（3）Calcium hypochlorite (7778-54-3)（原案作成：ICCA日本企業）

1）曝露状況

本化学物質は殺藻薬、殺菌剤、防臭剤、酸化剤、漂白剤などとして使用される。消費者曝露や職業曝露は使用時や生産時の事故によって発生する可能性がある。主要経路は吸入及び経皮と考えられる。

2）環境影響

本化学物質は速やかに分解されるので、本化学物質自体が大気・水圏・土壌・底質に分布することはない。本化学物質は加水分解性及び反応性が高く，生成される分解物はその物性から生物濃縮性が低いと推測される。水生生物に対する急性毒性では、藻類の EC_{50} は 0.075 mg/L（海水、24時間）、ミジンコの LC_{50} は 0.005 mg 遊離塩素/L（淡水、24時間）である。魚類の LC_{50} は、淡水で 0.06 mg 総残留塩素/L 未満（96時間）と推定され、また、海水では 0.032 mg 総残留酸化体/L（96時間）であった。慢性毒性では、マイクロコズム試験で得られた動物プランクトン群集に対する影響の NOEC は 0.0015 mg 総残留塩素/L（淡水、24日間）で最も低く、軟体動物の NOEC は 0.062 mg 総残留酸化体/L（海水、15日間）、魚類の NOEC は 0.005 mg/L（淡水、133日間）であった。

3）健康影響

水中でカルシウムイオン（Ca^{2+}）と次亜塩素酸イオン（ClO^-）に解離し、Ca^{2+}により適用部位は強アルカリ性になる。ClO^-については、次亜塩素酸ナトリウムや塩素ガスへの曝露による毒性と共通しており、WHO や EU のリスク評価プログラムにおいて国際的な評価が行われている。また、OECD 高生産量化学物質点検プログラムにおける塩素(CAS No 7782-50-5)に関する評価文書も参考になる。

本化学物質の経口毒性データは主に次亜塩素酸ナトリウムか塩素ガスを用いた試験から得られている。また、生物系（pH6～8）において最も豊富な活性化学物質は次亜塩素酸（HOCl）であり、ClO^-と平衡状態にある。ラットに経口投与した $HO^{36}Cl$ は速やかに吸収され、96時間後に ^{36}Cl は血漿、骨髄、精巣、皮膚、腎臓、肺に分布し、投与量の約 50%が排泄（主に尿中）された。HOCl は酵素的な代謝を受けない。

次亜塩素酸カルシウムを用いたラットの単回経口投与毒性試験での LD_{50} は 790 mg/kg であった。塩素ガスの致死濃度はラットで約 500 ppm 以上（10分間以上）であった。種々のヒトの吸入毒性試験から、塩素ガスの急性無毒性濃度（NOAEC）は 0.5 ppm（1.5 mg/m³）と判断された。

本化学物質はウサギの皮膚に対して腐食性、眼に対しては強い刺激性を有する。

ラットに飲水中 0、0.025、0.05、0.1、0.2 及び 0.4%の次亜塩素酸ナトリウムを投与した 13 週

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間反復経口投与毒性試験では、0.2%以上での雄と0.4%での雌において体重増加抑制が認められ、NOAELは、雄では遊離塩素として59.5 mg/kg/day（0.1%次亜塩素酸ナトリウム）、雌では遊離塩素として215.7 mg/kg/day（0.2%次亜塩素酸ナトリウム）と判定された。ラットに次亜塩素酸ナトリウムを13週間飲水投与した試験のNOAELは有効塩素で950 ppm（59.5 mg/kg/day）であった。

ラット及びマウスに塩素を飲水中0、70、140及び275 mg（遊離塩素相当）/L（雄ラットでは0、4.8、7.5及び13.9 mg/kg/day、雌ラットでは0、3.8、6.9及び13.2 mg/kg/day、また、雄マウスでは0、7.2、14.0及び22.5 mg/kg/day、雌マウスでは0、6.3、12.1及び19.8 mg/kg/day）を投与した一生涯試験では、最高用量でも投与の影響は認められず、反復投与毒性の有効塩素のNOAELはラットで14 mg Cl_2/kg/day、マウスで22.5 mg Cl_2/kg/dayと判定された。

ラットの雄には交配前56日間から、雌には交配前14日間から計66日間、HOCl溶液（pH 8.5）0、1、2及び5 mg/kg/day（有効塩素として、0、0.7、1.4及び3.5 mg/kg/day）を強制経口投与した一世代反復経口投与毒性試験では、反復投与及び生殖発生毒性に関する影響は認められなかった。また、公共水道を利用している妊婦を対象とした疫学調査でも生殖発生に対する影響はみとめられていない。

変異原性については種々の *in vitro* 試験で陰性または陽性の結果が示されているが、小核試験などの *in vivo* 試験では投与可能な最高用量においても陰性であったことから、本化学物質は *in vivo* では遺伝毒性を発現しないと判断された。

雌雄ラットと雌雄マウスに2.5 ppm（7.5 mg/m^3）までの塩素を1日6時間週5日で2年間吸入曝露した試験では、発がん性に関する影響は認められなかった。また、ラットやマウスに次亜塩素酸ナトリウムを飲水投与した、数種の長期間（85週～2年間）試験において、雌ラットでは13.2 mg/kg/dayまでの試験において6.9 mg/kg/dayで白血病発生率の増加（用量相関性はない）がみられたが、雄ラットと雌雄マウスへの発がん性に関する影響は認められなかった。また、疫学的調査ではヒトの腫瘍発生率と塩素処理飲用水摂取や次亜塩素酸塩曝露との間に因果関係は認められていない。

4）結論と勧告

健康影響についてはLPと勧告されたが、環境影響についてはFWと勧告され、塩素化副生成物を考慮した環境曝露評価を行うことが推奨された。

（4）3-Methoxy-3-methyl-1-butanol (56539-66-3)（日本政府作成）

1）曝露状況

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本化学物質はイソブチレンとメタノールを原料として製造され、塗料、インキ、シンナー、染料、洗剤、剥離剤、農薬原料、可塑剤原料等に広く用いられている。吸入及び経皮により消費者曝露の可能性がある。職業曝露の主要経路は吸入及び経皮と考えられる。

2）環境影響

本化学物質が大気や水圏に放出された場合にはそのまま停留する。土壌に放出された場合は大気に 29.4%、水圏に 9.3%、土壌に 61.3%分布する。本化学物質は易分解性試験(OECD TG 301C)ではパスレベルに達しなかったが、本質分解性試験では容易に生物分解し（OECD TG 302C)、水生生物における生物濃縮性は低い（生物濃縮係数 BCF：3.16、計算値)。水生生物に対する急性毒性では、藻類の EC_{50} は>1,000 mg/L（72 時間、OECD TG 201)、ミジンコの EC_{50} は>1,000 mg/L（48 時間、OECD TG 202)、魚類の LC_{50} は>100 mg/L（96 時間、OECD TG 203）であった。慢性毒性では、藻類の NOEC は 1,000 mg/L（72 時間、OECD TG 201)、ミジンコの NOEC は 100 mg/L（21 日間、OECD TG 211）であった。

3）健康影響

ラットの単回経口投与毒性試験（OECD TG 401）での LD_{50} は 4,300～4,500 mg/kg、ラットの単回経皮投与毒性試験（OECD TG 402）での LD_{50} は 2,000 mg/kg 以上と報告されている。

ウサギの皮膚に対して弱い刺激性、眼に対しては中程度の刺激性が認められている。モルモットでは皮膚感作性は認められていない。

雌雄ラットに 0、15、60、250 及び 1,000 mg/kg/day を強制経口投与した 28 日間反復経口投与毒性試験では、1,000 mg/kg/day において、雌雄で塩素の減少、雄でアルブミン－グロブリン比及び無機リンの増加がみられ、250 mg/kg/day の雄及び 1,000 mg/kg/day の雌雄で肝臓重量の増加が認められた。これらの結果から NOAEL は雄で 60 mg/kg/day、雌で 250 mg/kg/day と判定された。

ラットに交配前 2 週間及び交配期間、雄では計 47 日間、雌では妊娠期間及び分娩後哺育 4 日まで、0、8、40、200 及び 1,000 mg/kg/day を強制経口投与した経口投与簡易生殖毒性試験(OECD TG 421）において、200 mg/kg/day 以上で雄の腎臓重量の増加、1,000 mg/kg/day で雌の腎臓及び肝臓重量の増加が認められたが、病理組織学検査では雌雄とも腎臓及び肝臓に変化は認められなかった。これらの結果から反復投与毒性の NOAEL は雄で 40 mg/kg/day、雌で 200 mg/kg/day と判定された。生殖発生毒性に関する影響は認められず、生殖発生毒性の NOAEL は 1,000 mg/kg/day と判定された。

