

**Opinion of the Scientific Panel on Food Additives, Flavourings,
Processing Aids and Materials in Contact with Food**
on the food colour Red 2G (E128)
**based on a request from the Commission related to the re-evaluation
of all permitted food additives**

Question number EFSA-Q-2007-126

Adopted on 5 July 2007

SUMMARY

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food has been asked to re-evaluate all currently permitted food additives. The current opinion is a re-evaluation of the synthetic food colouring substance Red 2G following a pre-evaluation of the available data.

Red 2G has been evaluated previously by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 and 1981 and by the EU Scientific Committee for Food (SCF) in 1975. Both bodies allocated an Acceptable Daily Intake (ADI) for Red 2G of 0.1 mg/kg body weight (bw). It was also evaluated by the Nordic Council of Ministers in 2002. The present opinion briefly reports the major studies evaluated by these three bodies and describes the new data available in the literature since 1999 in more detail.

Red 2G is the common name for the mono-azo colour disodium 8-acetamido-1-hydroxy-2-phenylazo-naphthalene-3,6-disulphonate. In the European Union (EU), it is permitted only for use in breakfast sausages with a minimal cereal content of 6 % and burger meat with a minimum vegetable and/or cereal content of 4 %. In both foodstuffs a maximum level of 20 mg/kg is allowed (Directive 94/36/EC).

Considering specifically those foodstuffs in which Red 2G is permitted, and assuming they contain the maximum permitted level of 20 mg/kg, it can be calculated that consumption of 100 g breakfast sausages or 100 g burger meat could result in a dietary exposure of 2 mg Red 2G.

The Panel noted that Red 2G is extensively metabolised to aniline. The Panel noted that the current genotoxicity and carcinogenicity databases on Red 2G and its metabolite, aniline, are limited. However both genotoxic and carcinogenic effects have been observed in rodents treated with aniline.

The Panel based its evaluation on the conclusions of the EU Risk Assessment on aniline. This concluded that aniline should be considered as a carcinogen for which a genotoxic mechanism cannot be excluded, based on the following:

- aniline was genotoxic *in vivo* in rats and mice,
- there was insufficient mechanistic evidence to discount a genotoxic mechanism for the rodent carcinogenicity,
- based on similar metabolism of aniline in animals and humans a carcinogenic risk for man cannot therefore be excluded.

Therefore the Panel concluded that it would be prudent to regard Red 2G as being of safety concern since it is extensively metabolised to aniline. Based on these considerations, the Panel withdrew the ADI for Red 2G.

KEYWORDS:

Red 2G, Acid Red 1, Azophloxin, E 128, CAS 3734-67-6, Disodium 8-acetamido-1-hydroxy-2-phenylazo-naphthalene-3,6-disulphonate, Food colour.

BACKGROUND

According to the framework Directive 89/107/EEC¹ on food additives, the Scientific Committee on Food (SCF) must be consulted before the adoption of provisions likely to affect public health, such as the drawing up of lists of additives and the conditions for their use. Accordingly, all food additives have been evaluated for their safety by the SCF prior to their authorisation.

The framework Directive also requires that food additives must be kept under continuous observation and must be re-evaluated whenever necessary in the light of changing conditions of use and new scientific information.

Accordingly, the SCF has followed-up and re-evaluated additives when new scientific data had been requested or became otherwise available. However, due to lack of resources and to the heavy workload of the SCF, no high priority could be given to an additional, systematic follow-up of the evaluation of food additives.

There are a number of reasons why the Commission now wishes to start with a systematic re-evaluation of food additives:

- (a) The report from the Commission on Dietary Food Additive Intake in the European Union² has shown that the intake of some food additives possibly exceeds the Acceptable Daily Intake (ADI).
- (b) The Commission is currently preparing a proposal for an amendment of the framework Directive on food additives as announced in the White Paper on Food Safety. In this context, the Commission intends to introduce a requirement for a systematic re-evaluation of all authorised food additives into this legislation.
- (c) In the context of the amendment of Directive 95/2/EC on food additives other than colours and sweeteners, the Commission has agreed to present a status report about the re-evaluation of food additives to the European Parliament, in particular for those additives that were identified as possibly exceeding the ADI in the intake report.
- (d) Recently, a report entitled “Food additives in Europe 2000” has been submitted by the Nordic Council of Ministers to the Directorate-General Health and Consumer Protection. This report gives a good basis for prioritisation of additives for the re-evaluation mentioned above. It examines whether the safety evaluations on food additives done by the SCF are still valid and adequate in the light of present day standards for safety assessments. Furthermore, it examines whether significant new toxicological studies have been published since the latest evaluation of a substance by the SCF.

¹ Council Directive 89/107/EEC on the approximation of the laws of the Member States concerning food additives authorized for use in foodstuffs intended for human consumption, OJ L 40, 11.2.1989, p. 27

² COM(2001) 542 final

Synthetic colours were among the first additives to be evaluated, therefore, many of the evaluations are old. For some of these colours many new studies have become available and the results of these studies should be included in the evaluation. The report of the Nordic Council draws the same conclusion.

TERMS OF REFERENCE

The Commission asks the European Food Safety Authority to start a systematic re-evaluation of authorised food additives and to issue scientific opinions on these additives, taking into account the prioritisation as follows:

Colours as a group should be given the highest priority for re-evaluation for the reasons outlined above.

ASSESSMENT

Chemistry

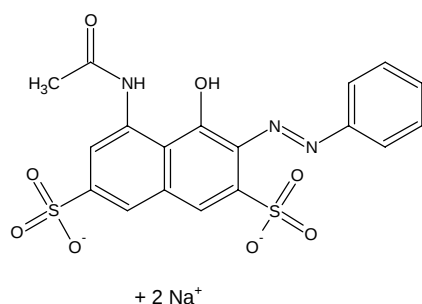
Description: Red powder or granules
Functional class: Colour
Colour class: Mono-azo
Synonyms: At least 87 synonyms are in use (ChemIDplus advanced, via internet, 2006). The most commonly used synonyms in published literature are Red 2G and Acid Red 1.

CAS Registry number: 3734-67-6
EINECS number: 223-098-9
Colour Index number: 18050

Chemical name: Disodium 8-acetamido-1-hydroxy-2-phenylazo-naphthalene-3,6-disulphonate

Chemical formula: $C_{18}H_{13}N_3Na_2O_8S_2$

Structural formula:



Physico-chemical properties

Red 2G has a molecular weight of 509.43 Da. It is soluble in water and sparingly soluble in ethanol.

