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**Toxicity of Ambient Particulate Matter**
II. Acute toxicity study in asthmatic mice following 3-
day exposure to fine ammonium ferrosulfate, a model
compounds for secondary aerosol fraction of $PM_{10}$.
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# Preface

Toxicity studies on particulate matter components are conducted within the framework of a research program on adverse health effects of ambient particulate air pollution. These studies are part of the Dutch "Wintertime Smog Program and Traffic", performed by order and at the account of The Ministry of Housing, Spatial Planning and Environment. The first part of this program included epidemiological studies on health effects of ambient particulate matter (PM) and the possible role of traffic emissions. The second part of this program includes toxicity studies on ambient PM and has been designed in accordance with the simplified strategy of the health risk assessment model for ambient PM, i.e. the "Pentagon" model of possibly main critical fractions with respect to particle size and chemical composition.

The PM toxicity program is directed to cardiorespiratory toxicity, as an important pathogenic mechanism, in healthy animals and animal models mimicking various human cardiopulmonary diseases. In combination with the development and use of ambient PM deposition models and exposure-dose relationships, this work will be an important support for quantitative risk assessment in healthy people and risk groups with asthma airways.

The 1st phase of these toxicity studies has been started in 1996 and the results of the second 3-day inhalation study (with ammonium ferrosulfate) in healthy mice and mice with mild asthmatic symptoms are presented in this report. The results of this and successive studies performed in this framework will be integrated in a review report on the toxicity of secondary aerosols.

# Samenvatting

In dit tweede rapport over acute inhalatiestudies met modelcomponenten van fijnstof in de buitenlucht worden de resulaten gepresenteerd van een studie met ammoniumferrosulfaat in "astmatische"dieren. De keuze van een zout met een overgangsmetaal (ijzer) is ingegeven door de hypothese dat dit radicaalvorming kan versterken. Het diermodel voor astma is ingezet omdat astmatici een risicogroep lijken te zijn als het gaat om blootstelling aan fijnstof.

We hebben gezonde en "astmatische" muizen blootgesteld aan fijn (MMD = 459 nm; $4 \times 10^3$ deeltjes per $cm^3$) ammoniumferrosulfaat gedurende 4 uur per dag drie dagen lang. De gemiddelde massa concentratie bedroeg 250 $\mu g/m^3$. De muizen werden 1 dag na de laatste blootstelling opgeofferd om de pulmonaire reactie vast te stellen middels longspoelvloeistof (BALF) analyse, luchtwegreactiviteitsmetingen en histopathologie.
Er werden geen veranderingen van LDH, NAG en eiwitgehaltes in BALF gevonden als gevolg van de blootstelling aan fijne aerosolen. De veranderingen in TNF-$\alpha$ en celdifferentiatiebeeld waren marginaal en worden niet relevant bevonden.

Pathologisch onderzoek kon geen effect van de blootstelling aan aerosolen vaststellen. Wel was het effect van de behandeling met ovalbumine aantoonbaar, namelijk hypertrofie van Goblet cellen in bronchus epitheel en mononucleair infiltraat. Ook hier kon geen effecten van blootstelling aan fijnstof worden aangetoond. Dit geldt ook voor de niet-gecompromitteerde dieren. Ook had de test atmosfeer geen invloed op de luchtwegreactiviteit, een belangrijke uitleesparameter voor astma.
De hier gepresenteerde gegevens suggereren dat bij net iets hogere concentraties dan de in Nederland voorkomende concentraties en bij een korte blootstellingsduur geen pulmonaire effecten in normaal gezonde dan wel gecompromitteerde muizen (als model voor astma) optreden. Er zijn geen indicaties voor een versterking van de allergische reactie. In vergelijking met eerder experimenten waarbij aan ammoniumbisulfaat werd blootgesteld kon geen effect van het overgangsmetaal ijzer worden aangetoond.

# Summary

In this second report on acute inhalation studies with model compounds for secondary particulate matter results are presented of a study with fine ammonium ferrosulfate aerosol in asthma animals. We hypothesized that a aerosol with a transitional metal can produce more enhance symptoms of asthma. We have exposed healthy and mice to fine (MMD = 459 nm; $4.1 \times 10^3$ particles per $cm^3$) ammonium ferrosulfate for 4 hr a day on 3 consecutive days. The mean actual mass concentration was 250 $\mu g/m^3$. Mice were sacrificed 1 days post-exposure to determine their pulmonary response using bronchoalveolar lavage fluid (BALF) analysis, airway reactivity measurements and histopathology.

No changes in NAG, LDH and protein levels in BALF were observed following exposure to fine ammonium ferrosulfate. Changes in TNF-$\alpha$ and cell differentials were marginal and considered not being biologically relevant. Gross and histopathological examination showed no treatment-related abnormalities in lungs except for a hypertrophy of Goblet cells in bronchus epithelium and mononuclear infiltrate in asthma mice. This latter observation was a result of the ovalbumin treatment. Ovalbumin-specific serum IgE levels were undetectable in healthy mice and significantly increased in asthma mice. Exposure to fine particles did not lead to alteration in tracheal reactivity in mice in which asthma-like symptoms were induced.

