

# 1,3-ビス(アミノメチル)ベンゼンの チャイニーズ・ハムスター培養細胞を用いる染色体異常試験

## In Vitro Chromosomal Aberration Test of

## 1,3-Bis(aminomethyl)benzene on Cultured Chinese Hamster Cells

### 要約

既存化学物質安全性点検に係る毒性試験調査事業の一環として、1,3-ビス(アミノメチル)ベンゼンの培養細胞に及ぼす細胞遺伝学的影響を評価するため、チャイニーズ・ハムスター培養細胞(CHL/IU)を用いて試験管内染色体異常試験を実施した。

連続処理(24および48時間)、短時間処理(6時間)のS9 mix 非存在下においては、50%を越える増殖抑制濃度、すなわち0.33 mg/mlの濃度を最高処理濃度とした。また、短時間処理のS9 mix 存在下では、50%を越える増殖抑制濃度、すなわち0.47 mg/mlの濃度を最高処理濃度とした。最高処理濃度の1/2および1/4を、それぞれ中濃度および低濃度として設定した。連続処理では、S9 mix 非存在下における24時間および48時間連続処理後、短時間処理ではS9 mix 存在下および非存在下で6時間処理(18時間の回復時間)後、標本を作製し、検鏡することにより染色体異常誘発性を検討した。

CHL/IU細胞を24時間および48時間連続処理した高濃度群(0.33 mg/ml)において、細胞毒性のため十分な細胞数を分析できなかったが、その他の処理群においては、染色体の構造異常や倍数性細胞の誘発作用は認められなかった。短時間処理では、S9 mix 存在下および非存在下で6時間処理したいずれの処理群においても、染色体の構造異常や倍数性細胞の誘発作用は認められなかった。

以上の結果より、1,3-ビス(アミノメチル)ベンゼンは、上記の試験条件下で、試験管内のCHL/IU細胞に染色体異常を誘発しないと結論した。

### 方法

#### 1. 使用した細胞

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

#### 2. 培養液の調製

培養には、牛胎児血清(FCS: JRH BIOSCIENCES)を10%添加したイーグルMEM(日水製薬(株))培養液を用いた。

#### 3. 培養条件

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

#### 4. 被験物質

1,3-ビス(アミノメチル)ベンゼン(略号: BAB, CAS No.: 1477-55-0, ロット番号: 30817, 三菱瓦斯化学(株)製造, (社)日本化学工業協会提供)は、無色透明液体で、水、アセトンおよびジメチルスルホキシドに可溶、融点14.1°C、沸点247°C、蒸気圧 $1.6 \times 10^{-3}$  mmHg(25°C)、分子式C<sub>8</sub>H<sub>12</sub>N<sub>2</sub>、分子量136.22、純度99.8%(不純物を0.2%含む、うち水分(0.018%))の物質である。被験物質原体は安定であり、溶媒中(注射用水および純水)では、0.825~50.00 mg/mlの濃度範囲で4時間は安定であった。

#### 5. 被験物質の調製

被験物質の調製は、使用のつど行った。溶媒は注射用水((株)大塚製薬工場)を用いた。原体を溶媒に溶解して原液を調製し、ついで原液を溶媒で順次希釈して所定の濃度の被験物質調製液を作製した。被験物質調製液は、すべての試験において培養液の10%(v/v)になるように加えた。染色体異常試験に用いた被験物質調製液の濃度は、許容範囲内(溶媒中の平均含量が添加量の90.0~110%)の値であった。なお、濃度の記載について、純度換算は行なわなかった。

#### 6. 細胞増殖抑制試験による処理濃度の決定

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

その結果、連続処理における50%の増殖抑制濃度を明らかに越える濃度(約60%の増殖抑制濃度)は、60%の増殖抑制濃度をはさむ2濃度より算出したところ、0.28 mg/mlであった。短時間処理のS9 mix 存在下およびS9 mix 非

存在下における50%の増殖抑制濃度を明らかに越える濃度は、それぞれ、0.47 mg/ml および 0.33 mg/ml であった (Fig. 1).