Manufacturing process

No data on the manufacture of Red 2G were available. Red 2G may be converted to the corresponding aluminium lake under aqueous conditions by reacting aluminium oxide with the colouring matter. Undried aluminium oxide is usually freshly prepared by reacting aluminium sulphate or aluminium chloride with sodium carbonate or sodium bicarbonate or aqueous ammonia. Following lake formation, the product is filtered, washed with water and dried (JECFA, 2004).

Specifications for Red 2G according to Commission Directive 95/45/EC and Codex Alimentarius are given below.

Description

Red 2G consists essentially of disodium 8-acetamido-1-hydroxy-2-phenylazonaphthalene-3,6-disulphonate and subsidiary colouring matters together with sodium chloride and/or sodium sulphate as the principal uncoloured components. Red 2G is described as the sodium salt. The calcium and the potassium salt are also permitted (EC, 1995). Aluminium lakes of Red 2G are also permitted.

Specification on assay and purity

Red 2G

Assay: $E_{1\text{cm}}^{1\%}$ 620 at ca. 532 nm in aqueous solution

Purity: Content not less than 80 % total colouring matters, calculated as the sodium salt.

Table 1. Specifications for Red 2G according to Commission Directive 95/45/EC and Codex Alimentarius

Purity	Commission Directive 95/45/EC	Codex Alimentarius
Water insoluble matter	≤ 0.2 %	≤ 0.2 %
Subsidiary colouring matters	≤ 2.0 %	≤ 2.0 %
- 5-acetamido-4-hydroxynaphthalene-2,7-disulphonic acid - 5-amino-4-hydroxynaphthalene-2,7-disulphonic acid	≤ 0.5 %	≤ 0.3 %
Unsulphonated primary aromatic amines	≤ 0.01 % (calculated as aniline)	≤ 0.01 % (calculated as aniline)
Ether extractable matter	≤ 0.2 % (under neutral conditions)	≤ 0.2 %
Arsenic	≤ 3 mg/kg	-
Lead	≤ 10 mg/kg	≤ 2 mg/kg
Mercury	≤ 1 mg/kg	-
Cadmium	≤ 1 mg/kg	-
Heavy metals (as Pb)	≤ 40 mg/kg	-

For the aluminium lake of Red 2G

According to EU legislation (EC, 1995), the above purity criteria for the pure substance also apply to the aluminium lake. In addition, the aluminium lake should contain no more than 0.5% HCl-insoluble material and no more than 0.2% ether-extractable material under neutral conditions. There are no additional specification requirements for the aluminium lake (EC, 1995).

Codex Alimentarius does not give specifications for aluminium lakes of Red 2G, other than reference to the General Specifications for Aluminium Lakes of Colouring Matters (JECFA, 2004). The Red 2G used in the production process should comply with the specifications as given above and the aluminium lake should contain not more than 2% water-soluble chlorides and sulphates calculated as sodium salts, not more than 0.5% hydrochloride acid-insoluble matter, 0.2% ether-extractable matter, 3 mg arsenic/kg and 5 mg lead/kg. According to EU specifications, unreacted aluminium oxide may also be present in the final product (not specified).

Methods of analysis in food

No formal method for analysis in food seems to have been described but there are methods available in the open literature, Red 2G in food colouring preparation can be analyzed by means of titration with titanous chloride. In this chemical analytical procedure the colour sample is dissolved in a sodium citrate or sodium hydrogen tartrate buffer and heated to boiling under a steady flow of carbon dioxide. This solution is titrated drop wise with a standardized titanous chloride solution. In this process the colour will act as its own indicator (Codex Alimentarius, via internet, 2006).

Reaction and fate in food

No data were available.

Exposure

Uses in foods and maximal permitted usage levels

Red 2G is exclusively permitted for use in breakfast sausages with a minimal cereal content of 6 %, and burger meat with a minimum vegetable and/or cereal content of 4 %. In both foodstuffs a maximum level of 20 mg/kg is allowed (EC, 1994).

Actual levels of use of Red 2G in foods

No data on actual levels of use of Red 2G were made available to the Panel.

Dietary exposure to Red 2G

Considering specifically those foodstuffs in which Red 2G is permitted, and assuming they contain the maximum permitted level of 20 mg/kg, it can be calculated that consumption of 100 g breakfast sausages or 100 g burger meat could result in dietary exposure of 2 mg Red 2G.

Toxicological data

Red 2G has been evaluated previously by the WHO/FAO Joint Expert Committee on Food Additives (JECFA) in 1977 and 1981 and the EU Scientific Committee for Food (SCF) in 1975. It was also evaluated by the Nordic Council of Ministers (TemaNord, 2002). The present opinion briefly reports the major studies evaluated in these opinions and describes the additionally reported new literature data in some more detail.

Absorption, Distribution, Metabolism, Excretion

JECFA describes several studies on the toxicokinetic aspects of Red 2G.

After administration of 250 mg/kg bw Red 2G to rats by gastric intubation, male and female urinary excretion was on average 61.8% and 71.5% of the dose. In 48 hours in males and females 42.2% and 56.4% of the dose was excreted as p-aminophenol. In 24 hours in males and females 9.2% and 2% of the dose was excreted as aniline, and 3% and 2.6% of the dose was excreted as unreduced dye.

Faecal excretions for males and females were 6.3% and 8.6% of the dose as p-aminophenol, 1% and 0.3% of the dose as aniline, and 0.1% and 1.6% of the dose as unreduced dye (no detail on which percentage of the dose was excreted in total) (Walker, 1971).

In rats intravenously injected with Red 2G on average 64% of the administered quantity was recovered from the bile in 6 hours (Priestly and O'Reilly, 1966). Biliary

excretion after intravenous administration of Red 2G was also reported by Ryan and Wright (1961).

Two metabolites were detected in the contents of a rat caecum after incubation with a solution of Red 2G at 37°C. These were, aniline, and 2-amino-8-acetamido-1-naphtho-3,6-disulphonic acid. After incubation of Red 2G with liver homogenate the same two metabolites were detected (Jenkins *et al.*, 1966a).

After incubation of caecal contents with Red 2G at 37°C darkening at the surface was observed. This was presumed due to oxidation of a sulphur-containing metabolite of Red 2G. Two groups of 12 rats were fed diets containing 0 or 0.51% Red 2G (255 mg/kg bw). 48.2% of the sulphur derived from Red 2G was excreted in the faeces. The fate of the other 51.8% of the sulphur or which sulphur-containing metabolite may have been involved was not clarified (Unilever, 1974).

