	0.448	0.094
Upper Limit	---	1.817	0.829
Weeks to First Observed Tumor	100	82	95

TABLE 5 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Liver: Hepatocellular Carcinoma[b]	12/39(0.31)	10/45(0.22)	7/43(0.16)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.722	0.529
Lower Limit	---	0.318	0.198
Upper Limit	---	1.620	1.306
Weeks to First Observed Tumor	86	88	105
Liver: Hepatocellular Carcinoma or Hepatocellular Adenoma[b]	12/39(0.31)	13/45(0.29)	13/43(0.30)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	0.939	0.983
Lower Limit	---	0.453	0.473
Upper Limit	---	1.981	2.071
Weeks to First Observed Tumor	86	88	105
Thyroid: C-Cell Carcinoma[b]	0/38(0.00)	0/46(0.00)	4/43(0.09)
P Values[c]	P = 0.017	N.S.	N.S.
Relative Risk (Control)[d]	---	---	Infinite
Lower Limit	---	---	0.825
Upper Limit	---	---	Infinite
Weeks to First Observed Tumor	---	---	105

TABLE 5 (CONCLUDED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Thyroid: C-Cell Carcinoma or C-Cell Adenoma[b]	0/38(0.00)	2/46(0.04)	4/43(0.09)
P Values[c]	P = 0.044	N.S.	N.S.
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	0.246	0.825
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	105	105
Thyroid: Papillary Adenoma, Follicular-Cell Adenoma, or Papillary Cystadenoma NOS[b]	0/38(0.00)	8/46(0.17)	16/43(0.37)
P Values[c]	P < 0.001	P = 0.006	P < 0.001
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	1.905	4.523
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	105	98

[a]Treated groups received doses of 0.1 or 0.2 percent in feed.

[b]Number of tumor-bearing animals/number of animals examined at site (proportion).

[c]The probability level for the Cochran-Armitage test is given beneath the incidence of tumors in the control group when P < 0.05; otherwise, not significant (N.S.) is indicated. The probability level for the Fisher exact test for the comparison of a treated group with the control group is given beneath the incidence of tumors in the treated group when P < 0.05; otherwise, not significant (N.S.) is indicated. For both Cochran-Armitage and Fisher exact tests a negative designation (N) indicates a lower incidence in the treated group(s) than in the control group.

[e]The probability level of the test for departure from linear trend is given beneath the control group when P < 0.05.

TABLE 6

ANALYSES OF THE INCIDENCE OF PRIMARY TUMORS AT
SPECIFIC SITES IN FEMALE MICE TREATED WITH 1,5-NAPHTHALENEDIAMINE[a]

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Lung: Alveolar/Bronchiolar Carcinoma[b]	0/49(0.00)	1/48(0.02)	3/46(0.07)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	0.055	0.638
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	89	91
Lung: Alveolar/Bronchiolar Adenoma or Alveolar/Bronchiolar Carcinoma[b]	0/49(0.00)	10/48(0.21)	5/46(0.11)
P Values[c]	N.S.	P = 0.001	P = 0.024
Departure from Linear Trend[e]	P = 0.005	---	---
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	3.037	1.347
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	89	91
Hematopoietic System: Leukemia or Malignant Lymphoma[b]	13/49(0.27)	19/50(0.38)	5/46(0.11)
P Values[c]	N.S.	N.S.	P = 0.045(N)
Departure from Linear Trend[e]	P = 0.011	---	---
Relative Risk (Control)[d]	---	1.432	0.410
Lower Limit	---	0.760	0.124
Upper Limit	---	2.781	1.117
Weeks to First Observed Tumor	57	63	105