雌ラットの妊娠 6-15 日に 0、250、500 及び 2,000 mg/kg/day を強制経口投与した試験では、2,000 mg/kg/day で妊娠ラットの自発運動の低下、流涎、歩行失調、筋弛緩、正向反射の消失が

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みられ、250 mg/kg/day 以上で体重増加の抑制及び摂餌量の減少が認められた。胎児については 2,000 mg/kg/day で体重の低値、骨格異常の増加、骨化遅延が認められた。これらの結果から、母体毒性の NOAEL は 250 mg/kg/day 以下、発生毒性の NOAEL は 500 mg/kg/day と判定された。

細菌を用いる復帰突然変異試験及びチャイニーズ・ハムスター培養細胞を用いる染色体異常試験では陰性あった。

4）結論と勧告

本化学物質は LP と勧告された。

（5）物質カテゴリー：Short Chain Alkyl Methacrylate Esters (4 chemicals: 97-63-2, 97-86-9, 97-88-1, 688-84-6)（原案作成：日本政府及び ICCA 米国企業）

本物質カテゴリーは、短鎖（C2～C8）で直鎖または不飽和の側鎖を持つ Alkyl methacrylate（Ethyl methacrylate (EMA)、iso-Butyl methacrylate (i-BMA)、n-Butyl methacrylate (n-BMA)、2-Ethylhexyl methacrylate (2-EHMA)）からなる。これらはエステルであり、色々な組織で非特異的なカルボシキルエステラーゼにより、Methacrylic acid（CAS 79-41-4）及びアルコールへと速やかに代謝される。C1 エステルである Methyl methacrylate (MMA)（CAS 80-62-6）の詳細な研究が OECD HPV Chemicals Programme でレビューされており、本カテゴリー物質についての参照となる。

1）曝露状況

本カテゴリー物質はポリマーの合成に使用され、それらのポリマーは自動車コーティング剤などとして使用されている。ポリマー製品の使用により吸入及び経皮による消費者曝露の可能性はあるが、極めて低い。また、製造過程やポリマーの使用によって吸入及び経皮による職業曝露の可能性がある。

2）環境影響

本カテゴリー物質は大気に放出された場合、主に大気にとどまる（96-99%）。EMA、i-BMA、n-BMA は水圏にも分布する（2-4%）。水圏に放出された場合は、EMA、i-BMA、n-BMA は主に水圏にとどまり（98%）、2-EHMA は水圏（35-66%）及び沈殿物（33-64%）に分布する。本カテゴリー物質は易分解性である(OECD TG 301C/D)。水生生物における生物濃縮性は、EMA、i-BMA、n-BMA では低く（生物濃縮係数 BCF：8-75、計算値）、2-EHMA では高い（生物濃縮係数 BCF：3217-11259）。水生生物に対する毒性は全般的に、EMA<i-BMA<n-BMA<2-EHMA の順に強くなる。水生生物に対する急性毒性では、藻類の EC_{50} は>110-7.68 mg/L（72 時間、OECD

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TG 201)、ミジンコの EC_{50} は>66-4.6 mg/L（48 時間、OECD TG 202)、魚類の LC_{50} は 100-2.78 mg/L（96 時間、OECD TG 203）であった。慢性毒性では、藻類の NOEC は 110-0.28 mg/L（72 時間、OECD TG 201)、ミジンコの NOEC は 18-0.12 mg/L（21 日間、OECD TG 211）であった。

3）健康影響

本カテゴリー物質についてラットの経口及びウサギの経皮 LD_{50} は 2,000 mg/kg 以上、2-EHMA を除く吸入 LC_{50} は 29 mg/l 以上と報告されている。2-EHMA の蒸気圧は低く（<1 hPa、20℃)、吸入は重要な曝露経路ではないと考えられる。

個々の化学物質ごとにウサギの皮膚に対して弱い刺激性が認められている。ウサギの眼に対して、EMA では弱い～中程度の刺激性が認められ、その他の化学物質では、あっても弱い刺激性が認められる程度である。モルモットにおける皮膚感作性について各化学物質で明確な結論は得られていない。臨床報告では EMA、n-BMA 及び i-BMA の皮膚感作性は陽性と報告されている。動物やヒトではメタクリル酸エステルは他のメタクリル酸エステルと交差反応を示すことから、陽性結果は他のメタクリル酸エステルによって引き起こされた反応の可能性も考えられる。メタクリル酸エステルのアクリル酸エステルとの交差反応の報告はないので、短鎖メタクリル酸エステルには弱い皮膚感作性があると考えられる。

雌雄ラットに交配前 2 週間及び交配期間を含め雄では計 44 日間、雌では分娩後哺育 3 日まで、0、30、100、300 及び 1,000 mg/kg/day の n-BMA を強制経口投与した反復投与毒性・生殖発生毒性併合試験（OECD TG 422）において、雄親では 100 mg/kg/day 以上で脾臓の絶対及び相対重量の減少が認められ、病理組織学検査では赤脾髄の萎縮が観察された。さらに、1,000 mg/kg/day で雄親の体重増加の抑制、摂餌量の減少、尿のケトン体及び潜血の増加、血液プロトロンビン時間の延長、血清尿素窒素及び腎臓の相対重量の増加が認められた。雌親では 1,000 mg/kg/day で体重増加の抑制、摂餌量の減少及び病理組織学的に脾臓の赤脾髄の萎縮が認められた。これらの結果から、反復投与毒性の NOAEL は雄で 30 mg/kg/day、雌で 300 mg/kg/day と判定された。雌親の生殖能については、1,000 mg/kg/day で黄体数及び着床数の減少が認められた。雄親の生殖能及び児の発生について投与による影響は認められなかった。これらの結果から、生殖発生毒性の NOAEL は 300 mg/kg/day と判定された。

ラットに交配前 2 週間及び交配期間を含め雄では計 44 日間、雌では分娩後哺育 3 日まで、0、30、100、300 及び 1,000 mg/kg/day の 2-EHMA を強制経口投与した反復投与毒性・生殖発生毒性併合試験（OECD TG 422）において、雄では 300 mg/kg/day で腎臓の絶対及び相対重量の高値が認められたことから反復投与毒性の NOAEL は 100 mg/kg/day、雌では 100 mg/kg/day で

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腎臓の相対重量の高値が認められたことから反復投与毒性の NOAEL は 30 mg/kg/day と判定された。また、雄親では交尾及び受胎能に影響が認められなかったことから生殖毒性の NOAEL は 1,000 mg/kg/day と判定され、雌親では 1,000 mg/kg/day で黄体数及び着床数の低値が認められたことから生殖毒性の NOAEL は 300 mg/kg/day と判定された。児については 300 mg/kg/day で新生児数の低値が認められたことから、発生毒性の NOAEL は 100 mg/kg/day と判定された。

ラットに 0、310、952 及び 1,891 ppm の n-BMA を 1 日 6 時間、週 5 日曝露した 4 週間反復吸入毒性試験（OECD TG 412）において、952 ppm 以上で雌雄に流涙、斜視、呼吸困難、背側鼻道の嗅上皮の退化が認められ、NOAEC は 1,832 mg/m³（310 ppm）と判定された。雌雄の生殖器官への影響は最高用量の 1,891 ppm でも認められなかった。物質カテゴリーの n-BMA 以外の化学物質（EMA、i-BMA、2-EHMA）については反復吸入曝露毒性のデータは無い。MMA（参照物質）の反復吸入曝露毒性に関するデータによると、MMA を 2 年間ラットに反復吸入させた場合の嗅覚細胞の病変に関する NOAEC は 104 mg/m³（25 ppm）であった。親エステルがカルボシキルエステラーゼにより加水分解され、Methacrylic acid を放出することで毒性が発現することが知られている。また、短鎖の Alkylmethacrylate 物質カテゴリーにおいて、嗅覚細胞の病変についての NOAEC または LOAEC はエステルのサイズに伴い増加する明白な傾向が認められている。嗅覚細胞の病変についての NOAEC は MMA と同程度の EMA で 119 mg/m³(25 ppm)、n-BMA と同程度の i-BMA で 1,832 mg/m³（310 ppm）と推定された。

雄マウスに交尾前 5 日間（6 h/day）、9,000 ppm までの MMA を反復吸入させた優性致死試験では、生殖能に対する影響は認められなかった。ラットの妊娠 6-20 日に 0、100、300、600 及び 1,200 ppm の n-BMA を吸入曝露した試験では、300 ppm 以上で母体毒性（体重増加抑制）が認められ、600 ppm 以上で児体重の低値がみられたが、胚致死や催奇形性は認められなかった。また、ラットの妊娠 6-20 日に 0、600、1,200、1,800 及び 2,400 ppm の EMA を吸入曝露した試験では、1,200 ppm 以上で母体重及び児体重の低値が認められたが、胚致死や催奇形性は認められなかった。ラットの妊娠 6-15 日に 0、99、304、1,178 及び 2,028 ppm の MMA を吸入曝露した試験では、99 ppm 以上で母体毒性（体重増加抑制）が認められたが、発生毒性に対する影響はみられなかった。ラットの妊娠 6-20 日に 0、50、100、200 及び 300 ppm の MMA を吸入曝露した試験では、300 ppm で母体毒性（体重増加抑制）が認められたが、発生毒性に対する影響はなかった。Butyl methacrylates（n-BMA 及び i-BMA）の代謝産物について、ラットとウサギへの iso-Butanol の反復経口投与、ラットへの iso-または tert-Butanol の反復吸入曝露の結果、発生毒性に関する影響は認められなかったが、ラットへの n-Butanol の反復吸入曝露では 8,000 ppm で胎児の骨格異常が認められた。