Rabbits were fed 500 mg/kg bw Red 2G in their diet. After a 48 hours urine collection the following metabolites were identified: total p-aminophenol (free and conjugated); p-aminophenylglucuronide, o-aminophenol, and aniline, (Daniel, 1962). The ratio of o-aminophenol to p-aminophenol after Red 2G administration was similar to the ratio found after aniline administration as previously examined by Parke (1960).

No binding of Red 2G to serum protein occurs (no further details) (Jenkins *et al.*, 1966b).

The SCF opinion does not specifically address toxicokinetics.

No new toxicokinetic data on Red 2G has been published since the TemaNord evaluation.

Acute oral toxicity

JECFA describes several acute oral toxicity studies which were conducted in a range of animal species.

The LD₅₀ values derived from the tests were: mouse, 7350 mg/kg bw; rat, more than 5000 mg/kg bw; guinea-pig, 4810 mg/kg bw; rabbit, more than 25 000 mg/kg bw (2 day exposure) and chicken, more than 10.000 mg/kg bw.

Apart from determining the LD₅₀ values several histopathological observations were made.

Administration of Red 2G to mice led to gross leptomeningeal vascular engorgement and focal subarachnoid haemorrhage. Histological examination of tissues of rats, guinea-pigs, and rabbits displayed extensive renal necrosis which was not seen in chickens. In rabbits haematological analysis did not show Heinz body production (Unilever, 1974).

In addition to these studies JECFA describes four acute (single dose) studies with aniline and phenylhydroxylamine, two of the metabolites of Red 2G. In the JECFA

evaluation these are described under the headings 'Special studies on aniline' and 'Special studies on haemotoxicity and haemopoiesis'.

In an acute human aniline poisoning methaemoglobin formation occurred followed by irreversible Heinz body appearance (Freifield *et al.*, 1937; Hughes and Treon, 1954; Rodek and Westhouse, 1952).

In 20 human subjects administration of single oral doses of aniline ranging from 25 to 65 mg/kg bw significantly increased methaemoglobin levels but no Heinz bodies were observed. No effects were observed at doses of 5 or 15 mg/kg bw (Jenkins *et al.*, 1972).

Aniline (no details on dosage) was administered to rats by stomach tube. Blood sampling every 30 minutes and further on in the study every 60 minutes gave a no-effect dose of 20 mg/kg bw (no details on which parameters were researched) (Jenkins *et al.*, 1972).

Groups of rats (6 per sex) were fed diets containing 0.098% aniline, or molecular equivalent levels of phenylhydroxylamine, or p-aminophenol. In rats that received aniline or phenylhydroxylamine, methaemoglobinaemia, Heinz bodies, and splenic enlargement were observed. The no-effect single oral dose of aniline was 20 mg/kg bw (Jenkins *et al.*, 1972).

The SCF opinion does not describe acute toxicity. In the TemaNord evaluation acute oral toxicity is not specifically addressed although some of the studies on aniline or haemotoxicity and haemopoiesis are briefly described.

No new data on acute oral toxicity has been published since the previous evaluations.

Short-term and sub-chronic studies

JECFA describes a broad range of short-term studies which have been subdivided under the headings 'short-term studies', 'special studies on aniline', and 'special studies on haemotoxicity and haemopoiesis'. This subdivision is maintained in this re-evaluation.

Short-term studies

A total of five short-term studies investigating Red 2G in mice and rats have been described.

Five groups of 15 male and 15 female mice were given diets containing 0, 0.01, 0.1, 1, or 2% Red 2G (equivalent to 14, 143, 1429, or 2858 mg/kg bw). 5 animals per group were haematologically investigated and fully autopsied at days 26, 55, and 96. A dose-dependent increase in the incidence of Heinz bodies was observed. Splenomegaly was observed in both sexes at 2% and in females at 1%. Relative liver weights were increased in females at 1% and 2% at day 26, and at 2% at days 55 and 96. Haemosiderin was increased in the liver (Kupffer cells) at 1% and 2%. In the spleen haemosiderin was increased at 2% at all test days, and at the other concentrations increased with treatment and duration. No adverse effects on growth or food consumption were evident (BIBRA, 1965).

In another mouse study 5 groups of 10 animals were fed 0, 0.02, 0.1, 0.5, or 1% Red 2G of the diet (equivalent to 0, 29, 143, 715, or 1429 mg/kg bw/d) for 6 weeks. At the top three concentrations the observed effects included increased Heinz body formation, methaemoglobinaemia, splenic enlargement and increased splenic macrophages (Unilever, 1974).

In a rat study six groups (5 per sex) received 0, 0.05, 0.1, 0.5, 1, or 2% Red 2G (equivalent to 0, 25, 50, 250, 500, or 1000 mg/kg bw/d) in their diet for 3 weeks. A further four groups (10 per sex) were given dietary concentrations of 0, 0.01, 0.05, or 0.1% Red 2G (equivalent to 0, 5, 25, or 50 mg/kg bw/d) for 2 months. Concerning these two studies JECFA also describes study results observed at doses of 2.5 and 5% although these are not included in the doses mentioned above.

At the 2.5 and 5% dose levels, retarded growth was associated with reduced food consumption after 9 days. Blood samples revealed macrocytosis, reticulocytosis and polychromasia with circulating normoblasts and a normoblastic marrow at a level of 5%. Heinz bodies were present at the 1 and 2% after 9 days and also at the 0.5% level after three weeks of exposure. Signs indicative of increased erythropoiesis were observed at doses from 0.1%. Significant splenomegaly was evident at 1 and 2%. Kidney weight was also increased from levels of 0.1%. Histological examination revealed increased haemosiderin in the liver (Kupffer cells), renal tubule cells, and spleen in some animals at 0.5 and 1%, and all animals at the 2% level (BIBRA, 1965).

Three groups of 12 rats received Red 2G in the drinking-water at levels of 0, 0.1, or 0.5%, corresponding respectively to 0, 0.02 and 0.1 ml/day (assuming an intake of water of 20 ml/day), for 100 days. At the 0.5% level after 10 days Heinz bodies were present but diminished with time. Heinz bodies were only occasionally seen at the 0.1% level. Spleens were enlarged slightly at the 0.1% and very much at the 0.5% levels. Histological examination of the spleens revealed increased erythropoiesis and red pulp engorgement at the drinking water concentration of 0.5%. At the 0.5% level in the liver haemosiderin was present in Kupffer cells and erythropoietic activity was also increased. No effect was observed on urine specific gravity (Jenkins *et al.*, 1966d; Gellatly *et al.*, 1966).

