ノニルフェノールのチャイニーズ・ハムスター培養細胞を用いる染色体異常試験

In Vitro Chromosomal Aberration Test of Nonylphenol on Cultured Chinese Hamster Cells

要約

既存化学物質安全性点検作業の一環として，ノニルフェノールの変異原性について染色体異常誘発性の有無を検討するため，チャイニーズ・ハムスター肺線維芽細胞株(CHL)を用いる*in vitro*染色体異常試験を行った．細胞増殖抑制試験結果を基に，細胞毒性が観察される濃度を最高用量として設定した．すなわち，連続24時間処理法で6.25, 12.5, 25.0および50.0 μg/ml，同48時間処理法で3.13, 6.25, 12.5および25.0 μg/ml，短時間処理法(6時間処理の+S9 mixおよび-S9 mix)で7.50, 15.0, 30.0および60.0 μg/mlの4用量(公比2)について染色体標本を作製した後，顕微鏡観察を実施した．細胞の増殖が強く抑制される用量まで検討した結果，連続処理法ならびに短時間処理法のいずれとも染色体異常，すなわち構造異常あるいは倍数性細胞の誘発は認められなかった．一方，連続処理法の陽性対照物質マイトマイシンC(MMC)および短時間処理+S9 mixの陽性対照物質シクロホスファミド(CP)は，いずれも染色体構造異常を高頻度に誘発した．従って，本試験条件下の*in vitro*試験系において，ノニルフェノールには染色体異常を誘起する可能性がないものと判断した．

材料および方法

1. 試験細胞株

哺乳類培養細胞を用いる染色体異常試験に広く使用されていることから，試験細胞株としてチャイニーズ・ハムスターの肺由来の線維芽細胞株(CHL)を選択した．昭和59年11月15日に国立衛生試験所から分与を受け，一部はジメチルスルホキシド(DMSO:MERCK社)を10%添加した後，液体窒素中に保存し，残りは3～5日ごとに継代した．なお，本染色体異常試験では解凍後継代数32の細胞を用いた．

2. 培養液の調製

Eagle-MEM培地(LIFE TECHNOLOGIES社)を1000 mlの精製水で溶解した後，2.2 gの炭酸水素ナトリウム(関東化学㈱)を加えた．1N塩酸を用いてpHを7.2に調整した後，メンブランフィルター(0.2 μm:Gelman Sciences社)を用いて加圧濾過除菌した．非働化(56℃，30分)済み仔牛血清(LIFE TECHNOLOGIES社)を最終濃度で10%になるよう加えた後，試験に使用した．

3. 培養条件

CO_2インキュベーター(FORMA社あるいは三洋電機特機㈱)を用い，CO_2濃度5%，37℃の条件で細胞を培養した．

4. S9 mix

製造後6ヵ月以内のキッコーマン㈱製S9 mixを試験に使用した．S9 mix中のS9は誘導剤としてフェノバルビタールおよび5,6-ベンゾフラボンを投与したSprague-Dawley系雄ラットの肝臓から調製されたものである．S9 mixの組成は松岡らの方法に従った[1]．

5. 被験物質

被験物質のノニルフェノール(ロット番号:F1132, CAS No.:25154-52-3)は分子式$C_{15}H_{24}O$，分子量220.36，純度99.0%以上の無色～黄色の粘調液体である．三井東圧化学㈱から提供された被験物質を使用した．試験終了後，被験物質提供元において残余被験物質を分析した結果，安定性に問題はなかった．

6. 被験物質溶液の調製

DMSOに被験物質を溶解して調製原液とした．調製原液を使用溶媒を用いて順次所定濃度に希釈した後，直ちに処理を行った(用時調製)．

7. 予備試験(細胞増殖抑制試験)

細胞培養用マルチプレートに細胞を播種し，培養3日後に被験物質溶液を処理した．連続処理法の場合，24あるいは48時間連続して処理を実施し，短時間処理法ではS9 mix存在下(+S9 mix)あるいは非存在下(-S9 mix)で6時間処理した後，新鮮な培養液に交換してさらに18時間培養を続けた．

細胞を10%中性緩衝ホルマリン液(和光純薬工業㈱)で固定した後，0.1%クリスタル・バイオレット(関東化学㈱)水溶液で10分間染色した．色素溶出液(30%エタノール，1%酢酸水溶液)を適量加え，5分間程度放置して色素を溶出した後，580 nmでの吸光度を測定した．各用量群について溶媒対照群での吸光度に対する比，すなわち細胞生存率を算出した．

