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TOXICOLOGICAL EVALUATIONS

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TOXICOLOGICAL EVALUATION

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1-(2,4-Dinitro-phenylazo)-2-naphthol

No. 223

CAS No. 3468-63-1

BG Chemie
Berufsgenossenschaft der chemischen Industrie

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1-(2,4-Dinitrophenylazo)-2-naphthol

1 Summary and assessment

Absorption of 1-(2,4-dinitrophenylazo)-2-naphthol via the human skin is low. According to in-vitro studies, absorption from mineral oil, castor oil and cream blusher formulations over a period of 24 hours was 0.007, 0.003 and 0.006%, respectively, of the administered radioactivity, whereas absorption from talc was less than 0.001%.

1-(2,4-Dinitrophenylazo)-2-naphthol is of low acute oral toxicity (LD_{50} rat oral > 4640 and > 15000 mg/kg body weight, depending on the source of information). In a 32-day feeding study conducted in rats, high concentration levels in the range from 2000 to 25000 ppm (equivalent to approx. 133 to 1667 mg/kg body weight) dose-dependently caused retarded body weight development, anaemia and leukocytosis.

When applied to the skin of the rabbit, 1-(2,4-dinitrophenylazo)-2-naphthol causes no irritation. In the rabbit eye, formulations containing 5% of the chemical are tolerated without any reaction. The undiluted substance causes minimal irritation to the rabbit eye.

1-(2,4-Dinitrophenylazo)-2-naphthol has no sensitising effect on the guinea pig skin when tested in the epicutaneous test according to Buehler.

Chronic administration of 1-(2,4-dinitrophenylazo)-2-naphthol at concentration levels of 0.025, 0.125 or 1% (equivalent to 12.5, 62.5 and 500 mg/kg body weight/day) in the food of beagle dogs for 2 years resulted in retarded body weight development, the high dose additionally causing anaemia and organ enlargement (thyroid gland, liver, spleen) as well as skin changes. At the 0.125% level, enlarged thyroid glands and increased liver weights were observed. All dose groups were found to have bilirubinuria.

Numerous groups of investigators have tested 1-(2,4-dinitrophenylazo)-2-naphthol in Salmonella/microsome studies conducted as standard-plate incorporation tests, preincubation tests or spot tests in one or several tester strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535, TA 1537 and TA 1538) in the absence and presence of metabolic activation and have predominantly found the chemical to be mutagenic. In L5178Y mouse lym-

phoma cells and in the HPRT test on V79 cells of the Chinese hamster, 1-(2,4-dinitrophenylazo)-2-naphthol causes no gene mutations in the absence or presence of metabolic activation. In the in-vitro chromosome aberration test in V79 cells of the Chinese hamster, 1-(2,4-dinitrophenylazo)-2-naphthol is not clastogenic either in the absence or presence of metabolic activation. The DNA repair test on primary rat hepatocytes gives negative results for 1-(2,4-dinitrophenylazo)-2-naphthol. In contrast, at 5 µg/ml, a level that is not cytotoxic, a 40-minute incubation with 1-(2,4-dinitrophenylazo)-2-naphthol causes DNA damage in primary rat hepatocytes in the comet assay. In vivo, 1-(2,4-dinitrophenylazo)-2-naphthol causes no chromosomal damage to bone marrow cells of the Chinese hamster after the maximum dose that can be administered by oral gavage. Despite the positive in-vitro results obtained in the Salmonella/microsome assay and the comet assay, 1-(2,4-dinitrophenylazo)-2-naphthol should not be considered mutagenic in the sense of the evaluation criteria set forth in the German Hazardous Substances Ordinance ("Gefahrstoffverordnung"), since in-vitro tests on mammalian cells and the mutagenicity study conducted in vivo gave negative results.

In two long-term feeding studies from the 1960s which were carried out over a period of more than 2 years, rats receiving 1-(2,4-dinitrophenylazo)-2-naphthol at concentrations of up to 1% were not reported to have shown any increase in substance-related tumour incidence. Similarly, no increased tumour incidences were seen in mice receiving dermal applications of 1-(2,4-dinitrophenylazo)-2-naphthol for 18 months. In a later, two-phase long-term feeding study in rats, in which the animals were given 1-(2,4-dinitrophenylazo)-2-naphthol in their feed at concentrations of 0.02, 0.05 and 0.1% during the first phase and 1-(2,4-dinitrophenylazo)-2-naphthol levels of 1% during the second phase, only the females of the 1% group, which at the same time exhibited a range of toxic and proliferative liver changes, were found to have an increase in hepatocellular adenomas and a small number of hepatocellular carcinomas. In the livers of the male rats that were given 1% 1-(2,4-dinitrophenylazo)-2-naphthol in their feed, increases were found neither in toxic or proliferative changes nor in neoplastic changes relative to controls. This suggests that the weak tumorigenic effect observed in female rats was caused only by high, hepatotoxic doses. When 1-(2,4-dinitrophenylazo)-2-naphthol was fed to mice at concentrations of 0.25% for 2 years and even more so at 1%, a concentration of 1-(2,4-dini-

trophenylazo)-2-naphthol which resulted in slightly reduced body weight gain and slightly increased mortality only in the male mice, a significant, dose-dependent increase in benign and malignant hepatocellular tumours was observed in the male mice but not in the females. Thus, following chronic oral administration at high concentration levels, 1-(2,4-dinitrophenylazo)-2-naphthol has a weak hepatocarcinogenic potential in female rats and in male mice, the liver carcinogenicity in female rats being accompanied by hepatotoxicity. When applied chronically to the skin, 1-(2,4-dinitrophenylazo)-2-naphthol is, however, not carcinogenic to mice.

In rats and rabbits, no teratogenic properties have been reported for 1-(2,4-dinitrophenylazo)-2-naphthol following administration by oral gavage. In a two-generation study in rats which were given the test substance in their feed, reproduction was not affected. The reproductive toxicity studies available are only of limited use for evaluation, because the publications lack details of the experimental setup.

No sensitising properties have been demonstrated for 1-(2,4-dinitrophenylazo)-2-naphthol in human studies.

2 Name of substance

2.1	Usual name	1-(2,4-Dinitrophenylazo)-2-naphthol
2.2	IUPAC name	1-(2,4-Dinitrophenylazo)-2-naphthol
2.3	CAS No.	3468-63-1
2.4	EINECS No.	222-429-4

3 Synonyms, common and trade names

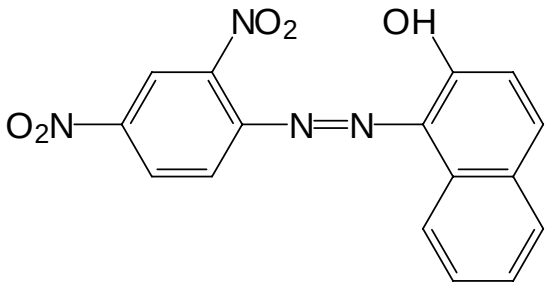
Brilliant Tangerine 13030
C.I. Pigment Orange 5
C.I. 12075
Calcotone Orange 2R
Carnelio Red 2G
Chromatex Orange R
D & C Orange No. 17

Dainichi Permanent Red GG
Dinitraniline Orange
Dinitroaniline (Orange, Orange ND-204, Red)
1-[(2,4-Dinitrophenyl)azo]-2-naphthalenol
Fastona Red 2G
FD&C Orange No. 17
Graphtol Red 2GL
Hansa Orange RN
Hansa Rot GG
Helio Fast Orange (RN, RT, 3RN, 3RT)
Irgalite Fast Red 2GL
Irgalite Red (PV8, 2G, 2GW)
Isol Fast Red 2G
Japan Orange 203
Lake Red 2GL
Light Orange R
Lutetia Fast Orange R
Monolite Fast (Orange 2R; Paper Orange 2R, Red 2G)
2-Naphthol, 1-((2,4-dinitrophenyl)azo)-
Nippon Orange X-881
Oralith Red 2GL
11048 Orange
Orange No. 203
Orange Pigment X
Permanent Orange
Permanent Orange (DN Toner, GG, HD, Toner, Toner RA-5650)
Permanent Red GG
Permansa Orange
Permaton Orange XL 45-3015
Permatone Orange
Pigment Fast Orange
Pigment Orange 5
Segnale Light Orange (RN, RNG)
Siegle Orange 2S
Silopol Orange R

