Doctoral Thesis

Musk xylene (MX)
Bioaccumulation, enzyme induction and developmental toxicity

Author(s):
Suter-Eichenberger, Regina

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Musk xylene (MX):
Bioaccumulation, enzyme induction
and developmental toxicity

A dissertation submitted to the
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for the degree of
Doctor of Natural Sciences

presented by

Regina Suter-Eichenberger

eidg. dipl. pharm.
born september 3rd, 1965
citizen of Beinwil am See (AG)
and Beromünster (LU)

accepted on the recommendation of
Prof. Dr. G. Folkers, examiner
PD. Dr. M. Schlumpf, co-examiner
Prof. Dr. W. Lichtensteiger, co-examiner

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1 SUMMARY

The use of scents is very old. Animal secrets, glands and other parts of organs with strong odor characteristics were first utilized for religious intentions and since antiquity also as remedies and fragrance ingredients. The odor of musk represents one of them. It mainly derives from glandular secrets of the musk deer which inhabits the Himalayas. Due to the enormous demand on natural musk, the number of animals was decreasing and finally had to be protected and replaced by other substances. Today synthetic musks are circulating, having just one property in common with the deer gland: the musky odor. There are neither similarities in chemical structure between natural and synthetic musk odorants nor between different synthetic musk groups. Musk compounds produced particularly by the industry belong to the groups of nitro musks (musk xylene (MX) and musk ketone (MK)), polycyclic musks (Galaxolide®, Tonalide® and Celestolide®) and to a smaller extent to macrocyclic musks. In 1987 the worldwide production of synthetic musks amounted to about 7000 tons per year. From 1987 up to 1996 the demand for nitro musks was decreasing by 23% to 12%. At the same time the amount of polycyclic musks was increasing by up to 85%. The decline in nitro musk production is mainly based on restrictions in the use of musk ambrette and on critical discussions about some environmental and toxicological characteristics of the nitro musks. In 1981, the occurrence of MX and MK was first reported in fish and mussels from the Tama river in Japan. In 1993, these two nitro musks were also detected in human breast milk and adipose tissue samples from Germany. Similar to some polyhalogenated compounds (pesticides, PCBs), nitro musks tend to bioaccumulate in fatty tissues, due to their lipophilic character and insignificant biodegradability. In contrast to the main human exposure route for polyhalogenated compounds which is oral uptake, nitro musks are absorbed percutaneously from perfumes and other cosmetics. Nitro musks deriving from detergents, reach the environment via waste water and sewage treatment plants and bioaccumulate in aquatic organisms. This second ecotoxicological route of exposure is oral and involuntary.

From studies investigating toxicity and carcinogenicity of MX in adult B6C3F1 mice and in adult Albino rats at rather high doses it was known that MX was inducing hepatic microsomal phase I (CYP1A2) and phase II enzymes in adult rats and CYP2B proteins
in adult mice. At that time nothing was known about possible effects on the offspring of exposed animals. Based on the lipophilicity of the compound one could assume that MX would reach the developing organism via placenta and milk. For this reason, we designed a developmental long term Long Evans rat model exposing the parent generation during at least 10 weeks according to OECD guideline for testing of chemicals in „one-generation reproduction toxicity study“ to relatively low MX concentrations, reflecting the more the naturally occurring situation.

In a first part (Suter-Eichenberger et al., 1998) we measured tissue concentrations of MX by GC-ECD in adult and developing Long Evans rats. 5-6 week old males and females were fed with MX-containing chow (0.001 g, 0.01 g, 0.03 g, 0.1 g MX/kg food pellets) for 10 weeks before mating. Treatment continued during pregnancy, birth and lactation. At 0.1 g MX/kg food, offspring at postnatal days 1 and 14 exhibited dose-dependent MX accumulations with 1/2-3/4 of adult female and 3-4 times adult male MX body fat levels. With a 0.1 g MX/kg diet the average MX intake is 7-8 mg/kg body weight per day. Milk levels were comparable to adult female adipose tissue values. Data indicate significant transplacental passage and exposure via maternal milk. In rats of both sexes fed with MX in adulthood, levels were highest in adipose tissue, in adrenal glands and ovary. Female tissue levels were 3.7-6.8 times higher as compared to male rats. This unexplained sex difference was absent in 14 day old offspring.

In a second part (Suter-Eichenberger et al., in press), 5-6 week old rats of both sexes were fed with MX containing chow (0.001 g, 0.01 g, 0.03 g, 0.1 g and 1.0 g MX/kg food pellets) corresponding to a daily intake of 0.07-0.08 mg MX/kg up to 70-80 mg MX/kg body weight. We found effects of MX on CYP1A1/1A2 enzyme and on CYP1A, CYP2B and CYP3A protein concentrations. MX induced CYP1A1/1A2 enzymes and proteins dose-dependently in adult and developing rats at postnatal day 1 and 14. The lowest effective maternal dose was 2-3 mg MX/kg/day. Western blot data of CYP2B and CYP3A proteins indicated the induction of both P450 enzyme proteins in developing rats at postnatal day 14 at the higher dose of 70-80 mg/kg/day. In contrast, CYP3A was found not to be induced in adult rats, indicating differential sensitivity to MX in development.

In a third part dealing with reproduction and reproductive outcome, the highest dose of 70-80 mg MX/kg, corresponding to 1.0 g MX/kg food pellets showed maternal toxicity and increased mortality of 14 day old rats by 48 % as compared to control group.
Additionally, 2 up to 14 day old rats had a decreased body weight, whereby male animals had been more affected. Dams exposed to 70-80 mg MX/kg in contrast, remained without any visible signs of health damage and toxicity, unless in pregnancy. Our results in comparison with other studies showed, that depending on treatment period, developmental state, dose and application of MX, considerable qualitative and quantitative differences of a defined toxic response can result. Thus, our choice of study design becomes more important since it rather reflects the naturally occurring situation.

Recent studies demonstrated that an amine metabolite, produced by nitroreductases in the intestinal flora, and not MX itself was accountable for the induction of hepatic liver enzymes in mice. If this is also true for other investigated toxic effects must be further examined.

No data on amine metabolites in human tissues are available so far. Until now studies focused on photosensitivity and dermal reactions. Due to the lack of other appropriate toxicological data, it is not yet possible to fully assess human health risk. Therefore, also a recently published study on environmental risk assessment is deficient, since possible effects of MX metabolites measured in waste water have not been considered.
Gebrauch von Parfümen und anderen Kosmetika als Hauptkontaminationsweg. Nitro-
moschusduftstoffe enthaltende Wasch- und Reinigungsmittel erreichen die Umwelt über
Abwasser und Kläranlagen und reichern sich in aquatischen Organismen an. Diese
zweite ökotoxikologische wichtige Art der Exposition erfolgt peroral und ist vom
betroffenen Individuum nicht gewollt.

Von Studien über akute und chronische Toxizität und Karzinogenität von hoch-
dosiertem MX in adulten B6C3F1 Mäusen und Albino Ratten war bekannt, dass MX
hepatische Phase I (CYP1A2) und Phase II Enzyme in adulten Ratten und CYP2B
Proteinkonzentrationen in adulten Mäusen zu induzieren vermag. Zu diesem Zeitpunkt
lagen keine Untersuchungen zu möglichen Effekten in Nachkommen von behandelten
Tieren vor. Aufgrund seiner lipophilen Eigenschaften wäre es jedoch möglich, dass MX
via Plazenta und Muttermilch in den sich entwickelnden Organismus gelangt. Aus
diesem Grund exponierten wir gemäß einer OECD Richtlinie für das Testen von
Chemikalien in einer „1-Generationen Reproduktionstoxizitätsstudie“ Long Evans (LE)
Ratten während mindestens 10 Wochen relativ tiefen MX-Konzentrationen.

In einem ersten Teil wurden MX Gewebekonzentrationen in adulten Ratten und ihren
Nachkommen mittels GC-ECD Analyseverfahren gemessen. 5-6 Wochen alte weibliche
und männliche Ratten erhielten MX enthaltende Futterwürfel (0,001 g, 0,01 g, 0,03 g,
0,1 g MX/kg Futter) während mindestens 10 Wochen vor der Verpaarung, während der
Schwangerschaft, Geburt und Laktation. 1 Tag und 14 Tage alte Nachkommen zeigten
eine dosisabhängige Anreicherung im Fettgewebe (Suter-Eichenberger et al., 1998),
wobei Konzentrationen erreicht wurden, welche 1/2-3/4 so hoch wie in adulten
weiblichen, und 3-4 mal so hoch wie in adulten männlichen Ratten waren. Milchwerte
waren vergleichbar mit Fettgewebekonzentrationen von weiblichen adulten Tieren. MX
überwindet problemlos die Plazentarschranke und wird auch in die Milch sezerniert.
Nach chronischer Fütterung mit 0,1 g MX/kg, entsprechend einer täglichen Aufnahme
von 7-8 mg MX/kg, wurden in adulten weiblichen und männlichen Ratten höchste
Konzentrationen im Fettgewebe, in der Nebenniere und im Ovar gefunden, wobei
Gewebekonzentrationen von Weibchen 3,7-6,8 mal höher waren als diejenigen von
männlichen Tieren. Diese Sexdifferenz in der Fett- und Gewebeakkumulation von MX
war in 14 Tage alten Nachkommen (noch) nicht vorhanden.

In einem zweiten Teil der Studie wurden 5-6 Wochen alte Ratten beiderlei Geschlechts
wiederum über die gleiche Zeitspanne mit MX enthaltendem Futter (0,001 g, 0,01 g,
0.03 g, 0.1 g und 1.0 g/kg Futter) behandelt, was einer täglichen MX Aufnahme von 0.07-0.08 mg MX/kg bis 70-80 mg MX/kg entspricht. MX induzierte CYP1A1/1A2 Enzyme und Proteine dosisabhängig in adulten Tieren und in deren 1 Tag und 14 Tage alten Nachkommen. Die tiefste noch effektive mütterliche Dosis betrug 2-3 mg/kg/Tag. Western Blots zeigten eine Induktion von CYP2B und CYP3A Proteinen in 14 Tage alten Ratten nach einer mütterlichen Dosis von 70-80 mg/kg/Tag, dagegen wurde in adulten Tieren bei der gleichen MX Dosierung nur eine Induktion von CYP2B Proteinen gefunden. In der Entwicklung stehende Ratten zeigen gegenüber MX eine im Vergleich zu erwachsenen Tieren unterschiedliche Sensitivität.

In einem dritten Teil wurde gezeigt, dass eine tägliche Dosis von 70-80mg MX/kg, entsprechend 1.0 g MX/kg Futter, die Mortalität von 14 Tage alten Ratten um 48 % im Vergleich zur Kontrollgruppe erhöhte. 2 bis 14 Tage alte Ratten wiesen auch ein vermindertes Körpergewicht auf, wobei männliche Tiere stärker davon betroffen waren. Die Muttertiere dagegen zeigten bei dieser MX Konzentration keine sichtbaren Gesundheitsprobleme. Die beobachteten Gewichtsunterschiede bei den MX exponierten Tieren lassen jedoch eindeutig auf eine maternale Toxizität schliessen.

Vergleiche mit Resultaten anderer Studien zeigen, dass durch die Behandlungsdauer und den Entwicklungszustand des Tieres, wie durch die Konzentration der Fremdsubstanz und ihre Applikationsart beträchtliche qualitative und quantitative Unterschiede bezüglich definierter toxischer Effekte entstehen. Wir wollten ein Entwicklungsstudien-Modell, welches die Umweltsituation am besten wiederspiegelt und somit aussagekräftigere Resultate erzielt.

Kürzlich durchgeführte Studien zeigten, dass nicht MX, sondern ein Aminmetabolit, welcher durch Nitroreduktasen in der Darmflora gebildet wird, für die Induktion von hepatischen Leberenzymen in Mausen verantwortlich ist. Inwiefern dies auch für andere nachgewiesene Effekte und für andere Spezies zutrifft, muss in weiteren Studien untersucht werden.

Bis anhin wurden keine Metabolitenmessungen im Menschen durchgeführt. Studien im Menschen konzentrierten sich bis anhin auf dermale Phototoxizitätseffekte und andere Hautreaktionen. Da weitere, aufschlussreiche toxikologische Studien fehlen, oder keinen Bezug auf MX Metaboliten nehmen, wie eine kürzlich zu MX publizierte Umweltrisikoabschätzung zeigt, ist es im Moment nicht möglich eine abschliessende Risikoabschätzung für den Menschen vorzunehmen.
3 INTRODUCTION

3.1 WHAT MAKES A MOLECULE SMELL?

3.3.1 Common principles of structure and a working model for odorant interaction

Clearly sensory activity in human olfaction is exclusively associated with volatile molecules. The higher limit of molecular weight found for odorants is around 300. Chemical reactivity of a ligand has little if any direct connection with olfactory activity since odorant molecules are uncharged and hardly require metabolic activation. Most, but not all odorous molecules contain both a strong hydrophobic and a relatively weak polar region. The latter, usually termed the "osmophore", is associated with a functional group such as carbonyl, hydroxyl or a limited variety of heteroatomic homologues. According to present knowledge, the triggering of a sensory impulse occurs by direct contact between the odorant and the constituents of the receptor membrane in the olfactory cilia. A signal is produced via the depolarization of the membrane and electronically conducted to the olfactory bulb, analysed in the brain and then confronted with stored recognition patterns. The initial and most important event in the biochemical stimulation process is the binding of the odorant molecule to the membrane-bound receptor protein R (Fig. 1), which seems to be an allostERIC enzyme consisting of a variable (v) and a constant region (c). Non-covalent binding leads to a reversible complex, activating the GTP binding-protein (G) and triggering a cascade of enzyme reactions. cAMP, produced by adenylate cyclase (C) activation, causes phosphorylation of protein kinase (P) inducing changes in ion-channel protein (IC) and ion gating. Consequently the neuronal membrane is depolarized and the output signal transmitted to the central nervous system via the olfactory bulb (Ohloff et al., 1991).
3.1.2 Structure-odor relationship of musk odorants

The term structure-odor relationship implies that certain compounds belong both to the same structural and olfactory class. But very often, within a given structural class, and even between two enantiomeric compounds, the most diverse odors can be detected. Inversely, a well-defined odor can be found within a large variety of very different structural types. This is the case with musk odorants, which contain such diverse structural classes as the macrocyclic musks, the aromatic nitro and non-nitro musks (see Fig. 2, 3 and 4). It would be futile to speculate on molecular parameters common to these three structural classes. It should be also clear, that, although the musk odor is very characteristic - it is referred to as warm, sensual, animal, natural-, there are substantial odor differences between the different musks. Only for the class of nitro-free aromatic musks the most important structural requirements for musk odor have been established in the context of SAR studies (Ohloff et al., 1991).

3.2 HISTORY OF MUSK ODOR

Since thousand of years men are fascinated by animal secrets, glands and other parts of organ with strong odor characteristics. It was supposed that the escaping odors are accompanied by magic power. So the animal drugs were first used for religious intentions. Since antiquity, based on principles of Hippocrates, Galen and Kriton, until the middle
of the 18th century different application forms are known, above all as remedy and as fragrance ingredients. Only the appearance of plaque and the awful stench of excrement during the 18th century changed the attitude towards animal drugs transitory and they were execrated because of their similar nature of scent (Corbin, 1984).

Musk odor has a wide range of natural origins in plants as well as in the animal kingdom. Some two dozen animal sources are listed in the literature, three of them have attained commercial importance: musk pods, the dried glands of the musk deer (*Moschus moschiferus*), which gave also the name to the common term „musk“; the glands of civet cat (*Viverra civetta*) from which civet extract is obtained; the glands of Louisiana muskrat (*Fiber zibethicus*) from which a processed commercial product is obtained. A musk odor has also been noted in reptiles such as alligators and tortoises, birds, insects, certain sea creatures (octopuses, snails and molluscs) and others. In most cases, the odorant serves to attract the other sex, but in some of these animals, the secretion is produced when danger threatens and often serves as a deterrent.

The occurrence of musk odors is more limited in the vegetable kingdom. The best known are ambrette seed (*Abelmoschus moschatus*) the root of angelica (*Angelica archangelica*), muskmelon (*Curcumis melo*) and the roots of the sambul plant found in Bokhara (Bedoukian, 1986; Mookherjee and Wilson, 1982).

The demand of natural musk was enormous and could not be satisfied any more. Probably the earliest report on the synthesis of compounds having musk-like odor appeared in 1759 in the *Actes de l'Académie de Berlin* which contained Morggraf's statement: „when oil of amber is treated with fuming nitric acid, a resinous material is obtained that possesses a musk odor“ (Bedoukian, 1986).

Baur is credited with the discovery and commercialization of nitrated compounds having strong musk odors. In a study about artificial musk, Baur described a process whereby toluene was butylated with butyl halide in the presence of aluminium chloride. The product boiled at 170-200° C was nitrated by addition of nitric and sulfuric acid to give a crystalline substance with strong musk odor. For this he obtained a patent (1888) and although the exact constitution of the product was apparently not known, it was popular and successful. 1891, three years after his original patent, Baur obtained another patent in which he identified his original musk as being trinitro-butyl-toluene (Baur, 1891). Continuing his studies, he discovered other musk compounds, which are used until today. He patented the nitro musks musk xylene (MX) in 1888, musk
ambrette (MA) in 1892 and musk ketone (MK) in 1894. Musk moskene (MO) was patented by Barbier (1932) and musk tibetene by Carpenter (1937) in the USA (Bedoukian, 1986). Besides nitro musks other musk compounds were synthesized. 1906, Walbaum was successful in isolating (-)-muscone, a macrocyclic ketone, as the sensorial active principle of the natural musk extract (Walbaum, 1906), which contains in addition alcohols and pyridine derivatives. While Ruzicka established the structure of (-)-muscone in 1926, he started the century of the macrocyclic musks (Ruzicka, 1926). This group consists of the natural musks and the synthetic macrocyclics. The third group encloses polycyclic musks or aromatic non-nitro musks which are today the most important class. Their original discovery goes back some 50 years (1948), when the first nitro-free (not macrocyclic) musk was synthesized, called Ambrai®. 1951, Fuchs developed the first polycyclic musk compound, Phantolide® (Rebmann et al., 1997). Common compounds of this group are today Fixolide®, Celestolide® and Galaxolide®.

3.3 CHEMICALS WITH MUSKY ASPECTS

3.3.1 Macrocyclic musks

This group consists of the natural musks, with trivial names like (-)-muscone (1), civetone (2) and ambrettolide (3), and the synthetic macrocyclics, e.g. ethylene brassylate (4) and 12-oxahexadecanolide (5), whose structures derived from natural musks. Due to their chemical structures macrocyclic musks contain ketones, lactones, ether-lactones and others.