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ラットに 60 日間、0、100、200、400 及び 800 mg/kg/day の EMA を腹腔内投与した神経毒性試験では 100 mg/kg/day 以上で自発運動が低下した。嗜眠、呼吸障害、自発運動の低下が 800 mg/kg/day でみられたが、一般毒性によると考えられた。

本カテゴリー物質は、変異原性について種々の *in vitro* 及び *in vivo* 試験で陰性の結果を示した。

4）結論と勧告

健康影響については LP と勧告された。EMA、i-BMA、n-BMA の環境影響について LP とされたが、2-EHMA の環境影響については FW と勧告され、環境曝露評価及び魚類への濃縮性調査を行うことが推奨された。

（6）物質カテゴリー：Gluconates (6 chemicals: 90-80-2, 299-27-4, 299-28-5, 526-95-4, 527-07-1, 18016-24-5)（原案作成：日本政府及び ICCA ベルギー企業）

本物質カテゴリーはグルコン酸（D-gluconic acid）とその誘導体（glucono-delta-lactone、sodium D-gluconate、calcium D-gluconate monohydrate、calcium D-gluconate anhydrous、potassium D-gluconate）からなる。これらのカテゴリー物質は、水中ではグルコン酸イオン（全てに共通）とそれぞれの陽イオンに容易に解離する。製造と使用法についてもカテゴリー物質間で相互に関連がある。ここでは陽イオンに関連した毒性影響は示されていない。

1）曝露状況

本カテゴリー物質は天然物であり、ほ乳類では D-gluconic acid と glucono-delta-lactone は、糖代謝の重要な中間体である。グルコン酸塩はブドウ糖酸化の際の代謝物である。グルコン酸塩は、体重 60 kg のヒトの体内で 1 日約 450 mg/kg 生産される。経口以外の経路で投与されたグルコン酸塩の多く（60-85%）がそのまま尿中に排泄される。本カテゴリー物質の多くが食品添加物として認められている。その他、工業的洗浄剤、金属の表面処理剤、繊維製品の漂白安定剤、アルミニウム加工剤、あるいはキレート剤としても使用されている。消費者曝露では経口及び経皮が主要経路と考えられる。閉鎖系で製造されるため、製造時の職業曝露は起こりにくいが、袋詰めされた製品を利用する際の飛沫により、吸入及び経皮経路の曝露が起こりうる。

2）環境影響

本カテゴリー物質は主に水圏（39-50%）と土壌（49-61%）に分布する。易分解性であり、水生生物における生物濃縮性も低いと考えられた。Sodium D-gluconate の水生生物に対する急性影響は認められず、藻類の EC_{50} は>1,000 mg/L、NOEC は 560 mg/L（72 時間、OECD TG 201）、ミジンコの NOEC は>1,000 mg/L（48 時間、OECD TG 202）、魚類の最大 0%致死濃度（LC_0）は>100 mg/L（96 時間、OECD TG 203）であった。

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3）健康影響

Potassium D-gluconate について、ラットの 14 日間経口投与毒性試験での LD_{50} は 6,060 mg/kg と報告されている。4 週間、6 ヶ月または 24 ヶ月の反復投与毒性試験においてグルコン酸塩の毒性影響は認められなかった。また、gluconic acid は皮膚と眼に対して刺激性を示さない。

ラットに、0、500、1,000 及び 2,000 mg/kg/day の sodium D-gluconate を強制経口投与した 28 日間反復経口投与毒性試験では、2,000 mg/kg/day で雄の胃境界縁壁の肥厚が認められ、NOAEL は雄で 1,000 mg/kg/day、雌で 2,000 mg/kg/day と判断されたが、胃境界縁壁はヒトにはない。また、ラットに、0、1.25、2.5 及び 5%（雄で 0、1,000、2,000 及び 4,100 mg/kg/day、雌で 0、1,000、2,000 及び 4,400 mg/kg/day）の sodium D-gluconate を混餌投与した 28 日間反復経口投与毒性試験では、最高用量でも毒性影響は認められなかった。

上記 sodium D-gluconate についての反復投与毒性試験において雌雄の生殖器に及ぼす影響は認められなかった。また、様々な種による glucono-delta-lactone についての発生毒性試験では全ての試験で投与による影響は観察されていない。

In vitro または *in vivo* での glucono-delta-lactone、sodium D-gluconate、calcium D-gluconate における遺伝子突然変異誘発性試験は陰性であった。

4）結論と勧告

本カテゴリー物質は LP と勧告された。

3　おわりに

本稿では、SIAM18 で合意された化学物質名および日本担当物質の初期評価文書について紹介した。SIAM で合意された化学物質の初期評価文書は出版され、また、インターネットの OECD web サイト（http://cs3-hq.oecd.org/scripts/hpv/）でも入手が可能である。

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表1　SIAM18で議論された物質の合意結果

CAS No.	物質名	担当国	結果
60-00-4	Edetic acid	DE:eu	HH: LP ENV: FW
64-02-8	Tetrasodium ethylenediaminetetraacetate	DE:eu	HH: LP ENV: FW
75-10-5	Difluoromethane	FR/ICCA	LP
79-11-8	2-Chloro-ethanoic acid	SE+ NL:eu	FW
96-18-4	1,2,3-Trichloropropane	US/ICCA	LP
98-07-7	Trichloromethylbenzene	DE/ICCA	LP
99-54-7	1,2-Dichloro-4-nitrobenzene	DE/ICCA	LP
101-54-2	4-Aminodiphenylamine	DE/ICCA	HH: － ENV: FW
110-65-6	But-2-yne-1,4-diol	DE:eu	HH: FW ENV: LP
110-85-0	Piperazine	SE:eu	FW
120-61-6	Dimethyl Terephthalate	US+IT	LP
122-99-6	Ethylene Glycol Phenyl Ether	US/ICCA	HH: FW ENV: LP
124-04-9	Adipic Acid	DE/ICCA	LP
140-88-5	Ethyl Acrylate	US/ICCA	LP
141-10-6	Pseudoionone	CH/ICCA	LP
793-24-8	N-(1,3-Dimethylbutyl)-N′-phenyl-1,4-phenylendiamine	JP/ICCA	FW
868-85-9	Dimethyl phosphonate	DE/ICCA	LP
2855-13-2	3-Aminomethyl-3,5,5-trimethylcyclohexylamine	DE/ICCA	LP
4979-32-2	N,N-Dicyclohexyl-2-benzothiazolesulfenamide	JP	FW
7778-54-3	Calcium hypochlorite	JP/ICCA	HH: LP ENV: FW
25321-14-6	Dinitrotoluene (isomers mixture)	DE/ICCA	HH: LP ENV: FW
31570-04-4	Tris(2,4-di-tert-butylphenyl)phosphite	UK/ICCA	HH: LP ENV: FW
56539-66-3	3-Methoxy-3-methyl-1-butanol	JP	LP
物質カテゴリー名（CAS No.）		**担当国**	**結果**
Amino tris(methylenephoshonic acid) and sodium salts (8 chemicals: 2235-43-0, 4105-01-5, 6419-19-8, 7611-50-9, 15505-05-2, 20592-85-2, 94021-23-5, one has no CAS No.)		UK/ICCA	LP
1-Hydroxy-1,1ethane diphosphonic acid and sodium and potassium salts (13 chemicals: 2666-14-0, 2809-21-4, 3794-83-0, 7414-83-7, 13710-39-9, 14860-53-8, 17721-68-5, 17721-72-1, 21089-06-5, 29329-71-3, 60376-08-1, 67953-76-8, 87977-58-0)		UK/ICCA	HH: LP ENV: FW