Silosol Orange RN
Siloton Orange RL
Syton Fast Red 2G
Tertropigment (Orange LRN, Red P2G)
Versal Orange RNL

4 Structural and molecular formulae

4.1 Structural formula



4.2 Molecular formula $C_{16}H_{10}N_4O_5$

5 Physical and chemical properties

5.1	Molecular mass, g/mol	338.30	
5.2	Melting point, °C	319 > 300	(Kozuka et al., 1980) (Ciba-Geigy, 1990)
5.3	Boiling point, °C	–	
5.4	Vapour pressure, hPa	No information available	
5.5	Density, g/cm³	1.7	(Ciba-Geigy, 1990)
5.6	Solubility in water	Insoluble (Hart et al., 1986; Ciba-Geigy, 1990)	
5.7	Solubility in organic solvents	No information available	
5.8	Solubility in fat	No information available	
5.9	pH value	7	(Ciba-Geigy, 1990)
5.10	Conversion factor	1 ml/m³ (ppm) $\triangleq$ 13.81 mg/m³ 1 mg/m³ $\triangleq$ 0.07 ml/m³ (ppm) (at 1013 hPa and 25 °C)	

6 Uses

As a pigment in coatings and printing inks (Hunger and Herbst, 1992) and in artists' colours (Orna and Pamer, 1985).

The use of 1-(2,4-dinitrophenylazo)-2-naphthol as a colour additive in externally applied cosmetics and pharmaceuticals has been prohibited by the US Food and Drug Administration (FDA) on account of the substance's weak hepatocarcinogenic activity in male mice and female rats (FDA, 1988). The Cosmetics Ordinance of the Federal Republic of Germany (Kosmetik-Verordnung der Bundesrepublik Deutschland) of October 1997 also no longer lists the substance because the EU Scientific Committee on Cosmetology (SCC) has had it delisted on account of its weak carcinogenic activity and in-vitro mutagenicity (induction of reverse mutations in *Salmonella typhimurium*; BgVV, 1997; Kosmetik-Verordnung, 1997).

7 Experimental results

7.1 Toxicokinetics and metabolism

The absorption of ^{14}C-labelled 1-(2,4-dinitrophenylazo)-2-naphthol (radiochemical purity 95 to 96%; no details of impurities) through half-thickness human abdominal skin (3 patients) was determined in vitro using Franz diffusion cells. For this purpose, formulations of 1-(2,4-dinitrophenylazo)-2-naphthol were prepared in mineral or castor oil and in cream blusher at 0.6% and in talc at 5%. Test substance recovery rates of 80 to 90% were seen in the oil formulations and 40 to 50% in cream blusher. Recoveries were not addressed in the talc studies. Volpo 20 (polyethylene glycol 20 oleyl ether) was employed to aid solubilisation in the receptor phase (isotonic saline, pH 7.6, 37 °C). The receptor phase was replaced at 4, 8 and 12 hours and every 12 hours thereafter up to 48 hours after the beginning of the study. Most of the applied dose was removed with toluene skin washes, although radioactivity was found in all skin preparations. Absorption of 1-(2,4-dinitrophenylazo)-2-naphthol after 24 hours was 0.007, 0.003 and 0.006% of the administered radioactivity for mineral oil, castor oil and cream blusher formulations, respectively, while it was less than 0.001% for talc (Franz, 1983).

7.2 Acute and subacute toxicity

In an acute oral toxicity study, 10 female Wistar rats (weighing 116 to 132 g) received a single dose of 1-(2,4-dinitrophenylazo)-2-naphthol by gavage as a 25-percent solution in sesame oil at the highest technically possible dose of 15000 mg/kg body weight. The observation period was 14 days. No clinical signs of toxicity or deaths occurred. Body weight gain was not affected. Thus the LD_{50} was greater than 5000 mg/kg body weight (Hoechst, 1973 a).

In a further study, groups of 5 male and 5 female rats (Tif.RAI; weighing 160 to 180 g) were given 1-(2,4-dinitrophenylazo)-2-naphthol as a 20-percent aqueous suspension in 2% carboxymethylcellulose once by oral intubation. The dose levels chosen were 2150, 3170 and 4640 mg/kg body weight. The observation period was 7 days. There were no deaths. Clinical signs of toxicity observed in all dose groups included dyspnoea, exophthalmus, curved position and ruffled fur. Higher doses resulted in more pronounced clinical signs. Autopsy revealed no substance-related changes. Thus the LD_{50} was > 4640 mg/kg body weight (Ciba-Geigy, 1974 a).

A subacute oral toxicity study of 1-(2,4-dinitrophenylazo)-2-naphthol (97.0% pure, admixtures: 1.5% tallow fat propylenediamine, 1.5% dimethyl (coconut oil alkyl) amine oxide) was carried out in groups of 10 male and 10 female Wistar rats (mean initial weights 92 and 87 g) as a 32-day feeding study. The concentration levels used in the study were based on the results of a 10-day preliminary study, in which 5% test substance in feed was very poorly absorbed by the animals. The levels were 0 (controls), 0.2, 1.0 and 2.5%, equivalent to 0, 2000, 10000 and 25000 mg/kg feed (approx. 0, 133, 667 and 1667 mg/kg body weight). Seven males and 3 females in the top dose group (2.5%) died after severe weight loss. The rats in the other dose groups also exhibited dose-dependent retardation of body weight gain. Only the females in the 0.2% dose group showed no effect on body weight gain. When haematology parameters were studied at the end of the study, all three dose groups were found to have dose-dependent reductions in red blood cell counts and haemoglobin levels and increased white blood cell counts. Differential blood counts revealed a marked increase in neutrophil counts in the groups given 1.0 and 2.5%. Urinalyses were without abnormal findings. At macroscopic and histopathological examination, all dose groups were found to have slightly increased iron levels in the

spleen. No other clearly substance-related alterations were seen (Hoechst, 1973 b). A *no effect level* could not be determined.

7.3 Skin and mucous membrane effects

The primary skin irritancy of 1-(2,4-dinitrophenylazo)-2-naphthol was studied in accordance with the “Appraisal of the safety of chemicals in food, drugs and cosmetics” (1959) of the US Association of Food and Drug Officials (AFDO). Groups of 3 male and 3 female rabbits of the Russian breed (weighing 1.5 to 2 kg) underwent exposure of the intact and the slightly scarified shaven skin of the flank. 1-(2,4-Dinitrophenylazo)-2-naphthol was applied as a 50-percent formulation in polyethylene glycol 400 under occlusive cover. The exposure period was 24 hours. Skin reactions were appraised immediately upon removal of the patch and 72 hours thereafter and evaluated according to Draize. The average irritation indexes for erythema and oedema formation were 0 for nonscarified skin. 1-(2,4-Dinitrophenylazo)-2-naphthol therefore proved to be not irritating to the nonscarified skin. When applied to scarified skin, the product caused very mild erythema in 5 out of 6 rabbits and very mild oedema in 2 out of 6 rabbits immediately after the end of exposure. None of the rabbits showed skin reactions at 72 hours after exposure. Taking into consideration the results obtained for scarified skin, the primary irritation index was calculated as 0.3, and therefore the product was evaluated by the investigators as being mildly irritating (Ciba-Geigy, 1974 b). Current criteria warrant evaluation of the chemical as not irritating on the basis of this result.

A further skin irritation study tested 1-(2,4-dinitrophenylazo)-2-naphthol on the mechanically depilated skin of 6 rabbits (of the “Gelb-Silber” breed). Exposure involved applying 0.5 ml aliquots of 10% and 5% dilutions in sesame oil once daily, 5 times in total, to the intact flank skin of each animal. No signs of irritation were observed (Hoechst, 1973 c).