Fig. 2: Chemical structures of macrocyclic musks.
Although an enormous amount of work has been done on the synthesis of macrocyclic compounds, only a few of them are on the market. Ethylene brassylate as a synthetic macrocyclic is one of the most important in the industry because of its low price and pleasant, powerful odor (Frater and Lamparsky, 1991). Exaltolide® derives from the oil of seeds and the roots of angelica (Angelica archangelica) and is also important for the industry because of the intensive, but fine musk note and very good fixative character. Today Exaltolide® is the most often used musk of the synthetic macrocyclics (about 150 tons in the year 1995). The group of macrocyclic musks surpass all other musks referring to the remarkable diffusive characteristics of odor and fixative character (Bedoukian, 1986; Rebmann et al., 1997).

### 3.3.2 Nitro musks

The main representatives of this group are musk xylene (MX) (6), musk ketone (MK) (7), musk ambrette (MA) (8), musk tibetene (MT) (9) and musk moskene (MM) (10). All of them are dinitro- and trinitro- substituted benzene derivatives.

![Chemical structures of nitro musks.](image)

The great advantage of the nitro musks is their attractive price. MX and MK are the most often used nitro-musks. For a long time nitro musks were the largest volume synthetic musks, but now they are displaced because of toxicological, physical (discoloration) reasons and the availability of very good non-nitro musks (Frater and...
Lamparsky, 1991). The artificial musks serve on the one hand as odor constituents of perfume compositions, and on the other hand as fixatives because of their high boiling point (lower volatility). The odor varies somewhat. Nearly all perfume compositions contain one or more of the above substances in different proportions. Very large quantities of musk aromatics are also consumed by the soap industry, but here discoloration problems arise.

MX accounts for possibly more than half of the nitro musks used in the industry, although it has a low intensity of odor but also a low price (Bedoukian, 1982). The worldwide MX consumption is approximately 1000 tons per year in detergents alone. It has a sweet musky odor and finds its major use in fragrances for fabric washing and household cleaning products. MK on the other hand has a unique odor of its own adding a powdery note to the musk. This unique odor is the key note in many well-known fine fragrances, as well as being widely used in cosmetic products.

3.3.2.1 Chemical and physical data of MX

(a) Molecular formula: \( \text{C}_{12}\text{H}_{15}\text{N}_3\text{O}_6 \)
(b) Relative molecular mass (MW): 297.30
(c) Description: Yellow crystals, plates or needles (from alcohol) with a musky odor (Lide, 1993).
(d) Solubility Virtually insoluble in water, soluble in diethyl ether and ethanol (Lide, 1993).
(e) Octanol/water partition coefficient (P) \( \log P, 5.20 \) (Helbling et al., 1994)

3.3.3 Polycyclic musks or aromatic non-nitro musks

This group constitutes today the most important class and is well represented by substances with trivial names like Celestolide\(^\text{®} \) (11), Galaxolide\(^\text{®} \) (12), Tonalide\(^\text{®} \) (13), Phantolide\(^\text{®} \) (14), Cashmeran\(^\text{®} \) (15). Due to their chemical structures they are tetralin, indan, isochroman or steroid derivatives.
These musks are in contrast to nitro musks non-discoloring and possess strong, long-lasting odors and high solubility. Like the macrocyclic musks they have remarkable diffusive characteristics and also few limitations are placed on their use in cosmetic fragrance formulations (Bedoukian, 1986; Frater and Lamparsky, 1991).

In 1987 the worldwide production of musks amounted to about 7000 tons per year. About 61% of the total tonnage were constituted by the polycyclic musks, 35% by the nitro musks and only the remaining 3-4% by the macrocyclic musks (Barbetta et al., 1988). In 1996 the relation shifted to the favour of polycyclic musks: 85% of the total tonnage are presented by the polycyclic musks, only 12% by the nitro musks and 3-4% by the macrocyclic musks (Rebmann et al., 1997). The decline in nitro musk production and the increase in polycyclic musk production is based primarily on the restrictions on the use and applications of musk ambrette (Barbetta et al., 1988). Furthermore, it may be assumed that the critical discussions about some environmental and toxicological characteristics of several nitro musks promoted their replacement by polycyclic musks.

### 3.4 OCCURRENCE OF SYNTHETIC NITRO MUSKS

Nitro musks are widely used as a fixative for fragrance ingredients in cosmetic products such as soaps, perfumes and lotions. (Betts et al., 1982; Yurawecz and Puma, 1983; Goh and Kwok, 1986; Müller, 1993; Schlatter, 1993; Sommer, 1993; Ippen, 1994). As a fixative, MX helps to preserve the scent of the desired fragrance. The concentration of MX in cosmetic products is typically in the range of 0.01 to 0.5%; the highest concen-
Concentration levels are in perfumes (Hood et al., 1996). MX is also used to perfume detergents (Bruze et al., 1985; Sommer, 1993; Ippen, 1994), occasionally fish food (Hahn, 1993), cigarettes (Nair et al., 1986) and incense sticks (Takiura et al., 1973). Some data on concentrations of synthetic nitro musks in consumer products are made up in Table 1 (Müller, 1997).

<table>
<thead>
<tr>
<th>Musk compound</th>
<th>Product</th>
<th>Concentration (mg/kg)</th>
<th>Positive / Total samples</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX</td>
<td>men's toiletries</td>
<td>&lt;90-280</td>
<td>1/9</td>
<td>(Betts et al., 1982)</td>
</tr>
<tr>
<td>MX</td>
<td>men's colognes</td>
<td>&lt;230-9000</td>
<td>11/32</td>
<td>(Goh and Kwok, 1986)</td>
</tr>
<tr>
<td>MX</td>
<td>toiletries</td>
<td>&lt;10-1450</td>
<td>2/21</td>
<td>(Müller, 1993)</td>
</tr>
<tr>
<td>MX</td>
<td>toiletries</td>
<td>&lt;2.5-13</td>
<td>4/23</td>
<td>(Sommer, 1993)</td>
</tr>
<tr>
<td>MX</td>
<td>laundry detergents</td>
<td>2.5-250</td>
<td>15/30</td>
<td>(Sommer, 1993)</td>
</tr>
<tr>
<td>MX</td>
<td>toiletries</td>
<td>&lt;10-220</td>
<td>3/150</td>
<td>(Welie, 1995)</td>
</tr>
<tr>
<td>MX</td>
<td>colognes</td>
<td>&lt;10-71</td>
<td>3/81</td>
<td>(Welie, 1995)</td>
</tr>
<tr>
<td>MX</td>
<td>shampoos</td>
<td>&lt;10-21</td>
<td>1/46</td>
<td>(Welie, 1995)</td>
</tr>
<tr>
<td>MK</td>
<td>men’s toiletries</td>
<td>&lt;90-9000</td>
<td>6/9</td>
<td>(Betts, 1982)</td>
</tr>
<tr>
<td>MK</td>
<td>men’s colognes</td>
<td>&lt;460-7000</td>
<td>5/32</td>
<td>(Goh and Kwok, 1986)</td>
</tr>
<tr>
<td>MK</td>
<td>toiletries</td>
<td>&lt;10-500</td>
<td>9/21</td>
<td>(Müller, 1993)</td>
</tr>
<tr>
<td>MK</td>
<td>toiletries</td>
<td>&lt;2.5-2200</td>
<td>14/23</td>
<td>(Sommer, 1993)</td>
</tr>
<tr>
<td>MK</td>
<td>lotions and creams</td>
<td>&lt;2.5-130</td>
<td>6/24</td>
<td>(Sommer, 1993)</td>
</tr>
<tr>
<td>MK</td>
<td>shampoos</td>
<td>&lt;2.5-720</td>
<td>7/13</td>
<td>(Sommer, 1993)</td>
</tr>
<tr>
<td>MK</td>
<td>laundry detergents</td>
<td>&lt;2.5-110</td>
<td>5/30</td>
<td>(Sommer, 1993)</td>
</tr>
<tr>
<td>MK</td>
<td>toiletries</td>
<td>&lt;10-150</td>
<td>14/150</td>
<td>(Welie, 1995)</td>
</tr>
<tr>
<td>MA</td>
<td>men’s toiletries</td>
<td>&lt;90-16000</td>
<td>8/9</td>
<td>(Betts, 1982)</td>
</tr>
<tr>
<td>MA</td>
<td>men’s colognes</td>
<td>&lt;230-4500</td>
<td>14/32</td>
<td>(Goh and Kwok, 1986)</td>
</tr>
<tr>
<td>MA</td>
<td>toiletries</td>
<td>&lt;2.5</td>
<td>0/23</td>
<td>(Sommer, 1993)</td>
</tr>
<tr>
<td>MA</td>
<td>laundry detergents</td>
<td>&lt;0.1-4.8</td>
<td>6/12</td>
<td>(Romann, 1993)</td>
</tr>
<tr>
<td>MA</td>
<td>colognes</td>
<td>&lt;10-330</td>
<td>2/81</td>
<td>(Welie, 1995)</td>
</tr>
<tr>
<td>MA</td>
<td>shampoos</td>
<td>&lt;10</td>
<td>0/46</td>
<td>(Welie, 1995)</td>
</tr>
<tr>
<td>MT</td>
<td>laundry detergents</td>
<td>0.1-6.8</td>
<td>7/12</td>
<td>(Romann, 1993)</td>
</tr>
<tr>
<td>MT</td>
<td>toiletries</td>
<td>&lt;10-110</td>
<td>1/150</td>
<td>(Welie, 1995)</td>
</tr>
<tr>
<td>MT</td>
<td>shampoos</td>
<td>&lt;10</td>
<td>0/46</td>
<td>(Welie, 1995)</td>
</tr>
<tr>
<td>MM</td>
<td>shampoos</td>
<td>&lt;2.5-630</td>
<td>2/13</td>
<td>(Sommer, 1993)</td>
</tr>
<tr>
<td>MM</td>
<td>laundry detergents</td>
<td>&lt;0.1-5.9</td>
<td>6/12</td>
<td>(Romann, 1993)</td>
</tr>
<tr>
<td>MM</td>
<td>toiletries</td>
<td>&lt;2.5-1080</td>
<td>2/23</td>
<td>(Sommer, 1993)</td>
</tr>
</tbody>
</table>

Table 1: Frequency and concentrations of nitro musks in consumer products. (MX musk xylene, MK musk ketone, MA musk ambrette, MT musk tibetene, MM musk moskene).
3.4.1 Natural occurrence

None of the nitro musks are known to occur as natural products.

3.4.2 Environmental occurrence

The occurrence of nitro musks in the environment was first reported for MX and MK, the two most often used nitro musks, in freshwater fish collected from the Tama river in Tokyo (Yamagishi et al., 1981). The results suggested that both compounds exist as bioaccumulation-type pollutants in the aquatic ecosystem (Yamagishi et al., 1983). At the same time an American working group also found MX and related substances in fish but attributed this to contamination of the investigation material by care products which were perfumed with nitro musks (Yurawecz and Puma, 1983). Quite a few studies followed, which analysed nitro musk concentrations in various fish probes (Müller, 1993; Rinkus and Wolf, 1993a and 1993b; Eschke et al., 1994; Rinkus et al., 1994; Rinkus and Wolf 1995; Gatermann et al., 1995). Polycyclic musk compounds in contrast, were not analysed and perhaps also not found in the environment and in fish until 1995 (Eschke et al., 1995). The highest concentrations of nitro musks were found in the effluents of wastewater. It could be shown that the use of detergents with large nitro musk concentrations caused these high water concentrations which are themselves the reason for the accumulation in fish. In 1998, Gatermann et al., detected for the first time metabolites of MX and MK in the effluents of wastewater, indicating a high metabolism activity of microorganisms in filter-beds of purification plants.

Tables 2 and 3 show concentrations of MX and MK in the abiotic environment and in aquatic organisms respectively (Tas et al., 1997).
<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Musk ketone (µg/l)</th>
<th>Musk xylene (µg/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tama River, Japan</td>
<td>18</td>
<td>median 0.26 (0.14-0.41)</td>
<td>median 0.035 (0.025-0.036)</td>
<td>(Yamagishi et al., 1983)</td>
</tr>
<tr>
<td>waste water</td>
<td>2</td>
<td>53</td>
<td></td>
<td>(Hahn, 1993)</td>
</tr>
<tr>
<td>River Lauchert, Germany</td>
<td>17</td>
<td>median 0.01 (nd-0.028)</td>
<td>median 0.0035 (0.0017-0.023)</td>
<td></td>
</tr>
<tr>
<td>waste water</td>
<td>19</td>
<td>median 0.03 (0.02-0.23)</td>
<td>mean 0.01 (0.01-0.03)</td>
<td>(Eschke et al., 1994)</td>
</tr>
<tr>
<td>River Ruhr, Germany</td>
<td>36</td>
<td>median 0.75 (0.22-1.3)</td>
<td>median 0.12 (0.03-0.31)</td>
<td></td>
</tr>
<tr>
<td>North sea (-10m), Germany</td>
<td>33</td>
<td>median 1.5 (0.57-2.4)</td>
<td>median 0.68 (0.09-1.7)</td>
<td>(Gatermann et al., 1995)</td>
</tr>
<tr>
<td>River Glatt, Switzerland</td>
<td>0.0083</td>
<td>0.00005-0.0025</td>
<td>0.00005-0.0025</td>
<td>(Müller et al., 1996)</td>
</tr>
<tr>
<td>waste water</td>
<td>MK</td>
<td>0.55</td>
<td>MX</td>
<td>0.15</td>
</tr>
<tr>
<td>-influent</td>
<td>2-amino-MK: nd</td>
<td>2-amino-MX: nd</td>
<td>4-amino-MX: nd</td>
<td></td>
</tr>
<tr>
<td>-effluent</td>
<td>MK</td>
<td>0.006</td>
<td>MX</td>
<td>0.01</td>
</tr>
<tr>
<td>Elbe (Hamburg), Germany</td>
<td>MK</td>
<td>0.25</td>
<td>MX</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>nd-0.004</td>
<td>2-amino-MX: nd-0.002</td>
<td>4-amino-MX: 0.001-0.009</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** Concentrations of musk ketone (MK) and musk xylene (MX) in the abiotic environment. Number of samples (n), median or mean values and range. nd: detection limit.
### Table 3: Concentrations of musk ketone (MK) and musk xylene (MX) in aquatic organisms. Number of samples (n), median or mean values and range. w.w.: wet weight; nd: detection limit.

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Musk ketone (mg/kg)</th>
<th>Musk xylene (mg/kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>-carp</td>
<td>31</td>
<td>median 0.002 (nd-0.027) muscle, w.w.</td>
<td>median 0.015 (0.0015-0.041) muscle, w.w.</td>
<td>(Yamagishi et al., 1983)</td>
</tr>
<tr>
<td>-shellfish</td>
<td>9</td>
<td>median 0.002 (nd-0.070) viscera, w.w.</td>
<td>median 0.002 (0.0014-0.14) viscera, w.w.</td>
<td></td>
</tr>
<tr>
<td>-trout from pond and River</td>
<td>44</td>
<td>median 0.002 (0.0009-0.026) w.w.</td>
<td>mean 0.68 (0.34-1.8) fat</td>
<td>(Hahn, 1993)</td>
</tr>
<tr>
<td>-mussels, Japan</td>
<td></td>
<td></td>
<td>mean 0.026 (0.011-0.082) w.w.</td>
<td></td>
</tr>
<tr>
<td>-trout (fish farm), Germany</td>
<td>26</td>
<td>median 0.07 (0.01-0.38) fat</td>
<td>median 0.07 (0.01-0.35) fat</td>
<td>(Rimkus and Wolf, 1993b)</td>
</tr>
<tr>
<td>-fish, Switzerland</td>
<td>9</td>
<td>mean 0.01-0.03 fat</td>
<td>mean 0.01-0.02 fat</td>
<td></td>
</tr>
<tr>
<td>-fish farm</td>
<td>46</td>
<td>mean 0.14 (0.02-0.33) fat</td>
<td>mean 0.33 (0.09-1.06) fat</td>
<td></td>
</tr>
<tr>
<td>-shrimp, German rivers</td>
<td>36</td>
<td>mean 0.03 (0.01-0.11) fat</td>
<td>&lt;0.005-0.09 w.w.</td>
<td>(Schlatter and Hunyady, 1993)</td>
</tr>
<tr>
<td>-fish from German rivers</td>
<td>50</td>
<td>mean 0.09 (&lt;0.01-0.38) fat</td>
<td>mean 0.02 (0.01-0.10) fat</td>
<td>(Rimkus and Wolf, 1995)</td>
</tr>
<tr>
<td>-fish from shrimp</td>
<td>22</td>
<td>mean 0.03-0.05 fat</td>
<td>mean 0.8 (&lt;0.01-0.35) fat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31</td>
<td></td>
<td>mean 0.01 fat</td>
<td></td>
</tr>
</tbody>
</table>

#### 3.4.3 Biological monitoring

Nitro musks have been found in human adipose tissue, blood and breast milk from Germany and Switzerland (Meier and Sedlacek, 1993; Liebl and Ehrenstorfer, 1993; Rimkus and Wolf, 1993c; Rimkus et al., 1994; Eschke et al., 1995b; Müller et al., 1996) (Tab. 4). In almost all analysed samples, MX and MK were found, with levels in human milk ranging from 0.005 to 1.22 mg/kg lipid for MX and from less than 0.001 to 0.24 mg/kg lipid for MK. The contamination of human adipose tissue was comparable: 0.005 to 0.288 mg/kg lipid for MX and from less than 0.001 to 0.22 mg/kg lipid for MK (Rimkus, 1998). MX was also detected in blood plasma and blood lipids (Helbling et al., 1994; Käfferlein et al., 1997). The MX concentrations could be compared with those of some organochlorine pesticides such as lindane, ß-HCH or dieldrin detected in the same samples (Rimkus et al., 1994).
<table>
<thead>
<tr>
<th>Material (origin)</th>
<th>Musk ketone (mg/kg lipid)</th>
<th>Musk xylene (mg/kg lipid)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk, Germany 1992-93 Schleswig-Holstein</td>
<td>n 23 mean 0.03 (0.01-0.09)</td>
<td>n 23 mean 0.08 (0.02-0.19)</td>
<td>(Rimkus et al., 1993c, 1994)</td>
</tr>
<tr>
<td>Milk, Germany 1992/1995 Schleswig-Holstein</td>
<td>5 0.005-0.015</td>
<td>5 0.01-0.03</td>
<td>(Rimkus and Wolf, 1996a)</td>
</tr>
<tr>
<td>Milk, Switzerland</td>
<td></td>
<td></td>
<td>(Meier et al., 1993)</td>
</tr>
<tr>
<td>Adipose tissue, Germany, 1992, women Schleswig-Holstein</td>
<td>13 mean 0.09 (0.01-0.09)</td>
<td>13 mean 0.035 (0.01-0.22)</td>
<td>(Rimkus et al., 1993c, 1994)</td>
</tr>
<tr>
<td>Adipose tissue, Germany, 1995 women Schleswig-Holstein</td>
<td>8 mean 0.013 (0.005-0.03)</td>
<td>8 mean 0.006 (0.05-0.01)</td>
<td>(Rimkus and Wolf, 1996a)</td>
</tr>
<tr>
<td>Adipose tissue, Germany, 1993 men Schleswig-Holstein</td>
<td>19 mean 0.02 (0.01-0.03)</td>
<td>19 mean 0.05 (0.02-0.09)</td>
<td>(Rimkus et al., 1994)</td>
</tr>
<tr>
<td>Adipose tissue, Germany 1993/95 men Schleswig-Holstein</td>
<td>6 mean 0.016 (0.005-0.03)</td>
<td>6 mean 0.027 (0.02-0.05)</td>
<td>(Rimkus and Wolf, 1996a)</td>
</tr>
<tr>
<td>Adipose tissue, women Switzerland</td>
<td>10 mean 0.026 (0.0028-0.173)</td>
<td>10 mean 0.038 (0.0067-0.106)</td>
<td>(Müller et al., 1996)</td>
</tr>
<tr>
<td>Adipose tissue, men Switzerland</td>
<td>5 mean 0.0039 (0.001-0.011)</td>
<td>5 mean 0.082 (0.013-0.288)</td>
<td>(Müller et al., 1996)</td>
</tr>
<tr>
<td>Blood Switzerland</td>
<td></td>
<td></td>
<td>(Heldling et al., 1994)</td>
</tr>
<tr>
<td>Blood Erlangen/Nürnberg Germany</td>
<td></td>
<td>11 0.012-0.049</td>
<td>(&lt;0.1-1.12)</td>
</tr>
</tbody>
</table>

Table 4: Concentrations of musk xylene (MX) and musk ketone (MK) in human milk, human adipose tissue and human blood (Rimkus, 1998).