TABLE 6 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Liver: Hepatocellular Carcinoma[b]	1/46(0.02)	25/49(0.51)	16/46(0.35)
P Values[c]	P = 0.001	P < 0.001	P < 0.001
Departure from Linear Trend[e]	P < 0.001	---	---
Relative Risk (Control)[d]	---	23.469	16.000
Lower Limit	---	4.156	2.683
Upper Limit	---	906.346	646.516
Weeks to First Observed Tumor	109	74	99
Liver: Hepatocellular Adenoma or Hepatocellular Carcinoma[b]	1/46(0.02)	28/49(0.57)	27/46(0.59)
P Values[c]	P < 0.001	P < 0.001	P < 0.001
Departure from Linear Trend[e]	P = 0.002	---	---
Relative Risk (Control)[d]	---	26.286	27.000
Lower Limit	---	4.741	4.874
Upper Limit	---	1030.801	1027.943
Weeks to First Observed Tumor	109	74	99
Stomach: Squamous-Cell Papilloma[b]	0/41(0.00)	3/47(0.06)	0/46(0.00)
P Values[c]	N.S.	N.S.	N.S.
Departure from Linear Trend[e]	P = 0.017	---	---
Relative Risk (Control)[d]	---	Infinite	---
Lower Limit	---	0.529	---
Upper Limit	---	Infinite	---
Weeks to First Observed Tumor	---	105	---

TABLE 6 (CONTINUED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Pituitary: Adenoma NOS, Chromophobe Adenoma or Acidophil Adenoma[b]	3/34(0.09)	4/35(0.11)	1/30(0.03)
P Values[c]	N.S.	N.S.	N.S.
Relative Risk (Control)[d]	---	1.295	0.378
Lower Limit	---	0.238	0.007
Upper Limit	---	8.188	4.424
Weeks to First Observed Tumor	109	105	106
Adrenal: Pheochromocytoma[b]	3/46(0.07)	0/44(0.00)	0/44(0.00)
P Values[c]	P = 0.040(N)	N.S.	N.S.
Relative Risk (Control)[d]	---	0.000	0.000
Lower Limit	---	0.000	0.000
Upper Limit	---	1.731	1.731
Weeks to First Observed Tumor	68	---	---
Thyroid: C-Cell Carcinoma[b]	0/44(0.00)	1/49(0.02)	6/45(0.13)
P Values[c]	P = 0.005	N.S.	P = 0.014
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	0.048	1.574
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	105	105

TABLE 6 (CONCLUDED)

TOPOGRAPHY:MORPHOLOGY	CONTROL	LOW DOSE	HIGH DOSE
Thyroid: C-Cell Adenoma or C-Cell Carcinoma[b]	0/44(0.00)	2/49(0.04)	8/45(0.18)
P Values[c]	P = 0.001	N.S.	P = 0.003
Relative Risk (Control)[d]	---	Infinite	Infinite
Lower Limit	---	0.267	2.250
Upper Limit	---	Infinite	Infinite
Weeks to First Observed Tumor	---	105	41
Thyroid: Papillary Adenoma, Follicular-Cell Adenoma, or Papillary Cystadenoma NOS[b]	2/44(0.05)	17/49(0.35)	14/45(0.31)
P Values[c]	P = 0.003	P < 0.001	P = 0.001
Departure from Linear Trend[e]	P = 0.025	---	---
Relative Risk (Control)[d]	---	7.633	6.844
Lower Limit	---	1.971	1.709
Upper Limit	---	64.662	58.827
Weeks to First Observed Tumor	80	105	91

[a]Treated groups received doses of 0.1 or 0.2 percent in feed.

[b]Number of tumor-bearing animals/number of animals examined at site (proportion).

[c]The probability level for the Cochran-Armitage test is given beneath the incidence of tumors in the control group when P < 0.05; otherwise, not significant (N.S.) is indicated. The probability level for the Fisher exact test for the comparison of a treated group with the control group is given beneath the incidence of tumors in the treated group when P < 0.05; otherwise, not significant (N.S.) is indicated. For both Cochran-Armitage and Fisher exact tests a negative designation (N) indicates a lower incidence in the treated group(s) than in the control group.