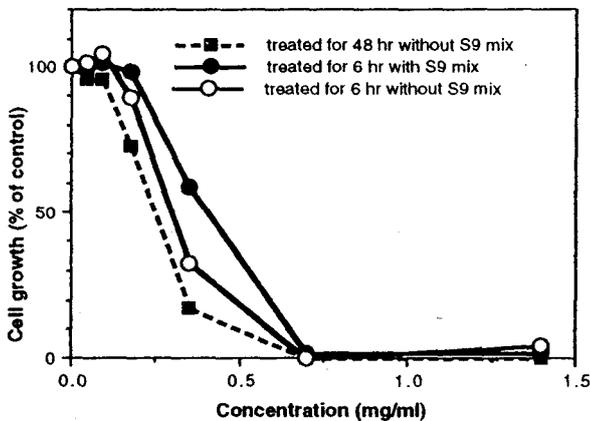


Fig.1 Growth inhibition of CHL/IU cells treated with 1,3-bis(aminomethyl)benzene

7. 実験群の設定

細胞増殖抑制試験の結果より、染色体異常試験で用いる被験物質の高濃度群を、連続処理および短時間処理のS9 mix 非存在下では 0.33 mg/ml、短時間処理のS9 mix 存在下では 0.47 mg/ml とし、それぞれ高濃度群の 1/2 の濃度を中濃度、1/4 の濃度を低濃度とした。陽性対照物質として用いたマイトマイシンCMC、協和醗酵工業(株)およびシクロホスファミド(CPA, Sigma Chemical Co.)は、注射用水((株)大塚製薬工場)に溶解して調製した。それぞれ染色体異常を誘発することが知られている濃度を適用した。

8. 染色体標本作製法

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

9. 染色体分析

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

10. 記録と判定

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

染色体異常を有する細胞の出現頻度について、フィッシャーの exact probability test 法により、溶媒対照群と被験物質処理群間および溶媒対照群と陽性対照群間の有意差検定(p<0.05)を行った。

被験物質の染色体異常誘発性についての最終判定は、石館ら<sup>2)</sup>の判定基準に従い、染色体異常を有する細胞の頻度が5%未満を陰性、5%以上10%未満を疑陽性、10%以上を陽性とした。

結果および考察

連続処理による染色体分析の結果を Table 1 に示した。

1,3-ビス(アミノメチル)ベンゼンを加えて24時間および48時間連続処理した高濃度群(0.33 mg/ml)では、細胞毒性のため十分な細胞数を分析できなかった。特に24時間連続処理では、構造異常が誘発(gapを含み27.3%)されたが、観察可能な細胞は33細胞と極端に少なく評価することができなかった。その他の処理群においては、染色体の構造異常および倍数性細胞の誘発は認められなかった。

短時間処理による染色体分析の結果を Table 2 に示した。

1,3-ビス(アミノメチル)ベンゼンを加えてS9 mix存在下および非存在下で6時間処理したいずれの処理群においても、染色体の構造異常および倍数性細胞の誘発作用は認められなかった。

従って、1,3-ビス(アミノメチル)ベンゼンは、上記の試験条件下で、試験管内のCHL/IU細胞に染色体異常を誘発しないと結論した。

文献

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- 2) 石館 基 監修, "<改訂>染色体異常試験データ集", エル・アイ・シー社, 東京, 1987.

Table 1 Chromosome analysis of Chinese hamster cells (CHL/IU) continuously treated with 1,3-bis(aminomethyl)benzene (BAB)\*\*without S9 mix

| Group                 | Concentration (mg/ml) | Time of exposure (hr) | No. of cells analysed | No. of structural aberrations |     |     |     |     |   |                   |       | Others <sup>3)</sup> | No. of cells with aberrations |            | Polyploid (%) <sup>4)</sup> | Judgement <sup>5)</sup> |     |
|-----------------------|-----------------------|-----------------------|-----------------------|-------------------------------|-----|-----|-----|-----|---|-------------------|-------|----------------------|-------------------------------|------------|-----------------------------|-------------------------|-----|
|                       |                       |                       |                       | gap                           | ctb | cte | csb | cse | f | mul <sup>2)</sup> | total |                      | TAG (%)                       | TA (%)     |                             | SA                      | NA  |
| Control <sup>1)</sup> |                       |                       | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0                 | 0     | 0                    | 0 ( 0.0 )                     | 0 ( 0.0 )  | 0.00                        |                         |     |
| Solvent               | 0                     | 24                    | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0                 | 0     | 0                    | 0 ( 0.0 )                     | 0 ( 0.0 )  | 0.00                        |                         |     |
| BAB                   | 0.08                  | 24                    | 200                   | 0                             | 1   | 1   | 0   | 0   | 1 | 0                 | 3     | 0                    | 3 ( 1.5 )                     | 3 ( 1.5 )  | 0.13                        | -                       | -   |
| BAB                   | 0.17                  | 24                    | 200                   | 0                             | 1   | 0   | 0   | 0   | 0 | 0                 | 1     | 0                    | 1 ( 0.5 )                     | 1 ( 0.5 )  | 0.13                        | -                       | -   |
| BAB                   | 0.33                  | 24                    | 33                    | 2                             | 10  | 11  | 0   | 0   | 1 | 0                 | 24    | 0                    | 9*(.27.3)                     | 8*(24.2)   | 0.00 <sup>6)</sup>          | Tox                     | Tox |
| MC                    | 0.00005               | 24                    | 200                   | 10                            | 46  | 124 | 6   | 2   | 2 | 0                 | 190   | 3                    | 97*(48.5)                     | 96*(48.0)  | 0.00                        | +                       | -   |
| Solvent <sup>1)</sup> | 0                     | 48                    | 200                   | 0                             | 0   | 0   | 1   | 0   | 0 | 0                 | 1     | 0                    | 1 ( 0.5 )                     | 1 ( 0.5 )  | 0.13                        |                         |     |
| BAB                   | 0.08                  | 48                    | 200                   | 0                             | 0   | 0   | 0   | 1   | 0 | 0                 | 1     | 0                    | 1 ( 0.5 )                     | 1 ( 0.5 )  | 0.13                        | -                       | -   |
| BAB                   | 0.17                  | 48                    | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0                 | 0     | 0                    | 0 ( 0.0 )                     | 0 ( 0.0 )  | 0.50                        | -                       | -   |
| BAB                   | 0.33                  | 48                    | 38                    | 0                             | 0   | 0   | 0   | 0   | 0 | 0                 | 0     | 1                    | 0 ( 0.0 )                     | 0 ( 0.0 )  | 2.90 * <sup>7)</sup>        | Tox                     | Tox |
| MC                    | 0.00005               | 48                    | 200                   | 0                             | 34  | 124 | 14  | 3   | 8 | 20                | 203   | 15                   | 101*(50.5)                    | 101*(50.5) | 0.38                        | +                       | -   |