### 3.5 PHARMACOKINETIC PARAMETERS OF SYNTHETIC MUSKS

#### 3.5.1 Humans

$^{15}$N-labelled MX was given in a single oral dose to three volunteers. The elimination half-lives amounted to 60, 67, and 94 days respectively. The *in vivo* human distribution pattern of MX in blood was the following: 10 % in cells, 8 % in VLDL and chylo-
microns, 13 % in LDL, 10 % in HDL and 59 % in the rest of the plasma most likely associated with proteins (Kokot-Helbling, 1995a). The investigation of dermal MX absorption from skin by different authors resulted in various absorption rates. MX absorption was determined in 9 healthy human volunteers in an in-vivo experiment by using $^{15}$N-labelled MX in 3 different matrices which were applied on an area of 50 cm$^2$ on the forearm and removed after 8 hours. The calculated absorptions were 3.1 % for after shave lotion, 0.9 % for body milk and approximately 0.3 % for cotton (Kokot-Helbling, 1995b). A report to RIFM (Research Institute for Fragrance Materials) determined human dermal absorption to be 0.5 % or less of the applied dose under simulated conditions of use (1 mg MX in a hydroalcoholic solution, in-vivo) and excretion was almost exclusively in the urine (Ford, 1998). In contrast, Hood et al., (1996) indicated in an in-vitro system a percutaneous total absorption after 24 hours from an oil-in-water emulsion and a volatile solvent of 22 % of the applied dose, whereby only 1-4 % of the absorbed dose was found in the receptor fluid, but 17.3-21.3 % in the skin. These two absorption rates were added due to results from further experiments, showing that most of the absorbed dose in skin after 24 hours will be systemically absorbed in vivo within one week (Chou et al., 1994 and 1996; Hood et al., 1996).

3.5.2 Experimental systems

$^3$H-labelled MX was given in a single oral dose to male Wistar rats (70 mg MX/kg). About 50 % of the dose was excreted into urine and feces by 24 hours and almost 87 % of the dose was recovered by 7 days. The excretion into urine and feces was about 10.3 % and 75.5 % respectively. About 2 % of the dose remained in the carcass, mainly in the adipose tissue after 7 days. The main metabolites observed derived from the reduction of the 2-nitro group (M-2) and then acetylation of the resulting amino group (M-4). At the second step, oxidation of the methyl group of 2-NH$_2$-MX proceeded with sterically less restriction due to the introduced amino group to 2-NH$_2$-3-CH$_2$OH-MX (M-5), 4-NH$_2$-3-CH$_2$OH-MX (M-7) and 2-NH$_2$-5-tert-BuOH-MX (M-6). The reduction of the nitro group at the 4 position was less than that at the 2 position, because the steric effect of adjacent bulky tert-butyl group was bigger than that of methyl groups. Except for M-8 no conjugated tert-butyl metabolites could be detected from bile samples. It is not clear whether or not hydroxylated metabolites such as M-5, M-6, M-7 and M-8 are
biotransformed to the corresponding acids with cytosolic dehydrogenases(s) depending on their solubility and steric effects. There was no evidence of carcinogenicity of metabolites. It has been assumed that the biological reduction of the nitro group proceeds to the amine through nitroso- and hydroxylamine intermediates, such as carcinogenic amines, acetylaminofluorene and 4-nitrobiphenyl (Minegishi et al., 1991).

![Diagram of proposed metabolic pathways of MX in rats (Minegishi et al., 1991)](image)

**Fig. 5: Proposed metabolic pathways of MX in rats (Minegishi et al., 1991)**

Dermal absorption from cosmetics and detergents via skin and clothes is considered to be the main uptake route. The concentration of musk xylene in cosmetic products is in the range of 0.01 to 0.5 %, with the highest concentration levels in perfumes. Inhalative absorption from cosmetics and detergents (less than 1-3 %) and food contamination via detergent residue uptake from waste water into fish play a minor role. Noteworthy is the absorption into milk and the passage into the placenta due to the lipophilic character, a molecular weight of around 300 and a high bioaccumulation potential in adipose tissue (Fig. 6).
The elimination rate and distribution of MX was studied in BALB/c mice and Wistar rats of both sexes after a single oral application by gavage of $^{15}$N-labelled MX. The half-lives for MX were 3.4 and 1.3 days in male and female mice respectively, and 1.6 days in rats (no significant sex difference) (Kokot-Helbling, 1995c). The organ distribution in mice, 5 or 10 days after treatment, principally followed the lipid contents of the various organs. In none of the investigated organs any particular accumulation was observed. In rats the distribution in blood, liver, muscle and brain also followed the lipid content, but in adipose tissue MX concentrations were around 1300 times higher than in blood. This kinetic effect can be explained with the model of the adipose tissue as a "deep compartment", from where MX is more slowly deliberated than it is metabolised in the liver. In this way the transfer out of the adipose tissue becomes the limiting step for elimination (Kokot-Helbling, 1995c). In contrast, Minegishi et al., (1991), discussed also an accumulation of MX in the liver after a single oral application of MX.

In a RIFM report after dermal administration of $^{14}$C-labelled MX material to rats, it was shown that 20 % of the applied dose was absorbed after 6 hours and virtually all the absorbed material was excreted in 96 hours after dosing primarily in the feces (Ford, 1998). In an in vitro study in hairless guinea pig skin (HGP) (Hood et al., 1996) total absorption after 24 hours (receptor fluid 25.8-32.1 % and skin 18.8-22.9 %) was 55 % from an oil-in-water emulsion and 45 % from a methanol vehicle. Throughout 24 hours, absorbed MX was not metabolized. HGP skin was found to be comparable to rat skin.
(Kraeling et al., 1995). In contrast, another in vitro RIFM study showed that less than 2 % of musk xylene is absorbed through full thickness rat skin under occlusion over 24 hours (Ford, 1998).

3.6 TOXICOLOGY OF SYNTHETIC NITRO MUSKS

3.6.1 Humans

3.6.1.1 Phototoxicity, photoallergy, irritation and sensitization reactions

Musk ambrette (MA), tested at 20 % in petrolatum on volunteers produced no irritation after a 48 hours closed-patch test and no sensitization reactions in a maximization test (Opdyke, 1975). MK at a concentration of 3.2 % and MT at a concentration of 2 % in petrolatum tested on volunteers produced both no sensitization reaction. Only MX at a concentration of 5 % in petrolatum produced a mild irritation after a 48 hours closed-patch test but no sensitization reactions (Opdyke, 1975). In 1979, Raugi was the first to recognize that MA in men's aftershave lotions was causing photosensitisation (Raugi et al., 1979). In unexposed patch test series MA was the only one of the 5 nitro musks which elicited a reaction without light (3 of 19). With exposure to light, MA was positive in all patients, MM in 3, and MX in 2. MK and MT caused no reactions. These findings suggest a cross-reactivity between MA, MM and MX (Cronin, 1984). Patch and photopatch tests with MA, MX and MK and in addition with solutions of these nitro musks that had been exposed to light for a period prior to patch testing showed, that only the photopatch tests with MA and with the photodecomposed MA gave positive results (Schmidt and Kingston, 1984). Further more a case with pigmented contact dermatitis from MM in cheek rouges was reported (Hayakawa et al., 1991).
3.6.2 Experimental systems

3.6.2.1 Acute, subacute and chronic toxicity

The acute toxicity of nitro musks in mammals and fish is low. The LD$_{50}$ values for different synthetic musks are shown in Table 5.

<table>
<thead>
<tr>
<th>Musk compound</th>
<th>LD$_{50}$ (g/kg)</th>
<th>Species</th>
<th>Administration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>4.8</td>
<td>rat</td>
<td>oral</td>
<td>(Opdyke, 1975)</td>
</tr>
<tr>
<td>MA</td>
<td>&gt;2.0</td>
<td>rabbit</td>
<td>dermal</td>
<td>(Opdyke, 1975)</td>
</tr>
<tr>
<td>MK</td>
<td>&gt;10.0</td>
<td>rat</td>
<td>oral</td>
<td>(Opdyke, 1975)</td>
</tr>
<tr>
<td>MK</td>
<td>&gt;10.0</td>
<td>rabbit</td>
<td>dermal</td>
<td>(Opdyke, 1975)</td>
</tr>
<tr>
<td>MT</td>
<td>&gt;6.0</td>
<td>rat</td>
<td>oral</td>
<td>(Opdyke, 1975)</td>
</tr>
<tr>
<td>MT</td>
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<td>dermal</td>
<td>(Opdyke, 1975)</td>
</tr>
<tr>
<td>MX</td>
<td>&gt;10.0</td>
<td>rat</td>
<td>oral</td>
<td>(Opdyke, 1975)</td>
</tr>
<tr>
<td>MX</td>
<td>&gt;15.0</td>
<td>rabbit</td>
<td>dermal</td>
<td>(Opdyke, 1975)</td>
</tr>
<tr>
<td>MX</td>
<td>&gt;4.0</td>
<td>mice</td>
<td>oral</td>
<td>(Maekawa et al., 1990)</td>
</tr>
</tbody>
</table>

Table 5: LD$_{50}$ of synthetic nitro musks in mammals and fish.

In MX treated B6C3F1 mice tremor was observed 3-18 hours after treatment with a single dose of 4000 mg/kg by gavage. No abnormal symptoms were found in the other groups (125, 250, 500, 1000, 2000 mg/kg). Macroscopic examination revealed no clear toxic lesions in any of the mice at the end of the study. From the result, the LD$_{50}$ of MX was considered to be >4000 mg/kg (Maekawa et al., 1990).

MK, MT and MX applied full strength to intact or abraded rabbit skin for 24 hours under occlusion was not irritating in contrast to MA with a moderate irritation. In the rabbit eye, MT produced slight conjunctival irritation, which disappeared within 72 hours (Opdyke, 1975). In a study with guinea pigs MA caused a high incidence of photoallergy. MX and MK could be classified as weak sensitizers. In addition MK was weakly phototoxic. MT and MM were negative under all test conditions (Parker et al., 1986).

Levels of 1.5-4 g MA/kg of diet to rats (approximately 75-200 mg/kg/day) caused progressive paralysis of the hind limbs beginning after 12-15 weeks and testicular atrophy (at ≥2.5 g/kg). At 0.5 g only minor effects were noted (Davis et al., 1967). The results of a dermal study of MA were remarkably similar to those of the oral study. Daily dermal doses of 80 or 240 mg MA/kg (and a positive control dosed orally with
1.5 g/kg MA in the diet) produced hind-limb weakness accompanied by primary
demyelination and distal axonal degeneration in selected regions of the central and peri-
pheral nervous system. Testicular atrophy was also seen at the 240 mg MA/kg/day level
(Spencer et al., 1984). In a 90-day dermal toxicity study rats were applied 7.5, 24, 75 or
240 mg MX or MK/kg/day and 7.5, 24 or 75 mg MT or MM/kg/day (no higher dose
because of the more limited solubility). As a positive control served MA at concen-
trations of 75 or 240 mg MA/kg/day. While MA was clearly neurotoxic and caused
testicular atrophy in male rats, the other compounds tested caused neither effects. The
only effects of application of these materials were some organ weight changes (liver,
kidney) at the higher doses, but these were not associated with histopathological chan-
ges in any of the investigated tissues (Ford et al., 1990).

The toxic effects of MA after dermal exposure (testicular effects at 240 mg/kg/day,
neurotoxicity at 80 and 240 mg/kg/day) could also be confirmed in a oral study (testi-
cular effects at 125 mg/kg/day, neurotoxicity at 75-200 mg/kg/day). Investigation of
dermal absorption revealed that upon dermal absorption, substantial biliary excretion
and enterohepatic circulation occurs. The highest organ concentration after dermal ap-
planation was in the gastrointestinal tract and the bulk of the dose is excreted in the
faeces. A similar mechanism is expected to occur after oral ingestion (Ford et al.,
unpublished observations, 1984).

The mechanistic base of testicular atrophy in male rats by MA is not investigated so far.
It is also unknown, whether MA is able to act in an estrogenic or antiandrogenic way.
Studies, testing a random screen of 20 organic man-made chemicals present in liquid
effluents by investigating their ability to inhibit binding of 17ß-estradiol to the fish
estrogen receptor, revealed no changes in receptor binding by MX and MK (Jobling et
al., 1995).

In a subchronic toxicity study for 17 weeks B6C3F1 mice were fed ad libitum at dietary
concentrations of about 50 to 760 mg MX/kg/day. All of the mice given 760 mg
MX/kg/day in the diet and all female mice and 80 % of male mice given 380 mg/kg/day
died during the study. No deaths occurred in any of the other groups. The absolute and
relative liver weights were increased slightly in all treated groups except after a dosage
of 50 mg/kg/day, but the increases were not dose related. Histologically, enlargement
and irregularity of liver cells were observed in male and female mice fed 190
mg/kg/day. There were no remarkable dose-related toxic lesions in any other organs of
any of the treated groups. From these results, the maximum long-term tolerable dose of MX was considered to be about 190 mg/kg/day. MX has been tested in a long-term toxicity study by feeding female and male B6C3F1 mice ad libitum with about 90 and 190 mg MX/kg/day for 80 weeks. The growth curves show that throughout the experiment there were no significant body-weight differences between the female groups, but a significant inhibitory effect of MX in the male group on growth from week 4 to week 80 after treatment with 190 mg/kg/day. The survival curves showed no differences between control and treated groups of either sex in the cumulative mortalities (Maekawa et al., 1990)

3.6.2.2 Mutagenicity, genotoxicity, cogenotoxicity and carcinogenicity

A mutation is a heritable change produced in the cell genotype. Such a change may be induced by a variety of agents, including foreign compounds. MA was found positive for mutagenicity in a Drosophila test (Base-Test) (Wild et al., 1983), while it was negative in the micronucleus genotoxicity test with human lymphocytes in vitro and in the human hepatoma cell line Hep G2 (Kevekordes et al., 1997) and in the in vitro SOS chromotest (Escherichia coli PQ37) (Emig et al., 1996). Mutagenic activity was further observed in Salmonella typhimurium TA100 requiring metabolic activation by rat liver postmitochondrial supernatant S-9, but not in TA98 (modified Ames plate incorporation assay) (Nair et al., 1986; Emig et al., 1996). MX and MK were negative in the modified Ames-test with different strains used in the presence and absence of S-9 (Nair et al., 1986; Api et al., 1995a and 1996; Api and San, 1995b). They also gave uniformly negative results in various short-term genotoxicity tests, in the mouse lymphoma assay, in the in vitro cytogenetics assay in Chinese hamster ovary (CHO) cells, in the in vitro unscheduled DNA synthesis (UDS) assay in primary rat hepatocytes, in an in vivo UDS assay, in the micronucleus test (MN) with human lymphocytes in vitro and in human hepatoma cell line Hep G2, and in the SOS chromotest using Escherichia coli PQ37 in the presence and absence of S-9 (Api et al., 1995a; Api and San, 1995b; Emig et al., 1996; Kevekordes et al., 1997). Also MM and MT gave negative results in the micronucleus test (MN) with human lymphocytes in vitro and human hepatoma cell line Hep G2, in the SOS chromotest and in the Ames-test in the presence and absence of S-9 (Emig et al., 1996; Kevekordes et al., 1997). In another study, MX and MK were
examined for their potency to induce toxifying enzymes in the liver of Sprague-Dawley rats, which is necessary to toxify different premutagens, pregenotoxicants and/or precarcinogens to the ultimate DNA damaging agents. Using an in vivo / in vitro model these isoenzyme inductions led to a metabolisation (toxification) of the pregenotoxicants benzo[a]pyrene and/or 2-aminoanthracene (cogenotoxicity) (Mersch-Sundermann et al., 1996a and 1996b).

Of all musks only MX has been tested in a long-term carcinogenicity study. Female and male B6C3F1 mice were fed ad libitum with 90 and 170 mg MX/kg for 80 weeks. The overall tumour incidences in all treated groups of both sexes were significantly higher than those in the corresponding controls. Combined malignant and benign liver cell tumours were clearly increased in both sexes. In males the incidence of Harderian gland tumours was also significantly greater in treated groups than in controls. A dose-dependency could not be seen (Maekawa et al., 1990). Due to the negative results in mutagenicity, the liver tumors of MX may be caused by a non-genotoxic mechanism.

3.6.2.3 Hepatic cytochromes P450 involved in drug metabolism

The cytochromes P450 are a superfamily of hemoproteins that are the terminal oxidases of the mixed function oxidase system. A recommended nomenclature system has been devised based on the evolutionary relationships of these oxidases (Nebert et al., 1991) by comparing the deduced amino acid sequences from the genes and by deviding into families, which are comprised of those P450s that share at least 40% identity (currently, 27 mammalian gene families are documented). Only 3 of these 27 families (CYP1, CYP2, and CYP3) are currently thought to be responsible for the majority of hepatic drug metabolism. The hepatic microsomal P450s catalyze the metabolism of a large number of lipophilic endogenous and exogenous compounds, but only a limited number of reactions including carbon hydroxylation, heteroatom oxygenation, dealkylation, epoxidation and reductive reactions are involved and with certain compounds, metabolites can be formed that inactivate the P450s (Guengerich, 1990). The metabolites formed from the majority of substrates by the P450s are more hydrophilic than the parent compounds and are thus more readily excreted from the body. In some cases, however, electrophilic metabolites are formed, which can react with cellular nucleophiles resulting in toxic or carcinogenic insult (Guengerich, 1988). It has become
apparent that significant differences exist between man and experimental species with regard to the catalytic activities and regulation of the expression of the hepatic drug-metabolizing P450s. In general terms, three basic differences were identified. First, different P450s in the various species may perform, with high specificity, the same metabolic function and even highly structurally related P450s may have different substrate specificities in different species. Second, the regulation of expression of related forms of P450 can vary among the various species, including man. The classic case of this is the sexual bimorphism observed for the metabolism of many compounds by rats that is not observed with other species, including man (Watkins, 1990). The third general difference is that, through gene duplication, species-specific P450s have evolved (Nebert, 1987).