その結果，いずれの処理法においても顕著な細胞増殖抑制が観察された(Fig. 1)．プロビット法あるいは対数確立紙を用いて算出した50%細胞増殖抑制濃度は連続24時間処理で23.2 μg/ml，同48時間処理で25.9 μg/ml，短時間+S9 mix処理で31.8 μg/ml，同-S9 mix処理で29.3 μg/mlであった．

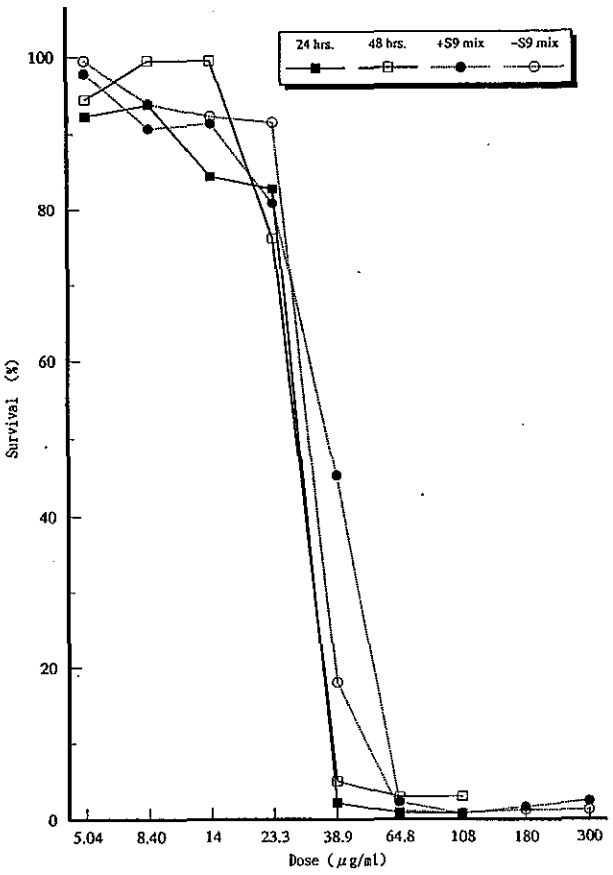

Fig. 1 Dose-survival curves of nonylphenol

8. 試験用量および試験群の設定

細胞増殖抑制試験結果を基に，染色体異常試験では連続24時間処理で50.0 μg/ml，同48時間処理で25.0 μg/ml，短時間処理法で60.0 μg/mlを最高用量とし，以下公比2で減じた計4用量ならびに溶媒対照群を設定した．

陽性対照として，連続処理法の場合，マイトマイシンC(MMC:協和醗酵工業㈱)を，24時間処理で0.05 μg/ml，48時間処理で0.025 μg/mlの用量で，短時間処理法の場合，シクロホスファミド(CP:塩野義製薬㈱)を，12.5 μg/mlの用量で試験した．

9. 染色体標本の作製

直径60 mmのプレートを用い，予備試験と同様に被験物質等の処理を行った．培養終了2時間前に，最終濃度で0.2 μg/mlとなるようコルセミド(LIFE TECHNOLOGIES社)を添加した．トリプシン処理で細胞を剥離させ，遠心分離により細胞を回収した．75 mM塩化カリウム水溶液で低張処理を行った後，固定液(メタノール3容:酢酸1容)で細胞を固定した．空気乾燥法で染色体標本を作製した後，1.2%ギムザ染色液で12分間染色した．

10. 染色体の観察

各プレートあたり100個，すなわち用量当たり200個の分裂中期像を顕微鏡下で観察し，染色体の形態的変化としてギャップ(gap)，染色分体切断(ctb)，染色体切断(csb)，染色分体交換(cte)，染色体交換(cse)およびその他(oth)の構造異常に分類した．同時に，倍数性細胞の出現率を記録した．染色体の分析は日本環境変異原学会・哺乳動物試験分科会[2]による分類法に従って実施した．