Abbreviations : gap : chromatid gap and chromosome gap, ctb : chromatid break, cte: chromatid exchange, csb : chromosome break, cse : chromosome exchange (dicentric and ring etc.), f : acentric fragment (chromatid type), mul : multiple aberrations, TAG : total no. of cells with aberrations, TA : total no. of cells with aberrations except gap, SA : structural aberration, NA : numerical aberration, MC : mitomycin C, Tox : toxic. 1) Water for injection was used as solvent. 2) More than ten aberrations in a cell were scored as 10. 3) Others, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations. 4) Eight hundred cells were analysed in each group. 5) Judgement was done on the basis of the criteria of Ishidate et al. (1987). 6) Seventy-six cells were analysed. 7) Sixty-nine cells were analysed. \* : Significantly different from solvent control at p<0.05. \*\* : Purity was 99.8%, water(0.018%) was contained as impurity.

Table 2 Chromosome analysis of Chinese hamster cells (CHL/IU) treated with 1,3-bis(aminomethyl)benzene (BAB)\*\* with and without S9 mix

| Group                 | Concentration (mg/ml) | S9 mix | Time of exposure (hr) | No. of cells analysed | No. of structural aberrations |     |     |     |     |   |                   |       | Others <sup>3)</sup> | No. of cells with aberrations |                    | Polyploid (%) <sup>4)</sup> | Judgement <sup>5)</sup> |    |
|-----------------------|-----------------------|--------|-----------------------|-----------------------|-------------------------------|-----|-----|-----|-----|---|-------------------|-------|----------------------|-------------------------------|--------------------|-----------------------------|-------------------------|----|
|                       |                       |        |                       |                       | gap                           | ctb | cte | csb | cse | f | mul <sup>2)</sup> | total |                      | TAG (%)                       | TA (%)             |                             | SA                      | NA |
| Control <sup>1)</sup> |                       |        |                       | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0                 | 0     | 0 ( 0.0 )            | 0 ( 0.0 )                     | 0.13               |                             |                         |    |
| Solvent               | 0                     | -      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0                 | 0     | 0 ( 0.0 )            | 0 ( 0.0 )                     | 0.00               |                             |                         |    |
| BAB                   | 0.08                  | -      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0                 | 0     | 0 ( 0.0 )            | 0 ( 0.0 )                     | 0.00               | -                           | -                       |    |
| BAB                   | 0.17                  | -      | 6-(18)                | 200                   | 0                             | 0   | 1   | 1   | 0   | 0 | 2                 | 0     | 2 ( 1.0 )            | 2 ( 1.0 )                     | 0.00               | -                           | -                       |    |
| BAB                   | 0.33                  | -      | 6-(18)                | 200                   | 1                             | 3   | 0   | 0   | 0   | 0 | 4                 | 0     | 3 ( 1.5 )            | 3 ( 1.5 )                     | 0.13 <sup>6)</sup> | -                           | -                       |    |
| CPA                   | 0.005                 | -      | 6-(18)                | 200                   | 0                             | 1   | 0   | 1   | 0   | 0 | 2                 | 0     | 2 ( 1.0 )            | 2 ( 1.0 )                     | 0.13               | -                           | -                       |    |
| Solvent <sup>1)</sup> | 0                     | +      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 1   | 0 | 1                 | 0     | 1 ( 0.5 )            | 1 ( 0.5 )                     | 0.25               |                             |                         |    |
| BAB                   | 0.12                  | +      | 6-(18)                | 200                   | 0                             | 0   | 0   | 1   | 0   | 0 | 1                 | 0     | 1 ( 0.5 )            | 1 ( 0.5 )                     | 0.25               | -                           | -                       |    |
| BAB                   | 0.24                  | +      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 1   | 0 | 1                 | 0     | 1 ( 0.5 )            | 1 ( 0.5 )                     | 0.50               | -                           | -                       |    |
| BAB                   | 0.47                  | +      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0                 | 0     | 0 ( 0.0 )            | 0 ( 0.0 )                     | 0.00               | -                           | -                       |    |
| CPA                   | 0.005                 | +      | 6-(18)                | 200                   | 4                             | 58  | 110 | 3   | 4   | 1 | 30                | 210   | 0                    | 94*(47.0)                     | 93*(46.5)          | 0.13                        | +                       | -  |