These preliminary data suggest that ammonium ferrosulfate in realistic concentrations and exposure duration as a model compound for the secondary fraction of ambient PM10 exert no pulmonary effects in normal healthy mice and in a mouse model of asthma, and there are no indications of enhanced allergic response. In comparison with a previous experiment in which animals were exposed to ammonium bisulfate, no additional effect of the transitional metal iron could be established.

# 1.    Introduction

The PM toxicity studies are conducted within the framework of a toxicity research program on health effects of relevant components of ambient PM. This program, conducted by RIVM, TNO Toxicology, and KUN Toxicology, is part of the Dutch "Wintertime Smog and Traffic Program". This is the second report on acute inhalation studies with model compounds of ambient particulate matter.

Recent experimental animal studies show that intratracheal instillation (high dosage of 2.5-5 mg/rat) as well as in vitro incubation of fine or coarse particles ($\sim$ 0.4-10 $\mu$m; mineral dusts, PM collected from ambient air, fly ash samples) cause (pulmonary) cytotoxicity, inflammation, and production of reactive oxygen species, also in an animal model for pulmonary hypertension (Hatch et al. 1985; Ghio et al. 1992; Ghio and Hatch 1993). The oxidative potency of these types of particles (iron ions added to $TiO_2$, ambient PM from various locations, residual oil fly ash) seems to be proportional with the content of first-row transition metals (such as iron, vanadium, and zinc) associated with the particles (Costa et al. 1994; Becker et al., 1996; Gilmour et al., 1996; Pritchard et al. 1996). These metallic components are able to participate in electron transfer and redox cycling reactions resulting in free radical generation ($O_2^-$, OH, lipid peroxides). Solubility or ionizability of these components seem to be a prerequisite for (oxidative) reactivity, as demonstrated with extracted PM samples (loosing their activity) and positive correlations of effects with the content of soluble components.

The studies described in this report were aimed to identify adverse effects of ammonium ferrosulfate aerosol in healthy and asthma (asthma) mice. We hypothesized that ammonium ferrosulfate particles as a model compound for secondary aerosol enhances inflammation and hyperresponsiveness in a mouse model that mimics asthma because the transitional metal iron increases the production of toxic radicals in the lung. We have exposed mice 4 hr/day for 3 consecutive days and investigated the effects of fine particles on the lung. In this study we have focused on biochemical, histopathological, cytological and immunological parameters indicative for cytotoxicity and asthmatic symptoms.

# 2.    Experimental

## 2.1    Testing facilities

The studies described in this report were conducted September 1996 at the department of Toxic Effects of the Laboratory of Health Effects Research, RIVM, the Netherlands.

## 2.2    Test material

Crystalline ammonium ferro(II)sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2.6H_2O$) was supplied by Aldrich-Chemie, Steinheim, Germany and has the following characteristics:

|                    |                                                      |
| ------------------ | ---------------------------------------------------- |
| CAS nr.            | : 7783-85-9                                          |
| Batch nr.          | : 06774-065                                          |
| Purity             | : 99 %                                               |
| Density            | : 1.864 kg/l                                         |
| Molecular weight   | : 392.14                                             |
| Melting point      | : 100°C.                                             |
| Solubility         | : 26.9 g/l                                           |
| Storage conditions | : room temperature, dark                             |
| Toxicity           | : irritating for skin, eyes and the respiratory tract |

## 2.3    Test animals

Specified pathogen free male BALB/c mice (6-8 weeks old) were obtained from the breeding colony of the National Institute for Public Health and the Environment, Bilthoven, the Netherlands. The mice were housed in macrolon cages and food and water was provided *ad libitum* during non-exposure periods. The acclimatisation period was at least 7 days. The room temperature was maintained at 22 ± 2°C and the relative humidity at 40-70% with a 12 hr light/dark cycle. The mice were fed a cereal-based rodent diet (SMR-A; Hope Farms, Woerden, the Netherlands). The day before the start of each substudy, the mice were identified with an earmark to be able to discriminate between sensitized and non-sensitized mice. One day prior to the challenge procedure each mouse was marked according to the allocation list with a unique number with was coded by placing stripes on the tail using felt-tips.

## 2.4    Location of the study

Mice arrived at the Institute's animal housing facilities (D6D) at least 7 days prior to the start of the study. The check for body weight variation, the random allocation and the sensitization procedure were performed in D6D. At day 33 groups of mice were transported to E2, where they were trained in the restraining tubes, challenged with ovalbumin and exposed to the test atmosphere. Part of the animals of each substudy was used for airway reactivity measurements, histopathology and serum collection and the rest was used for bronchoalveolar lavage.

