

**EVALUATION OF MUTAGENICITY STUDIES
DEVELOPED ON
(PFOS) PERFLUOROOCTANE SULFONATE**

PREPARED BY

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INTRODUCTION

Perfluorooctanesulfonate (PFOS) is one of the degradation products of Perfluorooctanesulfonylfluoride, a component of several product lines by 3M, widely used for several purposes, such as Surface Treatments, Surfactants and others. These degradation products can be found in the environment during several stages of a product's life cycle, such as during the industrial synthesis, the incorporation of the fluorochemicals into a finished product, during the distribution of the product to users, during the use of the product by consumers, and during disposal at all these stages (3M, August 31st, 1999).

Because of possible human exposure to these chemicals by different routes, direct or indirect, 3M has developed a set of toxicological studies on PFOS and other degradation products.

In the present Report we evaluate a set of mutagenicity/genotoxicity studies performed on PFOS, in order to express an opinion on the potential mutagenic/genotoxic risk posed by this chemical.

The set of mutagenicity/genotoxicity studies submitted to our evaluation consists of:

1. *Salmonella - Escherichia coli* /Mammalian Microsome Reverse Mutation Assay with PFOS. Covance Study No. 20784-0-409, completed on November 5, 1999.
2. Chromosome Aberrations in Human Whole Blood Lymphocytes with PFOS. Covance Study 20784-0-449, completed on October 25th, 1999.
3. Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures with PFOS. Covance Study No. 20784-0-447, completed on November 9, 1999.
4. *In Vivo* Mouse Micronucleus Assay on T-6295. Corning Hazleton Inc., No. 17403-0-455, completed on May 23rd, 1996.

EVALUATION

1. *Salmonella* - *Escherichia coli*/Mammalian Microsome Reverse Mutation Assay with PFOS

1.1 Identification of the test chemical

The test chemical evaluated in this study has been indicated as PFOS, FC-95, Lot 217, as white crystalline powder. No other identification properties are reported in the study. Purity of the test chemical has non been indicated.

The test chemical was dissolved in DMSO: the 2.00 µg/ml solution used for the dilutions employed in the assay was stable.

1.2 Tester strains

The strains used in the assay were *S.typhimurium* strains TA98, TA100, TA1535, TA1537 and *E.coli* strain WP2 uvrA.

1.3 Metabolic activation

The assay was performed in the absence and in the presence of a metabolic activation system. This was provided by S9 mix, liver homogenate, purchased by Molecular Toxicology, Inc. Batch 0872 (42.8 mg protein/ml). The homogenate was prepared from male Sprague-Dawley rats injected (i.p.) with Aroclor 1254 (200 mg/ml) at 500 mg/kg.

Data on composition of S9 are included in the Report.

1.4 Doses of the chemical

The tested doses of the chemical under evaluation were determined after a dose range finding study.

Tested doses were between 5,000 and 33.3 µg/ml (6 doses) in the presence of S9 mix and between 5,000 and 0.333 µg/plate (9 doses) in the absence of S9 for *S.typhimurium* 4 strains. As regards *E.coli* WP2 uvrA the doses tested were between 5,000 and 33.3 µg/plate (6 doses) both in the presence and absence of S9 mix. 1 exp. with 3 plates/dose was performed.

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1.5. Positive controls

Positive controls in the presence of S9 mix were benzo(a)pyrene for TA98; 2-Aminoanthracene for TA100, TA1535, TA1537 and WP2 uvrA. In the presence of S9 mix the positive controls were 2-nitrofluorene for TA98; Sodium Azide for TA100 and TA1535; ICR-191 for TA1537; 4-nitroquinoline-N-oxide for WP2 uvrA

1.6. Results

Results indicate that PFOS did not cause a positive increase in the mean number of revertants/plate in all tested strains and all conditions.
All positive controls were found mutagenic and comparable with literature data.

1.7. Comments

The study was performed in accordance with Good Laboratory Practice regulations laid down by the US-EPA.

The protocol employed in the assay was that one recommended by OECD, the "Bacterial Reverse Mutation Assay", referred to the test conditions and positive controls. However, it should be noticed that due to the negative results observed, the experiment needed to be repeated for a confirmation.