Abbreviations : gap : chromatid gap and chromosome gap, ctb : chromatid break, cte: chromatid exchange, csb : chromosome break, cse : chromosome exchange (dicentric and ring etc.), f : acentric fragment (chromatid type), mul : multiple aberrations, TAG : total no. of cells with aberrations, TA : total no. of cells with aberrations except gap, SA : structural aberration, NA : numerical aberration, CPA : cyclophosphamide. 1) Water for injection was used as solvent. 2) More than ten aberrations in a cell were scored as 10. 3) Others, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations. 4) Eight hundred cells were analysed in each group. 5) Judgement was done on the basis of the criteria of Ishidate et al. (1987). 6) Seven hundred and ninety-four cells were analysed. \* : Significantly different from solvent control at p<0.05. \*\* : Purity was 99.8%, water(0.018%) was contained as impurity.

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FOREWORD

INTRODUCTION

**1,3-Bis(aminomethyl)benzene (CAS No.: 1477-55-0)**

| Group                         | Concentration (mg/ml) | S9 mix | Time of exposure (hr) | No. of cells analysed | No. of structural aberrations |     |     |     |     |   |    |       |                      |         | No. of cells with aberrations |           | Polyploid (%)      | Judgement <sup>5)</sup> |   |
|-------------------------------|-----------------------|--------|-----------------------|-----------------------|-------------------------------|-----|-----|-----|-----|---|----|-------|----------------------|---------|-------------------------------|-----------|--------------------|-------------------------|---|
|                               |                       |        |                       |                       | gap                           | ctb | cte | csb | cse | f | mu | total | Others <sup>3)</sup> | TAG (%) | TA (%)                        | SA        |                    | NA                      |   |
| Control Solvent <sup>1)</sup> | 0                     | -      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0  | 0     | 0                    | 0       | 0 (0.0)                       | 0 (0.0)   | 0.13               |                         |   |
| BAB                           | 0.08                  | -      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0  | 0     | 0                    | 0       | 0 (0.0)                       | 0 (0.0)   | 0.00               | -                       | - |
| BAB                           | 0.17                  | -      | 6-(18)                | 200                   | 0                             | 0   | 1   | 1   | 0   | 0 | 0  | 2     | 0                    | 2       | 2 (1.0)                       | 2 (1.0)   | 0.00               | -                       | - |
| BAB                           | 0.33                  | -      | 6-(18)                | 200                   | 1                             | 3   | 0   | 0   | 0   | 0 | 4  | 0     | 3                    | 3       | 3 (1.5)                       | 3 (1.5)   | 0.13 <sup>6)</sup> | -                       | - |
| CPA                           | 0.005                 | -      | 6-(18)                | 200                   | 0                             | 1   | 0   | 1   | 0   | 0 | 2  | 0     | 2                    | 2       | 2 (1.0)                       | 2 (1.0)   | 0.13               | -                       | - |
| Solvent <sup>1)</sup>         | 0                     | +      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 0   | 1 | 0  | 1     | 0                    | 1       | 1 (0.5)                       | 1 (0.5)   | 0.25               |                         |   |
| BAB                           | 0.12                  | +      | 6-(18)                | 200                   | 0                             | 0   | 0   | 1   | 0   | 0 | 1  | 0     | 1                    | 1       | 1 (0.5)                       | 1 (0.5)   | 0.25               | -                       | - |
| BAB                           | 0.24                  | +      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 0   | 1 | 0  | 1     | 0                    | 1       | 1 (0.5)                       | 1 (0.5)   | 0.50               | -                       | - |
| BAB                           | 0.47                  | +      | 6-(18)                | 200                   | 0                             | 0   | 0   | 0   | 0   | 0 | 0  | 0     | 0                    | 0       | 0 (0.0)                       | 0 (0.0)   | 0.00               | -                       | - |
| CPA                           | 0.005                 | +      | 6-(18)                | 200                   | 4                             | 58  | 110 | 3   | 4   | 1 | 30 | 210   | 0                    | 94      | 94*(47.0)                     | 93*(46.5) | 0.13               | +                       | - |