## 2.5    Conduct of the study

The study was performed with 96 mice. Before exposure to the test material, half of the mice were asthma as described under 2.5. The animals were exposed to the test material for 4 hr a day on 3 consecutive days. Immediately after exposure, the animals were returned to their living cages, 4-6 animals per cage.

| Number of mice | Healthy | Asthma |
|---|---|---|
| Sham exposure | 16 | 16 |
| Ammonium ferrosulfate | 32 | 32 |

All mice were sacrificed 1-day post exposure. Mice were either used for bronchoalveolar lavage and serum collection or airway reactivity measurements and histopathology.

## 2.6    Sensitization and antigen challenge

Animals were sensitized by seven intraperitoneal 0.5 ml injections of 0.02 mg ovalbumin (Grade II; Sigma, St.Louis, MO) per ml saline vehicle on alternate days (days 0, 2, 4, 6, 8, 10 and 12). From day 34 these mice were exposed to ovalbumin (0.2 % w/v) aerosol for 5 min. at 8 consecutive days. On day 39 and 40 challenges were performed right after the exposure to the test material. The aerosol was generated with an ultrasonic nebulizer (Devilbiss 2000, particle size 5 μm) connected to a Plexiglas whole-body exposure chamber (27 l). The challenge was given in groups of maximal 6 animals (Hessel *et al.* 1995).

## 2.7    Exposure system

Mice were exposed in nose-only individual exposure units of the Institute's design. During exposure the animals were restrained in animal holders (modified Battelle tubes) which closely fit to the exposure chamber. The animals were allowed to breathe freely from the stream of fresh test atmosphere. During exposure animals were deprived from food and water.

## 2.8    Generation of the atmosphere[1]

The aerosol was generated by ultrasonic nebulization (Omron NE-U12, Omron Corporation, Tokyo, Japan) of an aqueous solution of ammonium ferrosulfate. The molarity of this solution determined the size of the final aerosol. Large droplets were removed by impaction. The main aerosol stream was led through a heating tube to evaporate the water. Water vapor was removed in a diffusion-drying chamber filled with silica beads. This resulted in solid sulfate particles. To improve the performance, the dryer was cooled with a cooling device. The final concentration was reached by diluting the aerosol stream with purified air.

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[1] A detailed generation report is added to the files of this study.

## 2.9     Analysis of the test atmosphere[2]

The mass concentrations of the test atmospheres were determined using a Real-time Aerosol
Monitor (RAM-1, MIE Inc., Bedford, Ma, USA) for fine particles.

Particle size, total number of particles and size distribution were determined with a scanning
mobility particle sizer consisting of an electrostatic classifier (TSI 3071, TSI Inc., St. Paul,
Min., USA) and a condensation nucleus counter (TSI 3022A, TSI Inc., St. Paul, Min., USA).
In addition, an aerodynamic particle sizer (APS, TSI 33, TSI Inc., St. Paul, Min., USA) was
used to check the number and size of particles above 500 nm.

Relative humidity and temperature of the test atmosphere were measured conventionally. The
morphology of the fine particles was visualized by scanning electron microscopy (SEM).
Freshly generated particles were sampled on a Teflon filter and processed for SEM.

## 2.10     Observations and measurements

The mice were visually daily inspected for reactions to treatment (sensitization and challenge
with ovalbumin and exposure to the test material).

## 2.11     Body weights

Body weights were recorded prior to the start of the study and just before sacrifice.

## 2.12     Bronchoalveolar lavage and measurements

Mice were anaesthetized with natriumpentobarbital. Each lung was lavaged three times with 40
ml/kg body weight with saline at 37 °C. These procedure was repeated and the collected fluid
(BALF) was centrifuged for 10 min. at 200 g. The supernatant was used for biochemical and
immunological measurements. The pellet was resuspended in saline and was used for total cell
number and cell differentials.

### 2.12.1     Biochemistry

Lactate dehydrogenase was determined according to a procedure by the Scandinavian
Committee on Enzymes (1974), N-acetyl glucosaminidase was determined according to
Yakata (1983) using a reagent kit (cat. no. 875406 obtained from Boehringer Mannheim
(Mannheim, Germany), total protein was determined according to Smith, et al. (1985) using a
reagent kit (Cat. no. 23225) obtained from Pierce (Oud-Beijerland, the Netherlands). LDH
was measured as a marker for cytotoxicity, NAG was determined as an indicator for
macrophage activation, and total protein levels in BALF were measured as an indicator for
increased permeability of the endothelium of the lung.

### 2.12.2     Immunology

A Cytoscreen immunoassay kit (Biosource international, Camarillo, CA) was used to determine
TNF-$\alpha$. This cytokine was measured as being an important mediator involved in the
communication network of the cells of the immune system. The primary cell sources of TNF-$\alpha$
are macrophages and will result in increased phagocytosis, enhancement of immune- and
inflammatory responses.