すべての標本をコード化した後，観察した．

11. 結果の解析

ギャップのみ保有する細胞を含めた場合(+gap)と，含めない場合(-gap)とに区別して染色体構造異常の出現頻度を表示した．

各試験群の構造異常を有する細胞あるいは倍数性細胞の出現頻度を，石館ら[3]の基準に従って判定した．染色体異常を有する細胞の出現頻度が5%未満を陰性(-)，5%以上10%未満を疑陽性(±)，10%以上を陽性(+)とした．最終的には再現性あるいは用量に依存性が認められた場合に陽性と判定した．

なお，統計学的手法を用いた検定は実施しなかった．

結果および考察

連続処理群での試験結果をTable 1に示した．ノニルフェノール処理群の場合，24時間ならびに48時間処理のいずれにおいても最高用量で強い細胞毒性作用が観察された．しかしながら，染色体の構造異常および倍数性細胞の誘発傾向は観察されなかった．一方，陽性対照物質のMMCで処理した細胞では染色体の構造異常の顕著な誘発が認められた．短時間処理群での試験結果をTable 2に示した．被験物質処理群の場合，+S9 mixならびに-S9 mixの最高用量で細胞毒性作用が認められたものの，いずれの用量においても染色体構造異常および倍数性細胞の誘発傾向は観察されなかった．また，陽性対照物質のCPで処理した細胞ではS9 mix存在下でのみ染色体の構造異常の顕著な誘発が認められた．以上の試験結果から，本試験条件下においてノニルフェノールの哺乳類培養細胞に対する染色体異常誘発性に関し，陰性と判定した．

文献

1) A. Matsuoka, M. Hayashi and M. Ishidate Jr., *Mutat Res.*, **66**, 277(1979).
2) 日本環境変異原学会・哺乳動物試験分科会編，"化学物質による染色体異常アトラス，" 朝倉書店，東京，1988，pp. 31-35.
3) 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，pp. 19-24.

Table 1. Chromosomal aberration test on CHL cells treated with nonylphenol [long-term treatment]

Compound	Dose (μg/ml)	Time of exposure (hr)	Number of cells analyzed	Number of cells with structural aberrations						Total [+gap] (%)	Total [-gap] (%)	Polyploid cells (%)	Final judgement
				gap	ctb	csb	cte	cse	oth				
DMSO*	0	24	200	0	0	0	1	0	0	0.5	0.5	0.0	-
Test Sub.	6.25	24	200	1	0	0	1	0	0	1.0	0.5	0.0	-
	12.5	24	200	0	0	0	0	0	0	0.0	0.0	0.0	-
	25.0	24	200	1	1	0	1	2	0	2.5	2.0	0.5	-
	50.0	24	Toxic										
MMC**	0.05	24	200	19	49	0	95	1	0	65.5	61.5	0.5	+
DMSO*	0	48	200	0	0	0	0	0	0	0.0	0.0	0.0	-
Test Sub.	3.13	48	200	1	0	0	0	0	0	0.5	0.0	1.0	-
	6.25	48	200	0	0	1	5	0	0	2.5	2.5	0.0	-
	12.5	48	200	0	1	0	0	0	0	0.5	0.5	0.0	-
	25.0	48	Toxic										
MMC**	0.025	48	200	14	47	0	91	6	0	60.0	59.0	1.0	+

*:Solvent control **:Positive control (mitomycin C)
ctb:chromatid break csb:chromosome break cte:chromatid exchange cse:chromosome exchange oth:others

Table 2. Chromosomal aberration test on CHL cells treated with nonylphenol [short-term treatment]

Compound	Dose (μg/ml)	S 9 mix	Time of exposure (hr)	Number of cells analyzed	Number of cells with structural aberrations						Total [+gap] (%)	Total [-gap] (%)	Polyploid cells (%)	Final judgement
					gap	ctb	csb	cte	cse	oth				
DMSO*	0	+	6	200	0	0	0	0	0	0	0.0	0.0	0.5	-
Test Sub.	7.50	+	6	200	0	0	0	0	0	0	0.0	0.0	0.0	-
	15.0	+	6	200	0	1	0	0	0	0	0.5	0.5	0.0	-
	30.0	+	6	200	0	0	0	2	1	0	1.5	1.5	3.0	-
	60.0	+	6	200	2	1	0	6	0	0	4.0	3.5	0.0	-
CP**	12.5	+	6	200	7	17	0	66	1	0	39.5	38.0	0.0	+
DMSO*	0	-	6	200	4	0	1	1	1	0	3.5	1.5	1.0	-
Test Sub.	7.50	-	6	200	1	0	0	2	1	0	2.0	1.5	1.0	-
	15.0	-	6	200	0	0	0	1	0	0	0.5	0.5	0.0	-
	30.0	-	6	200	1	0	0	1	0	0	1.0	0.5	0.5	-
	60.0	-	6	Toxic										
CP**	12.5	-	6	200	0	1	0	0	0	0	0.5	0.5	0.0	-