Abbreviations: gap : chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring, etc), f: acentric fragment (chromatid type), mu: multiple aberrations, TAG: Total no. of cells with aberrations, TA: Total No. of cells with aberrations except gap, SA: structural aberration, NA: numerical aberration, CPA: cyclophosphamide. 1) Water for injection was used as solvent. 2) More than 10 aberrations in a cell were scored as 10. 3) Others, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations. 4) Eight hundred cells were analysed in each group. 5) Judgement was done on the basis of the criteria of Ishidate et al. (1987). 6) Seven hundred and ninety four cells were analysed. \*: Significantly different from solvent control at p<0.05. \*\*: Purity was 99.8%, water (0.018%) was contained as impurity.

|                             |  |
|-----------------------------|--|
| <b>Test substance</b>       | : SOURCE: Mitsubishi Gas Chemical Company Inc. (obtained via Japan Chemical Industry Association)<br>PURITY: 99.8%<br>IMPURITY/ADDITIVE/ETC.: Water content = 0.018%<br>ANY OTHER INFORMATION:<br>Batch no: 30817<br>MW = 136.22<br>Other name: BAB<br>Melting point: 14.1°C<br>Boiling point: 247°C<br>Vapour pressure: 1.6 x 10 E-03 mm Hg<br>Soluble in water, acetone and dimethylsulphoxide |
| <b>Conclusion</b>           | : The substance is not clastogenic to the limit of cytotoxicity both with and without metabolic activation under the conditions of the test.   |
| <b>Reliability</b>          | : (1) valid with restrictions<br>Summary of a Japanese Guideline study. This summary is based on the concise summary of the Government assessment work on existing chemicals.  |
| <b>Flag</b><br>27.06.2001   | : Critical study for SIDS endpoint<br><span style="float: right;">(23)</span>  |
| <b>Type</b>                 | : Chromosome aberration  |
| <b>System of testing</b>    | : Chinese Hamster Ovary (CHO) cells  |
| <b>Test concentration</b>   | : -S9: 75 - 450 ug/ml<br>+S9: 200 - 800 ug/ml  |
| <b>Metabolic activation</b> | : with and without   |
| <b>Result</b>               | : negative   |
| <b>Method</b>               | : Directive 84/449/EEC, B.10   |

**Year** : 1989  
**GLP** : yes  
**Test substance** : as prescribed by 1.1 - 1.4  
**Method** : METHOD FOLLOWED:  
Guideline based upon:  
OECD Guideline No. 473 (May 26, 1983)  
EEC Method B 10 (67/548/EEC, adopted September 19, 1984)  
EPA Test Guideline (TSCA, FIFRA), subchapter R, part 798, subpart F,  
Genetic Toxicity § 798.5375 (revised July 1, 1986).

Study conducted in accordance with generally accepted scientific principles.

DEVIATIONS FROM GUIDELINE: At the first fixation time cells were fixed 6 hours post-dosing instead of at the protocolled 4 hours post-dosing. However, this amendment was not considered to seriously affect the experimental set-up of the study.

STATISTICAL METHODS:  
Chi-square test,  $P < 0.05$

**Test condition** : SYSTEM OF TESTING:  
Metabolic activation system:  
Species: Rat, Adult Wistar or Sprague Dawley.  
Charles River Wiga, Sulzfeld, FRG  
Organ: liver  
Induction substance: Aroclor 1254 in corn oil (20% w/v solution = 500 mg/kg bodyweight)  
Rats injected intraperitoneally with Aroclor 1254. After five days the rats were killed (access to food was denied for at least 12 hours before sacrifice). The livers were removed and washed in 0°C sterile 0.1M sodium phosphate buffer (pH 7.4) containing 0.1mM Na<sub>2</sub>-EDTA. The livers were then minced and homogenised in 3 volumes of phosphate buffer. The homogenate was centrifuged for 15 mins and the supernatant (S9) transferred to sterile ampoules. Storage in liquid nitrogen.

No. of metaphases analysed: At least 100 metaphase chromosome spreads per culture were examined.

ADMINISTRATION:  
Dosing:  
-S9 : 75, 200, 325 and 450 ug/ml  
+S9 : 200, 400, 600 and 800 ug/ml

+S9: A concentration of 400 ug/ml was tested at the first fixation time, concentrations up to 600 ug/ml at the second fixation time and 400 and 800 ug/ml at the third fixation time.