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[2] A detailed exposure report is added to the files of this study.

## 2.12.3   Cytology

Total number of cells and the viability were counted using a Bürker-Türk chamber. For differential cell counts cytospin preparations were made and stained with May-Grunwald Giemsa. Per cytospin preparation 400 cells were counted and the proportion of each cell type (macrophages, neutrophilic granulocytes, eosinophilic granulocytes and lymphocytes) was calculated based on total viable cells per BALF.

## 2.13   Serum IgE[3]

A sandwich micro-ELISA was used for the quantification of mouse IgE to ovalbumin. Microtiterplates were coated with anti-immunoglobulin solution (Monoclonal Rat Anti-mouse IgE, 2 µg protein/ml, clone LO-ME-2, Zymed) and incubated with diluted sera samples, Ovalbumin-DIG (DIG Antibody Labeling Kit, Boehringer Mannheim, Germany) and Anti-Digoxigenin-POD Fab fragments (Boehringer Mannheim, Germany), respectively. Finally, the peroxidase activity was visualized by an incubation in tetramethylbenzidine (TMB-$H_2O_2$ solution (6 mg per 100 ml sodiumacetate buffer + 20 µl of 30% $H_2O_2$)). The IgE levels are given as a percentage of a standard.

## 2.14   Airway reactivity

Airway reactivity was measured according to a method published by Garssen *et al.* (1990). Briefly, mice were killed with an intraperitoneal injection of 0.25 ml Nembutal (pentobarbitone sodium 60 mg/ml, Abbott Laboratories, North Chigago, Il, USA). The tracheas, resected in toto, were transferred to a petri dish containing a modified oxygenated Krebs'carbonate solution (118.1 mM NaCl; 4.7 mM KCl; 2.5 mM $CaCl_2.6H_2O$; 0.5 mM $NaHCO_3$; 1.0 mM $NaH_2PO_4$; 11.1 mM glucose). The trachea was prepared free of excess tissue under a binocular preparation microscope and a piece of approximately 6 mm length (8 trachea rings, just beneath the larynx) may taken. Tracheas were immediately slipped onto two supports in an organ bath, of which one was connected to an isometric transducer. The organ baths were filled with 20 ml of the Krebs' solution. The solution was continuously gassed with a mixture of 95% $O_2$ and 5% $CO_2$. The temperature was maintained at 37°C with a constant temperature-circulating unit.

Isometric tension was measured with a force displacement transducer (Servogor transducer, Harvard Bioscience, Boston, USA) and a two-channel recorder (Servogor type SE-120) and is expressed as changes in grams force. The optimal basic force is 1. The tracheas were allowed to equilibrate for at least 45 min. before methacholine was applied. During equilibration, the bath fluid was changed once every 15-min. Metacholine was prepared in Krebs' solution and kept on ice for the duration of the test. A cumulative contractile concentration-effect curve was determined for each trachea.

## 2.15   Histopathology

At autopsy the animals were anaesthetized and the trachea was removed for determination of airway reactivity. The lungs were removed and a canule, inserted in the rest of the trachea or in one of the bronchi, was fixed. The lungs were fixed intratracheally with 2% glutaraldehyde in a 0.1 M phosphate buffer at a pressure of 20 cm for one hour. After immersion fixation in

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[3] A detailed description of the procedure is added to the files of this study.

formalin the lungs were embedded in Paraplast and sliced in 5 μm sections. Slides were stained with haematoxylineosin and periodic acid Schiff-Alcian blue.

## 2.16 Statistical analysis

All values are expressed as the mean ± SD. All statistical analysis were performed using STATA (version 2.05, CRC, Oasis, Nieuwegein the Netherlands). The (log-transformed) data were analyzed using multiple one-way analysis of variance. The assumption of homogeneity of variance was tested with the Bartlett test. For some parameters, analysis of the control animals showed differences between the three sub-studies. Therefore responses of dose were analyzed separately. Since homogeneity of variance was not obtained for the IgE data, these data were analyzed using the Kruskal-Wallis non-parametric test.

Airway reactivity data were analyzed with an ANOVA with all data points of the concentration-effect curves. $EC_{20}$ (the concentration metacholine that induces 20 percent of the maximal effect), $EC_{50}$ and $EC_{80}$ as well as $E_{max}$ were calculated from fitted curves and these values were analyzed with a t-test.

## 2.17 Deviations from the protocol

The deviations encountered in the present study are not considered to have influenced the validity of the study. Data of BALF analysis of animal nr. 41 (treated with ovalbumin and ferrosulfate aerosol) were rejected because the values were far out of range of the rest of the data from this group.

## 2.18 Retention of the records

Raw data, the master copy of the final report and all other information relevant to the quality and integrity of the study, will be retained in the archives of the Laboratory of Health Effects Research of the RIVM for a period of at least 5 years after the reporting of the study.