*:Solvent control **: Positive control (cyclophosphamide)
ctb:chromatid break csb:chromosome break cte:chromatid exchange cse:chromosome exchange oth:others

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4.1.2.5.2 Respiratory tract

No data are available, although it can be predicted from its low chemical reactivity that nonylphenol is unlikely to be a respiratory allergen.

4.1.2.5.3 Summary of sensitisation

No human data are available. The results of several guinea pig maximisation tests suggest that nonylphenol does not have significant skin sensitising potential. No information on respiratory tract sensitisation is available, although it can be predicted from its low chemical reactivity that nonylphenol is unlikely to be a respiratory allergen.

4.1.2.6 Repeated dose toxicity

4.1.2.6.1 Animal data

There are no data for the inhalation or dermal routes. Two high-quality oral repeated dose studies in rats, of 28 and 90 days duration, have been conducted. The studies followed OECD guidelines and were in compliance with GLP. Additionally, the influence of nonylphenol on growth and cell proliferation and of the mammary gland has been investigated in the rat in a non-standard study involving subcutaneous administration.

In the 28-day study, groups of five male and five female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at nominal dose levels of 0, 25, 100 or 400 mg/kg/day (Hüls 1989). Clinical signs of toxicity, bodyweights and food consumption were recorded and towards the end of the study routine haematology, blood clinical chemistry and urinalysis examinations were made. A full necropsy was performed on all animals at termination. Adrenals, liver, kidneys and testes with epididymides were weighed and a limited range of major organs was examined microscopically.

There were no mortalities or treatment related clinical signs of toxicity. At 400 mg/kg/day, bodyweight gain was significantly reduced throughout the study, and by week four mean bodyweights were 26% and 13% less than the controls for males and females, respectively. The amount of food consumed and food utilisation was also reduced at 400 mg/kg/day for both sexes. For males only at 400 mg/kg/day there were slight differences in comparison with the controls for certain clinical chemistry parameters; urea and cholesterol levels were increased and glucose levels were reduced. Also, there were increases in the group mean relative kidney, liver and testes weights (all by about 20% compared with controls). Histopathological examination revealed hyaline droplet accumulation in the renal proximal tubules (an effect considered to be of no relevance to human health) and a minor vacuolation in the periportal hepatocytes for males at 400 mg/kg/day. Among the females at this level, there were no treatment-related changes in the organs.

For males and females at 25 and 100 mg/kg/day, there were no differences in any of the parameters examined that could be conclusively related to treatment. It should be noted that minor increases in comparison with the concurrent control group were reported for kidney, adrenal and liver weights and for the incidence of minimal hyaline droplet formation in the kidney among males at 25 and 100 mg/kg/day. However, all values were within the laboratory's historical control range (personal communication with study sponsor) and confirmatory changes were not seen for adrenal and liver weight or hyaline droplet formation in the 90-day study (see

below). Consequently these marginal changes could not be reliably attributed to nonylphenol treatment. Overall, this study identifies a NOAEL of 100 mg/kg/day for 28-day exposure.

In the 90-day study, groups of fifteen male and fifteen female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at concentrations of 0 (control), 200, 650 or 2000 ppm (Chemical Manufacturers Association 1997a, Cunny et al., 1997). Calculated nonylphenol intakes were about 0, 15, 50 and 140 mg/kg/day, respectively. Additionally, control and high dose satellite groups of ten animals of each sex were included; these were given a 28 day recovery period at the end of the 90-day exposure. Clinical signs of toxicity, bodyweights and food consumption were recorded and towards the end of the study routine haematology, blood clinical chemistry and ophthalmoscopy examinations were made. A full necropsy was performed on all animals at termination. A number of organs were weighed and histopathological examinations were conducted on a comprehensive range of organs and tissues. Also, oestrous cycles were monitored during week 8 and sperm motility, sperm number (in epididymis) and sperm morphology were evaluated at necropsy.