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Diethylene triamine penta (methlyene phosphonic acid and its sodium salts (12 chemicals: 15827-60-8, 22042-96-2, 61792-09-4, 68155-78-2, 93841-74-8, 93841-75-9, 93841-76-0, 94987-75-4, 94987-76-5, 94987-77-6, 95015-06-8, 95183-54-3)	UK/ICCA	LP
Ethylene glycols (5 chemicals: 107-21-1, 111-46-6, 112-27-6, 112-60-7, 4792-15-8)	CA/ICCA	HH:FW (107-21-1, 4792-15-8) and LP (111-46-6, 112-27-6) ENV: LP
Propylene glycol phenyl ether (3 chemicals: 770-35-4, 4169-04-4, 41593-38-8)	US/ICCA	HH: FW ENV: LP
Cadmium and cadmium oxide (2 chemicals: 74440-43-9, 1306-19-0)	BE:eu	FW
Short chain alkyl methacrylate esters (4 chemicals: 97-63-2, 97-86-9, 97-88-1, 688-84-6)	JP+US/ICCA	HH:LP ENV: FW (688-84-6) and LP (others)
Gluconates (6 chemicals: 90-80-2, 299-27-4, 299-28-5, 526-95-4, 527-07-1, 18016-24-5)	JP+BE/ICCA	LP
Maleic anhydride and acid (2 chemicals: 110-16-7, 103-81-6)	US/ICCA	LP
Soluble silicates (5 chemicals: 1312-76-1, 1344-09-8, 6834-92-0, 10213-79-3, 13517-24-3)	DE/ICCA	LP

担当国の略号は BE：ベルギー、CA：カナダ、CH：スイス、DE：ドイツ、FR：フランス、IT：イタリア、JP：日本、NL：オランダ、SE：スウェーデン、UK：英国、US：米国である。ICCA は国際化学工業協会協議会による原案提出を示す。eu は、欧州連合でのリスク評価をもとにしたことを示す。合意結果において、FW は追加の調査研究作業が必要であることを、LP は現状では追加作業の必要がないことを示す。HH はヒトへの健康影響、ENV は環境影響について示し、－は合意に達しなかったことを示す。

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Two-generation reproductive toxicity study of the rubber accelerator *N,N*-dicyclohexyl-2-benzothiazolesulfenamide in rats

Makoto Ema [a,*], Sakiko Fujii [b], Mariko Matsumoto [a], Mutsuko Hirata-Koizumi [a], Akihiko Hirose [a], Eiichi Kamata [a]

[a] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Tokyo 158-8501, Japan*
[b] *Safety Research Institute for Chemical Compounds Co. Ltd., Sapporo 004-0839, Japan*

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Abstract

Male and female Crl:CD(SD) rats were fed a diet containing rubber accelerator *N,N*-dicyclohexyl-2-benzothiazolesulfenamide (DCBS) at 0, 80, 600 or 4500 ppm throughout the study beginning at the onset of a 10-week pre-mating period and continuing through the mating, gestation, and lactation periods for two generations. At 4500 ppm, decreases in the body weight, body weight gain, and food consumption were found in F0 males and females. No changes in the estrous cyclicity, copulation index, fertility index, gestation index, delivery index, number of implantations, precoital interval, or gestation length were observed in any generation at any dose of DCBS. Delayed preputial separation at 4500 ppm as well as delayed vaginal opening and higher body weight at the age of vaginal opening at 600 and 4500 ppm were found in the F1 generation. A transient change in performance in a water-filled multiple T-maze was found at 600 and 4500 ppm in F1 females. There were no compound-related changes in number of pups delivered, sex ratio of pups, viability of pups, anogenital distance, surface righting reflex, negative geotaxis reflex, mid-air righting reflex, pinna unfolding, incisor eruption, or eye opening in the F1 and F2 generations. The body weight of F1 and F2 male and female pups was lowered at 4500 ppm. Reduced uterine weight of the weanlings was noted in the F1 generation at 4500 ppm and in the F2 generation at 600 and 4500 ppm. The data indicate that the NOAEL of DCBS for two-generation reproductive toxicity is 80 ppm (5.2 mg/kg bw per day) in rats.

Keywords: *N,N*-Dicyclohexyl-2-benzothiazolesulfenamide; Rubber accelerator; Two-generation reproductive toxicity; Developmental toxicity; Rat

1. Introduction

N,N-Dicyclohexyl-2-benzothiazolesulfenamide (DCBS) is a sulfenamide accelerator. The sulfenamide accelerator class of rubber accelerators has been manufactured in the USA for over 60 years [1]. Sulfenamide accelerator compounds are widely used in the manufacture of automotive compartments and industrial rubber products such as tires, hoses, conveyer belts, bushings seals, gaskets and windshield wiper blades, and the typical usage for sulfenamide accelerators is from 0.5 to 4 parts accelerator per every 100 parts of rubber [1]. Sulfenamide accelerator materials are shipped extensively throughout the world from manufacturing plants located in North America, South America, Europe, Asia and Africa [1]. DCBS was produced in Japan with an annual production level of about 1000 tonnes in 1990–1993 and 1900 tons in 2000–2003, and most of this amount was sold and handled domestically [2]. DCBS is used as an accelerator of vulcanization and is completely reacted in the vulcanizing process [2]. DCBS is regulated for use in articles in contact with food in Germany, but this compound is not regulated for use in FDA food contact applications [3]. Exposure of workers handling sulfenamide accelerator materials is likely to be highest in the area of materials packaging. During material packout at the manufacturing site and to a lesser degree during weigh-up activities at the consumer site, there is potential for skin and inhalation exposure. Although consumer exposure would be minimal, the most likely route of consumer exposure is skin contact from rubber or latex articles [1].

Only up to 6% biodegradation for DCBS was determined in a ready biodegradability test, and a measured log K_{ow} value of 4.8 suggests that DCBS may have a high bioaccumulation potential [2]. The possibility of such a chemical compound entering

* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

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into biological systems has aroused great concern regarding its toxicological potential. Generally, biological effects produced by chemicals should be studied in laboratory animals to investigate their possible influences on human health, and the results of animal tests of chemical toxicity are relevant to humans [4]. However, very little information on the toxicity of DCBS has been published. Vorobera (1969) [5] reported that the oral LD50 value was 8500 mg/kg bw in male mice and that repeated inhalation exposure of male rats for 15 days, daily, 2 h/day, at 350–400 mg/m^3 caused mucous membrane irritation. Although the toxic effects of DCBS have been briefly summarized by the European Chemical Bureau [6] and EPA [1], descriptions regarding the toxicity of DCBS are insufficient to assess the adverse effects of this compound. The EPA [1] noted that the oral LD50 values were 1077–10000 mg/kg bw in rats, the oral NOAEL for 44-day repeated dose toxicity was higher than 100 mg/kg bw per day in rats, and no effects on reproduction were observed at doses up to 400 mg/kg bw per day in rats. Toxicity studies including acute toxicity, in vitro genotoxicity, and repeat dose toxicity combined with reproductive/developmental toxicity studies of DCBS were performed as a part of the Safety Examination of Existing Chemical Substances and Chemical Safety Programmes by the Japanese Government [7]. These toxicity studies are summarized in the IUCLID Data Sets [8], OECD Screening Information Data Sets [2] and the Hazard Assessment Sheet [9]. We previously reported results of repeat dose toxicity combined with a reproductive/developmental toxicity screening test of DCBS showing that DCBS at 400 mg/kg bw per day possessed a deleterious effect on reproduction and development and caused a marked decrease in the number of live pups as well as a total loss of pups until postnatal day (PND) 4 [10]. The primary effects may be on the gestation index for dams and live birth index for pups, which both appear to be affected at multiple points along the female reproductive process; the viability of neonatal pups may also be affected. The previous study was performed in compliance with the OECD guideline for a Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test [11,12], but this screening test guideline does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. In order to further evaluate the reproductive and developmental toxicity of DCBS in rats, a two-generation reproductive toxicity study was conducted. We examined reproductive and developmental endpoints such as sexual development, estrous cyclicity, anogenital distance (AGD), physical and functional development, serum hormone levels, and sperm count and motility.

2. Materials and methods

This study was performed in 2006–2007 at the Safety Research Institute for Chemical Compounds Co. Ltd. (Sapporo, Japan) in compliance with OECD guideline 416 Two-generation Reproduction Toxicity Study [13] and in accordance with the principles for Good Laboratory Practice [14], "Law for the Humane Treatment and Management of Animals" [Law No. 105, 1 October 1973, revised 22 December 1999, Revised Law No. 221; revised 22 June 2005, Revised Law No. 68], "Standards Relating to the Care, Management and Refinement of Laboratory Animals" [Notification No. 88 of the Ministry of the Environment, Japan, 28 April 2006] and "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in the Testing Facility under the Jurisdiction of the Ministry of Health, Labour and Welfare" [Notification No. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, 1 June 2006].