Application: Monolayer cultures of the cells were used. The cells were exposed to test substance in F10 complete culture medium without serum, buffered with 20 mM HEPES (in the absence of CO<sub>2</sub>) (HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.) The cells were exposed for 2 hours, washed twice with 10 ml of Hank's Balanced Salt Solution and incubated in fresh culture medium for 6, 9 and 19 hours (harvest times 8, 11 and 21 hours respectively). Colchicine was added to the highest dose levels and the positive controls in the last 2 to 2.5 to halt cell division.

Positive and negative control groups and treatment:  
Negative control: The vehicle of the test article  
Positive controls:  
-S9: Ethylmethanesulphonate (EMS; CAS No. 62-50-0; purity 98%; Merck)

used as a direct acting mutagen at a final concentration of 8mM (solvent DMSO)  
+S9: Cyclophosphamide (CP; Cas No. 50-18-0; Endoxan-Asta, Asta-Werke, F.R.G)

DESCRIPTION OF FOLLOW UP STUDY: Duplication study carried out in the same manner as the original.

**Result**

**GENOTOXIC EFFECTS:**

Only in the presence of S9 at the second fixation time a statistically significant increase was induced at the concentration of 600 ug/ml. However, as this increase was observed only when gap-containing cells were included and as there was no evidence for a dose-response relationship, a biologically significant effect was not concluded.

**CYTOTOXIC CONCENTRATION:**

-S9: >333 ug/ml

+S9: >333 ug/ml

**STATISTICAL RESULTS:**

Total number of cells with aberrations; treatment/control comparison, (inclusive/exclusive gaps)

Second fixation time

| Dose (ug/ml) | S9 | Gaps | P-value (two-sided) | Decision at 95% confidence level |
|--------------|----|------|---------------------|----------------------------------|
| EMS (8Mm)    | -  | +    | <0.0004             | Significant                      |
|              |    | -    | <0.0004             | Significant                      |
| 600          | +  | +    | =0.0108             | Significant                      |
|              |    | -    | =0.1528             | Not significant                  |
| CP (5)       | +  | +    | <0.0004             | Significant                      |
|              |    | -    | <0.0004             | Significant                      |

Table 1: MXDA Frequency of chromosome aberrations

KEY:

A, B: Duplicates

g' = Chromatid gap, g'' = chromosome gap, b' = chromatid break, b'' = chromosome break, cb = centromere break, d' = chromatid deletion, f = fragment, f' = acentric fragment, dic = dicentric chromosome, tric = trivalent chromosome, r = ring chromosome, sp = spot of chromosome material, exch. = exchange figure, intra = chromosome intrachange, p = pulverized chromosomes, ma = multiple aberrations, poly = poly ploidy, endo. = endoreduplication

\* Significantly different from control group (Chi-square test): \* P < 0.05, \*\* P < 0.01 or \*\*\* P < 0.001

**a) First fixation time, -S9**

| Concentration<br>ug/ml                    | F10-HEPES |     |     | 450ug/ml |     |     |
|---|-----------|-----|-----|----------|-----|-----|
|   | A         | B   | A+B | A        | B   | A+B |
| Culture                                   |           |     |     |          |     |     |
| No. of cells scored                       | 100       | 100 | 200 | 100      | 100 | 200 |
| No. of cells with aberrations<br>(+ gaps) | 7         | 8   | 15  | 8        | 6   | 14  |
| No of cells with aberrations<br>(- gaps)  | 1         | 2   | 3   | 2        | 1   | 3   |
| g'  | 5         | 8   |     | 5        | 6   |     |
| g''                                       | 1         |     |     | 1        |     |     |
| b'  |           | 2   |     | 2        | 1   |     |
| b''                                       |           |     |     |          |     |     |
| f   |           |     |     |          |     |     |
| f'  | 1         |     |     | 1        |     |     |
| exch                                      |           |     |     |          |     |     |
| dic                                       |           |     |     |          |     |     |
| d'  |           |     |     |          |     |     |
| misc.                                     |           |     |     |          |     |     |
| Total aberrations (+ gaps)                | 7         | 10  |     | 9        | 7   |     |
| Total aberrations (- gaps)                | 1         | 2   |     | 3        | 1   |     |