There were no treatment-related mortalities or clinical signs of toxicity. At 140 mg/kg/day only, there were adverse effects on bodyweight gain, the amount of food consumed and food utilisation throughout the dosing period for both males and females. At 90 days, the mean bodyweights for both sexes at this exposure level were about 7% less than the controls. In the satellite group, some recovery of bodyweight and food consumption values was seen after exposure was discontinued. Haematology and ophthalmoscopy findings and oestrous cycle patterns were not affected by treatment. There was no evidence of effects on spermatogenesis. However, one interesting clinical chemistry change was seen among females from the 140 mg/kg/day group. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were markedly elevated in two females, which correlated with some histopathological changes reported in the liver (see below).

At necropsy, no treatment-related macroscopic findings were reported. Among the males killed at 90 days, there was a dose-related increase in group mean absolute (by 6, 9 and 13%, relative to controls, at 15, 50 and 140 mg/kg/day respectively) and bodyweight-related (by 8, 11 and 24%, respectively) kidney weight. In the recovery group, the bodyweight-related kidney weight among males was also increased, although the effect was less marked. However, this organ weight increase could not be correlated with any clinical chemistry or histopathological change and consequently this finding was considered unlikely to be of toxicological significance, particularly at 15 and 50 mg/kg/day where magnitude of the change was small. Also, ovary weight was slightly decreased in females from the 140 mg/kg/day group, in comparison with the controls, at 90 days. In contrast, the weight of this organ was slightly increased in the recovery group. Again, this difference could not be correlated with any histopathological change which, together with the inconsistency between the findings for the main and satellite groups, makes the interpretation of this finding uncertain. Bodyweight-related liver weight was increased at 90 days only in males at 50 and 140 mg/kg/day and females at 140 mg/kg/day, by about 10% compared with controls. This was considered likely to be an adaptive rather than toxicological response.

The only noteworthy microscopic changes were seen in the kidneys and liver. Among males at 140 mg/kg/day in both the main and satellite groups there was a decrease in the occurrence of renal tubular hyaline droplets/globules in comparison with the control group. The biological significance of this change is uncertain. Also, a lack of correlation with the findings of the 28-day repeated dose study, in which an actual increase in the incidence of renal hyaline droplets occurred, casts doubt on whether these changes should be considered to be related to treatment. Slight or moderate individual hepatic cell necrosis was seen in three females at 140 mg/kg/day; two of the affected females also had raised serum ALT and AST. This provides evidence that the liver may be a target organ for nonylphenol toxicity, although this evidence is weak in view of the mild nature of response and small number of animals affected.

The renal histopathological findings have been reviewed by a pathologist not involved in the original investigation (Hard 1998), because of a lack of coherence between the results of this study and a multigeneration study summarised below (NTP 1997). An increased incidence of deposits of intratubular mineralisation in the P3 (straight) segment of the proximal tubule at the outer stripe of the outer medulla/inner stripe of outer medulla (OSOM/ISOM) junction was seen in males at 140 mg/kg/day; 11 out of 25 from this group were affected, compared with 1 out of 25 control males.

Overall, a NOAEL of about 50 mg/kg/day can be derived from this study. At 140 mg/kg day there were reductions in bodyweight gain, food consumption and food utilisation together with evidence of morphological changes in the liver and possibly kidneys.

Further information on repeated dose toxicity can be derived from a good-quality multigeneration study (NTP 1997, see section 4.1.2.9.2 for full details of this study, including information on any findings in reproductive organs). Groups of thirty male and thirty female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at concentrations of 0 (control) 200, 650 or 2000 ppm over three generations. Calculated nonylphenol intakes were, respectively, about 0, 15, 50 and 160 mg/kg/day during non-reproductive phases. The F_0 generation were exposed for 15 weeks, the F_1 and F_2 generations from soon after birth to about 20 weeks of age and the F3 generation from birth to about 8 weeks of age.