2.1. Chemical and dosing

N,N-Dicyclohexyl-2-benzothiazolesulfenamide (DCBS, CAS No. 4979-32-2) was obtained from Ouchishinko Chemical Industrial Co. Ltd. (Tokyo, Japan). DCBS in the form of off white to tan granules is very slightly soluble in water and methanol but soluble in oil, and its melting point is 100–105 °C, density at 21 °C is 1230 kg/m^3, and molecular weight is 347 [3]. The DCBS (Lot no. 508001) used in this study was 99.7% pure, and it was kept in a sealed container under cool (1–8 °C) and dark conditions. The purity and stability of the chemical were verified by analysis using high-performance liquid chromatography before and after the study. Rats were given dietary DCBS at a concentration of 0 (control), 80, 600 or 4500 ppm. The dosage levels were determined based on the results of our previous dose-finding study in male and female rats fed a diet containing DCBS at 0, 1500, 3000, 6000 or 10,000 ppm (0, 83, 172, 343 or 551 mg/kg bw per day in males and 0, 126, 264, 476 or 707 mg/kg bw per day in females) for a total of eight weeks beginning 16 days before mating in males and a total of nine weeks in females throughout the mating, gestation and lactation periods beginning 16 days before mating. In that study, we found reduced body weight gain in males at 6000 ppm and higher and females at 3000 ppm and higher, reduced number of implantations at 6000 ppm and higher, decreased absolute and relative weight of the spleen in females at 6000 ppm and higher, reduced number of pups born at 10000 ppm, lowered body weight of pups at 6000 ppm and higher, and decreased absolute and relative weight of the spleen in male weanlings at 1500 ppm and higher and female weanlings at 3000 ppm and higher [15]. Dosed diet preparations were formulated by mixing DCBS into an appropriate amount of a powdered basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) for each dietary concentration. The control rats were fed a basal diet only. Analysis showed that DCBS was homogeneous in the diet and stable for at least 21 days in a room temperature, and formulations were maintained in a room temperature for no more than 21 days. Generally, diet was replaced every 1 week.

2.2. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies and historical control data are available. Male and female rats at 4 weeks of age were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for eight days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. One hundred and ninety two rats were randomly assigned 24/sex/group and all animals were assigned a unique number and ear tattooed prior to the start of the experiment. Animals were housed individually in suspended aluminium/stainless steel cages except during the acclimation, mating and nursing periods. From day 17 of pregnancy to the day of weaning, individual dams and litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan, Inc.).

Animals were reared on a basal diet or diet containing DCBS and filtered tap water *ad libitum* and maintained in an air-conditioned room at 22 ± 3 °C, with a humidity of 50 ± 20%, a 12-h light (8:00–20:00)/dark (20:00–8:00) cycle, and ventilation at 10–15 times/h.

2.3. Experimental design

Twenty-four rats (5-week-old males and females)/sex/group were fed a diet containing DCBS at 0, 80, 600 or 4500 ppm for 10 weeks prior to the mating period. Each female F0 rat was mated with a male rat of the same dosage group, with administration of DCBS in the diet continuing throughout the mating period. Administration of DCBS was continued throughout gestation and lactation. Twenty-four male and 24 female F1 weanlings (1 male and 1 female

in each litter) in each group were selected as F1 parents on PNDs 21–25 to equalize the body weights among groups. The day on which F1 parental animals were selected was designated as 0 week of dosing for the F1 generation. The administration of DCBS in the diet was not suspended during PNDs 21–25. F1-selected rats were administered DCBS in the diet with the respective formulation for 10 weeks prior to the mating period and mated as described above. Administration of DCBS in the diet was continued throughout the mating, gestation, and lactation periods. On PND 26, F1 weanlings not selected for breeding and all F2 weanlings were necropsied.

2.4. Mating procedures

Each female was mated with a single male of the same dosage group until copulation occurred or the mating period had elapsed. The mating periods for F0 and F1 animals were three weeks. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered evidence for successful mating. The day of successful mating was designated as day 0 of pregnancy. F1 females that did not mate during the 3-week mating period were cohabited with other males from the same group who had been proven to copulate. For F1 matings, cohabitation of siblings was avoided.

2.5. Parental data

All adult rats were observed twice a day for clinical signs of toxicity, and body weights and food consumption were recorded weekly. For females exhibiting evidence of successful mating, body weight and food consumption were recorded on days 0, 7, 14, and 20 of pregnancy and days 0, 4, 7, 14, and 21 of lactation. Daily vaginal lavage samples of each F0 and F1 female were evaluated for estrous cyclicity throughout the 2-week pre-cohabitation period and during cohabitation until evidence of copulation was detected. Females having repeated 4–6 day estrous cycles were judged to have normal estrous cycles. After weaning of their pups, parental female rats were necropsied at the proestrous stage of the estrous cycle. For each female, the number of uterine implantation sites was recorded.

2.6. Litter data

Once insemination was confirmed, female rats were checked at least three times daily at days 21–25 of pregnancy to determine the time of delivery. The females were allowed to deliver spontaneously and nurse their pups until PND 21 (the day of weaning). The day on which parturition was completed by 13:00 was designated as PND 0. Total litter size and the numbers of live and dead pups were recorded, and live pups were counted, sexed, examined grossly, and individually weighed on PNDs 0, 4, 7, 14, and 21. On PND 4, litters were randomly adjusted to eight pups comprising of four males and four females. No adjustment was made for litters of fewer than eight pups. Selected pups were assigned a unique number and limb tattooed on PND 4.

2.7. Developmental landmarks

All F1 and F2 pups were observed daily for pinna unfolding on PNDs 1–4, incisor eruption beginning on PND 8, and eye opening beginning on PND 12. One male and one female F1 and F2 pup selected from each dam was evaluated for the surface righting reflex on PND 5, negative geotaxis reflex on PND 8, and mid-air righting reflex on PND 18 [16]. All F1 offspring were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. Body weight of the respective F1 rats was recorded on the day of preputial separation or vaginal opening. The AGD was measured using calipers on PND 4 in all F1 and F2 pups, and the AGD per cube root of body weight ratio was calculated [17].

2.8. Behavioral tests

Spontaneous locomotor activity was measured with a multi-channel activity monitoring system (Supermex; Muromachi Kikai Co. Ltd., Tokyo, Japan) in 10 male and 10 female F1 rats selected from each group at 4 weeks of age. Rats were placed individually in transparent polycarbonate cages ($27.6W \times 44.5D \times 20.4H$ cm, CL-0108-1, Clea Japan Inc., Tokyo, Japan) under an infrared sensor that detects thermal radiation from animals. Spontaneous motor activity was determined for 10 min intervals and for a total of 60 min.

A test in a water-filled multiple T-maze was conducted in 10 male and 10 female F1 rats selected from each group at 6 weeks of age. The apparatus was similar to that described by Biel [18]. The water temperature of the maze was kept 22–23 °C. As a preliminary swimming ability test, each rat was allowed to swim three times in a straight channel on the day before the maze trial, and then tested in the maze with three trials per day for the next consecutive three days. The elapsed time between entry into the water at the starting point and touching the goal ramp as well as the number of errors were recorded. To prevent exhaustion of the rats, no animal was allowed to remain in the water for more than 3 min in any trial.

2.9. Termination/necropsy-adults

Parental rats were necropsied: males after the parturition of paired female, and females after weaning of their pups. Ages on the day of the scheduled terminal sacrifice were 19–20 weeks old in F0 males, 21–22 weeks old in F0 females, 18 weeks old in F1 males and 19–20 weeks old in F1 females. The proestrous stage of the estrous cycle was characterized by examination of the vaginal smears of female rats on the day of necropsy. A complete necropsy was performed on all rats found dead and those killed at the scheduled terminal sacrifice. Live rats were euthanized by exsanguination under ether anesthesia. The external surfaces of the rats were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. Weights of the brain, pituitary, thyroid, thymus, liver, kidney, spleen, adrenal, testis, epididymis, seminal vesicle (with coagulating glands and their fluids), ventral prostate, uterus and ovary were recorded. Weights of the thyroid and seminal vesicle were measured after fixation. Major organs were stored in 10% neutral buffered formalin. The testis and epididymis were fixed with Bouin's solution and preserved in 70% ethanol.

Histopathological evaluations in F0 and F1 adults were performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin: the liver, pituitary, thymus, thyroid, kidney, spleen, adrenal, bone marrow, mesenteric lymph node, Peyer's patches, testis, epididymis, seminal vesicle, coagulating gland, ventral prostate, ovary, uterus, vagina and mammary gland of all males and females in the control and highest dose (4500 ppm) groups and of females with abnormal estrous cycles, of males and females without evidence of copulation or insemination and of females with abnormal delivery or totally dead pups in all groups. Any organs or tissues of F0 and F1 adults showing gross alterations were evaluated histopathologically.

In ten each F1 females of the control and highest dose groups, the primordial follicles were counted [19]. The right ovary was fixed in 10% neutral buffered formalin and then dehydrated and embedded in paraffin in a longitudinal orientation by routine procedures. Sections were cut serially at 5 μm and every 20th one was serially mounted on slides and stained with hematoxylin and eosin. About 40 sections per ovary were used to determine the primordial follicles.

2.10. Termination/necropsy-pups

Following adjustment of litter size on PND 4, culled pups were euthanized by inhalation of carbon dioxide and subjected to a gross external and internal necropsy. No tissues from these pups were collected.