The test report does not include the identification data and the CAS no., as requested by the OECD protocol.

2. Chromosomal Aberrations in Human Whole Blood Lymphocytes with PFOS

2.1. Identification of the test chemical

The test chemical evaluated in this study was indicated as PFOS, FC-95, Lot #217, as white crystalline powder.

No other identification properties are included in the report. The purity of the test chemical was not indicated.

The test chemical was dissolved in DMSO: a solution was obtained with a 1.0% dilution of the DMSO stock solution (401 mg/ml).

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2.2. Biological material

Lymphocytes were obtained from human venous blood provided by a healthy, male adult donor.

For chromosome aberration assay, replicate cultures were used at each concentration, vehicle control and positive control.

In the presence and in the absence of a metabolic system, the treatment with the chemical lasted 3 hours; the cultures were later incubated for 22 hours, corresponding to 1.5 times the cell cycle time (15 hrs.).

2.3. Metabolic activation

The assay was performed in the presence and in the absence of a metabolic system. This was provided by S9 mix, liver homogenate, purchased by Molecular Toxicology Inc. Lot No.929. The homogenate was prepared from male Sprague-Dawley rats injected (i.p.) with Aroclor 1254 at 500 mg/kg 5 days before sacrifice.

Data on the composition of the S9 are included in the Report.

2.4. Doses of the chemical

Concentrations of the test chemical were between 27.2 and 400 $\mu\text{g/ml}$ (15 concentrations) in the first experiment.

The assay was repeated with concentrations between 12.5 and 559 $\mu\text{g/ml}$ (11 concentrations) without metabolic activation and between 12.5 and 449 $\mu\text{g/ml}$ (10 concentrations) with metabolic activation 100 cells/replicate flask for 4 concentrations of the treated cultures, the negative control, and one positive control were analysed. The mitotic index was evaluated in 1000 cells.

2.5 Positive and negative controls

Mitomycin C (MMC) was used as positive control at 3 concentrations (1-0, 1-5, 2.0 $\mu\text{g/ml}$) for the non-activated cultures, whereas Cyclophosphamide (CP) at 3 concentrations (25m 50, 75 $\mu\text{g/ml}$) was used for activated cultures.

For the chromosome aberration evaluation only one concentration/positive control has been evaluated.

DMSO at 10 $\mu\text{L/ml}$ was used as a negative control.

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2.6 Results

The toxicity, evaluated by means of the mitotic index, was around the order of 30% in the presence of metabolic activation and 8-92% in the absence of S9 mix.

The test article did not induce chromosomal aberrations, polyploidy or endoreduplication frequencies higher than the negative control cultures, whereas the positive control treated cultures demonstrated a higher frequency of induced chromosomal aberrations in the absence or presence of metabolic activation conditions.

2.7. Comments

The study was performed in accordance with the Good Laboratory Practice regulations laid down by the US-EPA.

The test report does not include the identification data and the CAS No.

3. Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures with PFOS

3.1 Identification of the test chemical

The test chemical evaluated in this study has been indicated as PFOS, FC-95, Lot #217, as white crystalline powder.

No other identification properties are included in the report. The purity of the test chemical has not been indicated.

The test chemical was dissolved in DMSO: a suspension/translucent liquid was achieved at 4010 µg/ml.

3.2 Biological material

Rat hepatocytes were obtained by perfusion of liver; the exposure period was of 19.6 - 20 hours; 23 hours after the beginning of treatments, cytotoxicity was assessed as cellular morphology and cell survival.

Triplicate cell cultures were used for UDS analysis, made by means of thymidine incorporation.

3.3 Metabolic activation

Hepatocytes are able to undergo metabolic activation by themselves.

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3.4. Doses of the chemical

Fifteen concentrations of PFOS were applied, ranging from 4000 µg/ml to 0.250 MG/ml; the chemical was cytotoxic down from 25 µg/ml.