Evidence of general toxicity was seen in adults of all generations, although there were no treatment-related clinical signs, mortalities or adverse effects on food consumption. At 160 mg/kg/day, bodyweight gain was reduced in comparison with controls in adults across all generations, with the terminal bodyweights being about 10% lower than the controls. Similar reductions in bodyweight gain were also seen at 50 mg/kg/day in F_1 females, F_2 males and F_3 females. Relative kidney weights were increased at 50 and/or 160 mg/kg/day in adult males of the F_0, F_1 and F_2 generations and also at 160 mg/kg/day in F_1 adult females. Histopathological examination revealed an increase, although often without a convincing dose-response relationship, in the incidence of renal tubular degeneration and/or dilatation in adult males from all generations and all nonylphenol treated groups; similar findings were reported for adult females at 160 mg/kg/day in the F_1, F_2 and F_3 generations and at 15 and 50 mg/kg/day in the F_3 generation. These data are given in **Table 4.13.**

Table 4.13 Number of animals with histopathological abnormalities in the kidney (n=10)

Males

Generation	Finding	Dose level (mg/kg/day)			
		0	15	50	160
F_0	Renal tubule degeneration	1	3	5	5
	Renal tubule dilatation	0	1	0	0
F_1	Renal tubule degeneration	1	2	7	8
	Renal tubule dilatation	1	1	0	2
F_2	Renal tubule degeneration	3	6	6	6
	Renal tubule dilatation	1	2	0	4
F_3	Renal tubule degeneration	0	7	10	2
	Renal tubule dilatation	0	0	3	3

Females

Generation	Finding	Dose level (mg/kg/day)			
		0	15	50	160
F_0	Renal tubule degeneration	3	3	0	0
	Renal tubule dilatation	0	0	1	0
F_1	Renal tubule degeneration	0	1	1	6
	Renal tubule dilatation	0	0	0	3
F_2	Renal tubule degeneration	1	2	0	4
	Renal tubule dilatation	0	0	0	1
F_3	Renal tubule degeneration	0	8	9	7
	Renal tubule dilatation	0	0	1	1

It is difficult to decide for certain whether or not this increased incidence of renal tubular degeneration and/or dilatation is related to treatment because these changes were not seen to the same extent in the 90-day study, which was conducted using the same strain of rats, and because a dose-dependent trend was not apparent in all generations/sexes. The lack of concordance between the studies cannot be explained on the basis of a slightly longer exposure period in the multigeneration study because kidney effects were seen in the F_3 generation which was exposed for only 8 weeks, nor on the basis of *in utero* and neonatal exposure because the effect also occurred in the F_0 generation. Giving special emphasis to the fact that the increased incidence occurred consistently across all four generations in the multigeneration study, it is considered that this cannot be dismissed as background variation. Consequently, a conclusion has been drawn from this study that there is a LOAEL for repeated exposure of 15 mg/kg/day, based on histopathological changes in the kidneys; this value will be taken forward to the risk characterisation.

The renal histopathological findings have been reviewed by a pathologist not involved in the original investigation (Hard, 1998). The presence of renal lesions in all nonylphenol exposed groups was confirmed, as was the lack of a consistent dose-dependent trend in all generations. The

predominant renal lesions were described as tubular mineralisation at the OSOM/ISOM junction, cystic tubules surrounded by fibrosis, or granular cast formation at the OSOM/ISOM junction.

A briefly reported oral (gavage) study investigating the testicular toxicity of nonylphenol (de Jager et al., 1999a) is summarised in the toxicity to reproduction section. In this study, mortality was observed at 100 (the lowest dose level tested), 250 and 400 mg/kg/day; 3, 15 and 18, respectively, out of 20 animals in each group died during a 10-week dosing period. No further information on these mortalities is available. The presence of mortality at such dose levels contrasts with the findings of the dietary administration studies (Hüls, 1989; Chemical Manufacturers Association, 1997a; NTP, 1997). The differences can probably be accounted for by the method of administration; gavage dosing is likely to produce higher peak concentrations of nonylphenol in the blood than dietary administration.

The influence of nonylphenol on growth and cell proliferation and of the mammary gland has been investigated in rats of the Nobel strain in two studies using non-standard methods. The Nobel strain of rat is particularly sensitive to oestrogenic activity. In the original study, groups of six female juvenile rats were exposed to nonylphenol by the subcutaneous route for 11 days, administered via osmotic minipumps implanted in the dorsal cervical region (Colerangle and Roy, 1996). The dose levels were 0 (DMSO vehicle control), 0.01 and 7.12 mg/day (0.05 and 35.6 mg/kg/day, assuming a bodyweight of 200 g). An additional group received diethylstilbestrol (DES) at 0.01 mg/day (0.05 mg/kg/day) for 11 days by an unspecified route. At the end of the exposure period the rats were killed and the abdominal mammary glands removed for evaluation. Mammary gland growth was assessed by counting the number of mammary structures (terminal ducts, terminal end buds or lobules) and cells in 16 mm^2 areas of the mammary gland. Cell proliferation and cell-cycle kinetics were evaluated using immunohistochemical techniques (reaction with antiproliferating cell nuclear antigen (PCNA)) which allowed cells in S, G1 and G0 phases to be identified. The labelling index (LI, proportion of cells in S phase) growth fraction (GF, proportion of cells in the G1 or S phase) were calculated.