The skin irritancy of 1-(2,4-dinitrophenylazo)-2-naphthol was also investigated in the Barail test by intracutaneous injection of 0.02 ml doses of sesame oil dilutions containing 10, 5, 1, 0.1, 0.01 or 0.001% of the test substance into the mechanically depilated flank skin of each of 6 “Gelb-Silber” rabbits. In all rabbits, the 10- and 5-percent dilutions led to a necrotic centre surrounded by a red halo at the injection site, whilst the 1% dilution caused

reactions of varying severity (reddening, severe reddening, swelling, necrosis). All lower concentrations were tolerated without reactions. According to the investigators, this sensitive test can only be considered positive, when concentration levels of 10^{-3} and below still produce clear signs of irritation (Hoechst, 1973 c).

The eye irritancy of 1-(2,4-dinitrophenylazo)-2-naphthol was studied in accordance with the “Appraisal of the safety of chemicals in food, drugs and cosmetics” (1959) of the US Association of Food and Drug Officials (AFDO) in groups of 3 male and 3 female rabbits of the Russian breed. Each animal had 100 mg of test substance instilled into the conjunctival sac. In 3 rabbits, the treated eyes were not washed whereas in the 3 other rabbits the treated eyes were rinsed with 10 ml lukewarm water about 30 seconds after treatment. The reactions were appraised according to Draize 24 hours and 2, 3, 4 and 7 days after treatment. No corneal or iridial irritation was noted at any time point of investigation for the rinsed and unrinsed eyes, whereas the conjunctival findings seen in 2 out of 3 animals with unrinsed eyes 24 hours after instillation gave an irritation index of 0.4 for the conjunctivae. The findings had cleared up completely by 48 hours after treatment. Therefore the investigators considered 1-(2,4-dinitrophenylazo)-2-naphthol as a minimal irritant to the rabbit eye (Ciba-Geigy, 1974 c). Current criteria warrant evaluation of the chemical as not irritating to the rabbit eye on the basis of this result.

A single instillation of 0.1 ml of sesame oil formulations containing 10 or 5% 1-(2,4-dinitrophenylazo)-2-naphthol into the conjunctival sac of the rabbit resulted in 3 out of 6 rabbits exposed to the 10% dilution developing mild reddening which persisted for 3 to 7 hours. The 5% formulation was tolerated without reactions by 6 rabbits (Hoechst, 1973 c).

7.4 Sensitisation

The sensitising potential of 1-(2,4-dinitrophenylazo)-2-naphthol (of unspecified purity) was investigated in 10 male Pirbright-White guinea pigs (initial body weight 312 to 390 g) in the Buehler patch test. Five animals served as controls. During the induction phase the animals each received 9 dermal treatments within 3 weeks with 0.5 ml of a 0.05% formulation in 2% starch gruel, a concentration identified in preliminary studies as being the lowest

concentration to cause primary irritation. All animals treated in this manner developed mild irritation in the form of hardly noticeable to mild reddening of the skin. Following a treatment-free period of 16 days, a single 0.5 ml dose of a 0.01% formulation, which was not capable of causing primary irritation, was applied to the skin, and the skin reactions were assessed 6, 24 and 48 hours later. Both this challenge and a second one carried out 48 hours later gave no indications that the chemical had sensitising properties (Hoechst, 1982).

7.5 Subchronic and chronic toxicity

Groups of 3 male and 3 female beagle dogs were fed 1-(2,4-dinitrophenylazo)-2-naphthol at concentration levels of 0.025, 0.125 or 1% of the diet for 2 years. Assuming that food consumption (dry food) was 50 g/kg body weight/day, this was equivalent to dose levels of 12.5, 62.5 and 500 mg/kg body weight/day. 1-(2,4-Dinitrophenylazo)-2-naphthol led to lower body weight gains in all animals treated. The animals given 1% 1-(2,4-dinitrophenylazo)-2-naphthol were found to have anaemia, increased thyroid, liver and spleen size and changes in the skin. The 0.125% group exhibited increased female thyroid and male liver weights. Furthermore, there were slight skin effects. All dose groups exhibited bilirubinuria. No histopathological changes were observed (no further details; Hazleton, 1964 a). A *no effect level* could not be determined in this study.

7.6 Genotoxicity

7.6.1 In vitro

1-(2,4-Dinitrophenylazo)-2-naphthol was tested for mutagenicity in a number of Salmonella/microsome assays (see Table 1), performed as standard plate incorporation, preincubation or spot tests using *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 with and without metabolic activation (S9 mix from rat, hamster or mouse liver induced with Aroclor 1254 or PCB).

Table 1. Salmonella/microsome assays with 1-(2,4-dinitrophenylazo)-2-naphthol

Test system	Concentration range tested[1] (µg/plate)	Metabolic activation system	Result[2]		References
			with metabolic activation	without metabolic activation	
1. Salmonella/microsome assay as standard plate incorporation test					
Salmonella typhimurium TA 98, TA 100, TA 1535, TA 1537, TA 1538	50–500 (95% pure)	S9 mix from Aroclor 1254-induced rat liver	positive (TA 98, TA 100, TA 1537, TA 1538)	positive (TA 98, TA 100, TA 1537, TA 1538)	Brown et al., 1979
Salmonella typhimurium TA 98, TA 100, TA 1535, TA 1537	0.2–400	S9 mix from Aroclor 1254-induced rat liver	positive (TA 98, TA 1537)	positive (TA 98, TA 100, TA 1535, TA 1537)	Muzzall and Cook, 1978, 1979
Salmonella typhimurium TA 98, TA 100	5–1000	S9 mix from PCB-induced liver (no further details)	positive (TA 98, TA 100)	positive (TA 98, TA 100)	Miyagoshi et al., 1983
Salmonella typhimurium TA 98, TA 100	0.1–100	S9 mix from PCB-induced hamster liver	positive (TA 98)	positive (TA 98)	Rojanapo et al., 1985
Salmonella typhimurium TA 98	0.1–100	S9 mix from Aroclor 1254-induced rat liver	negative (TA 98)	positive (TA 98)	Green and Pastewka, 1979, 1980
Salmonella typhimurium TA 98, TA 100, TA 1535, TA 1537	4–2500 (not crystallised)	S9 mix from Aroclor 1254-induced rat liver	negative (TA 98, TA 100, TA 1535, TA 1537)	not tested	Hoechst, 1979
Salmonella typhimurium TA 98, TA 100, TA 1535, TA 1537	0.016–5000 (not crystallised)	without metabolic activation system	not tested	positive (TA 98, TA 100, TA 1535, TA 1537)	Hoechst, 1979
Salmonella typhimurium TA 98, TA 100, TA 1535, TA 1537, TA 1538	0.0064–10000	S9 mix from Aroclor 1254-induced rat liver	positive (TA 98, TA 100, TA 1535, TA 1537, TA 1538)	positive (TA 98, TA 100, TA 1535, TA 1537, TA 1538)	Hoechst, 1980
2. Salmonella/microsome assay as preincubation test					
Salmonella typhimurium TA 98, TA 100	no data	S9 mix riboflavin (no further details)	no data	positive (TA 98)	Matsushima et al., 1978
Salmonella typhimurium TA 98	10–1000	S9 mix (no further details)	positive (TA 98)	positive (TA 98)	BFG, 1986 b
Salmonella typhimurium TA 98, TA 1535, TA 1538	10	S9 mix from Aroclor 1254-induced mouse liver	negative	positive (TA 98, TA 1538)	Milvy and Kay, 1978
3. Salmonella/microsome assay as spot test					
Salmonella typhimurium TA 98, TA 100, TA 1535, TA 1537	0.2–400	S9 mix from Aroclor 1254-induced rat liver	positive (TA 98, TA 1537)	positive (TA 98, TA 100, TA 1535, TA 1537)	Muzzall and Cook, 1978, 1979
Salmonella typhimurium TA 98, TA 1535, TA 1538	10	S9 mix from Aroclor 1254-induced mouse liver	negative*	positive* (TA 98, TA 1538)	Milvy and Kay, 1978
* The publication gives no separate results for the spot test and the preincubation test (see above).					
4. Salmonella/microsome assay (type of test not specified)					
Salmonella typhimurium TA 98	0.01–1000	–	–	positive (TA 98)	BFG, 1986 a

[1] Unless stated, publications give no data on the bacteriotoxic effects or the purity of the test substances used.
[2] Whenever specified in the references that only certain strains tested positive, these are mentioned here.