[d]The 95% confidence interval on the relative risk of the treated group to the control group.

[e]The probability level of the test for departure from linear trend is given beneath the control group when P < 0.05.

not. When incidences were combined so that the numerator represented mice with either a papillary adenoma, a follicular-cell adenoma, or a papillary cystadenoma of the thyroid, the Cochran-Armitage test indicated a significant positive association between dietary concentration and tumor incidence for both males ($P < 0.001$) and females ($P = 0.003$). These were supported by significant ($P \leq 0.006$) Fisher exact test results in each sex for comparisons of each dosed group to the control group. Based on these results, the administration of 1,5-naphthalenediamine was associated with the incidence of thyroid neoplasms in both male and female mice.

For females an increased incidence of hepatocellular carcinomas was also observed among the dosed mice. The Cochran-Armitage test indicated a significant ($P = 0.001$) positive association between dose and incidence. This was supported by significant ($P < 0.001$) comparisons of both the high and low dose to the control group using the Fisher exact test. Based on these results the administration of 1,5-naphthalenediamine was associated with the incidence of hepatocellular carcinomas in female mice.

For female mice, when the incidence of alveolar/bronchiolar adenomas and alveolar/bronchiolar carcinomas were combined, an increased incidence in the dosed groups was noted. The Fisher exact test was significant for both the high ($P = 0.024$) and low ($P = 0.001$) dose groups. The departure from linear trend was significant since tumor incidence was increased more in the low dose than in the high

dose group. In historical control data compiled by this laboratory for the NCI Carcinogenesis Testing Program, 17/275 (6 percent) of the untreated female B6C3F1 mice had an alveolar/bronchiolar neoplasm. Based upon these results the administration of 1,5-naphthalenediamine was associated with the incidence of alveolar/bronchiolar neoplasms in female mice.

For females the Fisher exact test comparing the incidence of leukemia or malignant lymphoma in high dose mice with that in the controls had a probability level in the negative direction of $P = 0.045$, a marginal result which was not significant under the Bonferroni criterion.

Also for females the Cochran-Armitage test showed a significant ($P = 0.040$) negative association between dose and the incidence of adrenal pheochromocytomas, but the Fisher exact tests were not significant.

In male mice the possibility of a negative association between dose and the incidence of malignant lymphomas or leukemia was noted.

Based upon these statistical results the administration of 1,5-naphthalenediamine was associated with the increased incidence of thyroid neoplasms in male mice and of thyroid neoplasms, of hepatocellular carcinomas, and of alveolar/bronchiolar neoplasms in female mice.

V. DISCUSSION

There were no significant positive associations between dietary concentrations of 1,5-naphthalenediamine and mortality in either sex of rats or mice. In all groups adequate numbers of animals survived sufficiently long to be at risk from late-developing tumors.

Several uterine neoplasms occurred in dosed female rats at higher incidences than in corresponding controls. There was a significant positive association between dietary concentration of the compound and the incidences of endometrial stromal polyps in female rats. In addition, the high dose to control Fisher exact comparison was significant. Endometrial stromal sarcomas were observed in two low dose and two high dose female rats, but not in controls. Uterine adenocarcinomas occurred at a higher incidence in the high dose female rat group than in the control group, but the difference in tumor incidence was not statistically significant.

The administration of 1,5-naphthalenediamine was associated with an elevated incidence of clitoral gland neoplasms in female rats. There was a significant positive association between the concentration of the chemical added to the diet and the incidence of either adenomas or carcinomas of the clitoral gland in female rats. The incidence of either of these neoplasms in the high dose female rat group was significant relative to the incidence in the control group.

Elevated incidences of thyroid neoplasms were observed among dosed mice. For mice of both sexes there were significant positive

associations between dietary concentration of 1,5-naphthalenediamine and the incidences of thyroid C-cell carcinomas. For the females the high dose to control Fisher exact comparison supported the finding; this was not true for males. When the mice were grouped so that the numerator of the incidence represented those animals with a papillary adenoma, a follicular-cell adenoma, or a papillary cystadenoma of the thyroid, the Cochran-Armitage test was significantly positive for both males and females and all the Fisher exact comparisons supported the findings.