The weanlings not selected to become parents were euthanized and necropsied as described for the adults. Organ weights of one male and one female F1 and F2 weanling selected from each dam was measured as described above for adults. The weights of the pituitary and thyroid were not determined in weanlings. All pups found dead before weaning were also necropsied.

In all male and female F1 and F2 weanlings whose organs were collected, histopathological evaluations of the thymus, liver and spleen in the control and 4500 ppm groups were performed after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin.

2.11. Hematological and blood biochemical parameters

On the day of the scheduled terminal sacrifice, blood samples were collected from the abdominal aorta of adult rats under ether anesthesia.

Hematological examinations were performed for 10 males and 10 females of F0 and F1 rats randomly selected from each group. Blood samples were analyzed for the following hematological parameters, using 2K-EDTA as an anticoagulant: white blood cell count (WBC) and differential leukocyte count.

Blood biochemical evaluations were performed for 10 males and 10 females of F0 and F1 rats randomly selected from each group. Serum samples obtained from centrifuged whole blood were analyzed for biochemistry parameters such as total protein, albumin and globulin.

2.12. Serum hormone levels

On the day of the scheduled terminal sacrifice, blood samples were collected from the abdominal aorta of adult rats. Eight males and eight proestrous females of the F0 and F1 generations from each group were selected randomly for blood collection. Hormone levels were determined by Panapharm Laboratories Co. Ltd. (Uto, Japan). Serum levels of testosterone, 5α-dihydrotestosterone (DHT), luteinizing hormone (LH), and follicle stimulating hormone (FSH) in males, and estradiol, progesterone, LH, and FSH in females were measured. The testosterone, DHT, estradiol, and progesterone concentrations were measured using a double antibody kit (Diagnostic Products Corp., Los Angeles, CA or Diagnostic Systems Laboratories Inc., Webster, TX). Serum concentrations of LH and FSH were measured using (rat LH)[125I] and (rat FSH) [125I] assay systems (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), respectively.

2.13. Sperm parameters

Sperm parameters were determined for all F0 and F1 male adults, except dead males, on the day of the scheduled terminal sacrifice. The right testis was used to count testicular homogenization-resistant spermatid heads. The right cauda epididymis was weighed and used for sperm analysis. Sperm motility was analyzed using a computer-assisted cell motion analyzer (TOX IVOS, Hamilton Thorne Biosciences, Beverly, MA). The percentage of motile sperm and progressively motile sperm as well as their swimming speed and pattern were determined. After the recording of sperm motion, the cauda epididymal fluid was diluted and sperm were enumerated using a hemacytometer under a light microscope. A sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the cauda epididymis. The sperm were stained with eosin and mounted on a slide glass. Two hundred sperm in each sample were examined under a light microscope, and the percentage of morphologically abnormal sperm was calculated.

2.14. Statistical analysis

Statistical analysis of offspring before weaning was carried out using the litter as the experimental unit.

Body weight, body weight gain, food consumption, length of estrous cycle, precoital interval, gestation length, numbers of implantations and pups delivered, delivery index, sperm parameters, hematological and blood chemical parameters, hormone levels, organ weight, organ/body weight ratio (relative organ weight), reflex response time, age displayed pinna unfolding, incisor eruption, and eye opening, age and body weight at sexual maturation, parameters of behavioral tests, AGD, AGD/cube root of body weight ratio, and the viability of pups were analyzed for statistical significance in the following way. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances. If the variances were equivalent, the groups were compared by one-way analysis of variance (ANOVA). If significant differences were found, Dunnett's multiple comparison test was performed. If the groups did not have equivalent variances, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pairwise comparisons were made by Mann–Whitney *U*-test. The incidence of pups with changes in clinical and gross internal observations, and reflex completion rate of pups were analyzed by Wilcoxon rank sum test. The number of primordial follicles in the control and highest dose groups was analyzed in the following way. Variance ratio was analyzed by *F*-test. Since the variance ratio was equivalent, the groups were compared by Student's *t*-test. The incidence of females with normal estrous cycles, copulation index, fertility index, gestation index, neonatal sex ratio, and completion rate of the reflex response were analyzed by Fisher's exact test.

The 0.05 level of probability was used as the criterion for significance.

3. Results

3.1. Clinical observations, body weight and food consumption during the pre-mating, mating, gestation, and lactation periods (F0 and F1)

There were no compound-related clinical signs of toxicity in either male or female F0 and F1 rats during the pre-mating, mating, gestation, or lactation periods. One F0 male at 80 ppm was euthanized in 11 weeks of dosing because of a moribund condition resulting from accidental injury in the home cage. One F1 female without any apparent clinical signs of toxicity died on day 5 of lactation in the control group, and no abnormal necropsy findings were found.

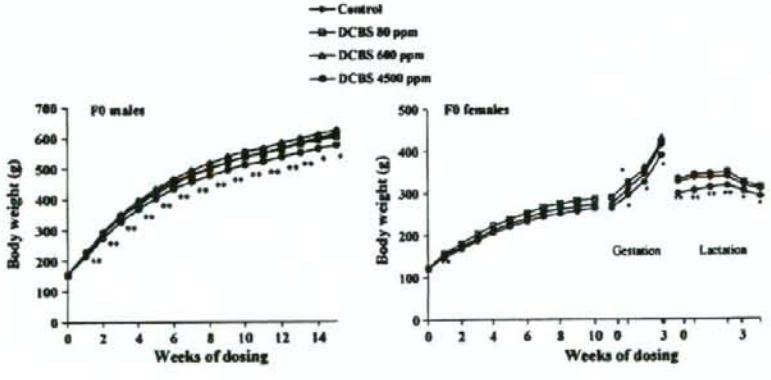

Fig. 1. Body weight of F0 males and females. *Significantly different from the control, $p < 0.05$. **Significantly different from the control, $p < 0.01$.

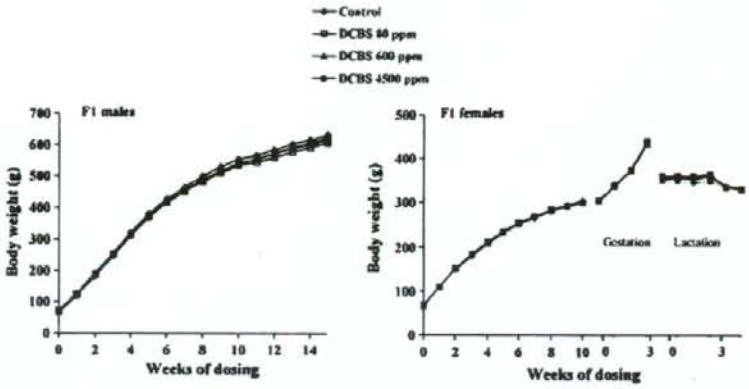

Fig. 2. Body weight of F1 males and females.

The body weights of F0 males and females during dosing are shown in Fig. 1. The body weight and body weight gain of male F0 rats were significantly lowered throughout the dosing period at 4500 ppm. At this dose, the body weight and body weight gain of F0 females were significantly reduced during the first week of dosing and throughout pregnancy and lactation. No compound-related changes in the body weight or body weight gain were noted in F0 males and females at 80 and 600 ppm.

Fig. 2 shows the body weights of F1 males and females during the dosing period. The body weight and body weight gain of F1 males and females exhibited no significant differences between the control and DCBS-treated groups.

There was a significant decrease in food consumption during weeks 1–8 and 13–14 of dosing in F0 males and during the first week of dosing and days 14–21 of lactation in F0 females at 4500 ppm. No significant changes in food consumption were observed in F0 rats of both sexes at 80 and 600 ppm (data not shown).

In F1 male rats, a significant decrease in food consumption was found during weeks 4–7 of dosing at 80 ppm, during week 6 of dosing at 600 ppm and during week 4 of dosing at 4500 ppm. No significant changes were observed in food consumption in F1 females at any dose (data not shown).

The mean daily intakes of DCBS were 5.2, 39 and 291 mg/kg bw in F0 males, 7.2, 54 and 416 mg/kg bw in F0 females, 5.9, 44 and 331 mg/kg bw in F1 males, and 7.4, 55 and 417 mg/kg bw in F1 females for 80, 600 and 4500 ppm, respectively.

3.2. Estrous cyclicity (F0 and F1 females)

Table 1 presents the estrous cyclicity of F0 and F1 females. All F0 females showed normal estrous cycles in all groups, and the length of the estrous cycles was not different between the control and DCBS-treated groups. Although one F1 female each in the control and 600 ppm groups displayed extended diestrous vaginal smears, no significant changes in the incidence of females having normal estrous cycles or length of the estrous cycles were observed.