Four groups of rats (24 per sex) were fed diets comprised of 80% sausage meat containing 0, 30 and 180 mg/kg of Red 2G respectively, leading to intakes of 100 or 600 times the assumed average daily dietary intake of Red 2G. In the rat, the meat containing 180 mg/kg of Red 2G increased erythropoiesis, splenic and red pulp haemosiderin, and red pulp reticular impregnation with iron. No effects were observed regarding growth, organ function and weights, blood picture, and liver histology (Jenkins *et al.*, 1966e).

Special studies on aniline

JECFA describes a single study examining the short-term effects of aniline.

In this human study a volunteer received aniline orally for 5 days (10 mg on days 1 and 2, and 25 mg on days 3, 4, and 5). No aberrations were seen in urine samples (urobilinogen, glucose and protein), or blood tests (haemoglobin, methaemoglobin, haematocrit, serum transaminases, alkaline phosphatase, thymol turbidity, serum proteins, serum bilirubin, or Heinz bodies) (Jenkins *et al.*, 1972).

Haemotoxicity and haemopoiesis

JECFA describes nine short-term studies which in its evaluation are categorised under the heading 'special studies on haemotoxicity and haemopoiesis'.

Rats were fed 1000-1500 mg/kg bw/d of Red 2G for 75 days. The mean peak Heinz body level was 80% falling to a maintained level of 30% (no further details for how to interpret these percentages). Furthermore a moderate though well controlled anaemia, pronounced reticulocytosis, and splenomegaly were noted (Rofe, 1957).

Male and female rats were fed 0 or 0.5% Red 2G (equivalent to 0 or 250 mg/kg bw/d) in their diets for 2 weeks. At weekly intervals before, during, and after administration ceased, 3 to 5 rats of each group were sacrificed and examined. The following effects were observed; methaemoglobinaemia, Heinz bodies, reticulocytosis, decreased haemoglobin, decreased haematocrit, decreased red cell count, erythropoiesis increases in liver, spleen, and bone marrow, and increased spleen weight. The results indicated that liver, spleen, and bone marrow respond to Red 2G with an increase in erythropoiesis (Jenkins *et al.*, 1980).

In a rat study animals received purified diets containing 0.1, 0.2, or 0.3% Red 2G (equivalent to 50, 100, or 150 mg/kg bw/d) or 0.004, 0.006, or 0.012% phenylhydroxylamine (equivalent to 2, 3, or 6 mg/kg bw/d). In both cases a linear relationship between intake of the substance and relative spleen weight was observed (Jenkins *et al.*, 1967; Gellatly and Burrough, 1967).

Rats were fed aniline, phenylhydroxylamine, or p-aminophenol at a level of 0.1% (equivalent to 50 mg/kg bw) for 13 days. p-Aminophenol had no effect on spleen weight whereas aniline and phenylhydroxylamine increased mean relative spleen weights by 60% and 500% respectively. After 11 days of phenylhydroxylamine administration a high incidence of Heinz bodies was also observed. Therefore, the toxic effects of feeding aniline were attributed to phenylhydroxylamine which is a metabolite of aniline (Gellatly and Burrough, 1966; Jenkins *et al.*, 1966c).

Three groups of six four-week old female rats were fed 0 or 0.5% Red 2G (equivalent to 0 or 250 mg/kg bw/day), or 0.093% aniline (46.5 mg/kg bw/day) in the diet for 19 days. Both substances caused a similar increase in spleen weight, accelerated erythropoiesis, and haemosiderin content (Jenkins and Campbell, 1966).

Rats were fed Red 2G (no details on dose or duration) and subsequently the amount of oxidation of haemoglobin to methaemoglobin was linearly related to the logarithm of phenylhydroxylamine concentration in blood samples. Based on the dose response curve it was estimated that for rat blood the no-effect dose is between 0.5 and 1 µg phenylhydroxylamine / ml blood. At all levels of Red 2G fed to rats, the proportion of Red 2G metabolized to phenylhydroxylamine was constant (Unilever, 1974). For human blood the *in vitro* no-effect concentration of phenylhydroxylamine ranged from 0.46 to 4.1 µg/ml blood (no details on which effects were determined) (Unilever, 1974).

In dogs it was shown that after an aniline injection, phenylhydroxylamine was the metabolite to produce most of the observed methaemoglobin (no further details on

methodology). The reaction is thought to occur by oxidation of phenylhydroxylamine to nitrosobenzene by oxygen and haemoglobin with conversion of haemoglobin to methaemoglobin (Kiese 1959).

In an *in vitro* experiment described by the BIBRA (1965) 100 or 200 mg% of Red 2G was added to samples of mouse, rat, and human blood. No Heinz bodies were seen.

In an another *in vitro* study human and rat blood samples were exposed to phenylhydroxylamine, a metabolite of aniline. Rises in methaemoglobin levels were observed. In rat blood samples the effect was the strongest (Jenkins *et al.*, 1972).

In the SCF opinion short-term and sub-chronic toxicity is not discussed. TemaNord refers to the JECFA evaluation on this subject.

No additional short-term / sub-chronic studies have been published.

Reproductive and developmental toxicity

In the JECFA evaluation two rat studies are described which focussed on reproductive and developmental toxicity.

In the first study, rats (46 per sex) received 0.2% Red 2G in the diet (equivalent to 100 mg/kg bw) for 18 weeks and then mated for 10 days. The progeny received the same dietary concentrations from birth and were mated at 16 weeks. The F2 generation finally was also weaned on the same diet. No effects were observed regarding litter size, litter weight, weaning weight, or at autopsy (Unilever, 1974).

In the second study, groups of 20 female rats were fed diets containing 0, 0.004, 0.02, or 0.2% Red 2G (equivalent to 0, 2, 10, or 100 mg/kg bw/d) on gestational days 0-19. At gestational day 21, fetuses of 15 rats were examined for visceral and skeletal malformations. The other 5 rats per group were allowed to litter and suckle the pups to weaning at 21 days. No fetotoxic or teratogenic effects were observed. Furthermore no abnormalities with regard to parturition or postnatal development occurred. The only significant effect observed was an increase in spleen weight and erythropoietic activity in dams at the highest dose level. Fetal spleens were normal (Cambridge *et al.*, 1980).

In the SCF opinion, the subject of reproductive and developmental toxicity of Red 2G are not specifically addressed. The TemaNord evaluation refers to the JECFA evaluation of these end-points.

No more recent studies have been published on this aspect.

