THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD PRODUCTS INTENDED FOR CONSUMERS

UPDATED RECOMMENDED STRATEGY

FOR

TESTING HAIR DYES FOR THEIR POTENTIAL GENOTOXICITY/MUTAGENICITY/CARCINOGENICITY

Adopted by the SCCNFP during the 24th plenary meeting
of 24-25 June 2003

1. Introduction

Within the framework of risk assessment presented by hair dye ingredients, the genotoxic/mutagenic/carcinogenic potential hazards are needed to be identified for the evaluation of their adverse effects which they may represent to consumers.
The European Cosmetic Industry uses a considerable number of hair dyes and the safety of many of them has not been assessed yet by Public Authorities. The reasons for this, include incomplete dossiers and non-conformation of experimental data to modern (current) methods of evaluation. The European Industry has submitted a list of these permanent hair dyes at levels of >2,000 kg per year in Europe. It is recommended that dossiers submitted for review conform to the "Notes of Guidance for Testing of Cosmetic Ingredients for Their Safety Evaluation" (http://europa.eu.int/comm/food/fs/sc/sccp/index_en.html) of 17th December 2000 produced by the SCCNFP, the critical data must conform to modern methods of determination (Updated Basic Requirements for Toxicological Dossiers to be Evaluated by the SCCNFP 0633/02).
A "Proposal for a Strategy for Testing Hair Dye Cosmetic Ingredients for Their Potential Genotoxicity/Mutagenicity" was adopted by the SCCNFP on June 4th 2002 (0566/02)(11). Comments to this SCCNFP's Opinion were received by the Cosmetic Industry on March 13th 2003, together with a proposal for a strategy to be applied in this sector of toxicological studies (27).

SCCNFP was requested to answer the following questions: :

* Is the strategy proposed by Industry in line with the "Strategy for Testing hair dye cosmetic ingredients for their potential genotoxicity/mutagenicity (SCCNFP/0566/02) which is part of the "Assessment Strategies for hair dyes" (SCCNFP/0553/02) adopted in December 2002?

* Are the data resulting from the proposed strategy efficient for the SCCNFP to perform the risk assessment of the potential genotoxicity/mutagenicity of individual substances as well as of mixtures of precursors (s) couplers (s) and oxidants of hair dyes, to which the consumer is exposed ?

2. Objective

A paper regarding the "Assessment Strategies for Hair Dyes", was adopted during the 22nd Plenary Meeting on 17th December 2002 (Opinion SCCNFP/0553/02)(16). In particular, for the risk assessment of hair dyes, the following must be submitted :

* The chemical specifications (purity and impurities) of the hair dyes used to generate the data must be defined and be representative of that used in commercial products;

* According to the intended use, the dyes/dye-precursors must be tested alone and/or in combination with other substances to simulate the conditions of use;

* *In vitro*, and those necessary *in vivo*, studies required to identify the nature and the level of the possible hazards of hair dyes;

* Data on genotoxicity – the studies must be consistent with internationally accepted guidelines (OECD, EU) and/or with modern testing strategies (doc. SCCNFP/0566/02,(11);

* All available data on carcinogenicity;

* Deviations from the above must be justified.

Safety assurance and with this, health protection of the consumer, can be applied only to those hair dyes and complexes that have been subject to the above assessment.

3. Hair dyes

Hair dyeing formulations belong to 3 categories, i.e. for temporary, semi-permanent and permanent colouring of hair. The products for temporary dyeing of hair generally comprise of water soluble acid dyes and water soluble pigments, which are deposited on the surface of the hair. These colours are removable by a single effective shampooing. The formulations for semipermanent dyeing of hair contain for the most part simple derivatives of nitroanilines, nitrophenylenediamines and nitroaminophenols. These dyes penetrate into the cuticle and partially into the cortex of the hair. As a result, the colouring effects of these dyes can resist 5-10 shampooings. The formulations for permanent hair colouring are marketed as two component kits. One component contains the dye precursors (such as *p*-phenylenediamine, 2,5-diaminotoluene, N,N-bis(2-hydroxymethyl)-p-phenylenediamine, p-aminophenol etc.) and couplers (such as resorcinol, chlororesorcinol, methyl resorcinol, α-naphthol, m-aminophenol, m-phenylenediamine, etc.) in an alkaline soap or syndet base, and the other component is a stabilised solution of hydrogen peroxide. The two components are mixed immediately prior to use. The precursors and peroxide diffuse into the hair shaft, where colour formation takes place after a cascade of chemical reactions. The dye precursors are oxidised by hydrogen peroxide to p-benzoquinone imines/diimines, which are reactive intermediates in the colour formation. The couplers, which are relatively stable to hydrogen peroxide, undergo rapid reaction with the intermediates resulting in dinuclear, trinuclear or polynuclear colour molecules. These molecules are too large to escape from hair structure. These dyes are also called oxidative hair dyes. Hydrogen peroxide in the oxidative hair dye formulations also serves as bleaching agent for the natural pigment of the hair. The colour formation (shades) is dependent on precursors present in the dyeing solution, its pH and the time of contact with the hair (17, 22, 23).All hair dyes are aromatic amines.