The test concentrations were in the range from 0.01 to 1000 µg/plate. In the absence of metabolic activation, 1-(2,4-dinitrophenylazo)-2-naphthol caused increases in revertant counts at all levels in strain TA 98. Positive results were also obtained for strains TA 100, TA 1537 and TA 1538, one group of investigators additionally obtaining positive results for strain TA 1535 (Muzzall and Cook, 1978, 1979). In the presence of metabolic activation, 1-(2,4-dinitrophenylazo)-2-naphthol also caused increases in revertant counts in strains TA 98, TA 100, TA 1537 and TA 1538, but these were far less pronounced than in the absence of metabolic activation (Brown et al., 1979; Green and Pastewka, 1979, 1980; Matsushima et al., 1978; Milvy and Kay, 1978; Miyagoshi et al., 1983; Muzzall and Cook, 1978, 1979). 1-(2,4-Dinitrophenylazo)-2-naphthol therefore proved to be clearly mutagenic in the Salmonella/microsome assay.

In mouse lymphoma cells (L5178Y), 1-(2,4-dinitrophenylazo)-2-naphthol concentrations of 0.65 to 5.0 mg/ml caused no increase in the rate of mutation. The assay was performed with and without metabolic activation (no further details; Litton Bionetics, 1977).

1-(2,4-Dinitrophenylazo)-2-naphthol (technical grade) was investigated in two independent series of the HPRT tests on V79 cells of the Chinese hamster in accordance with OECD guideline No. 476. The tests were carried out in the presence and absence of metabolic activation (S9 mix from Aroclor 1254-induced rat liver). After preliminary studies, in which the chemical proved to be cytotoxic from 250 µg/ml without metabolic activation and from 750 µg/ml with metabolic activation, the concentration levels chosen for the main study were 50, 75, 100, 150 and 250 µg/ml in the absence and 50, 100, 250, 500 and 750 µg/ml in the presence of metabolic activation. Without metabolic activation, concentrations in the range from 100 to 250 µl/ml were cytotoxic, while in the presence of metabolic activation cytotoxicity was observed from 500 to 750 µl/ml. 1-(2,4-Dinitrophenylazo)-2-naphthol caused no significant increase in mutation rate and, therefore, had no mutagenic effect in this test system (Hoechst, 1989 a).

V79 cells of the Chinese hamster were used in order to study the chromosome-damaging potential of 1-(2,4-dinitrophenylazo)-2-naphthol (technical grade; dispersion in DMSO) in accordance with OECD guideline No. 473, with and without metabolic activation (S9 mix from Aroclor 1254-induced rat liver). The exposure period was 4 hours, and assessments were perfor-

med at 7, 18 and 28 hours after the beginning of the study. Positive controls were treated with ethylmethane sulphonate or cyclophosphamide. Based on preliminary cytotoxicity studies, concentrations employed in the main study without metabolic activation were 200 (7 hours), 20, 100, 200, (18 hours) and 200 µg/ml (28 hours). Tests carried out in the presence of metabolic activation employed concentrations of 600 (7 hours), 60, 300, 600 (18 hours) and 600 µg/ml (28 hours). 1-(2,4-Dinitrophenylazo)-2-naphthol caused no chromosome aberrations in this test system (Hoechst, 1989 b).

The autoradiographic DNA repair test using primary rat hepatocytes gave negative results for 1-(2,4-dinitrophenylazo)-2-naphthol at a 3×10^{-3} M concentration (no further details; Williams et al., 1989).

1-(2,4-Dinitrophenylazo)-2-naphthol was investigated in a comet assay, which is based on visualisation of DNA by means of fluorescence microscopy. Single cells are embedded in agarose, cell proteins are removed and the DNA is subjected to electrophoresis. Undamaged DNA, due to its high molecular weight, migrates slowly in the electric field. In contrast, damaged DNA creates a comet-like image under the microscope because the small DNA fragments move towards the anode. The extent of migration in the electric field is proportional to the DNA damage. The comet assay was performed according to the protocol published by Singh et al. (1988), which allows the detection of strand breaks, alkaline-labile sites and incomplete excision repair sites. The studies were carried out with freshly isolated hepatocytes from female Wistar rats fasted overnight. The incubations with 1-(2,4-dinitrophenylazo)-2-naphthol were started within 2 hours after preparation of hepatocytes. The publication gives no information on analytical purity. Hepatocytes were suspended in RPMI medium (no further details) containing 1% foetal bovine serum and incubated at a final concentration of 10^6 cells/ml with an aqueous suspension of 1-(2,4-dinitrophenylazo)-2-naphthol at 0, 1 or 5 µg/ml in a 37 °C water bath for 40 minutes. It had previously been ascertained by trypan blue exclusion that the concentrations of 1-(2,4-dinitrophenylazo)-2-naphthol employed were not toxic to the hepatocytes. At the 5 µg/ml concentration level, 1-(2,4-dinitrophenylazo)-2-naphthol caused significant DNA damage. The effect persisted until the end of the incubation period of 80 minutes. The positive control used was the known hepatocarcinogen 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline, which caused significant DNA damage at a level of 2.13 µg/ml (Møller et al., 1998).

7.6.2 In vivo

1-(2,4-Dinitrophenylazo)-2-naphthol (technical grade) was tested in vivo for chromosome-damaging effects in bone marrow cells of the Chinese hamster in accordance with OECD guideline No. 475. The animals (males and females, respective mean body weights 32.7 and 29.9 g) received the maximum administrable dose of 5000 mg/kg body weight in sesame oil, as established in a preliminary study (no further details). The dose was split into two equal parts that were administered by oral gavage at an interval of 2 hours. Positive controls were treated with cyclophosphamide at a dose level of 50 mg/kg body weight. At 12, 24 and 48 hours after administration, the femoral bone marrow from groups of 5 male and 5 female hamsters was obtained subsequent to colcemid administration, and 50 metaphases/hamster were examined for aberrations. The animals had red-coloured faeces but showed no signs of toxicity 12 hours after administration of 5000 mg 1-(2,4-dinitrophenylazo)-2-naphthol/kg body weight, surviving until scheduled sacrifices. There were no indications of cytotoxicity in the form of a reduced mitotic index. Aberration rates, both including and excluding gaps, were not increased in males or females at any time point of investigation. Therefore 1-(2,4-dinitrophenylazo)-2-naphthol showed no clastogenic activity under these experimental conditions (Hoechst, 1990).

7.7 Carcinogenicity

The earliest carcinogenicity studies of 1-(2,4-dinitrophenylazo)-2-naphthol date back to the 1960s. Additional studies in rats and mice were later performed in the 1970s at the FDA's request.

Studies from the 1960s

Groups of 25 male and 25 female CD Charles River rats were fed 1-(2,4-dinitrophenylazo)-2-naphthol at concentration levels of 0 (controls), 0.025, 0.1 or 1% of the diet for 104 weeks. Assuming that food consumption was 100 g/kg body weight/day, the concentrations of 1-(2,4-dinitrophenylazo)-2-naphthol were equivalent to daily dose levels of 0, 25, 100 or 1000 mg/kg body weight. Some growth suppression was noted at the higher dose level. Furthermore, liver weights were increased in higher dose females. Dose-dependent depression of haematocrit and haemoglobin levels and red

blood cell counts were noted. The rats showed dose-related elevation of urine bilirubin levels. Neoplastic nodules of the liver and hepatocellular carcinomas were increased in high-dose female rats relative to controls. However, the increases were not statistically significant (no further details; Hazleton, 1964 b).

A further study reported no substance-related changes following 26-month dietary administration to rats of 1-(2,4-dinitrophenylazo)-2-naphthol at 0 (controls) and 80 ppm (equivalent to approx. 5 mg/kg body weight; no further details; Hazleton, 1966).