The question about the relevance of data from experimental studies for human arises. Since human in-vivo studies are out of question, experimental data have to give some information about the metabolic activity of a substance. Thereby, above all quantitative species differences must be taken into account. This means, that distinct metabolic routes are favoured in various species with a consequent difference in pharmacological or toxicological activity.

3.6.2.3.1 The CYP1A subfamily in rat and human

Only two genes to date have been identified in both humans and rodents (Nebert et al., 1991). Analysis of interspecies differences in P450 primary sequence showed a strong evolutionary conservation of mammalian 1A genes.

CYP1A1 is almost absent in untreated rats (and other experimental animals) of either sex at all stages of development. The enzyme can be induced to a high level in rat liver by any of a number of compounds, including β-naphthoflavone, 3-methylcholanthrene, benzo[a]pyrene, several other polycyclic aromatic hydrocarbons, 2,3,7,8-tetrachlorodibenzo-p-dioxin and related substituted dibenzo-p-dioxins and -furans, isosafrole, chlorpromazine and many polyhalogenated biphenyl congeners, particularly those lacking ortho-substitutes and those having 2,3,4-substitution on a single ring. CYP1A1 can be induced in fetal rat liver (Cresteil et al., 1986). All of these compounds also induce CYP1A2, although some are more selective for one or the other. CYP1A1 is expressed in most extrahepatic tissues. Hormonal status and sex differences do not
appear to affect induction of CYP1A1 (Guengerich, 1987). In man, CYP1A1 enzyme level appears to be very low and it is currently thought to be predominantly expressed in extrahepatic tissues. There is good evidence that it is inducible in extrahepatic tissues by cigarette smoke and polycyclic aromatic hydrocarbons (PAH) (Guengerich, 1993). The hypothesis has been presented that the extent of inducibility of this enzyme is correlated with the susceptibility to lung cancer (Kellerman et al., 1973). Extrapolation to man of information on P4501A1-dependent metabolism of xenobiotics in experimental animals may have little relevance since the current information about these forms indicates that large differences exist between man and the various species (Wrighton and Stevens, 1992).

CYP1A2 levels are low in untreated rats and remain relatively constant throughout life. No significant sex differences or its inducibility have been detected. CYP1A2 has considerable sequence homology with CYP1A1. The enzyme is readily induced by many compounds and plays a major role in the biactivation of a wide variety of carcinogens and mutagens (Wrighton and Stevens, 1992). Isosafrole and safrole induce CYP1A2 to a greater extent than CYP1A1. Compounds such as β-naphthoflavone, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin also induce CYP1A2, but to a lesser extent than CYP1A1. In extrahepatic tissues most of these chemicals induce CYP1A1 but not CYP1A2. Further CYP1A2 is not induced in fetal rat liver (Guengerich, 1987). In human liver, CYP1A2 is present at widely varying levels. This high level of interindividual variation led to speculation that 1A2 levels are regulated by genetic and/or environmental factors. Higher 1A2 levels and associated enzyme activities have been positively correlated to increased cigarette smoking. Although higher 1A2 levels may increase the bioactivation of carcinogens, there are also several studies linking 1A2 induction by dietary factors to a lower incidence of breast cancer (Michnovicz and Bradlow, 1990 and 1991). Unlike CYP1A1, CYP1A2 does not appear to be expressed in extrahepatic tissues (Wrighton and Stevens, 1992).

CYP1A1 is the only P450 for which a receptor-mediated mechanism of induction has been clearly demonstrated, via the Ah or TCDD receptor. The 5'-flanking region of the gene for this P450 contains several short sequence motifs, termed xenobiotic responsive elements, or XREs, that function as transcriptional enhancers when Ah receptor ligands, such as TCDD are added. These XREs bear some resemblance to the glucocorticoid-responsive element.
In contrast to CYP1A1, CYP1A2 lacks XREs in the proximal 5′-flanking region. Induction by PAHS has been shown to be mediated for the most part post-transcriptionally through enhanced stability and intranuclear processing of the 1A2mRNA precursor. The Ah receptor appears to be involved in this post-transcriptional induction (Porter and Coon, 1991).

3.6.2.3.2 The CYP2B subfamily in rat and human
Basal levels of CYP2B in untreated rats seem to be somewhat higher in younger animals, no sex difference has been observed (Guengerich, 1987). In untreated animals, CYP2B can be quantified in newborns but not in fetuses, but phenobarbital treatment induces CYP2B also in rat fetuses (Cresteil et al., 1986). Phenobarbital treatment of rats induces the expression of two forms of P450, CYP2B1 and CYP2B2 in the liver. In untreated animals, only CYP2B2 is expressed. Transplacental induction of CYP2B1 by phenobarbital and CYP2B2 mRNAs was not detectable in fetal rat livers prior to day 21 of gestation (Giachelli and Omiecinski, 1987). The mRNA’s of these two, CYP2B1 and CYP2B2, showed 97 % nucleotide similarity. The substrate specificities of these two P450s are overlapping, with the activity of CYP2B1 being much greater than that of CYP2B2 for the vast majority of substrates. Significant induction after phenobarbital treatment of either mRNA was not detected in several other tissues examined, although CYP2B2 was constitutively expressed in lung and testis (Gonzalez, 1989). Phenobarbital induces CYP2B1/2B2 primarily by increasing gene transcription. In humans, CYP2B6 codes for a P450 76 % similar to rat CYP2B1. However, whether the human CYP2B6 gene is expressed at the enzyme level has to be positively demonstrated. The role of the CYP2B subfamily in human drug metabolism may be limited, pointing to the fact, that the induction of the CYP3A subfamily in man by barbiturates may be responsible for many of the well-documented interactions that occur between barbiturates and other drugs (Wrighton and Stevens, 1992).

3.6.2.3.3 The CYP3A subfamily in rat and human
The various members of this subfamily have been extensively studied since they were shown to be responsible for the metabolism of a wide range of clinically and toxicologically important agents. CYP3A is inducible by steroids, macrolide antibiotics,
imidazole antifungals and phenobarbital. Currently, two genes, CYP3A1 and CYP3A2 have been identified in the rat exhibiting 89% amino acid sequence homology. CYP3A1 is not detectable in hepatic microsomes from untreated immature or adult male and female rats. Following dexamethasone or pregnenolone-16α-carbonitrile (PCN) treatment, expression of CYP3A1 is observed in all groups. Measurable amounts of constitutive CYP3A2 were detected in hepatic microsomes from immature and adult males as well as in immature but not in adult females. CYP3A2 expression is inducible by dexamethasone in immature rats of both sexes and in adult males, although dexamethasone is more effective as an inducer of CYP3A1. Constitutive expression and induction may therefore vary as a function of age (Cooper et al., 1993). Phenobarbital also induces preferentially CYP3A1 but also CYP3A2 (Gonzalez, 1989) like other compounds such as Aroclor 1254 and rifampicin. In contrast 3-methylcholanthrene modestly increases CYP3A2 levels but has no effect on CYP3A1 (Cooper et al., 1993).

Induction of CYP3A1 gene in rats by the glucocorticoids dexamethasone and pregnenolone 16α-carbonitrile is primarily the result of an activation of CYP3A1 transcription and an increase in mRNA (Simmons et al., 1987). The possible involvement of the glucocorticoid receptor, which controls a variety of genes in the liver might be of interest. Regulation of CYP3A1 seems to be quite distinct from the control of classical glucocorticoid-regulated genes such as tyrosine aminotransferase (TAT) (Schuetz et al., 1984), suggesting that either CYP3A1 gene activation may depend on other factors or that differences exist between these genes in their affinity for receptor-ligand binding to cis-acting DNA control elements (Gonzalez, 1989). Little is known about the regulation of expression of other rat 3A subfamily members.

The P450 3A subfamily in man appears to be composed of at least four genes, CYP3A3, 3A4, 3A5 and 3A7. The deduced amino acid sequences for CYP3A3 and CYP3A4 showed 97% similarity. The correlation of metabolic activity and CYP3A3/4 level demonstrated that human 3A forms are responsible for the majority of the metabolism of a large number of endogenous and exogenous compounds. In contrast to the exclusive expression of CYP3A2 in male adult rats, no human 3A subfamily member has been shown to be expressed exclusively in the male. CYP3A5 has been found to be polymorphically expressed with very limited metabolic capabilities in comparison to CYP3A3/4. In contrast to what has been reported for fetuses of experimental species, the human fetus is capable of metabolizing a large number of compounds. The major
form of P450 in the human fetus was shown to be CYP3A7 (30-50% of the total fetal P450) that has not been detected in adult liver (Wrighton and Stevens, 1992).

The regulation of expression of the members of rat and human 3A subfamilies seems to be dissimilar. In addition, not all substrates metabolized by the rat 3A subfamily are metabolized by the structurally related human 3A subfamily. Therefore extrapolation from experimental animals to man should be done with caution (Wrighton and Stevens, 1992).

3.6.2.3.4 Effects of MX on rat and mouse hepatic microsomal enzyme activities

After a daily intraperitoneal dose of 30 mg MX/kg for 5 days, Western blot analysis revealed that MX significantly induced CYP1A2 and to a far lesser extent, CYP1A1 in male Wistar rats, but showed no remarkable increase in Phase II enzyme activities and total cytochrome P450 content (Iwata et al., 1992 and 1993a). Higher intraperitoneal MX doses, 50, 100 or 200 mg MX/kg for 5 days, significantly increased activities of Phase I enzymes (7-ethoxycoumarin-O-deethylase and 7-pentoxyresorufin-O-depentylase), Phase II enzymes (DT-diaphorase and glutathione S-transferase (GST)) and total cytochrome P450 content, while erythromycin-N-demethylase, NADPH-cytochrome c-reductase and benzphetamine-N-demethylase activities remained unaffected. Relative liver weights and microsomal protein content were significantly increased after 100 mg MX/kg/day and Phase II enzyme activity of benzo[a]pyrene hydroxylase after a daily dose of 200 mg MX/kg. MX was therefore proposed to be a novel specific bifunctional inducer (induction of Phase I and II enzymes) for CYP1A2 as a Phase I enzyme and GST and DT-diaphorase as Phase II enzymes (Iwata et al., 1993b).

After a daily gavage dose of 1, 5, 10, 20, 50, 100 or 200 mg MX/kg for 7 days to male B6C3F1 mice, absolute liver weights were significantly increased at dosages of 20 mg/kg and higher, reaching a maximum of 65% increase at 200 mg/kg/day. MX treatment also increased the yield of total cytochrome P450 and microsomal protein content at a dosage of 20 and 50 mg/kg/day respectively. Higher doses of MX increased these parameters in a dose-dependent manner. 200 mg/kg/day MX increased microsomal activity for O-dealkylation of 7-ethoxy and 7-methoxyresorufin (EROD, MROD) 4- and 2-fold respectively, N-demethylation of erythromycin was increased after a dosage of 50 mg/kg/day. These results are consistent with increased CYP1A1, 1A2 and
3A protein levels determined by Western blotting. In contrast, no increase in O-dealkylation of 7-pentoxyresorufin (PROD) was observed following MX treatment 200 mg/kg/day, although CYP2B protein levels were increased about 25-fold over control. Furthermore, a single dosage of MX (200 mg/kg/day) increased CYP2B10 mRNA to a maximal level and with a time course similar to phenobarbital (PB). This increase in CYP2B protein without a concurrent increase in enzyme activity is suggesting that MX or metabolites of MX inhibit this enzyme. These results indicate, that MX is a PB-like inducer of mouse liver cytochrome P450 enzymes. Further experiments disclosed, that one of two amine metabolites of MX formed in vivo by nitroreduction in anaerobic intestinal flora, 4-amino-2,6-dinitro-1-t-butylxylene, referred to as p-NH2-MX was responsible for this enzyme inhibition, acting as a mechanism-based inactivator of mouse CYP2B10, while the 2-amino-2,6-dinitro-1-t-butylxylene, referred to as o-NH2-MX, reflecting the position of the amine substitution relative to the t-butyl function, is ineffective (Lehman-McKeeman et al., 1997b). The authors showed in an in vitro assay with PB-induced microsomes, that p-NH2-MX caused a time-and NADPH-dependent loss of PROD activity, and the inactivation rate was a pseudo-first-order process that displayed saturation kinetics. These characteristics are the hallmarks of mechanism-based inactivation (Halpert and Stevens, 1991; Halpert, 1995; Ortiz de Montellano, 1995).

Fig. 7: Structures of MX and monoamine metabolites
As mentioned earlier (see 3.6.2.2), although MX is uniformly negative in genotoxicity testing, it causes liver tumors in B6C3F1 mice (Maekawa et al., 1990) and is also capable of inducing cytochrome P450 enzymes in a manner similar to that of PB. This suggests that epigenetic mechanisms may be involved in the carcinogenic response. Northern blot analysis indicated that both amine metabolites of MX markedly induced CYP2B10 mRNA suggesting that the amine metabolites may contribute to the enzyme induction profile seen with MX treatment. In fact, in mice treated with antibiotics (neomycin, tetracycline, bacitracin) that eliminate intestinal flora, the formation of amine metabolites was prevented. In these animals no evidence of microsomal enzyme induction occurred, including no increases in liver weight, total cytochrome P450 content or CYP2B protein levels. These results indicate that the amine metabolites of MX are responsible for the enzyme induction seen after MX administration (Lehman-McKeeman et al., 1997c).

3.7 REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF NITRO MUSKS

Until now, no data are available concerning this topic. In a review published by Ford (1998), unpublished results from a developmental study conducted by RIFM were summarised. MX and MK were given orally by gavage once daily on days 7 through 17 of gestation at doses of 20, 60 and 200 mg/kg/day for MX and 15, 45 and 150 mg/kg/day for MK to pregnant female rats. Neither substance was selectively toxic to development. There were no adverse effects from MX on embryo-fetal development, even at the two higher doses, both of which produced toxic effects in the dams. The maternal no-observable-adverse effect level (NOAEL) for MX was 20 mg/kg/day. The developmental NOAEL for MX was stated by the authors as being 200 mg/kg/day. The endpoints on which the developmental analysis has been done remain unknown. For MK, adverse effects on embryo-fetal development (post-implantation loss and reduced fetal body weight) occurred only at the higher of the two doses that produced toxic effects in the dams. The maternal NOAEL for MK was 15 mg/kg/day. The developmental NOAEL for MK was 45 mg/kg/day (Christian et al., 1997a and 1997b). In addition a postnatal study was conducted for MK. Pregnant rats were treated orally with daily doses of 7.5 mg/kg MK at the beginning of the third week of pregnancy to allow
for a steady state. Detailed observations of pups included a comprehensive battery of behavioral tests and measurements of reproductive function. No effects on growth and development of foetus, neonates and pups were observed. Performance in specific behavioral tests and reproductive capacity of the offspring were normal following chronic developmental MX, providing a NOAEL of 7.5 mg/kg/day (Ford and Bottomley, 1997).

3.8 RISK ASSESSMENT

Toxicological studies normally are conducted with a small number of animals. Chemicals are administered in significantly higher concentrations than humans are exposed to. For this reason extrapolation to lower concentrations is the usual procedure. Therefore, due to extrapolation, dose-response curves in the low concentration ranges remain unknown. Figure 8 illustrates several possible dose-response relationships for a given chemical in the very low dose range ("black box").

![Figure 8: Possible dose-response relationships in the low dose range where no data are available ("black box"). The course of the curves are hypothetic, i.e. they have not been verified. It is recognised that the higher the distance from real data points the more extensive variability is observed for the extrapolation-based curves.

- a) Continuous dose response curve
- b) Rapidly reached threshold value
- c) Linear extrapolation NOEL to zero
- d) Mathematical function slowly reaching zero
- e) Mathematical function with an accelerated effect in lower dose ranges
3.8.1 Human safety assessment: NO(A)EL(C) and safety factor (SF)

Different methods for the extrapolation to lower concentrations exist today. An often used procedure is the definition of a concentration, where an effect can no longer be detected. This concentration is called the no-observed-effect-level (NOEL) or no-observed-adverse-effect-level (NOAEL), defining a clear toxic effect. A so-called tolerable concentration is established by applying an appropriate safety factor to the NO(A)EL. Environmental chemicals are predominantly classified by this method. The NO(A)EL is not an absolute value and varies using different test conditions (for example subacute toxicity study, cancerogenicity study etc.). It depends on the number of animals in the experiment and the distance between chosen concentrations. A factor of 10 is considered as being relatively inexact. The NO(A)EL gives no information about the course of a dose-response curve and does not consider pharmacokinetic parameters. Using safety factors, uncertainty factors like the fact that different species and breeding strains may show distinct compound susceptibilities are taken into account. Various criteria are used as the basis for the magnitude of the total safety factor, usually a factor of 100 to 1000 is proposed (Rees et al., 1990).

Species differences in toxicity are often related to differences in metabolism and disposition of a compound. Such differences are extremely important in the safety evaluation of compounds extrapolating toxicity data from animals to man and hence risk assessment (Timbrell, 1991). Due to pharmacokinetic differences, species comparisons should rather be done on plasma-or tissue concentrations (internal concentration) than on substance exposure concentrations. The knowledge of internal organ concentrations also enables to establish a no adverse effect concentration (NOAEC), that means the concentration or body burden, where no corresponding effect can be detected any more. In environmental risk assessment, besides the lack of data concerning human exposure to a chemical and possible toxic effects, very often no data are available on pharmacokinetic and metabolism parameters from humans and also from the experimental species. A definitive risk assessment concerning humans must be often carried out only on the basis of experimental data.

In contrast to other environmental chemicals, MX is investigated in more detail, even some human pharmacokinetic data are available. The known adverse effects of MA and the concluded questions about safety of structural related compounds have contributed to detailed investigations of the other nitro musks.
3.8.2 Environmental risk assessment (ERA)

A great number of publications indicate an increased interest in the environmental fate and effects of nitromusks (Rimkus and Wolf, 1995; Gatermann et al., 1995; Fernandez et al., 1996; Boleas et al., 1996). To assess the environmental risks for synthetic chemicals, a risk assessment strategy has been developed by the scientific community resulting in the development of European Union guidelines (EU, 1995). There are three environmental compartments that are traditionally assessed: aquatic, sediment and terrestrial. In contrast to human safety assessments, the ERA does not aim to protect one species, but aims to protect all species present in the environment, as well as ecosystem function (De Wolf and Kloepper-Sams, 1998).

The principal decision points involve a comparison of the predicted environmental concentration (PEC) with the predicted no-effect concentration (PNEC). Figure 9 shows that the PEC is calculated in the exposure assessment phase, and the PNEC in the effects assessment phase. The PEC is compared with the PNEC to obtain PEC/PNEC ratios or risk quotients in the risk characterization phase (De Wolf and Kloepper-Sams, 1998).