Oxidative hair dyes are resistant to fading by shampooing, but re-colouring of hair is required every 6 weeks approximately, due to hair growth. The time of contact of the dyeing solution with the hair scalp may vary from 15-45 minutes. Oxidative hair dyes represent the major segment of the hair colouring market.

In particular, the assessment of genotoxicity/mutagenicity/carcinogenicity potential of oxidative hair dyes is rather complex, because it involves dye precursors, intermediates, reaction and final product(s) at the same time. The hazard of the oxidative hair dyes must therefore be evaluated by testing the individual ingredients as well as by testing a combination of relevant ingredients, so that the genotoxic/mutagenic/carcinogenic potential of the novel reaction products formed during the application period can be evaluated.

4. Mutagenicity/Genotoxicity

4.1. Preamble

1. Mutation is defined as a permanent change of the amount or structure in the genetic material of an organism. These changes may result in heritable changes of the organism. These alterations may involve :

a. genes, block of genes and/or
b. whole chromosomes (structural and/or numerical)

2. Genotoxic properties may be defined as the potential of a chemical to react with DNA. Under certain circumstances (when lesions are not repaired or misrepaired) the induced lesions may be converted into mutations. Tests for detecting genotoxic potentials comprise :

a. DNA adduct determination;
b. Sister Chromatid Exchanges (SCEs)
c. Mitotic recombinations
d. Detection of unscheduled DNA synthesis that is indicative of nucleotide excision/mismatch/recombinational repair; DNA degradation.

3. During the risk assessment process of chemicals it is then necessary to pay attention to both genotoxicity and mutagenicity tests. Moreover, in addition to the detection of intrinsic genotoxic potentials of chemicals, the extent of human exposure should drive the need for an additional battery of tests (1, 2, 3).

The second value of the mutagenicity/genotoxicity testing also lies in its ability to identify chemicals that may, under certain exposure conditions, either cause cancer by a predominantly genotoxic mechanism or induce the initial phase of carcinogenic processes (19, 20, 21).

4.2. Testing Strategy

Adequate knowledge of the formulation of the oxidative hair dye must be taken into consideration before genotoxic/mutagenic testing is performed. Moreover, genotoxic/mutagenic testing of the mixture of precursor(s), coupler(s) and oxidant, to which the consumer is exposed, should be performed using several *in vitro* test methods.

There may be instances where alternative tests to those specified below are more appropriate: in such cases a scientific justification must be presented.

Stage 1 : *In vitro* tests

1. Tests for gene mutation

1.1. Bacterial Reverse Mutation Test (OECD 471, 21st July 1997; EC B.13/14, 19th May 2000) (19,24);

1.2. *In vitro* Mammalian Cell Gene Mutation Test (currently, the preferred choice is the mouse lymphoma assay; the CHO HGPRT assay has more shortcomings)(OECD 476 21st July 1997; EC B.17, 19th May 2000) (19,24).

2. Tests for clastogenicity

2.1. (preferred test) *In vitro* Mammalian Chromosome Aberration Test (OECD 473, 21st July 1977; EC B.10 19th May 2000)(19,24).

3. Tests for aneugenicity and non-disjunction

3.1. *In Vitro* Micronucleus Test (Guideline proposed to OECD,8).

4. Tests for DNA Repair

4.1. Unscheduled DNA Synthesis in Mammalian Cells *in vitro* (OECD 482, 23rd Oct. 1986; EC B.18, 18th November 1987) (4,19).