Groups of 50 male and 50 female CF1 Carworth mice had 0.1 ml of a 1-percent solution of 1-(2,4-dinitrophenylazo)-2-naphthol painted onto to the dorsal skin once a week for a period of 26 months. The study gave no indications that 1-(2,4-dinitrophenylazo)-2-naphthol was carcinogenic (no further details; Leberco, 1961).

In the context of a comparative study in mice, the carcinogenic potential of 14 different colour additives, including 1-(2,4-dinitrophenylazo)-2-naphthol under the name of “D&C Orange No. 17”, was investigated following repeated dermal application. The colour additives were tested in three series, encompassing 4, 5 and 5 dyes. Each test series included a vehicle control group, which was treated with the suspending agent, water. The first test series also included mice treated with 3,4-benzpyrene as positive controls. 1-(2,4-Dinitrophenylazo)-2-naphthol (97% pure) was tested in the second series. The colour additive groups and the vehicle control and 3,4-benzpyrene groups each comprised 100 (50 male and 50 female) ICR mice. Twice each week for a period of 18 months, a dose of 0.1 ml aqueous suspension containing 1 percent colour additive (equivalent to 1 mg/animal) was applied to the clipped dorsal skin (area of application approx. 6 cm^2) of each animal with an automatic syringe. A total of 300 (150 male and 150 female) mice in the vehicle control groups were treated with water in the same manner. The 50 male and 50 female animals serving as positive controls received twice-weekly dermal treatment with 0.1 ml of acetone solution containing 10 µg 3,4-benzpyrene. The hair on the dorsal area of each animal was clipped at study initiation and later on when required. The animals were assessed for behaviour and mortality on a daily basis. All mice that died intercurrently or were killed in a moribund condition and all those surviving to the end of the 18-month study period underwent necropsy, and all

main organs and tissues, skin from the area of treatment and any tissue masses suspected of being tumorous were preserved in formalin. Skin from the area of treatment and any grossly abnormal organs or tissues of all animals in the colour experimental groups and the 3,4-benzpyrene group and of 25 male and 27 female vehicle control animals were stained with haematoxylin and eosin (HE staining) and examined by light microscopy. In addition, 5 male and 5 female mice that survived to the end of the study were randomly selected from each of the experimental groups and the vehicle control group, and examination by light microscopy was carried out on the brain, pituitary gland, thyroid gland, thymus, liver, spleen, kidneys, adrenal gland, stomach, small intestines, large intestines, urinary bladder, axillary lymph nodes, testes, skin from the area of treatment and any grossly abnormal tissues.

Table 2. 1-(2,4-Dinitrophenylazo)-2-naphthol (D&C Orange 17): Carcinogenicity study in ICR mice, 50 male and 50 female mice/group, receiving twice-weekly dermal applications of 0.1 ml doses of a 0.1-percent suspension in water for a period of 18 months

Test group	Substance	Total dose applied per animal (mean dose)	Average experimental life (days)	Percent survival (male and female mice) after the indicated number of months			
				5	10	16	18
1	water	13.2 ml	507	96	83	64	46
2	3,4-benzpyrene	0.97 mg	no data	95	85	0	0
7	water	13.1 ml	469	98	84	67	48
10	1-(2,4-dinitrophenylazo)-2-naphthol	143.7 mg	489	98	92	65	50
13	water	12.5 ml	449	95	87	61	44
The data for the other 13 colour materials tested are not given here.							

The average experimental life of mice exposed to 1-(2,4-dinitrophenylazo)-2-naphthol was 489 days, which was longer than that seen in two out of the three vehicle control groups treated with water, and at the end of the study (at 18 months) survival among the group of mice treated with the colour additive was still 50%, which was slightly higher than the percentages (46, 48 and 44%) seen in the three vehicle control groups. Extramedullary haematopoiesis was found to occur at comparable rates in all experimental and control groups. It occurred at rates of 13, 18 and 22% in the three vehicle control groups and 13% in the group treated with 1-(2,4-dinitrophenylazo)-2-naphthol. Acanthosis, hyperkeratosis and dermatitis were noted to be so-

mewhat more frequent in the dosed animals than in the controls (no further quantitative data). Carson (1984) attributed these findings to a greater incidence of ectoparasitism in all of the dye-treated mice (no further details). The mice in all groups were found to have interstitial nephritis, cystitis, amyloidosis and bronchopneumonia. These findings were not attributable to application of the colour additives. The incidences of benign and malignant tumours observed in mice treated with 1-(2,4-dinitrophenylazo)-2-naphthol was comparable to vehicle controls. In contrast, 94 out of 100 mice treated with 3,4-benzpyrene developed gross skin lesions in association with the area of treatment in an average latent period of 245 days, all mice dying within 16 months (no data were given on the types of lesions or the incidences or types of tumours/animal or group).

Table 3. Tumour incidence

Test group	Substance	Male mice with tumours		Female mice with tumours	
		No. of animals	Type of tumour	No. of animals	Type of tumour
1	water	3 1	hepatic cell adenoma neoplasm in hindleg	1 1	mammary gland cystadenocarcinoma lymphosarcoma
2	3,4-benzpyrene	"ninety-four percent of the mice ... developed typical gross skin lesions associated with the area of treatment in an average latent period of 245 days" (no further details)			
7	water	1 1	hepatic cell adenoma anaplastic osteogenic sarcoma in subcutis	1	undifferentiated sarcoma in subcutis
10	1-(2,4-dinitrophenylazo)-2-naphthol	2 1 1	hepatic cell adenoma haemangiosarcoma in hindleg sebaceous gland carcinoma	3 1 1 1	mammary carcinoma wart-like growth, dorsal skin skin papilloma lymphosarcoma
13	water	4	hepatic cell adenoma	1	Harderian gland adenoma

Therefore, 1-(2,4-dinitrophenylazo)-2-naphthol was not carcinogenic to ICR mice after repeated dermal application (Hazleton, 1969; Carson, 1984).

Although no evidence of carcinogenicity related to 1-(2,4-dinitrophenylazo)-2-naphthol was found in the two feeding studies in rats and the two skin painting studies in mice, the FDA concluded that the sensitivity of the feeding studies was insufficient under current standards to provide the requisite demonstration of safety for ingested use of 1-(2,4-dinitrophenylazo)-2-naphthol and therefore required additional feeding studies in the February, 1977, order (FDA, 1986). Therefore, a further chronic feeding study in mice

and a combined chronic toxicity and carcinogenicity study with in-utero exposure of rats were carried out according to valid guidelines and in co-ordination with the FDA.

Studies from the late 1970s and early 1980s

In order to study the carcinogenic activity of 1-(2,4-dinitrophenylazo)-2-naphthol, a long-term feeding study was carried out in which groups of 60 male and 60 female Charles River albino (CD) rats were fed the dye in their diet at concentration levels of 0 (control group 1A), 0 (control group 1B), 0.02, 0.05 and 0.1% (equivalent to approx. 13, 33 and 67 mg/kg body weight/day, respectively). The batch of 1-(2,4-dinitrophenylazo)-2-naphthol used in the study was 97% pure, containing 0.29% dinitroaniline and 0.7% β-naphthol, according to analyses carried out by the FDA (1986). After a minimum feeding period of 60 days, the animals were mated by 1 : 1 pairing within each test group. Dietary administration of test substance was continued during mating, gestation, lactation and rearing. The pups were weaned from their mothers 21 days after delivery, litters were housed separately and feeding with the appropriate levels of 1-(2,4-dinitrophenylazo)-2-naphthol was continued for another 2 weeks. Subsequently, 2 male and 2 female F_1 rats per dose group were randomly selected from each litter for the long-term study such that each dose group and control group comprised 70 male and 70 female rats. The animals from each group were fed the appropriate diet for the entire duration of the chronic study, and all survivors/sex/group were sacrificed and necropsied at months 27 (males) and 28 (females). Ten animals/sex/group were sacrificed and necropsied after a treatment period of 12 months in order to study any potential chronic toxicity effects. The male rats treated with the intermediate and high doses of 1-(2,4-dinitrophenylazo)-2-naphthol had slightly lower body weights (not reduced more than 5%) than concurrent controls during the first year of the study. Body weights of all treated male and female rats were comparable with controls for the remainder of the study period. Aside from colouration of the coats of the treated animals, 1-(2,4-dinitrophenylazo)-2-naphthol produced no treatment-related effects on general physical condition or mortality nor on haematological, ophthalmological or urinalysis measurements. The following alterations in clinical chemistry parameters were described as being substance-related. Creatinine levels in treated animals were lower