The incidence of hepatocellular carcinomas in female mice was significantly associated with increased concentration of 1,5-naphthalenediamine. In addition, the high dose to control and the low dose to control Fisher exact comparisons were significant. The incidence of alveolar/bronchiolar adenomas was significant, relative to controls, in both the low dose and the high dose female mouse groups.

Under the conditions of this bioassay, 1,5-naphthalenediamine was carcinogenic in female Fischer 344 rats, causing clitoral and uterine neoplasms. 1,5-Naphthalenediamine was also carcinogenic for B6C3F1 mice, producing thyroid neoplasms in males and neoplasms of the thyroid, liver, and lung in females. Insufficient evidence was provided for the carcinogenicity of the compound in male Fischer 344 rats.

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Review of the Bioassay of 1,5-Naphthalenediamine*
for Carcinogenicity
by the Data Evaluation/Risk Assessment Subgroup
of the Clearinghouse on Environmental Carcinogens

June 29, 1978

The Clearinghouse on Environmental Carcinogens was established in May, 1976, in compliance with DHEW Committee Regulations and the Provisions of the Federal Advisory Committee Act. The purpose of the Clearinghouse is to advise the Director of the National Cancer Institute (NCI) on its bioassay program to identify and to evaluate chemical carcinogens in the environment to which humans may be exposed. The members of the Clearinghouse have been drawn from academia, industry, organized labor, public interest groups, State health officials, and quasi-public health and research organizations. Members have been selected on the basis of their experience in carcinogenesis or related fields and, collectively, provide expertise in chemistry, biochemistry, biostatistics, toxicology, pathology, and epidemiology. Representatives of various Governmental agencies participate as ad hoc members. The Data Evaluation/Risk Assessment Subgroup of the Clearinghouse is charged with the responsibility of providing a peer review of reports prepared on NCI-sponsored bioassays of chemicals studied for carcinogenicity. It is in this context that the below critique is given on the bioassay of 1,5-Naphthalenediamine for carcinogenicity.

The reviewer agreed with the conclusion in the report that 1,5-Naphthalenediamine was carcinogenic in treated female rats and in both sexes of mice. He noted that the study was conducted in a room in which other compounds were under test. Based on the experimental findings, he concluded that 1,5-Naphthalenediamine may pose a carcinogenic risk to humans. The reviewer moved that the report on the bioassay of 1,5-Naphthalenediamine be accepted as written. The motion was approved without objection.

Clearinghouse Members present:

Arnold L. Brown (Chairman), Mayo Clinic
Paul Nettesheim, National Institute of Environmental Health Sciences
Verne Ray, Pfizer Medical Research Laboratory
Verald K. Rowe, Dow Chemical U.S.A.
Michael B. Shimkin, University of California at San Diego
Louise Strong, University of Texas Health Sciences Center

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* Subsequent to this review, changes may have been made in the bioassay report either as a result of the review or other reasons. Thus, certain comments and criticisms reflected in the review may no longer be appropriate.

☆U.S. GOVERNMENT PRINTING OFFICE: 1978-260-899/3198

[redacted]

1. Chemical and Physical Data

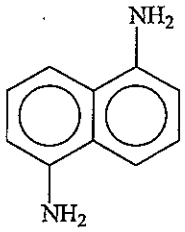
1.1 Synonyms and trade names

Chem. Abstr. Services Reg. No.: 2243-62-1

Chem. Abstr. Name and IUPAC Systematic Name: 1,5-Naphthalenediamine

Synonyms: 1,5-Diaminonaphthalene; 1,5-naphthylenediamine

1.2 Structural and molecular formulae and molecular weight



$C_{10}H_{10}N_2$ Mol. wt: 158.2

1.3 Chemical and physical properties of the pure substance

From Weast (1979), unless otherwise specified

(a) Description: Colourless crystals (Hawley, 1977)

(b) Boiling-point: Sublimes

(c) Melting-point: 190°

(d) Density: 1.4

(e) Solubility: Soluble in hot water, ethanol and diethyl ether; very soluble in chloroform, hot ethanol and hot diethyl ether

(f) Spectroscopic data: Infra-red and ultra-violet spectral data have been reported (Sadtler Research Laboratories, Inc., undated).