# 3.    Results

## 3.1    Analytical results of the test atmosphere

### 3.1.1    Actual concentration (#)

The actual concentration was determined by measuring the number of particles per volume and the results are summarized in Table 1.

**Table 1**    Actual exposures concentration ($\#/cm^3$) of fine ammonium ferrosulfate aerosol. Values are expressed as means ± SD.

| Day | Number concentration ($\#/cm^3$) |
|-----|----------------------------------|
| 1 | $4.9 \cdot 10^3$ |
| 2 | $3.6 \cdot 10^3$ |
| 3 | $3.7 \cdot 10^3$ |
| Mean | $4.1 \cdot 10^3 \pm 0.91 \; 10^3$ |

### 3.1.2    Particle size measurement

The results of the size measurements of the scanning mobility particles sizer are summarized in Table 2.

**Table 2**    Count Median Diameter (CMD) and Mass Median Diameter (MMD) as determined with SMPS of fine ammonium ferrosulfate aerosol. Values are expressed as means ± geometric standard deviation.

| Day | Particle Diameter (nm) CMD | MMD |
|-----|-----|-----|
| 1 | 230 ± 1.9 | 408 ± 1.5 |
| 2 | 256 ± 1.8 | 487 ± 1.5 |
| 3 | 247 ± 1.8 | 488 ± 1.5 |
| mean | 244 ± 1.8 | 459 ± 1.5 |

### 3.1.3    Mass measurements

The mass concentrations are presented in Table 3.

**Table 3**    The mean calculated and measured mass concentration of exposures to fine ammonium ferrosulfate aerosol. Values are expressed means ( 90% confidence limits).

| Day | Mass concentration $\mu g/m^3$ |
|-----|-------------------------------|
| 1 | 248 (114 - 322) |
| 2 | 246 (208 - 322) |
| 3 | 255 (208 - 322) |
| mean | 250 (177 - 322) |

### 3.1.4   Temperature and Relative Humidity

**Table 4**          The mean temperature and R.H. during. Values are expressed as means ± SD of 3 consecutive exposure days.

| | Temperature (°C) | RH (%) |
|---|---|---|
| control | 21.1 ± 1.7 | 61.6 ± 9.3 |
| ammonium ferrosulfate | 22.2 ± 2.1 | 47.6 ± 8.7 |

### 3.1.5     Shape of the particles

The fine aerosol test atmospheres appeared to consist of spherical shaped particles (Fig. 1)
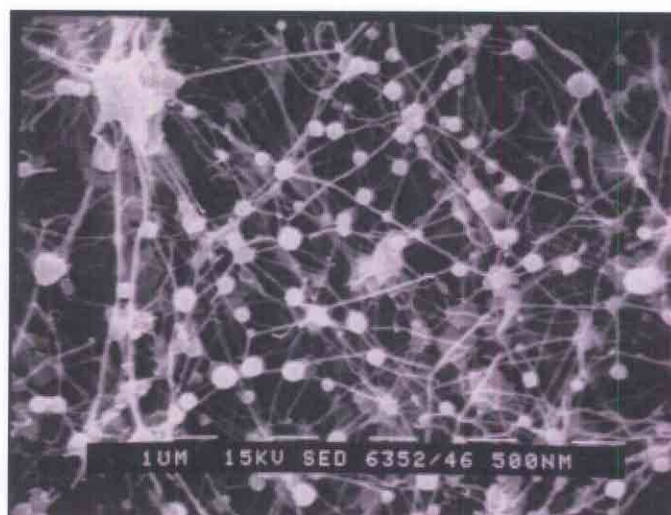


Fig.1    Scanning electron microscopic photographs of fine ammonium ferrosulfate particles collected filter.

## 3.2        Clinical observations

No abnormalities were observed in mice that were either sensitized plus challenged with ovalbumin, in mice that were exposed to the test material, or both.

## 3.3    Body weights

No differences in body weights were observed between controls and asthmatic mice. Neither were there treatment-related changes in the body weights 1-day post exposure.

**Table 5**      Body weights of healthy and asthma mice exposed to fine ammonium ferrosulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-7 for all groups).

| Type | control[a] | asthma |
|---|---|---|
| control | 30.3 ± 1.86 | 29.4 ± 1.11 |
| ammonium ferrosulfate | 29.2 ± 1.53 | 28.4 ± 2.39 |

a. Values are expressed as gram body weight

## 3.4 Bronchoalveolar lavage

### 3.4.1 Biochemistry

The results of the BALF protein, LDH and NAG measurements are shown in Table 6. No statistically significant differences between either control mice and mice exposed to ammonium ferrosulfate or between healthy and asthma mice could be established.