than the normal range of values for creatinine. Markedly reduced creatinine levels were seen in the males of all dose groups and the top-dose females at 24 months. Elevated mean fasted glucose levels were noted in the top-dose females at 12 months, in all males at 18 months and in male and female rats of the 0.1% group at 24 months. The increase in relative liver weights noted in the males and female treated with 0.1% 1-(2,4-dinitrophenylazo)-2-naphthol were interpreted as an indication that the liver was the main target organ for the test substance. A large number of neoplastic and non-neoplastic changes were observed in the tissues of both test and control animals. Occurring with comparable frequency and distribution, they were therefore not considered to be substance-related (no further details). In contrast, high-dose female rats (0.1%) showed a significant increase in the incidence of tumours of the lymphoreticular system (lymphosarcoma, reticulum cell sarcoma and leukaemia), though none of the individual tumour types was significantly increased in incidence compared with the controls (see Table 4; Bio/dynamics, 1982 a).

Table 4. Incidence of lymphoreticular tumours in rats fed 0.1% 1-(2,4-dinitrophenylazo)-2-naphthol in their diet for more than 2 years

Test group	Number of rats with lymphoreticular tumours/ total number of rats examined	
	Males	Females
Control group 1A	1/59	3/57
Control group 1B	0/58	0/55
0.1% 1-(2,4-dinitrophenylazo)-2-naphthol	1/60	7/55 (significantly greater than combined female control incidence)
These analyses did not include the rats sacrificed after 12 months' treatment since they were not found to have any tumours of importance.		

The FDA considered the dosage levels used in the study to be too low and therefore recommended that an additional long-term feeding study be carried out with 1% 1-(2,4-dinitrophenylazo)-2-naphthol (approx. 667 mg/kg body weight/day) using the same experimental design (in-utero exposure). A concurrent control group of 70 male and 70 female rats was included. The F_1 generation animals were administered the appropriate diet for 26 (males) and 30 (females) months. As in the first study, 10 males and 10 females from the test group and the control group were sacrificed and necropsied at 12 months. The rats treated with 1% 1-(2,4-dinitrophenyl-

azo)-2-naphthol had significantly elevated mean food consumption values relative to controls. Body weights of the females in this test group were similar to those of the female controls. In contrast, treated male rats gained less body weight compared with controls and on average weighed 18.5% less than control animals at study termination. Aside from test material colouration of the coats, the rats in the group receiving 1% 1-(2,4-dinitrophenylazo)-2-naphthol showed no clinical effects. Clinical chemistry tests also revealed no substance-related alterations except for discolouration of the urine. At most clinical pathology intervals, slight to statistically significant reductions in haemoglobin concentration, haematocrit and erythrocyte count were noted in treated animals, often with concomitant increases in reticulocyte count. Furthermore, increased deposition of pigment in the spleen of female rats was noted in the group treated with 1% 1-(2,4-dinitrophenylazo)-2-naphthol in addition to increased liver weights, which were also seen in rats fed 0.1% 1-(2,4-dinitrophenylazo)-2-naphthol. The increases in liver weights and the increased incidence of pericholangitis in the animals of the 1% group sacrificed at 12 months were considered to indicate that the liver was the target organ for 1-(2,4-dinitrophenylazo)-2-naphthol. The female rats receiving continuous dietary treatment with 1% 1-(2,4-dinitrophenylazo)-2-naphthol for 30 months were noted to have a significant increase in the incidence of benign hepatocellular adenomas or neoplastic nodules (see Table 5).

Beginning of Table 5

Table 5. Incidence of liver tumours in Charles River albino (CD) rats following administration of 1% 1-(2,4-dinitrophenylazo)-2-naphthol in the diet for 26 months (males) and 30 months (females); 70 male and 70 female rats/group at study initiation; interim sacrifice of 10 male and 10 female rats/group after a 12-month feeding period

Test group	Number of rats with liver tumours/ total number of rats examined			
	Adenomas	Carcinomas	All tumours	Investigator
Male rats				
Controls	1/56	0/56	1/56 (2%)	Bio/dynamics
1% 1-(2,4-dinitrophenylazo)-2-naphthol	0/56	2/56	2/56 (4%)	Bio/dynamics

Table 5. Incidence of liver tumours in Charles River albino (CD) rats following administration of 1% 1-(2,4-dinitrophenylazo)-2-naphthol in the diet for 26 months (males) and 30 months (females); 70 male and 70 female rats/group at study initiation; interim sacrifice of 10 male and 10 female rats/group after a 12-month feeding period

Test group	Number of rats with liver tumours/ total number of rats examined			
	Adenomas	Carcinomas	All tumours	Investigator
Female rats				
Controls	1/52	2/52	3/52 (6%)	Bio/dynamics
			3/70 (4%)[1)]	FDA
1% 1-(2,4-dinitrophenylazo)-2-naphthol	18/59	4/59	21/59 (36%)[2)]	Bio/dynamics
			21/70 (30%)[1), 2)]	FDA
Historical controls (12 groups), given only as a percentage, not in absolute numbers			(9.4%)	Bio/dynamics

[1)] The FDA included in its assessment all livers examined by light microscopy, including those from the animals sacrificed at 12 months.
[2)] One animal had both an adenoma and a carcinoma.

End of Table 5

In addition, the females in the group treated with 1% 1-(2,4-dinitrophenylazo)-2-naphthol were observed to have an increased incidence of proliferative, non-neoplastic ultrastructural alterations in the liver classified as eosinophilic and clear cell foci.

Table 6. Incidence of eosinophilic and clear liver foci in female Charles River albino (CD) rats after dietary administration of 1% 1-(2,4-dinitrophenylazo)-2-naphthol for 30 months

Test group	Number of female rats with liver foci/ total number of rats examined		
	Eosinophilic foci	Clear foci	Investigator
Controls	1/52	6/52	Bio/dynamics
1% 1-(2,4-dinitrophenylazo)-2-naphthol	11/59	14/59	Bio/dynamics

It was not stated whether alterations of this kind were also observed in treated male rats. In addition, the treated females were noted to have an increased incidence, relative to the control, of focal vacuolation, bile duct proliferation, pericholangitis and subacute, nonsuppurative hepatitis (Bio/dynamics, 1982 a). According to Dr. Squire's light microscopy studies, 27 out of 52 females in the group receiving 1% 1-(2,4-dinitrophenylazo)-2-naphthol had zonal necrosis and degeneration, but only one female control

animal exhibited hepatocellular hypertrophy. (Note: It is not clear from the results as described by the CTFA (1983) whether Dr. Squire's findings represent additional histopathological findings or diagnoses which differ from those reported by the Bio/dynamics pathologists). Light microscopy revealed that the animals treated with 1% 1-(2,4-dinitrophenylazo)-2-naphthol had no significantly increased incidence of gliomas of the brain and, in addition, that the incidence of mammary fibroadenomas in treated females was not significantly increased relative to the concurrent controls. The increased incidence observed in the previous rat feeding study of lymphoreticular tumours in the females fed 0.1% 1-(2,4-dinitrophenylazo)-2-naphthol was not seen in this study in which the rats were fed a ten times higher level (1%) of 1-(2,4-dinitrophenylazo)-2-naphthol (CTFA, 1983; FDA, 1986).