1.4 Technical products and impurities

No data were available to the Working Group.

2. Production, Use, Occurrence and Analysis

2.1 Production and use

(a) Production

1,5-Naphthalenediamine can be prepared by the reduction of 1,5-dinitronaphthalene (Sandridge & Staley, 1978) or by ammonolysis of 1,5-dihydroxynaphthalene. Both methods are believed to be used for its commercial production in Japan.

1,5-Naphthalenediamine is believed to be produced by two companies in the Federal Republic of Germany. It has been produced commercially in Japan since 1957; in 1979, two companies produced an estimated 50 thousand kg.

No evidence was found that 1,5-naphthalenediamine has ever been produced in commercial quantities in the US. Two thousand kg were imported through principal US customs districts in 1979 (US International Trade Commission, 1980).

(b) Use

1,5-Naphthalenediamine is believed to be used almost exclusively as an intermediate for the manufacture of 1,5-naphthalene diisocyanate and organic dyes. In Japan, an estimated 75% is consumed in the production of the isocyanate and 25% in dye synthesis.

1,5-Naphthalene diisocyanate, the subject of an earlier monograph (IARC, 1979), is used in Japan and western Europe in the production of polyurethane elastomers. The Society of Dyers & Colourists (1971) reports that 1,5-naphthalenediamine can serve as an oxidation base and that one dye can be prepared from it. No evidence was found that it is presently used commercially in these two applications. The nature of the dyes presently being produced in commercial quantities from 1,5-naphthalenediamine is not known.

2.2 Occurrence

1,5-Naphthalenediamine has not been reported to occur as a natural product. No data on its occurrence in the environment were available to the Working Group.

2.3 Analysis

An IARC manual (Egan *et al.*, 1981) gives selected methods for the analysis of aromatic amines. No information on quantitative methods of analysis for 1,5-naphthalenediamine were available to the Working Group.

3. Biological Data Relevant to the Evaluation of Carcinogenic Risk to Humans

3.1 Carcinogenicity studies in animals

Oral administration

Mouse: Groups of 50 male and 50 female B6C3F$_1$ mice, approximately seven weeks of age, were fed diets containing 1000 or 2000 mg/kg 1,5-naphthalenediamine (probably no more than 89% pure, with at least one unspecified impurity detected by thin-layer chromatography) for 103 weeks. The doses were selected on the basis of a range-finding study [see section 3.2(*a*)]. Groups of 50 mice of each sex served as matched controls. All animals in the study received food and water *ad libitum* and all were treated for parasites with 3 g/L piperazine adipate added for three days per week to the drinking-water for two weeks prior to treatment with the test chemical. The observation periods were 105-106 weeks for treated mice and 109 weeks for controls. There was no significant association between dose of 1,5-naphthalenediamine and mortality in animals of either sex; 58-82% of treated mice and 60-66% of controls survived the observation period. Statistically significant increases in tumour incidence were observed for the following neoplasms: (a) a dose-related increase ($P = 0.005$) in C-cell carcinomas of the thyroid gland in females: controls, 0/44; low-dose, 1/49; high-dose, 6/45 ($P = 0.014$); (b) dose-related increases ($P < 0.001$ and $P = 0.003$) in neoplasms of the thyroid gland (follicular-cell adenomas, papillary adenomas and papillary adenomas plus papillary cystadenomas) in 0/38 male controls, 8/46 low-dose males ($P = 0.006$), 16/43 high-dose males ($P < 0.001$), 2/44 female controls, 17/49 low-dose females ($P < 0.001$) and 14/45 high-dose females ($P = 0.001$); (c) an increase in hepatocellular carcinomas in females: controls, 1/46; low-dose, 25/49 ($P < 0.001$); high-dose, 16/46 ($P < 0.001$); and (d) an increase in alveolar/bronchiolar adenomas and carcinomas in females: controls, 0/49; low-dose, 10/48 ($P = 0.001$); high-dose, 5/46 ($P = 0.024$) (National Cancer Institute, 1978).