3.3. Reproductive effects (F0 parents/F1 offspring and F1 parents/F2 offspring)

The reproductive and developmental parameters for F0 parent/F1 offspring are presented in Table 2 . In F0 parent animals, all pairs in all groups copulated, although two females in the con-

Table 1
Estrous cyclicity of F0 and F1 females

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 females				
No. of females examined	24	24	24	24
Females with normal estrous cycles (%)[b]	100	100	100	100
Length of estrous cycles (days)	4.05 ± 0.16[a]	4.01 ± 0.06	4.04 ± 0.15	4.01 ± 0.06
F1 females				
No. of females examined	24	24	24	24
Females with normal estrous cycles (%)[b]	95.8	100	95.8	100
Length of estrous cycles (days)	4.21 ± 0.34	4.05 ± 0.21	4.25 ± 1.08	4.07 ± 0.24

[a] Values are given as the mean ± S.D.
[b] Incidence of females with normal estrous cycles (%) = (no. of females with normal estrous cycles/no. of females examined) × 100.

Table 2
Reproductive and developmental data for F0 parents/F1 offspring and F1 parents/F2 offspring

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 parents/F1 offspring				
No. of pairs	24	24	24	24
Copulation index (%)[b]				
Male/female	100/100	100/100	100/100	100/100
Fertility index (%)[c]	91.7	100	100	100
No. of pregnant females	22	24	24	24
Precoital interval (days)	2.4 ± 1.2[a]	2.8 ± 1.1	2.4 ± 1.0	2.4 ± 1.1
Gestation index (%)[d]	100	100	100	100
Gestation length (days)	22.1 ± 0.4	22.2 ± 0.4	22.0 ± 0.3	22.1 ± 0.3
No. of implantations	13.5 ± 2.1	13.9 ± 1.4	14.6 ± 1.3	13.2 ± 1.5
Delivery index (%)[e]	94.9	94.9	94.3	94.8
No. of pups delivered	12.8 ± 2.1	13.2 ± 1.6	13.8 ± 1.5	12.5 ± 1.7
No. of litters	22	24	24	24
Sex ratio of F1 pups[f]	0.528	0.554	0.506	0.525
Viability index during lactation (%)[g,h,i]				
Day 0	99.0	99.3	99.7	99.0
Day 4	98.7	98.2	96.6	97.6
Day 21	100	99.0	99.5	99.5
Male pup weight during lactation (g)				
Day 0	6.9 ± 0.5	6.7 ± 0.6	6.7 ± 0.6	6.6 ± 0.7
Day 4	11.2 ± 1.1	10.5 ± 1.2	10.5 ± 1.4	10.3 ± 1.0*
Day 7	18.6 ± 1.8	18.1 ± 1.7	17.7 ± 2.5	16.7 ± 1.6**
Day 14	37.2 ± 3.6	36.8 ± 2.4	36.0 ± 4.0	33.6 ± 2.5**
Day 21	62.3 ± 5.6	62.2 ± 3.7	60.2 ± 6.3	55.3 ± 4.8**
Female pup weight during lactation (g)				
Day 0	6.5 ± 0.5	6.3 ± 0.5	6.3 ± 0.5	6.3 ± 0.6
Day 4	10.9 ± 1.3	10.1 ± 1.4	10.0 ± 1.2	9.9 ± 1.0*
Day 7	18.1 ± 1.9	17.1 ± 2.3	17.2 ± 2.3	16.2 ± 1.4**
Day 14	36.3 ± 3.5	34.8 ± 3.6	35.0 ± 4.0	32.8 ± 2.6**
Day 21	60.7 ± 5.2	58.5 ± 6.0	58.2 ± 6.5	53.7 ± 4.5**
F1 parents/F2 offspring				
No. of pairs	24	24	24	24
Copulation index (%)[b]				
Male/female	100/100	100/100	91.7/100	100/100
Fertility index (%)[c]	95.8	91.7	91.7	100
No. of pregnant females	23	22	22	24
Precoital interval (days)	2.7 ± 1.0	2.6 ± 1.4	2.6 ± 1.2	2.8 ± 1.7
Gestation index (%)[d]	100	100	95.5	100
Gestation length (days)	22.3 ± 0.4	22.2 ± 0.4	22.1 ± 0.4	22.1 ± 0.3
No. of implantations	14.1 ± 3.2	13.5 ± 3.7	13.0 ± 4.2	14.3 ± 2.1
Delivery index (%)[e]	90.4	92.9	88.9	91.3
No. of pups delivered	12.7 ± 3.6	12.6 ± 3.7	12.0 ± 4.2	13.0 ± 2.4
No. of litters	23	22	21	24
Sex ratio of F2 pups[f]	0.488	0.516	0.557	0.522
Viability index during lactation (%)[g,h,i]				
Day 0	98.7	99.7	98.3	95.9
Day 4	95.9	94.2	93.1	88.4
Day 21	100[j]	100	97.0	97.7[j]
Male pup weight during lactation (g)				
Day 0	6.8 ± 0.9	6.7 ± 0.8	6.7 ± 0.5	6.7 ± 0.6
Day 4	11.0 ± 2.3	11.1 ± 2.6	10.0 ± 2.1	10.0 ± 1.4[j]
Day 7	18.5 ± 2.7[j]	18.4 ± 3.8	17.1 ± 2.8	15.9 ± 2.3[j,*]
Day 14	37.1 ± 4.0[j]	37.8 ± 6.3	35.5 ± 3.8	32.3 ± 4.1[j,**]
Day 21	62.5 ± 7.0[j]	63.4 ± 9.4	60.6 ± 5.6	53.5 ± 5.9[j,**]

Table 2 (*Continued*)

	DCBS (ppm)			
	0 (control)	80	600	4500
Female pup weight during lactation (g)				
Day 0	6.5 ± 1.0	6.3 ± 0.7	6.3 ± 0.4[k]	6.3 ± 0.7
Day 4	10.5 ± 2.3	10.5 ± 2.5	9.7 ± 2.0[k]	9.5 ± 1.5[l]
Day 7	17.6 ± 2.9[j]	17.7 ± 3.8	16.3 ± 2.8[k]	15.5 ± 2.2[l]
Day 14	35.9 ± 4.1[j]	36.6 ± 5.7	33.5 ± 4.9[k]	31.7 ± 3.9[l,*]
Day 21	59.6 ± 6.6[j]	60.7 ± 8.5	56.3 ± 7.0[k]	52.0 ± 5.7[l,**]

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = (no. of animals with successful copulation/no. of animals paired) × 100.
[c] Fertility index (%) = (no. of females pregnant/no. of females with successful copulation) × 100.
[d] Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e] Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[f] Sex ratio = total no. of male pups/total no. of pups.
[g] Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[h] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.
[i] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live pups on postnatal day 4 after cull) × 100.
[j] Data were obtained from 22 litters because one female that died on day 5 of lactation was excluded from the data.
[k] Data were obtained from 20 litters because one female had no female pups.
[l] Data were obtained from 23 litters because one female that experienced a total litter loss on day 3 of lactation was excluded from the data.
[*] Significantly different from the control, $p < 0.05$.
[**] Significantly different from the control, $p < 0.01$.

trol group did not become pregnant, and all pregnant females in all groups delivered live pups. There were no significant differences in the copulation index, fertility index, gestation index, pre-coital interval, gestation length, number of implantations, delivery index, number of F1 pups delivered, sex ratio of F1 pups, or viability of F1 pups during lactation between the control and DCBS-treated groups. No malformed F1 pups were found in any groups. A significantly lower body weight was observed in male and female F1 pups at 4500 ppm on PNDs 4, 7, 14 and 21.

The reproductive and developmental parameters for F1 parent/F2 offspring are also shown in Table 2. Two F1 males in the 600 ppm group did not copulate. One female in the control group and two females each in the 80 and 600 ppm groups did not become pregnant. One pregnant female in the 600 ppm group did not deliver. One dam in the control group died on day 5 of lactation, and her pups were euthanized. One dam experienced a total litter loss by PND 3 at 4500 ppm. No significant changes in the copulation index, fertility index, gestation index, pre-coital interval, gestation length, number of implantations, delivery index, number of F2 pups delivered, sex ratio of F2 pups, or viability of F2 pups during lactation were observed. Oligodactyly in one female of the control group and microphthalmia in one male at 80 ppm were observed. Body weights of F2 pups at 4500 ppm were significantly lowered on PNDs 7, 14 and 21 in males and PNDs 14 and 21 in females.

3.4. Developmental landmarks (F1 and F2)

Physical development of F1 and F2 pups is presented in Table 3. There was no significant difference in the age of male and female F1 and F2 pups that displayed pinna unfolding, or eye opening between the control and DCBS-treated groups. The completion of incisor eruption was delayed in male and female F1 pups at 80 ppm and in male and female F2 pups at 80 and 4500 ppm. The AGD and AGD per cube root of body weight ratio in male and female F1 and F2 pups were not significantly different between the control and DCBS-treated groups.