- **FDA assessment of the second rat feeding study with 1% "D&C Orange No. 17" and the hepatocarcinogenic potential of "D&C Orange No. 17" under the intended conditions of use by humans, and revocation of the "*de minimis* interpretation of the Delaney clause" by a court decision**

The discussion of the liver tumour findings from this study emphasised that only the incidence of benign hepatocellular adenomas, not that of hepatocellular carcinomas, was significantly increased and that this was only the case in female rats which also showed a spectrum of toxic and proliferative lesions. This suggests that the tumorigenic effect apparently occurring only at high doses that are toxic to the liver is likely to be due to an indirect mechanism.

Such a situation could not occur in humans because they would never be exposed to such high levels of "D&C Orange No. 17". Assuming that a woman used cream-based blusher/rouge, face powder and nail polish/enamel, all containing the maximum requested concentration of "D&C Orange No. 17", extremely frequently every day, the estimated "worst-case" absorption would be 0.015 to 0.021 µg. For a 53 kg woman, this represents an absorbed amount of 0.00029 to 0.00039 µg/kg body weight/day. This is approximately equivalent to a dietary intake of 0.000010 to 0.000014 ppm assuming a daily intake of 1.5 kg of food (CTFA, 1983). In the FDA's opinion, too, hepatocellular carcinomas by themselves were not significantly increased in female rats relative to controls. Referring to a 1979 report of

the Interagency Regulatory Liaison Group, the FDA considered it appropriate, however, to combine the incidences of malignant and benign hepatocellular tumours for analysis of such a study because all these lesions (neoplastic nodules, adenomas and carcinomas) could be concurrent or sequential stages in the neoplastic process. When carried out in this manner, data analysis showed that, at a level of 1%, 1-(2,4-dinitrophenylazo)-2-naphthol was a weak carcinogen in female CD rats in comparison with both concurrent and historical controls. As oral absorption of 1-(2,4-dinitrophenylazo)-2-naphthol is possible under the intended conditions of use, the Delaney Clause was considered to be applicable to risk assessment in the USA. However, the risk calculated for human use of "D&C Orange No. 17" is extremely low. For this reason, with reference to the "*de minimis* doctrine", the FDA decided not to apply the Delaney Clause to the external use of the colour additive as the external use of the product in drugs and cosmetics posed an extremely low risk to humans "of 1 in 19 billion" and the product could therefore be considered safe for humans under the intended conditions of use (FDA, 1986, 1987). This FDA rule was revoked by a court decision in 1988, the court stating in its conclusion that: "In sum we hold that the agency's *de minimis* interpretation of the Delaney Clause of the Color Additive Amendments is contrary to law. The listing decisions for D&C Orange No. 17 and D&C Red No. 19 based on that interpretation must therefore be corrected." Hence, "D&C Orange No. 17" had to be removed from the list of colour additives for external use in cosmetics and drugs in the USA (FDA, 1988).

A long-term feeding study was carried out in Charles River CD-1 mice in order to investigate the carcinogenic effect of 1-(2,4-dinitrophenylazo)-2-naphthol. Groups of 60 males and 60 females were exposed to 1-(2,4-dinitrophenylazo)-2-naphthol at dietary levels of 0.025, 0.25 or 1% (equivalent to approx. 36, 360 or 1460 mg/kg body weight/day) for 23 months (males) and 25 months (females). The test substance levels were selected on the basis of a preliminary 90-day study (no further details). The 1-(2,4-dinitrophenylazo)-2-naphthol used was of 97% analytical purity. According to FDA analyses, the 1-(2,4-dinitrophenylazo)-2-naphthol used also contained 0.29% 2,4-dinitroaniline and 0.7% β-naphthol. Two independent untreated groups of 60 male and 60 female Charles River CD-1 mice served as controls. The males in the highest dose group showed slightly depressed body weight gain relative to male control mice during the study. Body weight gain

in the other treated males and females was comparable to controls. Mortality in the male mice treated with the intermediate or high dose of 1-(2,4-dinitrophenylazo)-2-naphthol was slightly but not significantly increased in a concentration-dependent manner relative to the controls. In contrast, survival in the treated female mice was not reduced, except in the animals in the 0.25% group. Aside from 1-(2,4-dinitrophenylazo)-2-naphthol discolouration of the coats of the treated mice, there were no significant treatment-related effects on general physical condition, food consumption or incidence of palpable tissue masses. The mice in the 1% group often had slight but significant reductions in mean haemoglobin concentration, haematocrit and erythrocyte counts with concomitant reticulocyte counts relative to the corresponding controls in this study. Examination by light microscopy revealed pigment deposition in the brain, spinal cord, thyroid gland, heart and stomach in the animals of all groups treated with 1-(2,4-dinitrophenylazo)-2-naphthol. Severity of yellow-brown pigment deposition was seen to increase in a dose-related fashion. The animals in the high dose group additionally showed pigment deposition in the spleen, caecum and liver. Furthermore, the neurons of the brain and spinal cord of the male and female mice in the 1% group were noted to have an increased incidence of neuronal basophilia. Chronic myocarditis was diagnosed in the majority of treated animals.

Table 7. Incidence of chronic myocarditis in Charles River CD-1 mice after 23 (males) and 25 (female) months' dietary administration of 1-(2,4-dinitrophenylazo)-2-naphthol

Test group	Number of mice with chronic myocarditis/ total number of mice examined	
	Male mice	Female mice
Control group A	49/60	38/59
Control group B	57/60	44/59
0.025% 1-(2,4-dinitrophenylazo)-2-naphthol	46/58	33/58
0.25% 1-(2,4-dinitrophenylazo)-2-naphthol	39/60	31/60
1% 1-(2,4-dinitrophenylazo)-2-naphthol	55/60	54/59

The incidence of hepatocellular tumours (adenomas and carcinomas) was significantly increased in male mice in a dose-dependent manner. In female mice, only the 0.25 and 1% groups were noted to have increases in liver tumours (adenomas and carcinomas) compared with controls (see Table 8).

Table 8. Incidence of hepatocellular adenomas and carcinomas in Charles River CD-1 mice after 23 (males) and 25 (female) months' dietary administration of 1-(2,4-dinitrophenylazo)-2-naphthol				
Test group	Number of mice with liver tumours/ total number of mice examined			Investigator
	Adenomas	Carcinomas	Total number*	
Male mice				
Control group A	8/60	6/60	11/60 (18%)	Bio/dynamics
	7/60	3/60	10/60 (17%)	Dr. Squire
			8/58 (14%)	FDA
Control group B	6/60	4/60	9/60 (15%)	Bio/dynamics
	5/60	2/60	7/60 (12%)	Dr. Squire
			8/59 (14%)	FDA
0.025% 1-(2,4-dinitro-phenylazo)-2-naphthol	7/58	6/58	12/58 (21%)	Bio/dynamics
			11/58 (19%)	FDA
0.25% 1-(2,4-dinitro-phenylazo)-2-naphthol	7/59	8/59	14/59 (23%)	Bio/dynamics
			13/57 (23%)	FDA
1% 1-(2,4-dinitro-phenylazo)-2-naphthol	11/60	10/60	20/60 (33%)	Bio/dynamics
	12/60	7/60	19/60 (32%)	Dr. Squire
			19/56 (34%)	FDA
Female mice				
Control group A	1/60	0/60	1/60 (2%)	Bio/dynamics
Control group B	1/59	1/59	2/59 (3%)	Bio/dynamics
0.025% 1-(2,4-dinitro-phenylazo)-2-naphthol	2/60	0/60	2/60 (3%)	Bio/dynamics
0.25% 1-(2,4-dinitro-phenylazo)-2-naphthol	5/60	0/60	5/60 (8%)	Bio/dynamics
1% 1-(2,4-dinitro-phenylazo)-2-naphthol	3/59	2/59	5/59 (8%)	Bio/dynamics
* Three males in control group A and one male in each of the other groups had both adenoma and carcinoma.				