In the group receiving the highest dose of nonylphenol there was a 1.5-fold increase in the number of mammary structures and a 4-fold increase in the number cells/16 mm^2 area, compared with the vehicle control group. At the lowest level the number of structures was similar to the controls, but there was a 2-fold increase in the number of cells. DES caused a 6-fold increase in the number of cells. The LI was increased by 1.3 and 1.8 fold and GF by 1.2 and 2 fold in the nonylphenol low and high dose groups, respectively, in comparison with the vehicle control. DES had a much greater influence on the indices, with increases of 4 and 5 fold for the LI and GF. Cell cycle time was unchanged in the low dose group, slightly decreased (by about 10%) in the high dose group and markedly deceased (by more than half) in the DES group. This study shows that nonylphenol at dose levels of 0.05 and 35.6 mg/kg/day increases growth and proliferation activity in a dose-related manner in the mammary gland of the Nobel rat, although the effects at 0.05 mg/kg/day are marginal. The significance for human health of such a finding is unknown. Furthermore, the use of the subcutaneous route of administration and selection of the oestrogen-sensitive Nobel rat as the model casts doubts about the relevance of these findings to humans. Ashby and Odum (1998) draw attention to the fact that same positive control (DES) data reported for this study also appear in two other reports by Colerangle and Roy (1995 and 1997), and that the vehicle control data of the nonylphenol study is duplicated in the 1997 study. This raises some uncertainties as whether the control data were generated concurrently with the nonylphenol data and questions the validity of this study.

The Colerangle and Roy (1996) study was duplicated by Odum et al. (1999). Groups of ten female OVR^+ Noble rats were exposed to nonylphenol at dose levels of 0 (DMSO vehicle control), 0.073 or 53.2 mg/kg/day or DES at 0.076 mg/kg/day by the subcutaneous route for 11 days, administered via osmotic minipumps implanted in the dorsal cervical region. Mammary gland differentiation and mammary gland cell proliferation were assessed following similar methodology to Colerangle and Roy (1996), except that BRDU as well as PNCA staining was used (BRDU incorporation was considered to be a more sensitive and robust technique) and a more objective method was used to quantitate mammary gland changes. The quantitative determination of the numbers and areas of mammary gland structures showed no differences between the vehicle control and nonylphenol exposed groups, in contrast to the findings of the original study. DES, however, had a marked influence of the differentiation of mammary structures. Terminal ducts were completely absent and terminal end buds were present only in peripheral regions. Also, the numbers and areas of lobules were markedly increased in peripheral and central areas. The mammary gland cell proliferation assessment revealed, in comparison with the vehicle control group, no changes in the nonylphenol exposed groups, and a marked increase (about 4 fold in the lobules) in the DES group. This study shows the DES can induce growth and proliferation activity in the mammary gland of the Nobel rat, but failed to confirm the observation in the Colerangle and Roy (1996) study of such activity following nonylphenol exposure at similar dose levels.

4.1.2.6.2 Human data

The effects of nonylphenol exposure have not been evaluated in humans. There are two isolated case reports of leucoderma on the hands and forearms, with subsequent spreading to other areas, among Japanese workers exposed to alkaline detergents containing polyethylene alkylphenylether (Ikeda et al., 1970). The authors speculated that this might be caused by free octylphenol or nonylphenol, which were found to be present in the detergents. However, in the absence of corroborative reports from elsewhere, no firm conclusions regarding causality can be made.