**Table 6** BALF levels of NAG, LDH and protein in healthy and asthma mice exposed to fine ammonium ferrosulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-6 for all other groups).

| Type | NAG[a] | LDH[a] | Protein |
|---|---|---|---|
| control | 0.64 ± 0.12 | 122 ± 99 | 187 ± 55 |
| asthma | 0.72 ± 0.16 | 119 ± 51 | 195 ± 32 |
| ammonium ferrosulfate | 0.73 ± 0.11 | 132 ± 67 | 194 ± 34 |
| asthma + ammonium ferrosulfate | 0.72 ± 0.10 | 99 ± 59 | 193 ± 17 |

a. Activities are expressed as U/l BALF

### 3.4.2 Immunology

The results of BALF TNF-α measurements are shown in Table 7. Large inter-group variation was observed in all four groups.

**Table 7** BALF levels of TNF-α in healthy and asthma mice 1 day post exposure to fine ammonium ferrosulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n = 5-7 for all other groups).

| Type | TNF-α (pg/ml BALF) | |
|---|---|---|
| | control | asthma |
| control | 180 ± 193 | 306 ± 173 |
| ammonium ferrosulfate | 315 ± 202 | 289 ± 98 |

### 3.4.3 Cytology

The results of the cell measurements in BALF are summarized in Tables 8 and 9. No treatment-related effects were observed in total cell counts or the percentage of the different cell types that were distinguished.

**Table 8**  Viability of lavaged cells from lungs of healthy and asthma mice exposed to fine ammonium ferrosulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n = 5-7 for all other groups).

| Type | control[a] | asthma |
|------|-----------|--------|
| control | 93 ± 5 | 86 ± 7 |
| ammonium ferrosulfate | 91 ± 4 | 90 ± 10 |

**Table 9**  Total number of macrophages, neutrophils, and eosinophils plus lymphocytes in BALF of healthy and asthma mice exposed to fine ammonium ferrosulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n= 5-7 for all other groups).

| Type | Macrophages | | Neutrophils | | Eosinophils and Lymphocytes | |
|------|-------------|-------------|-------------|-------------|-------------|-------------|
| | control $\times 10^3$ cell/lung | asthma $\times 10^3$ cell/lung | control $\times 10^3$ cell/lung | asthma $\times 10^3$ cell/lung | control $\times 10^3$ cell/lung | asthma $\times 10^3$ cell/lung |
| control | 130 ± 81 | 74 ± 23 | 2 ± 2 | 2 ± 1 | 1 ± 1 | 4 ± 3 |
| Ammonium ferrosulfate | 155 ± 72 | 64 ± 29 | 2 ± 1 | 1 ± 0 | 2 ± 2 | 2 ± 2 |

To establish the effect of ferrosulfate on non-macrophages in asthma mice, slides were recounted. One hundred cells (or the maximum number of non-macrophages on a slide) were counted and the percentage of eosinophils, neutrophils and lymphocytes were calculated (Table 10). Since no effect was observed in these two groups and the number of eosinophils was almost zeroing per slide, the slides of the other two groups were not counted.

**Table 10**  Differential cell counts in BALF of asthma mice exposed to fine ammonium ferrosulfate during 4 hr/day for 3 consecutive days. Data are expressed as percentage (n=5-6).

| | Neutrophils | Eosinophils | Lymphocytes |
|------|-------------|-------------|-------------|
| Control | 58.3 ± 24.9 | 19.7 ± 20.4 | 15.5 ± 6.8 |
| Ammonium ferrosulfate | 53.9 ± 13.2 | 14.2 ± 11.1 | 27.3 ± 16.0 |

## 3.5 Serum IgE

The result of the serum IgE determinations is summarized in Table 11. Significant increased serum IgE directed against ovalbumin could be detected in mice that were sensitized and challenged with ovalbumin compared to their controls. No significant differences were observed between sham sulfate exposed mice. It should be noted that the variation in ovalbumin specific IgE levels ranged from 3 to 293 % of the standard within a group.

**Table 11**  Serum Ig-E levels (specific for ovalbumin) of asthma mice exposed to ammonium ferrosulfate during 4 hr/day for 3 consecutive days. Data are expressed as means ± SD (n = 5-7 for all other groups).

| Type | control[a] | asthma |
|---|---|---|
| control[a] | n.d. | 42.87 ± 43.98 |
| ammonium ferrosulfate | n.d. | 63.38 ± 113.32 |

a.  Mice that were not sensitized and challenged with ovalbumin had no detectable (n.d.) IgE directed against ovalbumin

b.  Values are expressed as percentage of a standard (see Experimental section)