3.5. Positive and Negative controls

As positive control, 2-AcetylAminoFluorene (2-AAF) was used at a final concentration of 0.10 µg/ml. Negative control was provided by DMSO at a concentration of 1%.

3.6. Results

The survival of the concentrations analysed of the test chemical from 25 µg/ml to 0.5 µg/ml was between 65 and 100%; the one of the 2-AAF was 88.5% at a concentration of 0.1 µg/ml.

The Mean Net Nuclear Grams (MNNG) of the treated cultures were not different from the vehicle control (DMSO = 1% survival) while 2-AAF treated cultures (0.1 µg/ml) presented a M.N.N.G. of 19.35.

In this study, PFOS was considered not able to induce UDS in rat hepatocytes treated *in vitro*.

3.7. Comments

The study was performed in accordance with the Good Laboratory Practice regulations laid down by the US-EPA.

The protocol is similar to the OECD Guideline 482.

The Report does not include identification data and CAS No. of the test chemical.

4. Mutagenicity test on T-6295n in an *in vivo* mouse micronucleus Assay

4.1. Identification of the test chemical

The test chemical evaluated in this study has been indicated as T-6295, as off-white mixture of powder and flakes,

There are no indications referring to the PFOS chemical.

The chemical was dissolved in corn oil, carboxymethyl cellulose and acetone.

4.2 Biological material

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Adult male and female mice, strain Crl: CD-1 (ICR) BR were used for this assay. After a preliminary range finding assay, 3 doses were employed in the main assay, namely 237.5 mg/kg - 475 mg/kg - 950 mg/kg. 5 males and 5 females were used for each dose and for treatment (24 - 48 -72 hours). In the highest dose (950 mg/kg) a few animals died and were replaced by others treated in the same way. Treatment was by oral gavage.

4.3. Positive and Negative Controls

Positive control was provided by 10 animals (5M and 5F) treated with Cyclophosphamide, 80 mg/kg and analysed 24 hours after treatment. Negative control was provided by 10 animals (5M and 5 F) treated with deionised water and analysed 24 hours after treatment. Bone marrow cells were analysed for the presence of micronuclei, the polychromatic erythrocyte (PCE) and the normochromatic erythrocyte (NCE).

1000 PCE per animal were scored and the no. of micronuclei was recorded. The spontaneous micronuclei frequency for this strain is about 0-0.4%.

4.4. Results

The chemical tested did not induce a significant increase in micronuclei induction frequency in all conditions and concentrations.

There was indication of some toxicity at 48 and 72 hours treatment, indicated by the ratio PCE:NCE.

The positive control (Cyclophosphamide) induced a significant increase in the percentage of micronucleated cells.

4.5. Comments

The study was performed in accordance with the Good Laboratory Practice regulations laid down by the Food and Drug Administration (FDA).

The protocol was similar to the OECD Guideline 474.

The Report does not include the information on the identification data and CAS no., as requested by OECD Guideline 474.

GENERAL CONSIDERATIONS

1. Perfluorooctanesulfonate (PFOS) was tested for the evaluation of its mutagenicity/genotoxicity in three *in vitro* systems, namely (i) the induction of gene mutations in bacterial cells, (ii) the induction of chromosome aberrations in human lymphocytes, (iii) the induction of UDS in rat primary liver cells, and in one *in vivo* system, namely (iv) the induction of micronuclei in bone marrow cells of mice. Besides some inadequacies present in the reports which could be corrected, due to the existence of data required, PFOS resulted a chemical classifiable as a non-mutagenic compound, thus representing no mutagenic risk. Data presented for this chemical allow its classification as a "probable non-mutagen/non-genotoxic *in vivo*", according to IPCS classification and proposal (1).
2. Mutagenicity/genotoxicity tests are useful predictive systems for both carcinogenicity and mutagenicity; they have been extensively validated in a set of international programmes during the last 30 years.