![Diagram of Tiered Approach](image)

Fig. 9: Tiered approach used in the EU TGD (1995)

A risk quotient greater than 1 indicates a potential for adverse effect. For a PEC/PNEC outcome less than 1, no additional testing is required (EU 1995), and the material can be considered safe. Assessment factors (AF) are used which can be considered somewhat
considered safe. Assessment factors (AF) are used which can be considered somewhat analogous to safety factors. AFs account for uncertainties such as species to species differences, acute to chronic toxicity ratios and extrapolations from laboratory toxicity tests to the field. AFs are greatest with modelled data and acute toxicity data and least with chronic and model ecosystem data (EU 1995).

The Dutch National Institute of Public Health and the Environment published an initial aquatic and soil ERA of MK and MX in accordance with the EU guidance (Tas et al., 1997). For both substances, PEC/PNEC ratios are at or below 0.1 for organisms in the aquatic environment, including sediment organisms, for soil organisms 0.5 for MK and 1.3 for MX. The PEC/PNEC ratio for soil organisms is considered to be overestimated due to the limited amount of data available. RIFM is currently generating additional experimental effects data from soil organisms.

3.9 REGULATION AND GUIDELINES

In 1974 the Council of Europe included MA at a level of 1 ppm and MX and MK without concentration limits in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. In 1965, MA was given GRAS status (Generally Recognized As Safe) (Opdyke, 1975). Spurred by reports in 1979 and 1980 that MA was a photosensitizer, the International Fragrance Association (IFRA) in Geneva, Switzerland, 1981 first limited the use of MA to 4% in new fragrance compounds, later forbade the use of MA in fragrance products (1985) and ordered a general veto in 1995. MA was removed from the GRAS list in the United States in 1984 and does not have any other food use status. MX does not have food use status and should not be used in lip products or flavours for oral hygiene products (RIFM, 1994). Since MA was detected in the aquatic environment (Yamagishi et al., 1981 and 1983) and has been reported to cause neurotoxic effects (Davis et al., 1967), MA and also the other nitro musks have not been used in Japanese products since 1982 on the basis of a voluntary restriction (Iwata et al., 1993a). In 1992 Germany defined a provisional standard value for MX at the level of 10 μg/kg foodstuff because of the measured concentrations of MX in fish probes from Danmark and Germany (Schlatter, 1993). Until now this standard value is not legally binding in the way of a maximum level (Bundestagsdrucksache). In 1995 Switzerland revised the ordinance of fragrance
materials (Verordnung über kosmetische Mittel, Vkos) in which maximum concentrations for nitro musks were allowed in the range of 50-500 mg/kg cosmetic product (BAG, 1996). These maximum concentrations are based on an average daily MX uptake of 11 μg/person or 160 ng/kg body weight (Kokot-Helbling, 1995d), which is postulated to be the maximal allowed daily intake for an adult person (BAG 1996). In addition, it is proposed to put MA on the list of forbidden cosmetic products. SWI (Schweizerische Seifen-u. Waschmittelindustrie) voluntarily excluded MX from any use in detergents in 1994. As a cosmetic MX is subject of the law on food in Switzerland for which the BAG (Bundesamt für Gesundheit) is responsible. Until now, the BAG did not decide on the safety of MX for consumers and for the environment.

For MX no IFRA guidelines currently exist on the use of MX in fragrance products (Wisneski and Havery, 1996). The IFRA itself has no scientific reason of limiting the use of MX and MK (Grundschober, 1997; Wagner, 1998), but submitted all nitro musks to the scientific committee for cosmetics (SCC) which advises the European Commission (Grundschober, 1997). FDA (Food and Drug Administration) is assessing human exposure to MX and will also be assessing human risk (Wisneski and Havery 1996). The International Agency for Research on Cancer (IARC), which belongs to the World Health Organization, came to the conclusion that MA and MX are not classifiable as carcinogens in humans (Group 3) (IARC monographs, 1996).

### 3.10 AIM OF THE STUDY

We designed our first experiments along studies investigating toxicity and carcinogenicity of MX (acute-, 14-day-, subchronic-, longterm toxicity) in adult B6C3F1 mice and in adult Albino rats at rather high doses (Maekawa et al., 1990; Ford et al., 1990). Effects on hepatic microsomal enzyme induction had been published (Iwata et al., 1992, 1993a and 1993b). The nitro musk compounds MX and MK detected in the environment since 1981 (Yamagishi et al., 1981) have been analysed also in human adipose tissue and in breast milk from Germany since the early nineties (Rimkus and Wolf, 1993c). The presence of MX in the environment, in human breast milk and human adipose tissue, indicate possible MX exposure during pre- and postnatal development. Because at that time, no developmental toxicity studies had been done, we designed a developmental Long Evans rat model with long term oral dosage according
to OECD guideline 415 for testing of chemicals in „one-generation reproduction toxicity study“ (OECD, 1983) at relatively low MX concentrations, reflecting more closely the naturally occurring situation.

Reprotoxicity including developmental toxicity is presently considered as being one of the most critical issues for testing environmental chemicals. Developmental studies on drugs, pharmaceutical chemicals and on environmental chemicals have shown that effects on the developing organism (especially during prenatal development) are often occurring at lower dosages than in adult animals (Schlumpf et al., 1995; vom Saal et al., 1998). In addition, they are often qualitatively different from effects in adults (Schlumpf et al., 1992 and 1994), insofar as they can affect other and/or additional systems, occur as delayed effects (being present only in the adult) or a non monotonous dose effect relationship is achieved.

A rat model was chosen, because the rat species is most often used in toxicology studies and the laboratory of developmental and environmental toxicology has a long time experience in handling rats. The Long Evans strain is easy to handle, the animals are not easily susceptible to diseases and reproduce without a high incidence of abortions. Although dermal absorption from cosmetics and detergents is the most important contamination route in humans, alimentary intake by food consumption or inhalative absorption is known as non voluntary route. MX was given orally via food pellets to rats in order to avoid stressful stimuli, in particular possible stress effects during pregnancy. Albino rats, treated orally and dermally with equal concentrations of MA, showed similar results concerning testicular effects and neurotoxicity (Ford et al., 1990).

1) Long Evans rat model for chronic low dosed MX: In our investigations we first determined internal MX concentrations in adipose tissue, where MX accumulates due to its lipophilic character, in milk and in other organs of adult parent and offspring generation following oral exposure to different MX doses. The internal dose is seen as a function of the external dose and represents the amount of the chemical which is actually absorbed by the organism. The model considers age, sex, species and individual differences in pharmacokinetic characteristics (Mattison, 1991). So, the comparison of organ tissue concentrations between different species becomes possible. Using internal doses can possibly achieve a more direct relation between tissue concentrations and alterations in organ function.
2) MX has been identified as an inducer of CYP1A1 and particularly of CYP1A2 microsomal enzymes in rat liver (Iwata et al., 1992). Acute high dose MX exposure caused a pleiotropic response in rat liver, such as an increase in liver weight, NADPH reductase and of a variety of Phase II conjugating enzymes (Iwata et al., 1993a and 1993b). MX furthermore markedly increased mouse CYP2B protein levels (Lehman-Mckeeeman et al., 1995 and 1997a). The main purpose of this chronic one generation study was to determine long term and low dose effects of MX exposure on cytochrome P450 enzymes in the adult parent generation of Long Evans rats and in their offspring. Three questions were intended to answer: 1. How does the pattern of P450 microsomal enzyme induction in chronically low dose exposed rats change as compared to the pattern described after acute short term high MX exposure, 2. are P450 microsomal enzymes already induced in young postnatal offspring of MX exposed dams, and 3. are identical sets of P450 enzymes induced in both parent and offspring generation?

3) A third part of possible MX influence on reproduction and reproductive outcome is still going on. Changes in physical development most often move along with changes in behavior. A decrease of body weight is the earlist indicator that some toxic action occurred (Alder, 1983). First results of a developmental toxicology study are given in part 3.
### 3.11 LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2,6-DNT</td>
<td>2,6-dinitrotoluene</td>
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<tr>
<td>2-NT</td>
<td>2-nitrotoluene</td>
</tr>
<tr>
<td>3-MC</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>AF</td>
<td>assessment factor</td>
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<tr>
<td>Ah</td>
<td>aryl hydrocarbon</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>ECD</td>
<td>electron capture detector</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERA</td>
<td>environmental risk assessment</td>
</tr>
<tr>
<td>EROD</td>
<td>7-ethoxyresorufin-O-deethylase</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
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<tr>
<td>GD</td>
<td>gestational day</td>
</tr>
<tr>
<td>GRAS</td>
<td>generally recognized as safe</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HGP</td>
<td>hairless guinea pig</td>
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<tr>
<td>IARC</td>
<td>international agency for research on cancer</td>
</tr>
<tr>
<td>IFRA</td>
<td>international fragrance association</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>lethal dose for 50% of the exposed population</td>
</tr>
<tr>
<td>LOAEC</td>
<td>lowest observed adverse effect concentration</td>
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<tr>
<td>LOAEL</td>
<td>lowest observed adverse effect level</td>
</tr>
<tr>
<td>MA</td>
<td>musk ambrette</td>
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<tr>
<td>MK</td>
<td>musk ketone</td>
</tr>
<tr>
<td>MM</td>
<td>musk moskene</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MROD</td>
<td>7-methoxyresorufin-O-demethylase</td>
</tr>
<tr>
<td>MT</td>
<td>musk tibetene</td>
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<tr>
<td>MX</td>
<td>musk xylene</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinic adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NOAEC</td>
<td>no-observed adverse effect concentration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NOAEL</td>
<td>no-observed adverse effect level</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PB</td>
<td>phenobarbital</td>
</tr>
<tr>
<td>PCN</td>
<td>pregnenolone-16α-carbonitrile</td>
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<tr>
<td>PEC</td>
<td>predicted environmental concentration</td>
</tr>
<tr>
<td>PN</td>
<td>postnatal day</td>
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<tr>
<td>PNEC</td>
<td>predicted no-effect concentration</td>
</tr>
<tr>
<td>PROD</td>
<td>7-pentoxyresorufin-O-depentylase</td>
</tr>
<tr>
<td>RIFM</td>
<td>research institute for fragrance materials</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>s.f.</td>
<td>safety factor</td>
</tr>
<tr>
<td>SAM</td>
<td>skin absorption model</td>
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<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>UDS</td>
<td>unscheduled DNA synthesis</td>
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<tr>
<td>XRE</td>
<td>xenobiotic responsive elements</td>
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4.1 ABSTRACT

Bioaccumulation of musk xylene (MX) was measured by GC-ECD in Long Evans rats. Males and females were fed with MX-containing chow (0.001, 0.01, 0.033, 0.1 g/kg food pellets) for 10 weeks before mating. Treatment continued during pregnancy and lactation. Offspring exhibited dose-dependent MX accumulation with 1/2-3/4 adult levels (at 0.1 mg/kg food) at days 1 and 14. Milk levels were comparable to adult female adipose tissue. Data indicate significant transplacental passage and exposure via maternal milk. In rats fed with MX in adulthood, levels were highest in adipose tissue with significant amounts in other organs (ovary, adrenal). Female tissue levels were 3.7-6.8 times higher. This unexplained sex difference was unrelated to lipid content and was absent in offspring.

4.2 INTRODUCTION

Musk odor has a wide range of natural origins in plants as well as in the animal kingdom. The term "musk" used for this group of odorants derives from the musk deer (*Moschus moschiferus*). The oily, powerfully smelling musk is produced in an abdominal gland, called musk pod of the male animal and acts to attract the females and to mark the territory (Mookerjee and Wilson, 1982). Since antiquity, the musk have been of high value as a remedy and as aphrodisiac and fragrance ingredients. When the increasing demand of natural musk could no longer be satisfied and the production of the original macrocyclic musk structure was too expensive (Ruzicka, 1926), cheaper substitutes were synthesized. Musk xylene, the first synthetic nitro musk, was discovered in 1891 by Baur (Baur, 1891), followed by musk ambrette, musk ketone, musk moskene and musk tibetene. In contrast to the natural musk extracts, containing macrocyclic ketones, alcohols and pyridine derivatives, nitro musks are dinitro and trinitro substituted benzene derivatives. The most often used nitro musks are musk xylene (MX) and musk ketone (MK). Because of their fragrance properties that are similar to natural musk extracts, synthetic musks are used world-wide in cosmetics and detergents (Opdyke, 1975; Sommer, 1993). In addition, some nitro musks were possibly used as food additives (Opdyke, 1975; Nair et al., 1986). In the fifties, additional synthetic musks, the polycyclic aromatic non-nitro musks (Galaxolide®, Tonalide®, etc.), were introduced (Frater and Lamparsky, 1991). In 1988, the worldwide production of musks amounted to about 7000 tons per year, 4200 tons of aromatic non-nitro musks, 2400 tons of nitro musks and 400 tons of macrocyclic musks (Barbetta et al., 1988).

Several studies indicated persistence of nitro musks in the environment and entrance into the food chain. MX and MK were identified in surface and wastewater as well as in freshwater fishes, mussels, shrimps and in rainbow trouts from aquaculture (Rimkus and Wolf, 1993a; Hahn, 1993; Müller, 1993; Eschke et al., 1994; Rimkus and Wolf 1995; Gatermann et al., 1995).

The low biodegradation rate, high bioconcentration factor (Rimkus et al., 1994) and high lipophilicity (MX: log K<sub>ow</sub> = 5.2) of these compounds (Kokot-Helbling, 1995a) is the basis of their persistence and bioaccumulation in the aquatic environment, as well as in human adipose tissue and breast milk. Human milk and fat tissue are excellent bioindicators for the contamination of humans by these compounds (Rimkus and Wolf, 1993c; Liebl and Ehrenstorfer, 1993; Meier and Sedlacek, 1993; Kypke-Hutter, 1993;
Rimkus et al., 1994; Eschke et al., 1995b; Müller et al., 1996). Musk xylene concentrations determined in human adult adipose tissue and milk samples range from 0.01-0.25 mg/kg fat. In fat tissues of children and newborns musk xylene was found in concentrations up to 0.6 mg/kg fat (Helbich, 1995).

The present knowledge of kinetics and toxicology of nitro musks are summarised by Ippen and Wisneski (Ippen, 1994; Wisneski and Havery, 1996; Brunn and Rimkus 1997). Most studies use short-term high dosage exposures, which do not reflect exposure in the environment. Studies on chronic low dose exposure and developmental toxicity of nitro musks are still missing.

In the present study, we analyzed MX concentrations in several organs and in milk of adult Long Evans rats and in their early postnatal offspring after chronic exposure to relatively low doses of MX.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals

Acetone (Ac308), dichlormethane (Cl340), n-hexane (He238), n-pentane (Pe100), petroleum ether b.p. 40-60° C (Et098), toluene (To081) were obtained from EGT Chemie, Tägerig, Switzerland. Florisil® 60-100 mesh ASTM (1.12994.0100) and sodium sulfate anhydrous (1.06639.0500) originated from Merck, Zurich, Switzerland. All chemicals were pesticide grade or equivalent. Quintozen (pentachloronitrobenzol) (P220-5) was obtained from Aldrich, Buchs, Switzerland. Musk xylene (1-tert.-butyl-3,5-dimethyl-2,4,6-trinitrobenzol, MX) was kindly provided by Dr.Ch.Grundschober, IFRA, Geneva, Switzerland.

4.3.2 Food pellets

Different amounts of musk xylene were dissolved in soya oil (Morga) and included into rat chow, in order to obtain final concentrations of 0.001 g/kg, 0.01 g/kg, 0.033 g/kg and 0.1 g MX/kg food pellets. Food pellets were prepared by Kliba Mühlen AG, Kaiseraugst, Switzerland. Control food pellets consisted of the same matrix (Kliba no. 343, including 1 % of soya oil).
Different charges of food pellets containing the two highest MX dosages were analyzed. The concentrations corresponded to 90-100% of the nominal value (data not shown).

4.3.3 Animals
Long Evans rats originally derived from the Møllegaard Breeding & Research Centre, Denmark, were bred in our colony. Groups of 3-4 animals of the same sex were housed in humidity- and temperature-controlled rooms (minimum relative humidity 50%, 22 ±1°C) with light/dark cycle (light on 02.00-16.00) and allowed free access to food and water.

4.3.4 Treatment
4.3.4.1 Tissues of adult rats
5-6 week old female and male rats were fed for a minimum of 10 weeks with either control or food pellets containing 0.1 g/kg MX. Rats were killed by decapitation. Adipose tissue, taken from the abdominal region, brain, kidney, liver, ovary, testes and adrenal gland from musk xylene exposed and control adult animals were dissected, briefly cooled on ice and stored at -80°C until analysis was performed.

4.3.4.2 Immature rats
5-6 week old female and male rats were fed for at least 10 weeks with either control or food pellets containing 0.001, 0.01, 0.033 or 0.1 g/kg MX. Receptive females were mated overnight. 2-3 sperm positive dams were kept per cage and isolated one day before parturition, at gestational day 22 (GD22). The day of birth (GD23) was defined as postnatal day 1 (PN1). Females were exposed to MX during pregnancy and lactation. Milk samples from stomach and liver of 1 day old suckling rats were taken and stored at -80°C until analysed. Milk and liver samples were only taken from animals with highest MX exposure (0.1 g/kg). Pups were killed at the age of 1 or 14 days (PN1 or PN14) by decapitation. At PN1, the liver was dissected; at PN14, adipose tissue samples were taken from the abdominal region.
4.3.4.3 Weight gain and determination of food intake in female and male rats
Animals were kept under same conditions as described before. According to OECD guideline number 415 for testing chemicals 5-6 week old female and male rats were fed during 10 weeks with control or food pellets containing different amounts of MX. Increase of weight and food intake were determined weekly.

4.3.5 Determination of musk xylene
4.3.5.1 Extraction
Prior to use all glassware was rinsed with acetone. The soxhlet cartridges were cleaned before use in a soxhlet apparatus with n-hexane.
Fat tissue (0.1-0.2 g), milk (0.1-0.2 g) and organ samples (liver 0.6-0.9 g, kidney 0.4-0.8 g, brain 0.3-0.6 g, adrenal gland 0.025-0.030 g, testes 0.3-0.6 g, ovary 0.070-0.080 g) were ground in anhydrous sodium sulfate (5-fold sample weight) for one hour to break up cells and then extracted in a soxhlet apparatus for 90 minutes with either 60 ml n-hexane or 60 ml 50 %-n- pentane/dichlormethane for 90 minutes, after adding 2.0 ml of a defined concentration of the internal standard quintozen. The solvent was rotary evaporated. The remaining fat in fat tissue and milk samples was dried (50° C) until weight stability was achieved, whereas samples containing organ extracts were rotary evaporated close to dryness.
Food analysis: 1 g of food pellets was pulverized. 0.1 g was ground in anhydrous sodium sulfate (5-fold sample weight) and then extracted in a soxhlet apparatus for 90 minutes with 60 ml of n-hexane, after adding 2.0 ml of a defined concentration of the internal standard quintozen. The solvent was rotary evaporated close to dryness.