The need to apply five *in vitro* tests for evaluating the genotoxic/mutagenic potential of hair dyes (precursors or combinations of different chemicals) has several justifications :

1. For a comprehensive coverage of the potential mutagenicity of a substance, information on (1) gene mutations, (2) structural chromosome aberrations (clastogenicity), (3) numerical chromosome aberrations (aneugenicity) (4) DNA-damages are required;

2. *In vitro* tests have a critical role in the testing strategy, as well as in the choice of appropriate animal models and tests for eventual future investigations;

3. Several aromatic amines are not mutagenic or are over-evaluated (false positive) in bacterial mutagenicity tests, whereas they may be identified as mutagens in a mammalian cell gene mutation test (18);

4. Two tests for gene mutation (bacterial and mammalian cell assays) are required when human exposure is expected to be extensive, as in the case of hair dyeing (2);

5. A highly positive correlation has been observed between the results of joint mutational and DNA repair tests with bioassays for carcinogenicity (13);

6. All *in vitro* tests for mutagenicity/genotoxicity so far considered, have been applied to thousands of chemicals, after intensive international validation studies. They are all based on a scientifically documented mechanistic set of changes involving genetic material, both structural and functional. Moreover, all mutagenicity/genotoxicity *in vitro* tests are currently carried out, as far as possible, according to internationally accepted protocols(19,20).

7. Tests for clastogenicity have different end points from tests for aneugenicity/non disjunction (8,14).A correlation of 80% has been found for the two tests (12).

All mutagenicity/genotoxicity studies should be carried out under the most rigorous protocols, such as those adopted by OECD or EC. (4,19,24).

Interpretation of results

1) If NEGATIVE results are obtained in Stage 1 screening tests, the substance or the combinations of the precursors (the intermediate and the final products) can be considered "not mutagenic" i.e. showing no evidence of intrinsic mutagenicity/genotoxicity.

2) If POSITIVE results are obtained in one or more Stage 1 screening tests, the substance or the combinations of the precursors (the intermediate and the final products) must be considered "*in vitro* mutagenic", i.e. showing evidence of intrinsic mutagenicity/genotoxicity.
Substances or combinations of the precursors (the intermediate and the final products) showing pronounced responses in several in vitro assays should be considered "mutagenic", if no relevant in vivo data are available.

3) If EQUIVOCAL results are obtained, further *in vitro* testing employing different cellular systems with the same endpoint should be considered. If this does not resolve the question the *in vivo* testing (Stage 2) is required.

Stage 2 : *in vivo* tests

The need and the choice of *in vivo* tests of Stage 2 should be justified by the results obtained with Stage 1 testing. Before undertaking any *in vivo* testing :

1. A review of the *in vitro* test results is needed. A particular *in vivo* test should be conducted only when it can be reasonably expected from all the properties of the test substance and the proposed test protocol that the specific target tissue will be adequately exposed to the test substance and/or its metabolites. If necessary, an investigation of toxicokinetics should be conducted before progressing to *in vivo* testing.

2. The toxicokinetic and toxicodynamic properties of the test substance in the conditions of mutagenic *in vivo* tests should be considered as far as possible before undertaking, or appraising, animal tests. Understanding these properties will enable appropriate test protocols to be developed, especially with respect to tissue(s) to be investigated, the route of substance administration and the highest dose tested. If little is understood about the systemic availability of a test substance at this stage, an experimental investigation may be necessary.

The following tests are those commonly preferred :

* Mammalian Erythrocyte Micronucleus Test (OECD 474, 21st July 1997; EC B.12, 19th May 2000) (19,24).

* Mammalian Bone Marrow Chromosome Aberration Test (OECD 475, 21st July 1997; EC B.11, 19th May 2000) (19,24).

* Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vivo* (OECD 486, 21st July 1997; EC B.39, 19th May 2000) (19,24).

* *In vitro* and *In vivo* Single Cell Gel/Comet Assay. (No OECD Guideline; 6). This test can be used to investigate the genotoxicity of chemicals, by detecting multiple classes of DNA damages at the level of the single cell. It may applied to cell cultures, to lymphocytes, to different tissues of an animal treated *in vivo*. Comet formation is due to primary DNA lesions: for the interpretation of test results one should elucidate whether the primary DNA damage is converted into biologically relevant chromosome or gene mutations(7,10).

5. Carcinogenicity

Long term effects (carcinogenic effects) have been found for several hair dye ingredients (17 hair dye ingredients have been included in Annex II of Directive 76/768/EEC). Carcinogenicity is assessed by the use of a large number of rats and mice which are treated with a substance under evaluation for the lifetime (approximately 2 years) and at the end analysed for the presence of different types of tumours in all organs and tissues.

There exists a draft OECD Guideline for an "*In vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay" (TG 965.DOC) which indicates that "the test measures the morphological transformation (MT) of cell colonies induced by chemicals. The MT is a recognised step in the multistage process of conversion of normal, finite life-span, non tumorigenic mammalian cells to a neoplastic stage. This assay has been shown to detect genotoxic and non-genotoxic carcinogens"(15). A similar Guideline has been published by the EC (1998) as B.21 "*In vitro* Mammalian Cell Transformation Tests" which refers to different mammalian cell lines(4). The "EPA-OPPTS 870.8800 Morphologic Transformation of cells in Culture" (1996) has also been published (Public Draft); it refers to Balb/c 3T3 mouse cells (12).