**b) First fixation time, +S9**

| Concentration<br>ug/ml                    | F10-HEPES |     |     | 400ug/ml |      |     |
|---|-----------|-----|-----|----------|------|-----|
|   | A         | B   | A+B | A        | B    | A+B |
| Culture                                   |           |     |     |          |      |     |
| No. of cells scored                       | 100       | 100 | 200 | 100      | 100  | 200 |
| No. of cells with aberrations<br>(+ gaps) | 7         | 5   | 12  | 8        | 8    | 16  |
| No of cells with aberrations<br>(- gaps)  | 1         | 1   | 2   | 3        | 3    | 6   |
| g'  | 7         | 4   |     | 4        | 6    |     |
| g''                                       |           |     |     | 2        |      |     |
| b'  | 1         |     |     | 3        | 1    |     |
| b''                                       |           |     |     |          |      |     |
| f   |           |     |     |          |      |     |
| f'  |           | 1   |     |          | 2    |     |
| exch                                      |           |     |     |          |      |     |
| dic                                       |           |     |     |          |      |     |
| d'  |           |     |     |          |      |     |
| misc.                                     |           |     |     |          | poly |     |
| Total aberrations (+ gaps)                | 8         | 5   |     | 9        | 9    |     |
| Total aberrations (- gaps)                | 1         | 1   |     | 3        | 3    |     |

c) Second fixation time, -S9

| Concentration<br>ug/ml                    | F10-HEPES |     |     | 200ug/ml |     |     | 325ug/ml |     |     | 450ug/ml |     |     | EMS 8mM |     |           |
|---|-----------|-----|-----|----------|-----|-----|----------|-----|-----|----------|-----|-----|---------|-----|-----------|
|   | A         | B   | A+B | A        | B   | A+B | A        | B   | A+B | A        | B   | A+B | A       | B   | A+B       |
| No. of cells scored                       | 100       | 100 | 200 | 100      | 100 | 200 | 100      | 100 | 200 | 100      | 100 | 200 | 100     | 100 | 200       |
| No. of cells with aberrations<br>(+ gaps) | 5         | 4   | 9   | 5        | 4   | 9   | 4        | 9   | 13  | 6        | 6   | 12  | 23      | 20  | 43<br>*** |
| No of cells with aberrations<br>(- gaps)  | 3         | 3   | 6   | 2        | 2   | 4   | 2        | 4   | 6   | 2        | 3   | 5   | 16      | 16  | 32<br>*** |
| g'  |           | 1   |     | 2        | 1   |     | 2        | 6   |     | 5        | 2   |     | 6       | 4   |           |
| g''                                       | 2         |     |     | 1        | 1   |     |          |     |     | 1        | 1   |     | 2       |     |           |
| b'  | 3         | 1   |     | 2        | 2   |     | 1        | 3   |     | 1        | 1   |     | 6       | 7   |           |
| b''                                       |           |     |     |          |     |     |          |     |     |          |     |     |         |     |           |
| f   |           |     |     |          |     |     |          |     |     |          |     |     |         |     |           |
| f'  |           | 2   |     |          |     |     | 1        | 1   |     | 1        | 2   |     | 10      | 10  |           |
| exch                                      |           |     |     |          |     |     |          |     |     |          |     |     |         |     |           |
| dic                                       |           |     |     |          |     |     |          |     |     |          |     |     |         |     |           |
| d'  |           |     |     |          |     |     |          |     |     |          |     |     |         |     |           |
| misc.                                     |           |     |     |          |     |     |          |     |     | poly     |     |     |         |     |           |
| Total aberrations<br>(+ gaps)             | 5         | 4   |     | 5        | 4   |     | 4        | 10  |     | 8        | 6   |     | 24      | 21  |           |
| Total aberrations<br>(- gaps)             | 3         | 3   |     | 2        | 2   |     | 2        | 4   |     | 2        | 3   |     | 16      | 17  |           |

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d) Second fixation time, +S9