4.3.5.2 Clean-up
All samples were dissolved in 2 ml of petroleum ether and transferred to the top of a 6 g Florisil® column. Florisil® was heated for six hours at 600° C and overnight at 130° C. It was standardized by adding one percent by weight of distilled water. Elution took place with 50 ml 20 %-dichlormethane/petroleum ether. The eluate was rotary evaporated nearly to dryness, a few milliliters toluene were added and concentrated to 2.0 ml. 1 µl of the sample volume was injected for quantitative determination with capillary gas
chromatography. When the concentration in a sample was too high, it was diluted before injection.

4.3.5.3 Gas chromatography and quantification

Residues were quantified using capillary gas chromatography on a VARIAN 3400 instrument (with VARIAN 8100 autosampler) equipped with a $^{63}$Ni electron capture detector (ECD) and a septum equipped programmable injector (SPI) (1 μl, temperature program). Separation was achieved on a 5 m deactivated fused silica retention gap with 30 m DB-5 capillary column (J.+W. Scientific, USA), 0.25 mm i.d., 0.25 μm ft. Helium and nitrogen were used for carrier gas and make-up gas, respectively. The temperature program was 110° C, 1 min., 15° C/min. to 170° C, 0 min., 3° C/min. to 210° C, 0 min., 20° C/min. to 300° C, 20 min.

Musk xylene was quantified by internal calibration (quintozen) and by relating peak areas in the sample GC pattern with those of identical retention times and known concentrations in a standard curve. No interfering peaks were noted in the analysis of treated organ samples.

Positive identification of the determined musk xylene and quintozen was confirmed by comparison of the retention times with those of standard solutions and by gas chromatography-mass spectrometry, GC/MS. (GC: Varian 3400 with fused silica DB-5, 30 m x 0.25 mm i.d.; MS: Finnigan SSQ710).

In fat tissue and milk samples the concentration of musk xylene was related to lipid content, in organ samples to sample weight.

4.3.5.4 Recovery rate and blank probe

Recovery rates were determined in adipose tissue and liver samples of control animals after addition of known concentrations of musk xylene and quintozen. Laboratory contamination was controlled by analyzing blank probes without and with matrix in the same way as sample probes. Samples and blanks were analysed simultaneously.
4.3.6. **Determination of percent body fat and tissue fat of liver and kidney of female and male adult rats**

7-8 week old female and male rats were fed for 10-15 weeks with food pellets containing 0.1 g/kg MX. White adipose tissue taken from defined regions of the body of female and male adult rats was weighted. Liver and kidneys of the same animals were ground in anhydrous sodium sulfate (5-fold sample weight) for one hour and then extracted in a soxhlet apparatus for 4 hours with 250 ml 50% n-pentane/dichlormethane. The solvent was rotary evaporated and the remaining fat was dried (50°C) until weight stability was achieved, resulting in the lipid content.

4.4 **RESULTS**

4.4.1 **Sample preparation and GC/ECD-detection**

GC/ECD chromatograms of MX and quintozen standard solutions showed purity and baseline separation of both substances. Known concentrations of MX and quintozen which are in the range of the expected sample values were added to adipose tissue and liver samples of control animals. The recovery rates were 90-100% for both substances. Due to its nitro groups MX is detectable with great sensitivity by ECD. 0.2 pg of MX (SPI injector, 1 µl) in a standard solution could be detected. Two different types of blank probes, i.e. (1) without matrix and (2) with matrices of different tissues and different tissue quantities, yielded peak areas of MX that were not statistically different from each other (Fisher's test p = 0.832). The mean sample concentration of MX of both blank groups was 1123 pg MX/sample (2.0 ml) +/- 950 (n = 44, +/- S.D., range 180 pg-3500 pg MX/sample (2.0 ml)). It represents the mean background value. Figure 10 shows examples of chromatograms of a blank probe without matrix (A), a sample probe of kidney tissue of a control animal (B) and a sample probe of kidney tissue of an animal treated with 0.1 g/kg MX (C). In all chromatograms a peak with a relative retention time (rrt) of 1.154 is found.
Fig. 10: GC/ECD chromatograms of organ extracts dissolved in toluene. A: blank probe without matrix, undiluted; B: sample probe of kidney tissue of a 14 day old male control animal, undiluted; C: sample probe of kidney tissue of a 14 day old male animal whose mother was treated with 0.1 g MX/kg food pellets, diluted (1:20). 30 m DB-5, carrier gas: helium; SPI-injector 1 μl; MX, musk xylene; IS, internal standard quintozen. Relative retention times (rrt) of MX are 1.154.
4.4.2 Musk xylene accumulation in different tissues

MX concentrations were determined in different organs of adult female and male rats fed during a minimum of 10 weeks with food pellets containing 0.1 g MX/kg (Fig. 11). MX concentrations were significantly higher in all tissue samples of female rats than in male rats. Milk samples taken from the stomach of one day old suckling pups whose mothers got 0.1 g MX/kg chow during at least 10 weeks before and during pregnancy and during lactation were in the range of MX concentrations measured in adipose tissue of female rats (Fig. 11 B).

![Fig. 11: MX concentrations in various tissues of adult female (F) and male (M) Long Evans rats treated with food pellets containing 0.1 g/kg MX during 10 weeks (concentrations in mg/kg tissue or mg/kg lipid). A: CNS, central nervous system: F 2.42 ±0.25; M 0.39 ±0.11 KID, kidney: F 3.82 ±1.71; M 1.03 ±0.24 LIV, liver: F 4.41 ±0.52; M 0.65 ±0.08 OV, ovary: F 17.35 ±3.92; TE, testes: M 0.38 ±0.29 ADR, adrenal gland: F 23.08 ±3.77; M 4.32 ±2.80 B: FAT, adipose tissue: F 161.99 ±24.20; M 36.90 ±9.62 Milk, milk taken from stomachs of 1 day old suckling pups: 218.91 ±52.27 Each bar represents the mean ±S.D. (n = 4).
In order to elucidate the possible basis of the gender difference, we determined the amount of tissue fat in kidney and liver in percent of organ weight and the amount of adipose tissue in percent of body weight in female and male adult rats, fed during 10-15 weeks with food pellets containing 0.1 g/kg MX (Fig. 12). Adipose tissue in percent of body weight tended to be higher in male rats, whereas no sex difference in the amount of kidney and liver tissue fat was observed.

**Fig. 12**: Total adipose tissue in percent of body weight and tissue fat of kidney and liver in percent of organ weight in female and male adult rats treated with food pellets containing 0.1 g/kg MX during 10-15 weeks. Each bar represents the mean ± S.D. (n = 3).

Body weight gain tended to be slightly higher in MX-treated animals of both sexes, but the trend did not reach statistical significance (Mann-Whitney U test: females p = 0.091; males p = 0.560, Fig. 13). Daily food intake did not differ between MX-treated animals and controls. Males consumed in the first 4 weeks on average 23-24 g/day and in the last four weeks 25-26 g/day, females in the first 4 weeks on average 18 g/day and in the last four weeks 18-19 g/day.
Fig. 13: Body weight gain in female and male control animals and in animals treated with food pellets containing 0.1 g/kg MX during 10 weeks. Results are means ± S.D. (MX 0.1 male n = 9; C, control male n = 7; MX 0.1 female n = 10; C, control female n = 8).

MX concentrations of adipose tissue of 14 day old suckling male and female offspring were analysed after pre- and postnatal exposure to MX (Fig. 14). MX adipose tissue concentrations were dose-dependent. In contrast to the situation in adult animals, no sex difference was observed. Measurements of MX in the liver of one day old male and female offspring of dams treated with 0.1 g/kg MX confirmed the absence of a sex difference in MX tissue at early postnatal stages.
**Fig. 14:** MX concentrations in adipose tissue of 14 day old female (F) and male (M) rats and in liver of 1 day old female (F) and male (M) rats from mothers exposed to different concentrations of MX in chow (MX concentrations in mg/kg lipid or mg/kg liver respectively). Results are means ±S.D..

Adipose tissue: 
- MX 0.001 g/kg: F 0.53 ±0.04; M 0.57 ±0.14 (n=4); 
- MX 0.01 g/kg: F 6.38 ±0.67; M 5.50 ±0.55 (n=4); 
- MX 0.033 g/kg: F 26.94 ±6.16; M 22.19 ±4.69 (n=4); 
- MX 0.1 g/kg: F 115.26 ±29.66; M 120.48 ±39.36 (n=8)

Liver: 
- MX 0.1 g/kg: F 2.27 ±0.55; M 2.36 ±0.82 (n=3)

### 4.5 DISCUSSION

#### 4.5.1 Musk xylene analysis

Similar to organochlorine pesticides and PCBs, sample preparation of the relatively nonpolar and lipophilic MX for GC/ECD analysis is done by soxhlet extraction with a variety of solvents. With this technique most of the organic compounds are stable and sample contamination is under control. The cartridges used were identified as important contamination sources and had to be cleaned beforehand. The extracts were cleaned-up by Florisil® adsorption chromatography (Stijve and Cardinale, 1974; Rimkus, personal communication, 1994). This method can be used successfully without a gel permeation chromatograph (GPC), however, it is more time-consuming than GPC.
The idea of soxhlet extraction is to extract MX together with the lipids, since lipophilic compounds such as MX are homogeneously distributed in body fat. The amount of extracted fat and the concentrations of the investigated compounds can be influenced by the solvent (de Boer and Wester, 1988). n-Hexane as a non-polar solvent will completely extract the main non-polar, free lipids in adipose tissue. In other organ tissues, the non-depot fat or bound lipids form a considerable pool of the total lipid, that needs a more polar solvent for complete extraction. Therefore, liver tissue was extracted with two different solvent-systems, n-hexane and 50 % n-pentane/dichlormethane, for different time periods. Neither the solvent nor the extraction time had an influence on MX concentration values, and the recovery rates were comparable. The advantage of the more polar solvent is the lower boiling-point, which enables a careful sample treatment during soxhlet extraction and the rotary evaporation of the solvent nearly without vacuum.

Due to its nitro groups, MX is detected with great sensitivity by ECD, with a background MX of 0.6 pg/sample (2.0 ml). The absolute quantity of background MX did not depend on the amount of matrix, which indicates that there possibly exist additional sources of sample contamination by MX. However, the absolute background level did not distort the results, since it was 10 times smaller than the lowest MX concentration measured in adipose tissue of 14 day old rat offspring (group fed by MX 0.001 g/kg food pellets, both values expressed as pg/μl).

4.5.2 Accumulation of musk xylene in different tissues in relation to age and sex

The tissue distribution of MX was studied in adult rats fed with the highest dose of the compound during adulthood. As could be expected, the highest MX concentrations were found in fat tissue. However, it should be noted that significant concentrations of MX were also present in a number of additional organs, in particular in the ovary and in the adrenal gland of both sexes. These findings may be of interest with regard to further investigations in reproductive and endocrine toxicology. The relative tissue distribution of MX appeared to be similar in adult male and female rats (with the possible exception of ovary vs. testis), however, absolute tissue levels of females exceeded by 3.7 to 6.8 times those of males in all tissues studied. There are indications that MX levels in
adipose tissue samples from women tend to be somewhat higher than those of men (Rimkus et al., 1994), while no gender differences were observed in human blood levels (Kokot-Helbling, 1995a).

Tissue concentrations of lipophilic substances can be correlated with the lipid content of a given organ. Yet, adult MX-exposed rats did not exhibit sex differences in adipose tissue as percentage of body weight, or in tissue fat concentrations of kidney and liver as percentage of organ weight. Hence, the pronounced sex difference cannot be explained by differences in lipid content. Daily MX intake calculated from food consumption was similar in both sexes with approximately 7-8 mg/kg in males and 8 mg/kg in females in the first and last 4 weeks. In rats fed on a diet of 0.1 g MX/kg food pellets for 70 days, this yields a total MX exposure from food of 560 mg/kg body weight. Based on the percentage of tissue fat (15 and 17 % of total body weight in females and males, respectively, Fig. 12), an approximate value of MX retention can be calculated from the mean MX concentrations in parenchymatous organs and fat tissue. Female rats retained about 40 mg MX/kg body weight (Fig. 11) or about 7.2 %, whereas male rats with the identical exposure retained only 8 mg MX/kg body weight or 1.4 %. A slower elimination half-life of MX in females could provide another explanation for the higher tissue levels in this sex. However, results of an investigation of the elimination rate of MX after oral application in Wistar rats suggest a longer half-life in males (1.8 days) rather than females (1.1 days; Kokot-Helbling, 1995c).

MX metabolism has been analyzed only in male rats (Wistar rats) (Minegishi et al., 1991). The first step of the main metabolic pathway is the reduction of the nitro group to an amino group at the 2 position, followed by acetylation of the amino group. Anaerobic intestinal bacteria and reduced nitroreductase activity in the liver both contribute to the metabolic reduction (Levin and Dent, 1982; Lehman-McKeeman et al., 1997a). MX is mainly excreted via the bile and undergoes enterohepatic circulation. The reduced form is reabsorbed and returns to the liver where it can be activated by N-oxidation catalyzed primarily by CYP 1A2 (Butler et al., 1989). Prolonged MX treatment elicits a significant induction of ethoxresorufin-0-deethylase (EROD) activity corresponding mainly to CYP450 1A1, and of methoxyresorufin-0-deethylase (MROD) activity, representing mainly CYP450 1A2, in male and female rats, without sex difference (Suter et al., 1995 and 1996). Thus, the sex difference in MX tissue levels of adult rats so far remains unexplained.
It is interesting to note that no sex difference in MX tissue levels was seen in immature offspring studied at postnatal days 1 (liver) and 14 (adipose tissue). In developmentally exposed 2 week old males, adipose tissue levels were higher than in males treated in adulthood. Whether tissue levels of developmentally exposed males would remain elevated until adulthood upon continued exposure, and whether a sex difference would later develop also in developmentally exposed animals, is presently not known. However, the data indicate that the influence of sex on MX bioaccumulation should be investigated more thoroughly.

Immature offspring of rats exposed to MX before and during pregnancy and during lactation exhibited a dose-dependent accumulation of MX. The increase was relatively higher in the upper dose range as compared to the lower dose range, suggesting an enhanced accumulation at higher doses. In one day old pups of both sexes, the MX concentration in liver already attained nearly half the value of adult female liver. At 14 days of age, the rather high load in fat tissue exceeded adult male fat tissue concentrations by a factor 3 to 4 and reached 1/2 to 3/4 of the female MX load. The neonatal liver data point to a considerable transplacental passage of MX. Thereafter, MX exposure continues via the maternal milk. Milk levels were comparable to adipose tissue levels of adult females. With respect to both, transplacental passage and secretion into milk, MX appears to behave similar to PCBs (Meier and Sedlacek, 1993).

In human adipose tissue and milk samples, MX concentrations range between 0.01 and 0.25 mg/kg lipid (Rimkus and Wolf, 1993c; Liebl and Ehrenstorfer, 1993; Rimkus et al., 1994; Müller et al., 1996). In fat tissue of children and newborns, MX was found in concentrations up to 0.6 mg/kg lipid (Helbich, 1995). Thus, the lowest MX dose used in the present study (0.001 g/kg food) yields MX concentrations in lipid in the upper human range, whereas 200-480 times human levels (upper range) are reached after the highest dose (0.1 g/kg food).

Our developmental study in rats demonstrates efficient absorption and loading of MX through oral uptake of the compound in adult rats, and significant bioaccumulation of MX during prenatal and early postnatal life. Data on liver enzyme induction in early postnatal rat pups (Suter-Eichenberger et al., in preparation) indicate that the load in offspring results from a combination of exposure via placental transfer and milk. In contrast, recent data on adult humans and animals (Brunn and Rimkus, 1997) suggest that oral absorption of MX is a minor path in adulthood. In vitro and in vivo studies in
guinea pigs and rats show an efficient transcutaneous absorption of the lipophilic compound MX (Ford et al., 1990; Hood et al., 1996). Transcutaneous exposure in adult humans through cosmetics spread on the skin was calculated to be around 4 to 5 times higher than the median oral intake (Brunn and Rimkus, 1997). Rats exposed to MX in adulthood exhibited a marked sex difference in MX levels of several organs. The background of this sex difference and the consequence of developmental exposure to MX require further investigation.

4.6 ACKNOWLEDGEMENTS

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5 CYP450 ENZYME INDUCTION BY CHRONIC ORAL MUSK XYLENE IN ADULT AND DEVELOPING RATS

5.1 ABSTRACT
Developmental and adult toxicity of musk xylene was studied in Long Evans (LE) rats fed with chow containing musk xylene (MX) in food pellets in concentrations of 1 mg, 10 mg, 33 mg, 100 mg and 1000 mg MX per kg chow corresponding to a daily intake of 0.07-0.08 mg MX/kg up to 70-80 mg MX/kg body weight. Adult male and female rats were MX exposed for a minimum of 10 weeks before mating. Exposure continued throughout pregnancy, birth and lactation. The effects of MX on CYP1A1/1A2 were studied in liver microsomes by EROD (7-ethoxyresorufin-O-deethylase) for CYP1A1 and by MROD (methoxyresorufin-O-demethylase) for CYP1A2 activity and by Western blotting. MX induced these enzymes dose dependently in adult and developing rats at PN (postnatal day) 1 and 14. The lowest effective maternal dose was 2-3 mg MX/kg/day. Western blot data of CYP2B and CYP3A indicated the induction of both P450 enzyme proteins in developing rats at PN 14 at the higher dose of 70-80 mg MX/kg/day. In contrast, upon high MX exposure CYP2B but not CYP3A was found to be induced in adult first generation male and female rats, indicating differential sensitivity to MX in development.

5.2 INTRODUCTION

MX, a synthetic nitro musk, is used world-wide in cosmetics and detergents because of its pleasant fragrant properties similar to natural musk. The low degradation rate of the compound, its high bioconcentration factor (Rimkus et al., 1994) and high lipophilicity (MX logK_{ow} 5.2) led to high persistence and bioaccumulation in the environment. The total annual volume of use for MX in the EU and US is 50 tons and 62 tons respectively (Ford, 1998). As a consequence of its bioaccumulation potential, MX was banned in Japan in the eighties. Since, the fragrant compound has been primarily analyzed in European aquatic environment, in fish, mussels, shrimps and in human adipose tissue and breast milk (Rimkus and Wolf, 1993c; Müller et al., 1996; Rimkus and Brunn, 1996b; Gattermann et al., 1998). The chemical properties and behavior of MX in the environment calls for toxicological clarification. Most studies on toxicology of MX done so far (Ippen, 1994; Wisneski and Haverty, 1996; Brunn and Rimkus, 1997) use acute high dosage exposures by intraperitoneal injection or gavage application, which do not reflect exposure of humans and wildlife in the environment. Studies on chronic or subchronic low dose exposure (Maekawa et al., 1990) and on developmental toxicity of nitro musk are very sparse (Ford, 1998).