Genotoxicity

Red 2G:

JECFA mentions that a series of microbial mutagenicity assays were conducted. In these assays, food grade samples of Red 2G weakly induced repairable DNA damage and base-substitution mutations only after metabolic activation with rat-liver microsomes. Azo-reduction products were non-mutagenic (products not specified)

(Haveland-Smith *et al.*, 1979; Haveland-Smith and Combes, 1980a, 1980b). The JECFA comments that the effects were observed only at very high concentrations of 10 mg/ml whereas no activity was detectable at 1 mg/ml.

The TemaNord evaluation refers to the studies described by JECFA and in addition reports of several more recent genotoxicity studies.

In a study by Edwards and Combes (1984) the genotoxicity of urine and faecal extracts from rats treated orally with 800 mg/kg bw Red 2G was studied in two strains of *Salmonella typhimurium* with and without activation by liver microsomes and/or a beta-glucuronidase-sulphatase preparation. No significant mutagenic activity was observed.

Four other studies are mentioned but no details about these studies or the results are given. These studies consist of a study in *Salmonella typhimurium* (Edwards and Combes, 1983), a *Drosophila* somatic eye mutation test (Edward and Combes, 1981), and studies in CHO cells (EPA, 1989), and other cell cultures (EPA, 1988).

The SCF opinion does not discuss genotoxicity.

No additional information on the genotoxic properties of Red 2G has become available since the previous evaluations.

Aniline and its metabolites:

Two recent documents have thoroughly reviewed the genotoxic activities of aniline, the EU Risk Assessment Report on aniline (EU, 2004) and a publication on aniline and its metabolites (Bomhard and Herbold, 2005). Both documents basically review the same studies.

A number of studies have been conducted on the potential of aniline to induce point mutations. Although positive effects were observed in mammalian cells, these were relatively weak and confined to (very) high concentrations. Furthermore, the test system was considered to be possibly prone to false-positives, and due to the high concentrations it was assumed that the effects were due to unphysiological treatment rather than to a mutagenic effect of aniline itself. It was nonetheless concluded that a point mutagenic potential cannot at this time be excluded (Bomhard and Herbold, 2005, and references therein).

A potential for chromosomal aberrations was observed in various studies *in vitro* with or without metabolic activation, but mostly at relatively high concentrations only. In a range of *in vivo* studies, aniline was capable of inducing an increase in the rate of micronuclei in somatic cells of rats and mice, although at dose levels at which strong toxicity was reported (2 x 470 mg/kg bw in mice; 300 mg/kg bw in rats). In *in vivo* tests in rats but not mice, chromosome aberrations were increased in bone marrow cells, also at high doses (>300 mg/kg bw) (Bomhard and Herbold, 2005, and references therein).

Tests for DNA damage yielded both positive and negative results within some test systems/endpoints across a large database. When reproducible results from validated studies with clearly defined endpoints were taken into consideration the potential for

DNA damage of aniline was very weak if not negligible (Bomhard and Herbold, 2005, and references therein).

For most metabolites of aniline no genotoxic activities were observed or the available data were too limited, equivocal or of questionable relevance. Only p-aminophenol was found to induce clastogenic effects in non-mammalian and mammalian cells, both *in vitro* and *in vivo*, and notably at concentrations and dose levels below overt toxicity (LOAEL of (2 x) 107 mg/kg bw). In addition, a potential for point mutagenic activity in mammalian cells *in vitro* could not be excluded for p-aminophenol, albeit the results were observed at cytotoxic concentrations, (Bomhard and Herbold, 2005, and references therein).

Based on the same dataset in the EU Risk Assessment Report (EU, 2004) it was concluded that aniline should be classified as a category 3 mutagenic substance. In concordance with the review by Bomhard and Herbold (2005), for many studies the methodological limitations were identified.

Chronic Toxicity / Carcinogenicity

One mouse study and two rats studies were conducted and are described in the JECFA evaluation.

Mice (40 per sex) were fed Red 2G at concentrations of 0, 0.005, 0.025, 0.125, or 0.625 % (equivalent to 0, 7, 36, 179, or 893 mg/kg bw/d) of the diet for 20 months. At the two highest dose levels animals displayed enlarged and darkened spleens. Furthermore, increased deposition of iron was observed in the spleen and the kidneys, and erythropoiesis was accelerated. There was no sign of colour related carcinogenicity (no further details). Two-year survival was better than 75% (Unilever, 1974). At the highest dose level the theoretical maximum exposure to aniline amounted to approximately 180 mg/kg bw/d.

In a rat study animals (40 per sex) were fed a diet containing 0.004, 0.016, 0.064, or 0.16% Red 2G (equivalent to 2, 8, 32, or 80 mg/kg bw/d) for 2 years. At the highest concentrations, spleens were enlarged which ultimately led to necrosis of splenic elastica. The spleen was also darkened due to iron storage resulting from haemolysis of red blood cells. Up to a dietary level of 0.16% there was no evidence of carcinogenicity (no further details). Two-year survival was better than 50% (Unilever, 1974). At the highest dose level the theoretical maximum exposure to aniline amounted to approximately 16 mg/kg bw/d.

In another rat study animals (30 of each sex) were fed Red 2G daily at concentrations of 0 or 0.5% of the diet (equal to 0 or 250 mg/kg bw/d) for 2 years. At the 0.5% level there was enlargement and darkening of the spleen which could be attributed to accelerated splenic erythropoiesis, increased splenic haemosiderin deposition, and extensive degeneration of splenic elastica. Urinalysis and blood chemical examination revealed no adverse effects on kidneys and liver. Two-year survival was better than 50% (Unilever, 1974). At the administered dose of 250 mg Red 2G/kg bw, the theoretical maximum exposure to aniline amounted to approximately 50 mg/kg bw.

The SCF opinion does not discuss chronic toxicity or carcinogenicity. The TemaNord evaluation refers to the JECFA evaluation on this subject.

No more recent data on chronic toxicity or carcinogenicity of Red 2G are available. In a recent EU risk assessment report however, the carcinogenic properties of aniline have been evaluated (EU, 2004). This report describes a few studies in which aniline produced higher incidences of sarcomas in the spleen of rats.