In 2001 OECD published a detailed review paper (5) describing three cell lines test assays (SHE, Balb/c 3T3, C3H 10T1/2).
This concludes that SHE cell transformation assay is believed to mimic early steps of carcinogenesis, while the Balb/c 3T3 and the C3H 10T1/2 cell transformation systems are likely to mimic later stages(5).

6. Strategy for Testing hair dye ingredients and reaction products for their potential mutagenicity/genotoxicity/carcinogenicity

Following the positive comments received after the publication of the " Proposal for a Strategy for Testing Hair Dye Cosmetic Ingredients for their Potential Mutagenicity/Genotoxicity (SCCNFP/0566/02)" a recommended strategy has been developed for testing hair dyes. . .

Stage 1 Testing Assays (*in vitro*)

* Bacterial Reverse Mutation Assay (OECD 471);

* *In Vitro* Mammalian Chromosome Aberration Test (OECD 473);

* *In Vitro* Mammalian Cell Gene Mutation Test (OECD 476);

* DNA Damage and Repair/Unscheduled Synthesis in Mammalian Cells *in vitro* (OECD 482);

* *In Vitro* Mammalian Micronucleus Test (UK-EEMS)(8);

* *In Vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay (OECD TG495).

These six assays cover the *in vitro* evaluation for mutagenicity/genotoxicity and also the non genotoxic/carcinogenic potential.

The need to include the *in vitro* Micronucleus Test in the Stage 1 *in vitro* tests is supported by many national Institutions, as it allows to specifically identify those chemicals which might induce genetically relevant harmful effects as a consequence of the induction of aneugenic and/or numerical chromosomal mutations.

The inclusion of the "*In Vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay" in the Stage 1 *in vitro* tests allows the identification of potential non-genotoxic carcinogens among hair dye ingredients, as suggested also by the Industry.

Stage 2 Testing Assays (*in vivo*)

The aim of the *in vivo* assays is to ascertain whether a genotoxic/mutagenic effect shown *in vitro* may also occur in somatic cells under in vivo conditions.

The selection of the *in vivo* assays cannot be defined *a priori* and depends on the positive results observed in the *in vitro* assays.

As previously stated, all considerations presented in Section 4 of this document must be taken before the decision of the *in vivo* tests to be performed.

We are aware that a need may arise to apply several *in vivo* mutagenicity/genotoxicity tests to clarify the potential for *in vivo* somatic cells. It is unnecessary to discuss such issue in the details. Flexibility specifically applies to Stage 2 *in vivo* test methods.

It is the Applicant's responsibility to select the suitable Stage 2 *in vivo* studies. In all cases the methods selected must be among those adopted by OECD/EC, or by a recognised group of scientists. The rationale of the choice must be justified on a case-by-case having regard to the chemical structure, its metabolism, the expertise available and the results from earlier (*in vitro*) tests.

In the case of a positive result *in vitro*, the *in vivo* test should use the same end point and the tissue, if possibly.

Most permanent hair dyeing technology involves the oxidatively induced coupling of primary intermediates and couplers (dye precursors) to form dyes within the hair (17).The problem of identifying possible harmful compounds formed during the application of this technology is very complex.

One possibility is represented by the isolation and identification of the reaction products, during a simulation of the in-use conditions, their exposure and bioavailability and to submit them to tiered genotoxicity testing, according to their relevance.

During the application of a permanent oxidative hair dye, kinetics of the disappearance of the basic ingredients, the couplers and the primary intermediates, as well the appearance of the reaction products can be determined. In the case of a relevant systemic exposure, an animal mutagenicity/genotoxicity/carcinogenicity model should be developed, able to evaluate the reaction products in an *in vivo* situation.

7. OPINION of the SCCNFP

Based on the available scientific information present in the literature and provided by the Cosmetic Industry, the SCCNFP is of the opinion that :

> The strategy proposed by Industry for testing Hair Dyes, although in line with the SCCNFP's Doc. 0566/02, is insufficient. It does not provide complete information on the potential for mutagenicity/genotoxicity/carcinogenicity, based on the evaluation of all relevant endpoints known to be responsible for direct toxic effects of concern (mutation and cancer);
>
> In order to perform a risk assessment on the potential for mutagenicity/ genotoxicity/carcinogenicity of individual Hair Dyes as well as their reaction products, a complete set of six *in vitro* test assays is recommended as a basis of the evaluation, as defined in section 6 of this opinion.

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