## 3.6   Airway reactivity

Concentration-effect curves of *in vitro* reactivity of the trachea to methacholine in mice, in which asthma-like symptoms were induced, were not influenced by exposure to any of the exposures, irrespective the type of analysis. The concentration-effect curves of methacholine in non-asthma mice were unexpectedly high compared to previous results of our laboratory in which mice were not strained in exposure tubes. The effects of handling in this study were so high that they were not distinguishable from "asthma-mice". The effects in normal and asthma mice were not considered to be caused by the exposure to particles. Neither the sensitivity or the reactivity was changed compared to their controls.
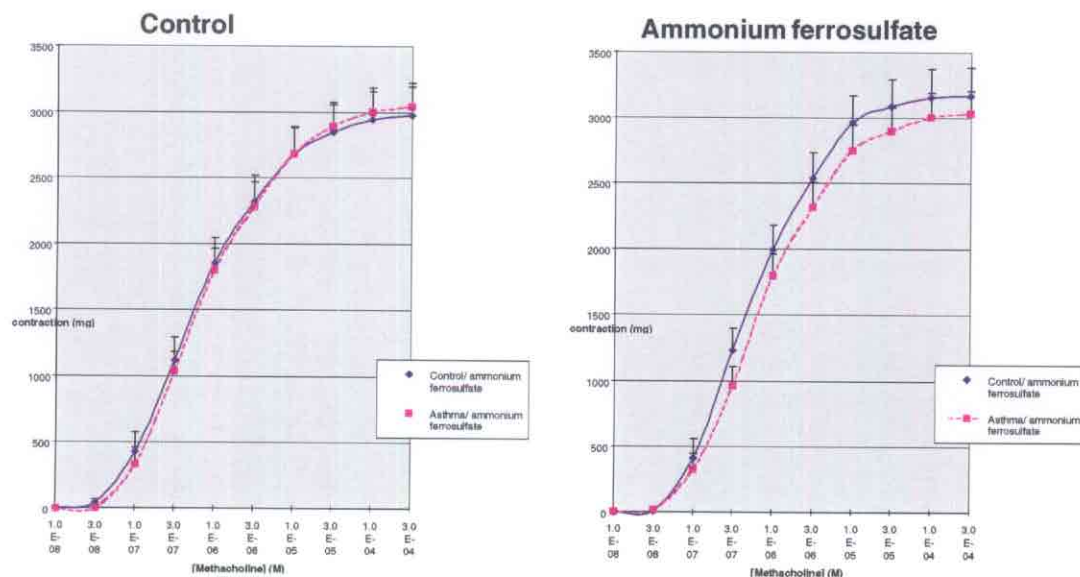


Fig. 2 Tracheal responsiveness to methacholine measured in healthy and asthma mice exposed to clean air or ammonium ferrosulfate aerosol atmospheres.

# 3.7    Pathology

## 3.7.1    Autopsy
Macroscopic examination of the mice at autopsy did not reveal abnormalities.

## 3.7.2    Histopathology
No abnormalities were observed in lungs of healthy, sham-exposed or ammonium ferrosulfate-exposed animals.

Hypertrophy of Goblet cells in the relatively large bronchioli was observed in all asthma mice (either sham exposed or mice exposed to ferrosulfate), although the variation within a group was substantial. In two animals of each group demonstrable mucus was spotted in the bronchiolar lumen. In addition, minimal to moderate mononuclear cell infiltrate was observed near the bifurcations of these large airways. One sham exposed asthma mouse had a brochiolo-alveolar adenoma. A second animal of this group showed alveolar edema throughout the lungs and a third animal had a focal perivascular infiltrate of eosinophilic leucocytes.

In three asthma mice exposed to the test atmosphere minimal inflammation in the nasal epithelium was sighted.

**Table 12**      Semiquantitative registration of hypertrophy of bronchiolar epithelium and mononuclear cell infiltrate in asthma mice exposed to fine ammonium ferrosulfate. Data expressed as incidences.

| Type | Infiltrate | Hypertrophy |
|---|---|---|
| **control** | | |
| slight | 3/6 | 0/6 |
| moderate | 3/6 | 5/6 |
| marked | 0/6 | 1/6 |
| **ammonium ferrosulfate** | | |
| minimal | 3/6 | 0/6 |
| slight | 0/6 | 0/6 |
| moderate | 3/6 | 6/6 |

# 4.  Discussion and Conclusions

Associations between exacerbation of asthma and particulate matter have been demonstrated in several recent epidemiological studies, but a causal relationship is still lacking (Gamble and Lewis, 1996). The present study was designed to peruse these observations in a mouse model that mimics asthma. This report describes the results of short-term inhalation study with mice. Healthy and asthma mice were exposed to an aerosol of ammonium ferrosulfate. We hypothesized that a model compound for secondary ambient particulate matter (sulfate) in combination with a transitional metal (iron) that has the potential to generate oxygen radicals would result in at least an inflammatory response and lung damage. Second, this model aerosol might exercbate the asthmatic symptoms provoked in mice. Moreover, we wanted to compare the results with those of a previous study with ammonium bisulfate. In the foregoing study exposure concentrations and particle size ($2.7 \times 10^3$ and $3.6 \times 10^4$ particles per $cm^3$ and 78 and 972 $\mu g/m^3$) were used. In the present study, we have exposed the animals to $4.1 \times 10^4$ particles per $cm^3$ (250 $\mu g/m^3$),