MX has been identified as an inducer of CYP1A and particularly of CYP1A2 microsomal enzymes in rat liver (Iwata et al., 1992). Acute high dose MX exposure caused a pleiotropic response in rat liver, such as increase in liver weight, NADPH reductase and of a variety of phase II conjugating enzymes (Iwata et al., 1993a and 1993b). MX furthermore markedly increased mouse CYP2B protein levels (Lehman-McKeeman et al., 1995 and 1997a).

The main purpose of this chronic one generation study was to determine long term and low dose effects of MX exposure on cytochrome P450 enzymes in the adult parent generation of Long Evans rats and in their offspring. Oral administration of MX via food was chosen in order to avoid stressful stimuli, in particular possible stress effects during pregnancy. An earlier study on adult and developmental bioaccumulation of MX in rats revealed sex-dependent MX concentrations in different organs and in fat tissues of male and female rats with considerably higher concentrations in females (Suter-Eichenberger et al., 1998). Milk concentrations in females were found to be similar to levels in female fat tissues. Independent of the sex, a dose dependent increase of MX levels in fat tissues of developing offspring at postnatal day 14 was found. The present
study was intended to answer two questions: 1. How does the pattern of P450 micro-
somal enzyme induction in chronically low dose exposed rats compare to the pattern
described after acute short term high MX exposure, and 2. are P450 micro-somal
enzymes already induced in young postnatal offspring of MX exposed dams? Offspring
are exposed to the cosmetic chemical during the comparatively short but possibly more
sensitive pre- and perinatal period.

5.3 MATERIALS AND METHODS

5.3.1 Animals
Female and male Long Evans rats (Møllegaard Breeding & Research Centre, Denmark)
were bred in our colony. Groups of 3-4 animals of the same sex were housed in
humidity and temperature controlled rooms (relative humidity 50%; 22 ±1° C) with
regular light/dark cycle (light on 02.00-16.00) and allowed free access to food and
water.

5.3.2 Food pellets containing MX
The preparation of food pellets with different concentrations of musk xylene was done
by Provimi Kliba AG, Kaiseraugst, Switzerland. Different amounts of MX were
dissolved in soya oil (Morga) to achieve the following MX concentrations in chow: 1
mg/kg; 10 mg/kg; 33 mg/kg; 100 mg/kg, 1000 mg/kg. The control food pellets consisted
of the same matrix (Provimi Kliba no. 343, including 1 % of soya oil). MX concen-
trations in food pellets were chosen according to our previous kinetic study and verified
regularly. (Suter-Eichenberger et al., 1998).

5.3.3 Chemicals and reagents
MX kindly provided by Ch.Grundschober, IFRA, Geneva, Switzerland, was essentially
100 % pure as determined by gas chromatography equipped with a 63Ni electron capture
detector (Suter-Eichenberger et al., 1998). Resorufin (Na-salt), 7-ethoxyresorufin and 7-
pentoxyresorufin were obtained from Sigma, Buchs, Switzerland, 7-methoxyresorufin
from Molecular Probes Inc, Eugene, USA, and NADPH (tetrosodium salt) from GEBrU GmbH, Gaiberg, Germany. All other chemicals used were of highest analytical grade.

Antibodies to rat CYP1A1 and CYP1A2 (rabbit) used for ELISA and for part of the Western blot analyses were obtained from Amersham Life Science, Zurich, Switzerland. Antibodies to rat CYP1A1/2, CYP2B1/2, CYP3A1/2 and positive controls used for Western blotting experiments were a gift by Dr. F. Waechter, Novartis Crop Protection, Basel, Switzerland. Secondary antibodies used for ELISA and Western blotting experiments were either goat anti-rabbit IgG horseradish peroxidase conjugates (Promega Corporation, Zurich, Switzerland), or goat anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM) horseradish peroxidase conjugates (Sigma, Buchs, Switzerland). ELISA plates were 96-well MaxiSorp F8 unframed immuno modules from Nunc, Roskilde, Denmark. All other reagents used for immunoblotting procedures were of the highest electrophoresis grade available.

5.3.4 MX-exposure schedules

5.3.4.1 Analysis of adult female and male rats following chronic oral exposure to different concentrations of MX

5-6 week old female and male rats were fed for a minimum of 10 weeks (10-11 weeks) with either normal rat chow (food pellets) or with food pellets containing 10 mg MX/kg, 100 mg MX/kg or 1000 mg MX/kg food pellets according to OECD Guideline 415 for testing of chemicals (one generation reproduction toxicity study). Animals were tested at the age of 16 to 22 weeks.

In the analysis of recovery from enzyme inductive effects in long term MX (100 mg/kg food pellets) exposed rats the following four groups of animals were studied: (1) control group on control diet throughout 18 weeks, (2) one group on the 100 mg MX/kg diet for 18 weeks. Two additional groups received (3) 100 mg MX/kg food pellets for 16 weeks followed by 2 weeks of control food or (4) 100 mg MX/kg food pellets for 10 weeks followed by 8 weeks of control diet.
5.3.4.2 Analysis of female and male offspring at PN 14 after pre- and postnatal exposure to MX

5-6 week old female and male rats were fed for at least 10 weeks with control pellets or food pellets containing 1 mg MX/kg, 10 mg MX/kg, 33 mg MX/kg, 100 mg MX/kg or 1000 mg MX/kg chow. Females were then mated overnight with exposed male rats matched for MX diet and age. 2-3 sperm positive dams were kept together in cages and isolated one day before parturition (gestational day 23 (GD23)). The day of birth (GD 23) was defined as postnatal day 1 (PN1). MX treatment continued during pregnancy and after birth. Pups were killed at the age of 14 days and their livers analysed for enzyme induction.

5.3.4.3 Cross-fostering experiment with pups of chronically exposed female and male rats (MX 100 mg/kg food pellets)

In a cross-fostering experiment 5-6 week old female and male rats were fed for at least 10 weeks with either control pellets or food pellets containing 100 mg MX/kg. Females were mated with males exposed to the identical MX diet and gave birth after 23 days. Within the first 12 hours after birth, untreated pups were given to exposed dams and pups of MX exposed dams were left to an untreated dam. The four different treatment groups were: control pups, pups pre- and postnatally exposed to MX, prenatally MX-exposed pups, and postnatally MX-exposed pups. Pups studied at PN1 (prenatal exposure) were sacrificed within 12 hours after birth and pups of the postnatal or pre-and postnatal MX exposure groups were studied at the age of 14 days postnatal.

5.3.5 Liver microsomal preparation of adult and young rats

Rats were sacrificed by decapitation. The liver was quickly removed, washed in ice-cold homogenising medium (10 mM phosphate buffer pH 7.4) to remove excess blood, gently blotted dry with tissue paper (Lake, 1987), weighed and transferred to a homogenising vessel after mincing the tissue in ice-cold phosphate buffer with scissors. All steps were carried out at 0-4°C. An appropriate amount of homogenising medium was added to obtain a final 25 % (w/v) homogenate. Livers of 1 day old male and female
pups of the same litter were pooled, livers of 14 day old pups were prepared individually or in groups of 3-4 animals of the same sex and litter. Microsomes were prepared by differential centrifugation according to procedures outlined by Lake (1987). The final microsomal pellet was resuspended, diluted to an appropriate tissue concentration (1 g liver/ml) and stored at -80° C.

5.3.6 Measurement of enzyme activity
EROD-, MROD- and PROD- (7-Pentoxysterorufin-O-depentylase) activities were determined spectro-fluorometrically with a Perkin Elmer Luminescence spectrometer LS50 according to Burke et al. (1985). Enzyme sample sizes used in these assays ranged from 30-120 µl, according to substrate and activity of the sample. Briefly, microsomes were incubated for 1 min in 200 mM Tris/HCl buffer pH 8.5 in the presence of either 1.2 M (final concentrations) 7-ethoxyresorufin, 7-methoxyresorufin or 7-pentoxyresorufin at 37° C. Resorufin generation was monitored over 2 min. at an excitation wavelength of 540 nm and an emission wavelength of 586 nm. The reaction was calibrated by the addition of 200 pmol resorufin standard to each incubation. Reactions were run under linear conditions of both time and protein concentration. The results are expressed as pmol resorufin generated per min per mg protein. Microsomal protein content was determined according to Bradford (1976), adapted for a Cobas Fara autoanalyzer (Roche), using bovine serum albumin as a standard.

5.3.7 Electrophoresis and immunoblotting procedures
Samples of liver microsomes for SDS-PAGE were expressed as µg liver equivalents (1 µg liver in 1 µl); they ranged from 60-100 µg corresponding to 0.4-1.5 µg microsomal protein. SDS-PAGE was performed on 10 % polyacrylamide gel (Laemmli, 1970). Microsomal proteins were transferred electrophoretically to nitrocellulose with a mini-trans-blot apparatus (Bio Rad Trans Blot SD). The nitrocellulose treated with blocker (5 % nonfat dried milk) for 1 hour at room temperature was then incubated overnight (approximately 17 hours) at 4° C in a 1/1000 dilution of anti-rat cytochrome P450 antibody diluted in blocker. For colour development, the nitrocellulose was incubated with IgG, IgA, IgM horseradish peroxidase conjugate (1/2000 dilution in blocker) at room
temperature for 1 hour. After each incubation stage unbound antibodies were removed by washing once with RIPEA (Tris 20 mM, NaCl 60 mM, Triton-X-100 0.4 %, SDS 0.4 %, EDTA 2 mM, Deoxicholate 0.4 %) and twice with Tris buffered saline and Tween\textsuperscript{TM}-20 (0.05 %) solution. NEN\textsuperscript{TM} detection reagent (NEN Life Science Products, Boston, Massachusetts, USA) utilizes the bound horseradish peroxidase to catalyze the oxidation of luminol, in the presence of hydrogen peroxide and an enhancer. Blots were evaluated by image analysis on a computing densitometer modul 300A (Molecular Dynamics). As positive controls (a gift by Dr. F. Waechter, Novartis AG, Basel) for CYP1A1/2 served liver microsomes from male rats dosed 25 mg/kg 3-MC (3-methylcholanthrene) for 4 days i.p.; for CYP2B1/2 PB liver microsomes from male rats dosed 75 mg PB (phenobarbital)/kg for 4 days i.p.; and for CYP3A1/2 microsomes from male rats dosed 50 mg PCN (pregnenolone-16α-carbonitrile)/kg for 4 days i.p.. Liver microsome samples of the positive controls corresponded to 3-15 µg liver equivalents.

5.3.7.1 Quantification of CYP450 with ELISA

96-well microtitre plates were coated with 100 µl of various concentrations of microsomal proteins (0.1-1.6 µg) in Tris buffer pH 7.4 (Tris) for 3 hours at room temperature. The plates were washed three times with Tris-Tween\textsuperscript{TM} 20 (0.2 %) and blocked with 100 µl Tris containing 10 % BSA for 1 hour at room temperature. 70 µl of the first antibodies (CYP1A1/1A2) were added after dilution in Tris containing 2 % BSA (1:2/1:100 respectively). Incubation was carried out overnight at 4\degree C (approximately 17 hours). Then the wells were washed three times with Tris-Tween\textsuperscript{TM} 20 (0.2 %) and incubated for 90 min at room temperature with an anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (70 µl, 1/2000 diluted in Tris containing 2 % BSA). The plates were washed again three times with Tris-Tween\textsuperscript{TM} 20 (0.2 %) and once with distilled water. Tetramethylbenzidine (TMB) was used as a chromogenic substrate for horseradish peroxidase. Wells were incubated with 100 µl of a mixture of 20 mM TMB and 200 mM H\textsubscript{2}O\textsubscript{2} in citrate buffer pH 3.95 in a ratio of 1:20 at room temperature. After 9-10 min, the reaction was stopped by addition of 100 µl 1M H\textsubscript{2}SO\textsubscript{4}. The absorbance at 450 nm was read on an ELISA plate reader (Anthos 2001). Absorbances of exposed animals were compared with those of control animals at protein concentrations where both groups lay in the linear range of serial dilutions.
Microsomal protein content was determined in Western blotting and ELISA experiments according to Bradford (1976) with a dual beam spectrophotometer UV-160 A (Shimadzu), using bovine serum albumin as a standard.

5.3.8 Data analysis
Data were analyzed for statistical significance either by analysis of variance followed by Fisher’s test or by Mann-Whitney test depending on sample size. For statistical analysis of Western blot instrument readings, two-tailed Wilcoxon rank sum test was used. All analyses were conducted with Systat statistical application (Systat, Inc., Evanston, IL USA).

5.4 RESULTS
5.4.1 Adult male and female rats
Adult first generation MX exposed rats were clinically indistinguishable from animals on control chow. No change in weight gain (Suter-Eichenberger et al., 1998) was observed and MX exposure produced no consistent and/or dose dependent effects on liver weight and microsomal protein in animals of both sexes (Tab. 6). Table 6 also shows a conversion of the different MX-concentrations in chow (1-1000 mg MX/kg chow) to the approximate intake of MX per kg body weight and day in adult male and female rats, based on our kinetic investigation (Suter-Eichenberger et al., 1998).
Table 6: General hepatic effects of MX treatment in adult animals

<table>
<thead>
<tr>
<th>Dosage/animal(^a) (MX mg/kg/day)</th>
<th>Dosage (MX mg/kg food pellet)</th>
<th>Sex</th>
<th>Liver wt. (g) in % of control</th>
<th>L/BW (%)(^b) in % of control</th>
<th>n</th>
<th>Microsomal protein (mg/g liver) in % of control</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>male</td>
<td>100.0 +/- 5.9</td>
<td>100.0 +/- 5.1</td>
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<td>100.0 +/- 5.1</td>
<td>(9)</td>
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<tr>
<td>0</td>
<td>0</td>
<td>female</td>
<td>100.0 +/-10.6</td>
<td>100.0 +/- 6.6</td>
<td>(12)</td>
<td>100.0 +/- 7.2</td>
<td>(9)</td>
</tr>
<tr>
<td>0.7-0.8</td>
<td>10</td>
<td>male</td>
<td>94.9 +/- 3.3</td>
<td>92.8 +/- 1.2(^*)</td>
<td>(3)</td>
<td>102.7 +/- 10.1</td>
<td>(3)</td>
</tr>
<tr>
<td>0.7-0.8</td>
<td>10</td>
<td>female</td>
<td>106.8 +/-13.3</td>
<td>115.5 +/-11.7(^*)</td>
<td>(3)</td>
<td>91.7 +/-6.8</td>
<td>(3)</td>
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<tr>
<td>7-8</td>
<td>100</td>
<td>male</td>
<td>103.0 +/- 7.6</td>
<td>99.8 +/- 7.6</td>
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<td>104.0 +/-10.1</td>
<td>(11)</td>
</tr>
<tr>
<td>7-8</td>
<td>100</td>
<td>female</td>
<td>98.3 +/- 4.3</td>
<td>100.4 +/- 5.9</td>
<td>(11)</td>
<td>114.0 +/-16.5(^*)</td>
<td>(11)</td>
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<tr>
<td>70-80</td>
<td>1000</td>
<td>male</td>
<td>126.4 +/- 3.1(^*)</td>
<td>110.3 +/- 3.0(^*)</td>
<td>(4)</td>
<td>100.5 +/- 5.8</td>
<td>(3)</td>
</tr>
<tr>
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<td>1000</td>
<td>female</td>
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<td>109.6 +/- 8.3</td>
<td>(3)</td>
<td>89.6 +/-10.1</td>
<td>(3)</td>
</tr>
</tbody>
</table>

\(^a\) Daily MX uptake (male: mean body weight 300-380 g, 23-26 g food pellets/day; female: mean body weight 210-250 g, 18-19 g food pellets/day) (Suter-Eichenberger et al., 1998)

\(^b\) Liver to body weight ratio, with liver weight expressed as a percentage of total body weight

Results are means +/- S.D. of 3-12 animals

Asterisk indicates statistically different from control (* p<0.05) using Mann-Whitney test

n Number of animals in brackets
MX exposure significantly affected CYP1A1 (EROD) and CYP1A2 (MROD) activities in adult LE rats exposed subchronically (10 to 18 weeks) to 7-8 mg MX/kg/day. In both sexes EROD and MROD activities were increased 2.5- and 4-fold, respectively (Fig. 15). Control levels were about 3 pmol/min/mg protein with considerable variation. PROD activity, indicating CYP2B enzyme induction, yielded only an increase of 1.7-fold in male and 2.0-fold in female rats (Mann-Whitney test p < 0.05, n=4, data not shown).

![Fig. 15: EROD-(7-ethoxyresorufin-O-deethylase) and MROD-(7-methoxyresorufin-O-deethylase)-activity in liver microsomes of adult male and female rats exposed to 7-8 mg MX/kg/day (100 mg MX/kg food pellets) or control chow during 10-11 weeks. Each bar represents the mean ± S.D. of 6-8 animals. Absolute control levels for EROD-activity: male 24.0 ±4.1; female 36.9 (pmol/min/mg protein), for MROD-activity: male 25.4 ±6.2; female 27.6 ±6.8 (pmol/min/mg protein) Asterisks indicate statistical significance from control using Mann-Whitney test (*p < 0.05).](image)

Recovery of EROD/MROD enzyme activity and enzyme protein levels after MX-induced induction was measured in adult rats of both sexes that were fed for either 10 or 16 weeks on chow containing 100 mg MX/kg, followed by a feeding period with control chow during either 2 weeks or 8 weeks on control chow. These animals were compared with rats fed for 18 weeks on MX containing chow or control chow (Fig. 16 A,B). Already 2 weeks after cessation of MX-exposure, EROD-/MROD-activity in
male and female rats (Fig. 16 A,B) significantly decreased and enzyme protein levels of male rats (CYP1A1, CYP1A2) were no longer significantly different from control levels (Tab. 7). Enzyme protein values in female rats also declined but were still significantly above control after 2 weeks cessation of MX-exposure (p < 0.05, Mann-Whitney U test), reaching control levels only after more than 2 weeks on control diet (Tab. 7).