The various sources on liver tumour incidences in male mice (Pathology Report in Bio/dynamics, 1982 b; CTFA, 1983; histopathological examination by Dr. R.A. Squire (CTFA, 1983)) and the FDA (1986) statement give slightly different tumour incidences. The FDA attributed this to the fact that FDA assessment was based solely on livers the tissues of which were deemed suitable for examination by light microscopy by FDA pathologists. No other significant neoplastic or non-neoplastic ultrastructural alterations were noted in the treated mice. Approximately 2 years' dietary administration of 1-(2,4-dinitrophenylazo)-2-naphthol at concentration levels of 0.025, 0.25 or 1% to Charles River CD-1 mice resulted in a significant dose-related increase in benign and malignant hepatocellular tumours in the males,

but not the females, in the intermediate and high dose groups (Bio/dynamics, 1982 b; CTFA, 1983; FDA, 1986).

In the following, the biological significance is discussed of the liver tumours noted in the feeding study in Charles River CD-1 mice. The CTFA (1983) contested the biological significance of the liver tumours noted to occur more frequently in male CD-1 mice, giving the following reasons. Liver tumours are found to be more frequent in many strains of mice due to concurrent retrovirus infection. The male mice were only found to have an increased incidence of liver tumours in conjunction with severe hepatotoxicity in the highest dose group fed 1% 1-(2,4-dinitrophenylazo)-2-naphthol, a level which exceeded the maximum tolerated dose. The incidence of hepatocellular tumours in male mice was statistically significantly increased only in relation to concurrent control data but not in comparison with the controls from 7 long-term feeding studies that were also sponsored by the CTFA to investigate other chemicals in CD-1 mice. These control groups had incidences of male mouse hepatocellular tumours that ranged from 8.5 to 26.2%. The CTFA argued that the incidence of hepatocellular tumours in male mice fed the highest level of 1-(2,4-dinitrophenylazo)-2-naphthol (1%) was 31.7%, based on light microscopic re-examination of the liver histopathology slides by Dr. Squire. Although liver tumour incidence was significantly greater ($p = 0.006$ by Fisher's exact test) than the incidence of 14.2% in the combined control groups A and B, it was not significantly greater than the general range of incidence (8.5 to 26.2%) of liver tumours in male controls in the other 7 feeding studies. According to the CTFA, the high and spontaneous incidence of liver tumours in male CD-1 mice therefore suggested that no biological significance could be attributed to the increased incidence of liver tumours that occurred in the feeding study with 1-(2,4-dinitrophenylazo)-2-naphthol (CTFA, 1983).

Note: The CTFA's argumentation was based solely on the liver tumour incidences established by Dr. Squire's light microscopic examination of the slides. The FDA, on the other hand, later only re-examined those livers from the 2-year CD-1 mouse feeding study with 1-(2,4-dinitrophenylazo)-2-naphthol by light microscopy that were deemed suitable by FDA pathologists (FDA, 1986). This explains why the numbers of animals differ.

Table 9. Number of male mice with benign liver tumours in the two control groups relative to the total number of control mice examined				
Control groups		Total number		Investigator
A	B	Absolute figures	%	
11/60	9/60	20/120	16.7	Bio/dynamics
10/60	7/60	17/120	14.2	Dr. Squire
8/58	8/59	16/117	13.7	FDA

The CTFA's arguments were refuted by the FDA on the following grounds. The group treated with 1% 1-(2,4-dinitrophenylazo)-2-naphthol exhibited slight reduction in body weight gain, slightly increased mortality and yellow colouration of the animals' coats. No other alterations of biological significance were noted that would have been attributable to the administered chemical. Therefore, apart from liver tumours, no significant substance-related effects were observed in the animals. The deaths observed in the dose group given 1% 1-(2,4-dinitrophenylazo)-2-naphthol were not due to systemic toxicity. Hence, the maximum tolerated dose of 1-(2,4-dinitrophenylazo)-2-naphthol was not exceeded in the study. The argument that very many strains of mice show a high incidence of spontaneous liver tumours did not apply to the CD-1 mice used in the study because that strain of mouse has a relatively low spontaneous incidence of liver tumours compared with most other strains. This is evident from the data for 16 control groups (> 900 male and > 900 female mice) of this strain which were made available to the FDA (1986) by the CTFA in 1983.

Table 10. Spontaneous incidence of hepatocellular adenomas and hepatocellular carcinomas in CD-1 mice, given as percentages (> 900 males and > 900 females; according to FDA, 1986)			
	Carcinomas (%)	Adenomas (%)	Total (%)
Males	9.65 ± 4.25	5.12 ± 3.92	approx. 15
Females	0.63 ± 1.03	1.17 ± 1.21	approx. 2

Thus, the incidence of hepatocellular tumours (adenomas and carcinomas) among the male CD-1 mice in the basic control was 15% and, hence, in the range of the concurrent control groups in the feeding study with 1-(2,4-dinitrophenylazo)-2-naphthol. In contrast, the incidences of benign or malignant liver tumours among male mice given 1% 1-(2,4-dinitrophenylazo)-2-naphthol were 33% according to Bio/dynamics and 34% according to FDA

analysis, whereas both Bio/dynamics and the FDA found 23% for the group given 0.25% 1-(2,4-dinitrophenylazo)-2-naphthol. Thus, the incidence of liver tumours among male mice treated with 1-(2,4-dinitrophenylazo)-2-naphthol was increased not only relative to the concurrent control groups in the 1-(2,4-dinitrophenylazo)-2-naphthol feeding study but also relative to the basic control data for that strain of mouse. Hence, the significantly increased and dose-related incidence of hepatocellular adenomas and hepatocellular carcinomas seen among male CD-1 mice at the intermediate and high dose in the feeding study were caused by the 1-(2,4-dinitrophenylazo)-2-naphthol administered to the animals in their food (FDA, 1986).

7.8 Reproductive toxicity

Studies were carried out to test 25 synthetic colour additives, including 1-(2,4-dinitrophenylazo)-2-naphthol, for teratogenic activity in rats and rabbits. The studies comprised a two-generation study in rats and were carried out at commercial contract research laboratories using a standardised protocol fortified with additional control groups.

Dosing was accomplished by gavage during organogenesis in the rat and rabbit teratogenicity studies. The doses were selected on the basis of the highest no effect levels observed in previous 2-year feeding studies in rats and dogs. There was no evidence of substance-related skeletal or soft tissue abnormalities in the foetuses from dams treated during organogenesis (no further details given in the abstract; Burnett et al., 1974).

In the two-generation study, 1-(2,4-dinitrophenylazo)-2-naphthol was administered to the rats in their diet, as were the other 24 colour additives. The doses administered in this study were based on the 1, 10, 30 and 100-fold multiples of the A.D.I. (acceptable daily intake), or of the projected “safe dose” determined from data of previous long-term feeding studies in rats and dogs. Human usage level of the colour additive was also a factor in determining the concentration levels used in the diet. No details of the dose levels of the individual colour additives, including 1-(2,4-dinitrophenylazo)-2-naphthol, were given in the available abstract. However, dose levels never exceeded 1000 mg/kg body weight. The rats’ reproductive performance gave no indication of adverse effects of the colour additive down to the F_{2b} generation (no further details given in the abstract; Pierce et al., 1974).

7.9 Effects on the immune system

No information available.

7.10 Neurotoxicity

No information available.

7.11 Other effects

No information available.

8 Experience in humans

Patch tests were carried out between 1980 and 1985 in a total of 365 patients suffering from cosmetic dermatitis (n = 166), facial melanosis (n = 3) or non-cosmetic dermatitis or eczema (n = 196) in order to test for sensitivity to 1-(2,4-dinitrophenylazo)-2-naphthol. The substance was applied as a 1-percent formulation. The reaction results were negative in all patients and a control group (n = 51; Itoh et al., 1986).

Eight patients suffering from pigmented contact dermatitis caused by Brilliant Lake Red R showed no cross-sensitivity between 1-(2,4-dinitrophenylazo)-2-naphthol and Brilliant Lake Red R (1-phenylazo-2-hydroxy-3-naphthalenecarboxylic acid). The test concentrations used were 1% in petrolatum. The skin reactions were read according to the ICDRG (International Contact Dermatitis Research Group) classification 24 hours after the patches were removed (Kozuka et al., 1980).

9 Classifications and threshold limit values

No information available.

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