4.1.2.6.3 Summary of repeated dose toxicity

No useful human data are available. In a multigeneration study in the rat involving oral exposure via the diet for up to 20 weeks, a LOAEL for repeated dose toxicity of 15 mg/kg/day was identified, based on histopathological changes in the kidneys (tubular degeneration or dilatation), although such changes were not apparent at this dose level in a 90-day dietary exposure rat study. At higher dose levels the liver may also be a target organ; minor histopathological changes in the liver (vacuolation in the periportal hepatocytes or occasional individual cell necrosis) were seen at doses of 140 mg/kg/day and above in some dietary studies. The oral toxicity of nonylphenol appears to be enhanced when dosed by gavage, with mortalities being reported at dose levels of 100 mg/kg/day and above. No studies involving dermal or inhalation exposure have been conducted. Nonylphenol has been reported to induce cell proliferation in the mammary gland of the Nobel rat following subcutaneous exposure at levels down to 0.05 mg/kg/day, but this finding could not be reproduced in a duplicate study; furthermore, there are doubts about the relevance of this finding to humans and regarding the validity of the original study.

4.1.2.7 Mutagenicity

Only data from *in vitro* test systems and animals are available.

4.1.2.7.1 Studies in vitro

Two pre-incubation bacterial mutagenicity (Ames) tests have been conducted. Both were negative. The first cannot be fully appraised because only a summary report is available (Hüls 1984). *Salmonella typhimurium* strains TA1537, TA 1538, TA 98 and TA 100 were exposed to nonylphenol at concentrations to 5000 µg/plate, both in the presence and absence of metabolic activation (Aroclor induced rat liver S9). The same *Salmonella* strains were used in the second study, together with *Escherichia coli* strain WP2urvA (Shimizu et al., 1985). Concentrations up to 100 µg/plate were tested, both in the presence and absence of metabolic activation (polychlorinated biphenyl induced rat liver S9), and toxicity was reported at the highest concentrations tested. A limitation of both studies is that the results of neither appeared to have been confirmed by a second independent experiment.

In a well-conducted *in vitro* mammalian cell gene mutation test, following OECD guideline 476 and in compliance with GLP, the potential for nonylphenol to induce mutations at the HPRT-locus was investigated in Chinese hamster V79 cells (Hüls, 1990). The exposure period was 5 hours, and a range of concentrations up to 2.5 µg/ml (without metabolic activation) or 1.25 µg/ml (with metabolic activation) were tested. At higher concentrations there was no cell survival. The results were confirmed by independent experiment. The test was negative.

4.1.2.7.2 Studies in vivo

Two micronucleus studies are available.

In the most recent study, conducted according to OECD guideline 474, groups of 5 male and 5 female NMRI strain mice received a single intraperitoneal dose of 50, 150 or 300 mg/kg (Hüls, 1999b). Appropriate positive (cyclophosphamide) and negative (vehicle) control groups were included. The highest treatment level was chosen as the maximum tolerated dose, based on the results of a preliminary study. Bone marrow was sampled 24 hours after treatment. There was a second sampling time of 48 hours for additional groups receiving either nonylphenol at 300 mg/kg or only the vehicle. Toxicity was elicited at 150 and 300 mg/kg, seen as clinical signs such as sedation, squatting posture, abnormal gait and piloerection. There was no consistent effect on the polychromatic to normochromatic erythrocyte (PCE/NCE) ratio. No increases in the frequency of micronucleated PCEs were seen in the nonylphenol exposed groups; thus the tests is considered to be negative. The anticipated response was seen in the positive control group. Although the PCE/NCE ratio was not affected, the fact that the study was conducted at the maximum tolerated dose and using the intraperitoneal route of administration, it can be presumed that exposure of the bone marrow to nonyl phenol was maximised. Accordingly, a high level of confidence can be given to this negative result.

An earlier micronucleus test was conducted using the oral route of administration (Hüls, 1988). In accordance with the OECD guideline, groups of five male and five female mice of the NMRI strain received a single oral dose of nonylphenol at 500 mg/kg. The dose level was chosen as the maximum tolerated dose. No evidence was presented to support this choice, but it is noted that it is greater that a reported oral LD_{50} of 307 mg/kg/day for mice. Appropriate positive and negative controls were included. Bone marrow was sampled at 18, 48 and 72 hours. There were no increases in the frequency of micronuclei at any of the sampling times and the test was declared negative. The PCE/NCE ratio was not affected by nonylphenol, which raises concerns about adequacy of exposure of the bone marrow to the test substance. The toxicokinetic information suggests that the systemic bioavailability of nonylphenol following oral administration is