Reflex ontogeny in F1 and F2 pups is shown in Table 4. All male and female F1 pups in all groups completed the surface righting reflex on PND 5, negative geotaxis reflex on PND 8, and mid-air righting reflex on PND 18. In F1 pups, no significant difference was observed in the response time of the surface righting reflex or the negative geotaxis reflex between the control and DCBS-treated groups. Of the F2 pups, one female did not complete the surface righting reflex and one male did not complete the mid-air righting reflex at 80 ppm, one female did not complete the mid-air righting reflex at 600 ppm, and one female did not complete the negative geotaxis reflex at 4500 ppm; however, no significant difference was found between the control and DCBS-treated groups in the completion ratio and response time for these reflexes.

Table 5 presents data on sexual development in F1 rats. Although a significant delay in the age of preputial separation in males was noted at 4500 ppm, the body weight at the age of preputial separation was not significantly different between the control and DCBS-treated groups. In females, a significantly delayed age of vaginal opening and a higher body weight at the age of vaginal opening were found at 600 and 4500 ppm.

3.5. Behavioral effects (F1)

Spontaneous locomotor activity in 10 min intervals for a total of 60 min was not significantly different between the control and DCBS-treated groups in male and female F1 rats (data not shown).

Fig. 3 shows the results of the water filled T-maze test in F1 males and females. The pre-test swimming trials in the straight channel on the first day of the T-maze test revealed that all F1

Table 3
Physical development in F1 and F2 pups

	DCBS (ppm)			
	0 (control)	80	600	4500
F1 pups				
No. of litters examined	22	23	24	24
Age at pinna unfolding (days)				
Male	2.7 ± 0.5[a]	2.7 ± 0.5	2.9 ± 0.3	2.7 ± 0.5
Female	2.6 ± 0.6	2.6 ± 0.6	2.9 ± 0.4	2.7 ± 0.5
Age at incisor eruption (days)				
Male	10.2 ± 0.6	10.8 ± 0.6**	10.3 ± 0.6	10.5 ± 0.4
Female	10.1 ± 0.6	10.7 ± 0.7**	10.2 ± 0.7	10.2 ± 0.6
Age at eye opening (days)				
Male	14.5 ± 0.6	14.5 ± 0.5	14.7 ± 0.5	14.6 ± 0.5
Female	14.4 ± 0.6	14.5 ± 0.7	14.4 ± 0.4	14.5 ± 0.5
AGD				
Male pup AGD (mm)	5.60 ± 0.28	5.50 ± 0.28	5.51 ± 0.41	5.54 ± 0.28
Male pup AGD/($BW^{1/3}$)	2.51 ± 0.09	2.52 ± 0.08	2.52 ± 0.12	2.55 ± 0.09
Female pup AGD (mm)	3.02 ± 0.11	2.95 ± 0.14	2.99 ± 0.14	2.96 ± 0.14
Female pup AGD/($BW^{1/3}$)	1.36 ± 0.05	1.37 ± 0.06	1.39 ± 0.04	1.38 ± 0.04
F2 pups				
No. of litters examined	23	22	21	23
Age at pinna unfolding (days)				
Male	2.7 ± 0.8	2.7 ± 0.7	2.8 ± 0.6	2.7 ± 0.5
Female	2.7 ± 0.8	2.7 ± 0.8	2.8 ± 0.4[c]	2.6 ± 0.6
Age at incisor eruption (days)				
Male	9.7 ± 0.7[b]	10.6 ± 0.9**	9.9 ± 0.6	10.3 ± 0.8*
Female	9.8 ± 0.7[b]	10.4 ± 0.8*	10.0 ± 0.6[c]	10.4 ± 0.9*
Age at eye opening (days)				
Male	14.4 ± 0.7[b]	14.6 ± 0.8	14.3 ± 0.7	14.6 ± 0.6
Female	14.3 ± 0.6[b]	14.4 ± 0.8	14.4 ± 0.5[c]	14.5 ± 0.7
AGD				
Male pup AGD (mm)	5.54 ± 0.51	5.60 ± 0.55	5.39 ± 0.56	5.47 ± 0.38
Male pup AGD/($BW^{1/3}$)	2.50 ± 0.12	2.53 ± 0.14	2.51 ± 0.12	2.55 ± 0.08
Female pup AGD (mm)	2.93 ± 0.19	2.91 ± 0.22	2.88 ± 0.19[c]	2.85 ± 0.18
Female pup AGD/($BW^{1/3}$)	1.34 ± 0.04	1.34 ± 0.06	1.35 ± 0.03[c]	1.35 ± 0.05

[a] Values are given as the mean ± S.D.
[b] Data were obtained from 22 litters because one dam that died on day 5 of lactation was excluded from the data.
[c] Data were obtained from 20 litters because one female had no female pups.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

rats in each group could swim satisfactorily, and no significant changes in the elapsed time to traverse the straight channel were observed. In males, no significant differences were observed between the control and DCBS-treated groups in the elapsed time and number of errors in on days 2–4 of the T-maze test. In females, a significantly longer elapsed time at 600 and 4500 ppm and more errors at 4500 ppm were noted on day 2 of the T-maze test. There were no significant differences in the elapsed time or number of errors on days 3 and 4 of the T-maze test in female rats between the control and DCBS-treated groups.

3.6. Necropsy and histopathology (F0, F1 and F2)

There were no compound-related gross lesions or microscopic alterations in the reproductive organs of F0 and F1 males and females showing reproductive difficulties. No compound-related gross lesions or remarkable microscopic alterations of tissues and organs, including the reproductive organs, were noted in F0 and F1 males and females in the highest dose group and dead animals before the scheduled terminal sacrifice. In the histopathological examinations of the ovary in F1 females, no significant difference was noted in the number of primordial follicles (mean ± S.D.) between the control (323 ± 57) and 4500 ppm (255 ± 109) groups. There were no compound-related gross lesions or microscopic alterations in male and female F1 and F2 pups, including pups that died before weaning (data not shown).

3.7. Organ weights (F0 adults)

The body weight at the scheduled terminal sacrifice was significantly lowered at 4500 ppm in males and females. Sig-

Table 4
Reflex ontogeny in F1 and F2 pups

	DCBS (ppm)			
	0 (control)	80	600	4500
F1 pups				
No. of pups examined (male/female)	22/22	24/24	24/24	24/24
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Surface righting reflex response time (s)				
Male	2.1 ± 1.6[a]	1.5 ± 0.5	2.4 ± 2.3	1.8 ± 1.2
Female	2.8 ± 3.4	1.6 ± 0.6	1.9 ± 0.9	3.4 ± 3.9
Negative geotaxis reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Negative geotaxis reflex response time (s)				
Male	14.5 ± 8.0	15.4 ± 8.2	13.8 ± 6.4	16.0 ± 7.5
Female	15.3 ± 6.8	14.1 ± 6.0	15.4 ± 6.2	18.3 ± 7.6
Mid-air righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
F2 pups				
No. of pups examined (male/female)	22/22	22/22	21/20	23/23
Surface righting reflex completion rate (%)				
Male/female	100/100	100/95.5	100/100	100/100
Surface righting reflex response time (s)				
Male	2.5 ± 1.6	2.2 ± 1.8	1.7 ± 0.5	2.1 ± 1.9
Female	2.6 ± 1.8	2.4 ± 2.0[b]	2.5 ± 1.7	3.2 ± 4.5
Negative geotaxis reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/95.7
Negative geotaxis reflex response time (s)				
Male	15.3 ± 6.3	17.2 ± 7.4	14.4 ± 5.7	16.1 ± 4.9
Female	16.9 ± 7.2	14.0 ± 6.5	12.6 ± 8.1	16.0 ± 6.2[c]
Mid-air righting reflex completion rate (%)				
Male/female	100/100	95.5/100	100/95.0	100/100

Surface righting reflex on postnatal day 5, negative geotaxis reflex on postnatal day 8 and mid-air righting reflex on postnatal day 18 were examined three times.
Completion rate (%) = (number of animals showing all successful responses of thee trials/number of animals examined) × 100.
[a] Values are given as the mean ± S.D.
[b] Data were obtained from 21 pups.
[c] Data were obtained from 22 pups.

Table 5
Sexual development in F1 males and females

	DCBS (ppm)			
	0 (control)	80	600	4500
Male preputial separation				
No. of males examined	24	24	24	24
Age (days)	41.3 ± 1.6[a]	41.4 ± 1.6	41.8 ± 1.6	42.8 ± 1.5**
Body weight (g)	226.9 ± 20.3	226.5 ± 18.5	228.3 ± 17.0	229.6 ± 17.5
Female vaginal opening				
No. of females examined	24	24	24	24
Age (days)	29.6 ± 1.0	30.0 ± 1.7	31.2 ± 1.7**	31.1 ± 1.3**
Body weight (g)	104.6 ± 9.4	109.1 ± 10.6	112.1 ± 13.8*	112.3 ± 9.1*

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.