In a study in rats (NCI, 1978), doses of aniline hydrochloride equivalent to 174 and 361 mg aniline per kg bw/d were given in the diet for 103 weeks. Statistically significant increases in the incidence of splenic haemangiosarcomas and the combined incidence of splenic fibrosarcomas and sarcomas not otherwise specified (NOS) were seen in male rats. In body cavity/multiple organs (not further specified) the increase in incidence of fibrosarcomas and sarcomas NOS combined was also statistically significant in males. Although in females statistical significance was not obvious, the observed incidences of fibrosarcomas and sarcomas NOS were considered indicative of a compound-related carcinogenic effect because of the rarity of these tumours. Based on the above, the original authors concluded that aniline (hydrochloride) was carcinogenic to both male and female rats.

The same research group performed a similar study in mice. In this study splenic haemangiosarcomas and malignant lymphomas were also observed in both males and females, but incidences were low and found in approximately equal numbers in controls. It was thus concluded that no evidence of carcinogenicity could be demonstrated at doses of more than 700 and more than 1500 mg/kg bw/d (EU, 2004, and reference therein).

In the second study in rats (CIIT, 1982), animals were given aniline hydrochloride in the diet for 104 weeks, equivalent to aniline doses of 7, 22, and 72 mg/kg bw/d. Of 130 animals exposed, 35 high-dose males had spleen tumours (3 fibrosarcomas, 21 stromal sarcomas, 1 capsular sarcoma, 6 haemangiosarcomas, 3 osteogenic sarcomas, and 1 lymphoreticular neoplasm), 9 of which were reported in premature deaths. In the mid-dose group a spleen tumour was found in a single male. In females no spleen tumours were found other than a single haemangiosarcoma in the high-dose group. In both males and females no tumours were found at the lowest dose or in the control group. From the study description, it is unclear whether other organs were also examined for tumours.

In several occupational aniline exposure studies in humans aniline could not be clearly associated with tumour induction (EU, 2004).

Intolerance / Allergenicity

None of the previous evaluations discusses intolerance or allergenicity nor were any studies identified which had been published since the TemaNord evaluation.

Allocation of an ADI by the SCF and the JECFA

JECFA

JECFA in its evaluation monograph of 1981 (JECFA 1981a) refers to two long-term studies on which the ADI may have been based; a mouse study with a NOAEL of 0.025% of the diet (26-43 mg/kg bw/d) (Unilever, 1974), and a rat study with a NOAEL of 0.016% of the diet (8 mg/kg bw/d) (Unilever, 1974). In the JECFA *Technical Report Series* of 1981 however, the Committee unambiguously allocated Red 2G an ADI of 0-0.1 mg/kg bw (JECFA, 1981b).

Based on the data described above, the final ADI was probably based on the long-term rat study with the NOAEL of 8 mg/kg bw/d. Using a safety factor of 100 and rounding up the outcome would lead to the ADI of 0-0.1 mg/kg bw.

SCF

The SCF in its evaluation allocated an ADI of 0-0.1 mg/kg bw/d. No details on study, NOAEL, or safety factor are given (SCF, 1975).

DISCUSSION

Concerning chemistry aspects, the Panel noted the following issues:

- no information was available describing the manufacture of Red 2G,
- the specifications and purity criteria only defined about 80% of the material of commerce. From the definition, it could be assumed that the missing 20% would probably be accounted for by sodium chloride or sodium sulphate, but this is never described explicitly,
- the specifications for purity of Red 2G permit unsulphonated aromatic amines to be present in concentrations up to 100 mg/kg Red 2G. Given the maximal allowed concentration of Red 2G that can be added to food (20 mg/kg food), the concentration of these amines in food would be up to 2 µg/kg food. Due to the association of these amines with genotoxicity, confirmation that the presence of unsulphonated aromatic amines in the final product is unavoidable due to the method of manufacture of Red 2G could be needed,
- no details or specifications were found characterising the chemical nature of the aluminium lake of Red 2G,
- no formal method of chemical analysis of Red 2G in food has been described. The method as laid down in the Codex Alimentarius seems to apply to food colouring preparations in general and is highly unspecific.

The Panel noted that the design of various studies in the previously evaluated dataset would not be in full compliance with current regulatory requirements. However, from the study descriptions available, the Panel considered that the quality of these studies was sufficient and there was no concern that the database was inadequate.

The toxicokinetic fate of Red 2G has been investigated. The Panel noted that pre-systemic and systemic azo-reduction yields 2-amino-8-acetamido-1-naphto-3,6-

disulphonic acid and aniline. Aniline is further oxidized to p-aminophenol and o-aminophenol. A range of other aniline metabolites are formed after oral dosing of aniline but these have not been detected in studies following administration of Red 2G. p-Aminophenol (free and conjugated) was quantitatively the most important metabolite in urine following administration of Red 2G.

Based on excretion of Red 2G metabolites in bile and urine, at least 50-70% of the dose is reduced at the azo-bridge. In urine and faeces of rats less than 5% of unchanged Red 2G could be detected which indicates that azo-reduction may be more extensive. Urinary excretion of the aniline moiety takes place mainly in the form of aminophenols (in total 42-56% of the dose). Faecal excretion of these products was also observed although to a lesser degree than in urine. No details were found regarding the fate of 2-amino-8-acetamido-1-naphtho-3,6-disulphonic acid. In effect, the fate of about 30-50% of an oral dose of Red 2G which is converted to 2-amino-8-acetamido-1-naphtho-3,6-disulphonic acid has not been elucidated. However, after oral administration of Red 2G to rats, 48.2% of the sulphur derived from Red 2G was excreted in the faeces. This sulphur is most probably in the form of 2-amino-8-acetamido-1-naphtho-3,6-disulphonic acid or further metabolites of this compound. The fate of the remaining 51.2% of the sulphur was not determined.

Following these observations the Panel had concerns about the exposure to aniline resulting from metabolism of Red 2G.

The Panel noted that the main effects observed after single-dose and short term oral exposure of humans and rats to Red 2G or its metabolites were consistent with the haematotoxic effects caused by aniline and its metabolites. Although some of the previously evaluated studies are inadequately described, the NOAELs or LOAELs would not affect the value of the current ADI and thus there is no reason to re-evaluate these studies.

The increase in spleen weight or erythropoietic activity observed in dams was not seen in fetuses. Based on the available studies the Panel concluded that reproduction and development were not the critical end-points for Red 2G.

The main Red 2G-induced long-term effects found were splenomegaly, accelerated erythropoiesis, splenic haemosiderin deposition, and iron deposition in the spleen due to haemolysis. It is not clear if in the chronic studies with Red 2G, haematological changes (e.g. red blood picture, methaemoglobin, and Heinz body formation) have been monitored, although these effects were observed in acute and short-term studies.

The Panel noted that Red 2G is found to induce DNA damage and base-substitution mutations, although only at very high concentrations in vitro.