| Concentration<br>ug/ml                    | F10-HEPES |     |     | 200ug/ml |     |     | 400ug/ml |     |     | 600ug/ml |     |     | CP<br>5 ug/ml |     |           |
|---|-----------|-----|-----|----------|-----|-----|----------|-----|-----|----------|-----|-----|---------------|-----|-----------|
|   | A         | B   | A+B | A        | B   | A+B | A        | B   | A+B | A        | B   | A+B | A             | B   | A+B       |
| Culture                                   |           |     |     |          |     |     |          |     |     |          |     |     |               |     |           |
| No. of cells scored                       | 100       | 100 | 200 | 100      | 100 | 200 | 100      | 100 | 200 | 100      | 100 | 200 | 100           | 100 | 200       |
| No. of cells with aberrations<br>(+ gaps) | 1         | 2   | 3   | 4        | 2   | 6   | 2        | 5   | 7   | 6        | 7   | 13* | 15            | 21  | 36<br>*** |
| No of cells with aberrations<br>(- gaps)  | 1         | 1   | 2   | 2        | 1   | 3   | 1        | 2   | 3   | 2        | 4   | 6   | 11            | 15  | 26<br>*** |
| g'  |           | 1   |     | 2        | 1   |     | 1        | 5   |     | 3        | 2   |     | 2             | 4   |           |
| g''                                       |           |     |     | 1        |     |     |          |     |     | 1        | 1   |     | 2             | 3   |           |
| b'  | 1         | 1   |     | 1        | 1   |     | 1        | 2   |     | 2        | 3   |     | 6             | 2   |           |
| b''                                       |           |     |     |          |     |     |          |     |     |          |     |     |               |     |           |
| f'  |           |     |     |          |     |     |          |     |     |          |     |     |               |     |           |
| f''                                       |           |     |     | 1        |     |     |          |     |     |          | 1   |     | 4             | 11  |           |
| exch                                      |           |     |     |          |     |     |          |     |     |          |     |     | 3             | 5   |           |
| dic                                       |           |     |     |          |     |     |          |     |     |          |     |     |               |     |           |
| d'  |           |     |     |          |     |     |          |     |     |          |     |     |               |     |           |
| misc.                                     |           |     |     |          |     |     | poly     |     |     |          |     |     |               |     |           |
| Total aberrations (+ gaps)                | 1         | 2   |     | 5        | 2   |     | 2        | 7   |     | 6        | 7   |     | 17            | 25  |           |
| Total aberrations (- gaps)                | 1         | 1   |     | 2        | 1   |     | 1        | 2   |     | 2        | 4   |     | 13            | 18  |           |

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e) Third fixation time, -S9

| Concentration<br>ug/ml                    | F10-HEPES |      |     | 450ug/ml       |     |     |
|---|-----------|------|-----|----------------|-----|-----|
|   | A         | B    | A+B | A              | B   | A+B |
| Culture                                   | 100       | 100  | 200 | 100            | 100 | 200 |
| No. of cells scored                       | 100       | 100  | 200 | 100            | 100 | 200 |
| No. of cells with aberrations<br>(+ gaps) | 3         | 4    | 7   | 3              | 5   | 8   |
| No of cells with aberrations<br>(- gaps)  | 0         | 2    | 2   | 1              | 3   | 4   |
| g'  | 2         | 2    |     | 2              | 2   |     |
| g''                                       | 1         |      |     |                | 1   |     |
| b'  |           |      |     | 1              | 1   |     |
| b''                                       |           |      |     |                |     |     |
| f'  |           | 2    |     |                | 2   |     |
| exch                                      |           |      |     |                |     |     |
| dic                                       |           |      |     |                |     |     |
| d'  |           |      |     |                |     |     |
| misc.                                     |           | poly |     | 2 poly<br>endo |     |     |
| Total aberrations (+ gaps)                | 3         | 4    |     | 3              | 6   |     |
| Total aberrations (- gaps)                | 0         | 2    |     | 1              | 3   |     |

f) Third fixation time, +S9

| Concentration<br>ug/ml                       | F10-HEPES      |     |     | 400ug/ml         |        |     | 800ug/ml |      |     |
|--|----------------|-----|-----|------------------|--------|-----|----------|------|-----|
|  | A              | B   | A+B | A                | B      | A+B | A        | B    | A+B |
| Culture                                      | 100            | 100 | 200 | 100              | 100    | 200 | 100      | 100  | 200 |
| No. of cells scored                          | 100            | 100 | 200 | 100              | 100    | 200 | 100      | 100  | 200 |
| No. of cells with<br>aberrations<br>(+ gaps) | 5              | 5   | 10  | 10               | 3      | 13  | 5        | 9    | 14  |
| No of cells with<br>aberrations<br>(- gaps)  | 3              | 2   | 5   | 5                | 1      | 6   | 2        | 3    | 5   |
| g'   | 1              | 3   |     | 5                | 1      |     | 3        | 6    |     |
| g''  | 1              |     |     |                  | 1      |     |          |      |     |
| b'   |                | 1   |     | 1                |        |     |          | 1    |     |
| b''  |                |     |     |                  |        |     |          |      |     |
| f'   |                |     |     |                  |        |     |          |      |     |
| f''  | 2              | 1   |     | 1                | 1      |     | 2        | 2    |     |
| exch   | 1              |     |     | 3                |        |     |          |      |     |
| dic  |                |     |     |                  |        |     |          |      |     |
| d'   |                |     |     |                  |        |     |          |      |     |
| misc.  | 2 endo<br>poly |     |     | 3 endo<br>3 poly | 2 endo |     | 4 endo   | endo |     |
| Total aberrations (+<br>gaps)                | 5              | 5   |     | 10               | 3      |     | 5        | 9    |     |
| Total aberrations (-<br>gaps)                | 3              | 2   |     | 5                | 1      |     | 2        | 3    |     |