Rat: Groups of 50 male and 50 female Fischer 344 rats, approximately seven weeks of age, were fed diets containing 500 or 1000 mg/kg 1,5-naphthalenediamine (same sample as used above) for 103 weeks. The doses were selected on the basis of a range-finding study [see section 3.2(*a*)]. Groups of 25 rats of each sex served as matched controls. All animals under study received food and water *ad libitum,* and all were treated for parasites with piperazine adipate added for three days to the drinking-water (followed by three days of plain tap-water and three subsequent days of piperazine adipate) two weeks prior to treatment with the test chemical. The observation periods were 106-107 weeks for treated rats and 109-110 weeks for controls. There was no significant association between dose of 1,5-naphthalenediamine and mortality of animals of either sex: 74-80% of treated rats and 64-68% of controls survived the observation period. A statistically significant, dose-related increase ($P = 0.003$) in the incidence of adenomas plus carcinomas of the clitoral gland was observed: controls, 1/24; low-dose, 3/50; high-dose, 13/50 ($P = 0.021$) (National Cancer Institute, 1978). [The Working Group noted that the increase in the incidence of clitoral gland tumours was only marginally significant, and that histological section of this organ was performed only when it showed gross abnormality.]

3.2 Other relevant biological data

(a) Experimental systems

Toxic effects

No LD_{50} values were available to the Working Group.

In eight-week subchronic feeding studies, male and female Fischer 344 rats and $B6C3F_1$ mice received up to 3.0% 1,5-naphthalenediamine in the diet. Some deaths were observed in treated groups fed 0.3% or more. Mean body weight gain was depressed by 3-22%. No compound-related lesions were observed in chronic studies with 1,5-naphthalenediamine in rats and mice (highest dose, 0.1% in rats and 0.2% in mice) (National Cancer Institute, 1978).

Effects on reproduction and prenatal toxicity

No data were available to the Working Group.

Absorption, distribution, excretion and metabolism

No data were available to the Working Group.

Mutagenicity and other short-term tests

1,5-Naphthalenediamine (same sample as used in the carcinogenicity tests) was mutagenic to *Salmonella typhimurium* strain TA100 without metabolic activation (Dunkel & Simmon, 1980).

(b) Humans

No data were available to the Working Group.

3.3 Case reports and epidemiological studies of carcinogenicity in humans

No data were available to the Working Group.

4. Summary of Data Reported and Evaluation

4.1 Experimental data

1,5-Naphthalenediamine (technical grade) was tested in one experiment in mice and in one experiment in rats by dietary administration. It produced adenomas of the thyroid in male mice and carcinomas and adenomas of the thyroid and lungs and carcinomas of the liver in female mice. The experiment in rats was inadequate for evaluation.

1,5-Naphthalenediamine (technical-grade) was mutagenic to *Salmonella typhimurium.*

4.2 Human data

1,5-Naphthalenediamine has been produced commercially since at least 1957. Its use as an intermediate in the manufacture of 1,5-naphthalene diisocyanate and of dyes could result in occupational exposure.

No case report or epidemiological study was available to the Working Group.

4.3 Evaluation

There is *limited evidence* for the carcinogenicity of 1,5-naphthalenediamine in experimental animals.

No evaluation of the carcinogenicity of 1,5-naphthalenediamine to humans could be made.

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