No signs of Red 2G-induced carcinogenicity were observed in mice at dose levels of approximately 900 mg/kg bw/d, or in rats at dose levels of at least 80 mg/kg bw/d. The Panel noted that assuming 100% release of aniline these doses of Red 2G would be equivalent to aniline doses of 158 mg/kg bw/day in mice and 14 mg aniline/kg bw/day in rats. The Panel noted that for the rats these amounts of aniline from Red 2G would be significantly lower than the doses that induced spleen tumours, mainly in male rats, in other studies on aniline itself.

The EU Risk Assessment describes potential arguments for both genotoxic and non-genotoxic mechanisms by which the splenic tumours could arise (EU, 2004, and reference therein). The EU Risk Assessment concluded the following on mutagenicity and carcinogenicity:

“Aniline is negative in routine bacterial mutation tests. In mammalian cell cultures positive effects were obtained with respect to chromosomal effects, SCE and possibly for gene mutations. In general, stronger effects are induced in the presence of an exogenous metabolic activation system than in the absence. *In vivo*, in bone marrow assays with mice a negative response was found in one investigation on chromosomal aberrations, whereas weak positive effects were found in micronucleus tests, which, however, were limited to high-doses in the toxic range. In rats induction of micronuclei was found at several doses.”

...

” The mutagenicity *in vitro* and *in vivo* of aniline is supported by *in vivo* studies showing DNA strand breaks and DNA adducts in different organs. Furthermore mutagenicity data of a metabolite (4-aminophenol) and a structurally related substance (azobenzene) strengthen the evidence for mutagenicity of aniline in somatic cells of animals.

In two carcinogenicity studies on the F344 rat, aniline produced dose-dependently higher incidences of spleen sarcomas in males. A few splenic tumors observed in female rats were also considered to be related to aniline treatment. Aniline is genotoxic *in vivo* in rats and in mice. It can be assumed that the genotoxicity is responsible for tumor initiation and development, but this did not necessarily include a scientific plausible proof that the underlying mechanism of carcinogenicity is based on the genotoxic activity. Other mechanisms are also discussed to be involved in tumor development. Until now, it is not possible to demonstrate a plausible mode of action indicating the existence of a threshold mechanism.”

...

“As far as known aniline is metabolised similarly in rat and man. Therefore a certain carcinogenic risk for man cannot be excluded for all exposure scenarios. Although available human data do not support a carcinogenic risk to humans this holds true only for the described circumstances. From the limited human database a final assessment of human cancer risk is not possible.”

Based on these considerations, the EU Risk Assessment ultimately concluded that:

- based on the available data on mutagenicity and due to the possible findings in several *in vitro* and *in vivo* tests, especially in the bone marrow micronucleus test with rats, aniline was classified as a category 3 mutagen,
- in accordance to the EU criteria for classification and labelling, aniline was classified as carcinogenic, category 3.

The Panel noted that Red 2G is extensively metabolised to aniline. The Panel noted that some issues were raised by the EU Risk assessment report, with respect to the mode of action of aniline, particularly that the positive findings *in vivo* both for mutagenicity and carcinogenicity were usually associated with general and/or target organ toxicity. Nevertheless the EU Risk assessment concluded that aniline should be classified as a category 3 substance for both mutagenicity and carcinogenicity. Based on these considerations, the Panel concluded that a genotoxic potential of Red 2G cannot be excluded.

The Panel noted that at the time of the previous JECFA and SCF evaluations, it would have been reasonable to conclude that Red 2G could be assessed using a threshold approach based on the toxicokinetic and genotoxicity data available at that time.

In order to give some guidance on the margin of exposure between the dose of aniline shown to cause tumours in rats and the estimated human dietary exposure, the Panel has estimated the BMDL10^{*} for splenic tumours caused by aniline in male rats. The Panel has used the EPA Benchmark Dose Software (version 1.4.1) and the data from both the CIIT and NCI carcinogenicity studies cited in the EU report. The results from all the models are shown in Annex I.

Based on the data available, the Panel calculated BMDL10 values that ranged from 29 to 35 mg/kg bw/day. These values were used to estimate the margin of exposure for aniline resulting from the intake of Red 2G. The Panel assumed a 100% release of aniline from Red 2G and estimated the exposure to aniline from consumption of 100 g of a meat product coloured with Red 2G at its maximum permitted level of 20 mg/kg to be 0.006 mg/kg bw (0.033 mg Red 2G/kg bw of which 18.3% is aniline on a molecular weight basis) for a 60 kg individual.

Based on these assumptions, the margins of exposure for aniline in adults would be in the range of 5000 to 6000.

CONCLUSIONS

For the current re-evaluation of Red 2G, the Panel has reconsidered several existing evaluations of Red 2G and its metabolite aniline supplemented by several studies which have been published since 1999.

The Panel based its evaluation on the conclusions of the EU Risk Assessment on aniline. This concluded that aniline should be considered as a carcinogen for which a genotoxic mechanism cannot be excluded, based on the following:

- aniline was genotoxic *in vivo* in rats and mice,
- there was insufficient mechanistic evidence to discount a genotoxic mechanism for the rodent carcinogenicity
- based on similar metabolism of aniline in animals and humans a carcinogenic risk for man cannot therefore be excluded.

Therefore the Panel concluded that it would be prudent to regard Red 2G as being of safety concern since it is extensively metabolised to aniline. Based on these considerations, the Panel withdrew the ADI for Red 2G.

The Panel considered that should the tumour inducing mechanism of aniline be further elucidated, shown to be thresholded and/or its relevance for man discounted, Red 2G could be re-evaluated once again.

* The BMDL10 stands for “Benchmark Dose lower limit for a benchmark response of 10%” and is defined as the lower limit of the 95% confidence interval on the dose of aniline estimated to cause a 10% incidence of tumours in a given species (here in rats).

In order to give some guidance concerning dietary exposure to Red 2G, the Panel has used the benchmark dose approach to calculate the margin of exposure between the lower limit of the 95% confidence interval on the dose of aniline estimated to cause a 10% incidence of tumours in rats and the estimated human dietary exposure. Based on the assumptions that there could be 100% release of aniline from Red 2G and that 100 g of meat product coloured with Red 2G at the maximum permitted level of 20 mg/kg is consumed daily, the margins of exposure for Red 2G in adults would be in the range of 5000 to 6000.

DOCUMENTATION PROVIDED TO EFSA

The pre-evaluation document was drafted by Bas Koomen and Wim Mennes (RIVM, The Netherlands) under EFSA contract number AFC/ADD/tender/02/2004.