Several strategies have been proposed to use a small battery of test methods with the highest probability to produce positive and easily interpretable results. In 1995, IPCS (ASHBY, J. *et al.*) proposed the scheme reported in Fig.1. IPCS' strategy is based on the use as a first tier of the two most employed *in vitro* test methods for analysing the potential for the induction of gene mutation and of chromosome aberrations, namely (1) the Reverse Mutation Assay using Bacteria (OECD Guidelines 471 and 472) and (2) *In Vitro* Mammalian Chromosome Aberration Test (OECD Guideline 473). OECD Guidelines for performing these two *in vitro* tests have been revised several times by the group of experts on the basis of development of techniques and methodologies, as well as on the basis of experience developed during the years on the use of the two methodologies. The present estimate of the amount of chemicals tested by these two methodologies amounts to more than 10,000 assays. Several *in vitro* genotoxicity tests are currently available. The combination of two *in vitro* tests:

- bacterial reverse mutation test (or *n vitro* mammalian cell gene mutation test for specific chemicals, for which a scientific justification must be provided)
- *in vitro* mammalian cell chromosome aberration test

provide in general, sufficient evidence of mutagenic and/or genotoxic potential. (2)

Depending on the results, other *in vitro* or *in vivo* tests may be required. Use of *in vivo* tests is limited to confirmation of a mutagenic activity already observed *in vitro*. The scheme of testing chemicals of different use for their mutagenic/genotoxic potential proposed by several different agencies bases its scientific rationale on the

scheme proposed by ICPS, as the latter allows identification of the two types of mutagenic/genotoxic hazards of the chemicals, namely carcinogenic or somatic cell mutagen and genetic or germ cell *in vivo* mutagen.

The same scheme has been accepted by a set of national health protection agencies, among which the Department of Health of the United Kingdom, whose Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment, in 1989 stated as follows:

"Where a chemical is not proposed for human ingestion and where it is expected that its use will be limited, and the degree of human exposure is small or containable, it is acceptable to limit investigation to two tests, one to detect gene mutation in bacteria, and the second to detect clastogenic effects in mammalian cells". (3)

IPCS' scheme for testing for mutagenicity/genotoxicity is exemplified in Fig.2 developed in those cases in which the results of the first two *in vitro* tests produced both or only one positive result, thus indicating a certain potential to induce mutagenic effects.

It should also be considered that the results of the mutagenicity are not valid at 100% level; the same can be said for all the toxicological studies, when defining the toxicological hazard and the possible risk for consumers.

However, in order to illustrate the value of such tests, in the sector of mutagenicity, it is possible to evaluate the probability of one chemical to be carcinogenic or non-carcinogenic on the basis of the results of mutagenicity tests. The other predictive value of the mutagenicity tests, namely the probability of the prediction of the germ, cells mutagens is at present not possible due to scarcity of data.

N.LOPRIENO *et al*, have investigated the relationships between the mutagenicity testing results of the two *in vitro* assays, one *in vivo* assay and the results of carcinogenicity assays. The results are reported in Fig.3 which shows the accuracy of the correct identification of chemicals for a reliable number of assays, derived from the analysis of a total of 3762 chemicals (see Fig.4) (4)

Since fluorochemicals residuals of different chemical structure (and biological reactivity?) are known to be present in a fluorochemical product and since some of them have been found in plant effluent or in groundwater at different level concentrations, it seems reasonable to develop a full programme of testing for mutagenicity/genotoxicity potential of all possible derivatives which have been found in the environment or which could also be predicted to produce to some extent a specific human exposure.

Among them, for instance N-EtFOSA and N-MeFOSA, PFHS, N-EtFOSE alcohol and N-MeFOSE alcohol, etc.

The mutagenicity/genotoxicity testing programme should include, for each chemical, the two basic *in vitro* complementary tests, namely:

- the induction of gene mutation in bacterial cells (4 *Salmonella* strains and 1 *E.coli* strain);
- the induction of chromosome aberrations in a mammalian cell line (Hamster Ovary Cells or human lymphocytes).

The results of these two tests, if negative on the basis of PFOS' results, could reinforce the concept of the absence of a mutagenic/genotoxic risk in connection with a potentially human exposure to these chemicals.

All types of results obtained with a testing of many fluorochemical derivatives should moreover contribute to the establishment of a data-base system for further considerations of the toxicological profile of these specific chemicals.

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REFERENCES

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