Ambient sulfate is nearly all in the accumulation mode (0.1 - 0.5 $\mu m$) of the trimodal distribution. The average sulfate aerodynamic geometric and volume based diameter have been reported to be 0.48 and 0.37 $\mu m$, respectively (Whitby, 1977). Concentrations under heavy polluted conditions can be as high as 100 $\mu g/m^3$. Ambient concentrations of Fe range from approximately 0.5 to 15 $\mu g$ per cubic meter of air in both rural and urban areas (Schroeder et al., 1987). The size of the solid spherical shaped sulfate particles in the previous and present studies were roughly 0.46 $\mu m$ MMD. The higher mass concentration used in this study, equivalent to an iron concentration of 35$\mu g/m^3$, was mainly due to differences in molecular weight values between ferrosulfate and bisulfate.

NAG, LDH, total protein, and TNF-$\alpha$ levels in BALF were unaffected by exposure to the test atmosphere, meaning that no macrophage activation, lung injury and edema have been evoked by the ferrosulfate exposures. Both histopathologic examination and counting of inflammatory cells in BALF fluid showed a minimal inflammation in asthma mice as well as decreased total cell counts, which seem to be macrophage-related. However, the inflammation was not changed by exposure to ferrosulfate particles, since results of eosinophils, neutrophils and lymphocyte counts were similar in the exposed and non-exposed asthma groups. Histopathologic examination showed no particle-related effects in either healthy or asthma mice. Control groups showed clean, healthy lungs. The method to induce symptoms that refer to asthma proved to be effective: both hypertrophy of bronchiolar epithelium and mononuclear cell infiltrate were observed. Moreover, significant ovalbumin specific IgE levels could be detected in asthma mice whereas IgE could not be detected in healthy mice. However, the variations in these levels were rather high which might implicate that the asthmatic status of the animals were variable as well. No differences were observed for airway reactivity in healthy and asthma mice. A separate study has shown that handling of the animals itself causes changes in airway reactivity. For instance, airway reactivity decreased by increasing the time of training in the exposure tubes. Another to be identified factor will be responsible for the relatively high reactivity of healthy non-asthma mice. We feel confident to state that ammonium ferrosulfate did not influence this parameter at exposure levels used in this study.

The results of this study are fully in line with our previous study in which we could not demonstrate any effect due to exposure to ultrafine and fine ammonium bisulfate in healthy

and asthma mice. This means that the addition of the transitional metal does not result in adverse health effects at the utilized exposures. At present, no peer reviewed papers have been published that can be used for comparison. In the past several studies with other sulfate aerosol have been performed. Schlesinger (1984) and Amdur *et al.* (1978) have shown that the irritant potency of inhaled sulfates (MMD $\approx$ 0.4 $\mu$m) in rabbits and guinea pigs was related to the deposition of [$H^+$] on the mucus lining the conducting airways. In human exposure studies, Utell *et al.* (1983) could not demonstrate an effect on specific airway conductance with concentrations of these sulfates up to 1 mg/m$^3$ alone, but bronchoconstrive action of a carbachol aerosol was enhanced by the acidity of the sulfates. Moreover, similar exposure concentrations of $H_2SO_4$ and $NH_4HSO_4$ decreased the airway conductance in asthmatics, showing that asthmatics are more sensitive to acid aerosol than healthy subjects. However, none of these studies were aimed to identify potency due to differences in diameter or surface of the test aerosol and the concentrations used in these studies were far above reality.

The same holds for studies with ferric sulfate and ammonium sulfate in rats reported by Phalen *et al.* (1980). Rats were exposed to 0.4-0.6 $\mu$m MMAD salt aerosol at mass concentrations up to 4 mg/m$^3$ and the influence of these exposures on the half-life time of monodisperse polystyrene latex was studied. Only ferric sulfate showed a statistically significant slowing of short-term clearance at relative low humidity only. Notwithstanding the high exposure concentrations this might still have implications for susceptibility to infection. Kleinman *et al.* (1981) have used ferric sulfate aerosol, emitted by for instance power plants, at a nominal concentration of 75 mg/m$^3$ (MMAD 2 $\mu$m) to exposure normal and asthmatic human volunteers for 2 hr. Only five out of 38 subjects showed minimal decrements of lung function and nine subjects exhibited significant improvement in function. This improvement was mainly found in non-or infrequent smokers.

Collectively, these preliminary data of this study suggest that ammonium ferrosulfate model particles exert only marginal if any pulmonary responses but no clear inflammatory effects in a mouse model of allergic asthma and there seem to be no indications of enhanced allergic response following exposure to PM.

# 5.    References

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