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ACKNOWLEDGEMENT

The Panel wishes to thank the members of the Additives working group:

Fernando Aguilar, Dimitrios Boskou, David Gott, Sandro Grilli, Werner Grunow, Karolina Hulshof, John Chr. Larsen, Catherine Leclercq, Jean-Charles Leblanc, Wim Mennes, Alicja Mortensen, Dominique Parent-Massin, Annette Pöting, Iona Pratt, Ivonne Rietjens, Gerrit Speijers, Paul Tobback, Fidel Toldrá.

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ANNEX 1. BENCHMARK DOSE (BMD) MODELLING OF THE MALE RAT TOTAL SPLENIC TUMOUR DATA ON ANILINE.

The Benchmark Dose (BMD) approach was originally put forward as an alternative to the NOAEL/LOAEL determination for non-cancer health effects because it provides a more quantitative alternative to the first step in the dose-response assessment than the NOAEL/LOAEL. The BMD is based on a mathematical model being fitted to the experimental data within the observable range and estimates the dose that causes a low but measurable response (the benchmark response BMR) typically chosen at a 5 or 10% incidence above the control. The BMD lower limit (BMDL) refers to the corresponding lower limits of a one-sided 95% confidence interval on the BMD. Using the lower bound takes into account the uncertainty inherent in a given study, and assures (with 95% confidence) that the chosen BMR is not exceeded.

For the evaluation of human and experimental animal data the EFSA Scientific Committee has proposed to use the BMD methodology to derive a reference point on the dose-response curve for compounds that are both genotoxic and carcinogenic, and the Committee considered the use of the BMDL, calculated for a BMR of 10% (BMDL10), to be an appropriate reference point. Such a value is the lowest statistically significant increased incidence that can be measured in most studies, and would normally require little or no extrapolation outside the observed experimental data (EFSA 2005).

The Committee considered the long term toxicity and carcinogenicity studies on aniline in rats performed by the NCI (1978) and CIIT (1982) as the most adequate studies for dose-response modelling. The Committee performed BMD modelling on the occurrence of total splenic tumours in the male rats combined from these two studies (Table 1). It was not clear to what extent tumours found at the intermediate sacrifices were included in the reporting. Therefore modelling was performed on both scenarios.

Table 1. Combined male rat total splenic tumour data on aniline from the NCI (1978) and CIIT (1982) studies.

Dose (mg/kg bw/day)	Number of animals per group. Interim sacrifices included	Number of animals per group. Interim sacrifices excluded.	Number of total splenic tumours reported
0	155	115	0
7	130	90	0
22	130	90	1
72	130	90	35
174	50	50	33
360	46	46	35

The US EPA BMD software (BMDS) version 1.4.1 (available from http://cfpub.epa.gov/ncea/cfm/nceatools_human.cfm) was used for modelling the spleen tumour dose-response in the male rats. For carcinogenicity data, a number of models are available in the BMDS, and model fitting, determination of goodness-of-fit, and comparing models to decide which one to use for obtaining the BMDL10 have been outlined by Filipsson *et al.* (2003). The following dose-response models were fitted to the dose-incidence data:

- Gamma multihit model
- Log-logistic model
- Multistage model
- Log probit model
- Quantal linear model
- Weibull model

The BMD and BMDL values for an extra 10% risk compared to the background were estimated by performing 250 iterations.

The acceptability of a model can be based on several criteria. The likelihood ratio tests can be used to judge 1) whether there is a dose-response relationship, i.e. the likelihood value of the “reduced” model should be significantly different from the likelihood value of the “full” model ($p < 0.05$; p becomes higher as the likelihood value for the reduced model approaches the likelihood value of the full model), and 2) the likelihood value of the “fitted” model should not be significantly worse than the value provided by the “full” model ($p < 0.05$; p becomes higher as the likelihood value for the fitted model approaches the likelihood value of the full model). The full model is the model that assumes no dose-response function (its parameters are simply the frequencies per dose level) and the reduced model assumes that the probability of an adverse effect would be the same at each dose level (Filipsson *et al.*, 2003).

In addition, the BMDS provides statistics for the goodness of the fit using the Pearson Chi-square Test. This test is similar to the test of the “fitted” versus the “full” model using the likelihood ratio test. The lower the chi-square value the better the fit and the calculated p -value should be significantly larger than 0.05, which in this case was chosen to represent a rejection level (Filipsson *et al.*, 2003).

The BMD10 and BMDL10 values, as well as the associated statistics for the models used, are presented in Table 2 and 3.

Table 2. BMD10 and BMDL10 calculations based on total splenic tumours in male rats from the NCI (1978) and CIIT (1982) carcinogenicity studies on aniline. Animals at interim sacrifices were included.

Model	Log (likelihood)	p-value	Chi-square	p-value	Accept	BMD10 (mg/kg bw per day)	BMDL10 (mg/kg bw per day)
Full model	-139						
Gamma multi-hit	-147	0.003	14.5	0.006	No	42	33
Log-logistic	-143	0.06	8.2	0.08	Yes	43	35
Multi-stage	-151	<0.001	19.3	0.0007	No	34	25
Log-probit	-141	0.21	5.6	0.23	Yes	42	35
Quantal-linear	-153	<0.001	18.9	0.002	No	26	20
Weibull	-148	<0.001	16.1	0.003	No	39	31
Reduced model	-284.2	<0.001					

The accepted BMDL10 values for the male rats were in both cases 35 mg aniline/kg bw per day, representing the best fit.

Table 3. BMD10 and BMDL10 calculations based on total splenic tumours in male rats from the NCI (1978) and CIIT (1982) carcinogenicity studies on aniline. Animals at interim sacrifices were excluded.

Model	Log (likelihood)	p-value	Chi-square	p-value	Accept	BMD10 (mg/kg bw per day)	BMDL10 (mg/kg bw per day)
Full model	-123						
Gamma multi-hit	-134	0.0003	15.5	0.0006	No	31	22
Log-logistic	-130	0.01	11.6	0.02	No	32	25
Multi-stage	-135	0.0001	18.1	0.003	No	22	19
Log-probit	-128	0.07	9.8	0.08	Yes	34	29
Quantal-linear	-136	0.0001	18.1	0.003	No	22	19
Weibull	-135	0.0001	19.5	0.006	No	28	20
Reduced model	-251	<0.001					

The only accepted BMDL10 value for the male rats was 29 mg aniline/kg bw per day, representing the best fit.

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