Fig. 16 A,B: Recovery of EROD/MROD-activities in liver microsomes of adult male (A) and female (B) rats, fed for 18 weeks with control or MX containing chow (100 mg MX/kg food pellets corresponding to daily intake of 7-8 mg MX/kg). EROD/MROD was analysed in animals after 16 weeks on MX (100 mg/kg) chow followed by 2 weeks of...
control chow and in a second group of animals 10 weeks on MX (100 mg/kg) chow followed by 8 weeks of control chow. Bars represent means ± S.D. of 2-4 animals. Asterisks indicate statistical significance in combined male and female samples from EROD/MROD values of 18 weeks MX (7-8 mg/kg/day) exposed animals using Mann-Whitney U test. (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>C18 wks</th>
<th>MX 18 wks</th>
<th>MX 16 wks</th>
<th>MX 10 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A1</td>
<td>(3) 0.434 +/-0.004*</td>
<td>(2) 0.805 +/-0.021</td>
<td>(2) 0.502 +/-0.040*</td>
<td>(3) 0.415 +/-0.028*</td>
</tr>
<tr>
<td>1A2</td>
<td>0.373 +/-0.012*</td>
<td>0.598 +/-0.078</td>
<td>0.414 +/-0.009*</td>
<td>0.391 +/-0.022*</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A1</td>
<td>(4) 0.430 +/-0.010*</td>
<td>(3) 0.976 +/-0.014</td>
<td>(3) 0.556 +/-0.031*</td>
<td>(4) 0.455 +/-0.052*</td>
</tr>
<tr>
<td>1A2</td>
<td>0.501 +/-0.021*</td>
<td>0.972 +/-0.081</td>
<td>0.531 +/-0.005*</td>
<td>0.513 +/-0.033*</td>
</tr>
</tbody>
</table>

Table 7: Recovery of enzyme protein induction: CYP1A1 and CYP1A2
Means ±S.D. of 2 – 4 animals, determination of 1A1 and 1A2 by ELISA (OD at 450 nm). Identical samples of liver microsomes were used for protein analysis as shown in Figure 16 A,B. Asterisks indicate statistical significance in female and combined male and female samples from CYP1A1 and CYP1A2 protein values of 18 weeks MX (7-8 mg/kg/day) exposed animals (Mann-Whitney U test, *p < 0.05). If the number of animals was less than 3, male and female samples were combined (number of animals in brackets).

Microsomal levels of CYP1A, 2B and 3A proteins were evaluated for different MX exposure levels by Western blotting. Figure 17 shows a representative Western blot for adult male and female rats. MX appears as a strong CYP1A and CYP2B inducer in adult rats in comparison to the model inducers MC-3 and PB. Table 8 summarizes the results of a semi-quantitative evaluation (absolute instrument readings) of Western blots in pooled values of adult male and female adult rats. Western blots of CYP1A, CYP3A and CYP2B were evaluated by image analysis on a computing densitometer (modul 300A, Molecular Dynamics). In adult animals CYP1A protein levels were significantly induced over control levels in a dose-dependent manner from MX 7-8 mg/kg to MX 70-80 mg/kg. No change occurred in CYP3A protein levels, whereas a marked induction of CYP2B protein was seen at the highest MX (70-80 mg/kg) dose.
Fig. 17: Representative Western blots for CYP1A, 2B and 3A protein levels following MX treatment in adult male and female rats. For detection of CYP1A and 3A 60 µg and for CYP2B 90 µg of liver equivalents were used in samples of control and MX exposed animals (MX dosage in mg/kg/day). Positive controls of liver microsomes: A: 3-MC 5.0 µg; B: PCN 12.0 µg; C1: PB 12.5 µg, C2: PB 7.5 µg liver equivalents. Sample probes of liver microsomes: 1 Control male; 2 male MX 0.7-0.8 mg/kg; 3 male MX 7-8 mg/kg; 4 male MX 70-80 mg/kg; 5 Control female; 6 female MX 0.7-0.8 mg/kg; 7 female MX 7-8 mg/kg; 8 female MX 70-80 mg/kg

The second band in CYP2B following MX treatment has not been identified.
<table>
<thead>
<tr>
<th>Dosage (mg/kg/day)</th>
<th>CYP1A</th>
<th>CYP3A</th>
<th>CYP2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>202.3 +/-68.2</td>
<td>913.0 +/-261.2</td>
<td>6.7 +/-4.7</td>
</tr>
<tr>
<td></td>
<td>(3F, 3M)</td>
<td>(4F, 4M)</td>
<td>(3F, 3M)</td>
</tr>
<tr>
<td>MX 0.7-0.8</td>
<td>441.5 +/-168.0</td>
<td>1096 +/-291.1</td>
<td>58.5 +/-24.3</td>
</tr>
<tr>
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<td>(3F, 3M)</td>
<td>(3F, 3M)</td>
<td>(3F, 3M)</td>
</tr>
<tr>
<td>MX 7-8</td>
<td>916.3 +/-228.7*</td>
<td>1050.3 +/-289.8</td>
<td>38.2 +/-17.9</td>
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<tr>
<td></td>
<td>(3F, 3M)</td>
<td>(3F, 3M)</td>
<td>(3F, 3M)</td>
</tr>
<tr>
<td>MX 70-80</td>
<td>1436.5 +/-160.8**</td>
<td>1507.8 +/-256.0</td>
<td>1233.8 +/-304.1***</td>
</tr>
<tr>
<td></td>
<td>(3F, 3M)</td>
<td>(4F, 4M)</td>
<td>(3F, 3M)</td>
</tr>
</tbody>
</table>

Table 8: Semiquantitative determination of CYP1A, CYP2B and CYP3A in adult rats. Means ±S.E.M. of absolute instrument readings. Pooled values of males and females, number of males (M) and females (F) in brackets. Asterisks indicate statistical significance from control compared by two-tailed Wilcoxon rank sum test (***p < 0.001, **p < 0.005, *p < 0.01).

5.4.2 PN 14 male and female offspring

Liver weights (in % of controls) tended to be lower in offspring of dams exposed to MX but the effect was not dose dependent and did not reach statistical significance (Tab. 9).

EROD and MROD activities measured in 14 day old male and female controls were 50% and 20% respectively of adult control values. Offspring of dams treated with different concentrations of MX showed a dose-dependent induction at low maternal dosages between 2-3 mg (1.6 to 1.8-fold of EROD and MROD, respectively) and 7-8 mg MX/kg/day (3.0-fold for EROD and 3.7-fold for MROD (Fig. 18 A,B), i.e. in a similar range as had been observed in adult rats. In the lowest concentration ranges of 0.7-0.8 mg and 0.07-0.08 mg MX/kg/day, EROD was not induced (MROD not analysed). 0.7-0.8 mg MX/kg/day was therefore considered as developmental NOAEL for P450 enzyme induction.
<table>
<thead>
<tr>
<th>Maternal dosage (MX mg/kg/day)</th>
<th>Dosage (MX mg/kg food pellet)</th>
<th>Sex</th>
<th>Liver wt. (g) in % of control</th>
<th>L/BW (%) in % of control</th>
<th>n</th>
<th>Microsomal protein (mg/g liver) in % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>male</td>
<td>100.0 +/-12.3</td>
<td>100.0 +/- 5.8</td>
<td>(39)</td>
<td>100.0 +/-16.2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>female</td>
<td>100.0 +/-14.0</td>
<td>100.0 +/- 6.0</td>
<td>(47)</td>
<td>100.0 +/-18.7</td>
</tr>
<tr>
<td>0.07-0.08</td>
<td>1</td>
<td>male</td>
<td>84.0 +/- 7.1*</td>
<td>96.8 +/- 5.6</td>
<td>(8)</td>
<td>75.8 +/-20.6*</td>
</tr>
<tr>
<td>0.07-0.08</td>
<td>1</td>
<td>female</td>
<td>84.2 +/- 6.6*</td>
<td>94.7 +/- 5.8*</td>
<td>(8)</td>
<td>87.0 +/-13.9*</td>
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<tr>
<td>0.7-0.8</td>
<td>10</td>
<td>male</td>
<td>96.2 +/-21.2</td>
<td>99.3 +/- 6.7</td>
<td>(14)</td>
<td>84.6 +/-16.2*</td>
</tr>
<tr>
<td>0.7-0.8</td>
<td>10</td>
<td>female</td>
<td>99.3 +/-27.5</td>
<td>98.8 +/- 8.0</td>
<td>(13)</td>
<td>91.4 +/-14.3*</td>
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<tr>
<td>2-3</td>
<td>33</td>
<td>male</td>
<td>90.6 +/-18.7</td>
<td>96.6 +/- 5.7</td>
<td>(12)</td>
<td>98.4 +/-10.8</td>
</tr>
<tr>
<td>2-3</td>
<td>33</td>
<td>female</td>
<td>99.5 +/-24.6</td>
<td>99.7 +/- 9.1</td>
<td>(11)</td>
<td>105.7 +/- 6.9</td>
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<tr>
<td>7-8</td>
<td>100</td>
<td>male</td>
<td>95.9 +/-10.3</td>
<td>99.6 +/- 7.3</td>
<td>(31)</td>
<td>90.7 +/-15.8</td>
</tr>
<tr>
<td>7-8</td>
<td>100</td>
<td>female</td>
<td>98.7 +/-13.0</td>
<td>100.6 +/- 5.6</td>
<td>(39)</td>
<td>91.4 +/-20.6</td>
</tr>
<tr>
<td>70-80</td>
<td>1000</td>
<td>male</td>
<td>84.3 +/-14.6</td>
<td>103.1 +/- 9.9</td>
<td>(4)</td>
<td>80.0 +/-17.3</td>
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<tr>
<td>70-80</td>
<td>1000</td>
<td>female</td>
<td>90.9 +/-22.2</td>
<td>100.1 +/- 6.9</td>
<td>(13)</td>
<td>90.4 +/-32.9</td>
</tr>
</tbody>
</table>

Table 9: General hepatic effects of MX treatment of 14 day old animals (PN14)

a Maternal daily MX uptake (female: mean body weight 210-250 g, 18-19 g food pellets/day (Suter-Eichenberger et al., 1998)

b Liver to body weight ratio, with liver weight expressed as a percentage of total body weight

Results are means +/- S.D. of 3-47 animals (3-13 litters)

Asterisk indicates statistically different from control (* p<0.05) using ANOVA followed by Fisher's test

n Number of animals in brackets
Fig. 18A,B: EROD/MROD-activities in liver microsomes of 14 day old male (A) and female (B) offspring of dams exposed for at least 10 weeks before pregnancy, during pregnancy and after birth to different concentrations of MX in chow or to control chow. Maternal dosage in mg/kg/day: 0.07-0.08; 0.7-0.8; 2-3; 7-8; 70-80 (corresponding MX concentration in food pellets see Table 4). Each bar represents the mean ±S.D. of 5-21 animals (3-10 litters).

Absolute control levels for EROD-activity: male 11.7 ±2.9; female 13.4 ±3.8 (pmol/min/mg protein), for MROD-activity: male 6.5 ±1.6; female 6.6 ±1.6 (pmol/min/mg protein). Asterisks indicate statistical significance from control using ANOVA followed by Fisher's test (*p < 0.05).
Prenatal vs postnatal and combined pre-and postnatal induction of EROD-/MROD-activities were compared in 1 and 14 day old offspring of dams exposed to 7-8 mg MX/kg/day (Fig. 19 A,B). In 1 day old pups (GD23 = PNI) of dams exposed before (10 to 15 weeks) and during pregnancy to 7-8 mg MX/kg/day or to control chow (Figure 19 A), EROD-activity (pooled males and females) was induced 3-7-fold, whereas MROD-activity was hardly detectable in MX-exposed and control animals. At gestational day 22, 1 day before birth, EROD-/MROD-activity was not detectable in male and female (pooled) samples of exposed control fetuses (data not shown).

In a cross-fostering study, offspring of exposed (7-8 mg MX/kg/day) dams were given to control dams (prenatal exposure) and control offspring to exposed dams (postnatal exposure) within 12 hours after birth. All pups were killed at the age of 14 days. Prenatally MX exposed pups exhibited control levels of EROD-/MROD-activity at day 14, whereas pups receiving MX from postnatal day 1 on (through milk) exhibited comparable enzyme induction as pups that had been exposed pre-and postnatally to MX until day 14 (Fig. 19 B).

**Fig. 19 A:** Prenatal exposure to MX: EROD/MROD activity
Fig. 19 B: Pre- and/or postnatal exposure to MX: EROD/MROD activity

A: Pups of exposed (MX 7-8 mg/kg/day) and control dams were killed within 12 hours after birth at postnatal day 1 (PNI). MROD-activity at PNI in control pups was not detectable (0.1 ±0.2 pmol/min/mg protein). MROD-activity of MX exposed animals was very low (0.9±0.5 pmol/min/mg protein).

Each bar represents the mean ± S.D. of 3 animal litters (pooled male and female samples). Asterisks indicate statistical significance from control (* p < 0.05) using Mann-Whitney U test.

B: EROD/MROD-activity following pre- or postnatal MX exposure. In a cross-fostering study with maternal MX dose of 7-8 mg/kg/day corresponding to 100 mg/kg MX in food pellets, pups of an exposed dam were given to an untreated dam and from an untreated dam to an exposed dam. 4 groups of treatment: Control animals (Control - Control), animals pre- and postnatally exposed to MX (MX7-8 - MX7-8), animals prenatally exposed to MX (MX7-8 - Control) and animals postnatally exposed to MX (Control - MX7-8). Due to the absence of sex differences in enzyme activities (see Fig. 18 A,B), results are means of male and female samples.

Each bar represents the mean of 8-14 animals (3-5 litters). Asterisks indicate statistical significance from control (p<0.05) using ANOVA followed by Fisher's test.

Microsomal protein levels of CYP1A, 2B and 3A were evaluated by Western blotting in 14 day old offspring of dams exposed to different MX concentrations. Figure 20 and Table 10 show representative Western blots and semiquantitative data (pooled males and females). In developmentally exposed PN14 offspring, MX was also found to be a strong inducer of CYP1A and CYP2B. CYP1A1 protein was induced dose-dependently from a maternal dose of 7-8 mg MX/kg to 70-80 mg MX/kg. The highest dose level was also studied with separate antibodies distinguishing between CYP1A1 and CYP1A2. Both enzyme proteins were increased (Tab. 10). CYP2B was again induced only by the highest MX dose, as in adult rats. In contrast to the adult stage, an induction of CYP3A was seen in PN14 offspring of rats exposed to the highest MX dose (70-80 mg/kg).
Fig. 20: Representative Western blots for CYP1A, 2B and 3A protein levels following MX treatment in PN14 male and female rats. For detection of CYP1A and 3A 60 µg and for CYP2B 100 µg of liver equivalents were used in samples of control and MX exposed animals. Positive controls of liver microsomes: A: 3-MC 3-3.1 µg; B: PCN 7.5 µg; C1: PB 6.3 µg, C2: PB 3.1 µg liver equivalent. Sample probes of liver microsomes: 1 Control male; 2 male MX 0.7-0.8 mg/kg; 3 male MX 7-8 mg/kg; 4 male MX 70-80 mg/kg; 5 Control female; 6 female MX 0.7-0.8 mg/kg; 7 female MX 7-8 mg/kg; 8 female MX 70-80 mg/kg

The second band in CYP2B blots following MX exposure has not been identified.
Table 10: Semiquantitative determination of CYP1A, CYP2B, CYP3A, CYP1A1 and CYP1A2 in PN14 rats. Mean ±S.E.M. of absolute instrument readings. Pooled values of males and females, number of males (M) and females (F) in brackets. Asterisks indicate statistical significance from control compared by two-tailed Wilcoxon rank sum test (***p < 0.001, **p < 0.005, *p < 0.01).

5.5 DISCUSSION

The present investigation demonstrates that for the first time chronic oral MX significantly induces P450 enzymes in adult male and female rats and in their offspring. Offspring displayed inductive patterns of microsomal enzymes different from adult. Adult rats were kept for a minimum of 10 up to 22 weeks on a chow containing different concentrations of MX (1 to 1000 mg MX/kg chow). The lowest MX concentration corresponding to a daily intake of 0.07-0.08 mg MX/kg body weight and day yielded MX concentrations in body fat of up to 0.55 mg MX/kg lipid in 14 day old rat offspring (Suter-Eichenberger et al., 1998). This level is in the upper range of MX
concentrations in human body fat and milk (Rimkus and Wolf, 1993c; Liebl and Ehrenstorfer 1993; Rimkus et al., 1994; Helbich, 1995; Müller et al., 1996). The highest dose used in this study (1000 mg/kg/day) with a daily intake of 70-80 mg/kg initiated increased neonatal mortality (Suter-Eichenberger, thesis) without visibly affecting parent generation adult animals.

5.5.1 Effects on adult animals

Chronic oral MX exposure of adult male and female rats revealed no dose related effects on general hepatic parameters such as absolute and relative liver weights and microsomal protein levels. Preliminary liver histology in animals exposed to the highest MX dose of 70-80 mg/kg/day disclosed no hepatocellular changes (data not shown). Likewise no significant differences were detected between control and MX exposed mice in a chronic oral study of 17 weeks in the 50-100 mg/kg/day dose range (Maekawa et al., 1990). In contrast, MX 100 mg/kg given intraperitoneally for just 5 consecutive days to male Wistar rats, produced a marked increase in relative liver weight and microsomal protein levels (Iwata et al., 1993a). Similarly, a 7 day acute MX administration by oral gavage of 200 mg/kg in mice produced significant general hepatic changes (Lehman-McKeeman et al., 1997b). Thus, acute short term (5-7 days) single high doses of MX appear to lead to significant changes in liver weight parameters, while continuous dietary intake at comparable dose levels over 10 to 17 weeks is not effective in inducing general hepatic changes in mice and rats (Tab. 6).

Exogenous chemicals can induce cytochrome P450 enzymes that metabolize a variety of xenobiotics and endogenous chemicals, in a process often mediated by transcriptional activation of the respective gene or inhibition by specific inhibitors. Exposure to MX can also lead to an induction of liver P450 enzyme activities, as shown after acute high dose exposure (Iwata et al., 1992 and 1993b). EROD activity is considered to be specific for CYP1A1 and MROD for CYP1A2 in rodents (Namkung et al., 1988; Nerurkar et al., 1993). Under chronic MX exposure (7-8 mg MX/kg/day) MROD-activity levels in adult male and female rats were increased 4-fold and EROD-activity 2.5-fold over control values (Fig. 15). This induction of CYP1A1 and 1A2 is comparable to values reached with 3 to 5 times higher acute doses of the chemical. Chronic oral exposure in the high dose range (70-80 mg MX/kg/day) increased EROD
activity to 3 times higher values than observed following 7-8 mg MX/kg/day (data not shown).

While CYP1A proteins increased in line with the induction of EROD and MROD enzyme activities, only recently Lehman-McKeeman et al. (1997a and 1997b) reported on a 25-fold increase in CYP2B protein levels after an acute 7 day 200 mg/kg dose of MX without a concomitant increase in the CYP2B specific PROD. A single high dose of 200 mg/kg MX also increased CYP2BmRNA to a maximal level, with a time course similar to that seen after PB, but enzyme activity was inhibited. In our chronic oral MX exposure study at the 7-8 mg and 70-80 mg level, MX also dose dependently induced CYP2B enzyme protein, whereas PROD activity was hardly increased. The most specific inhibitors of cytochrome P450 enzymes are mechanism based inactivators. Such chemicals are substrates for the target enzyme which are metabolized to intermediates that inactivate the enzyme. The lack of functional CYP2B activity despite induced enzyme protein suggested some type of inhibition of its catalytic function (Lehman-McKeeman, 1997c). The ability of antibiotic treatment to eliminate the enzyme inhibition implicated amine metabolites of MX formed by nitroreduction in anaerobic intestinal flora as a basis of the inhibitory effect (Rowland et al., 1985 and 1986). The amine metabolite p-NH2-MX was found to be a mechanism-based inactivator of PROD activity (Lehman-McKeeman et al., 1997c). The same metabolite has also been identified as a main metabolite of MX in rats (Minegishi et al., 1991). MX has been classified as a PB-like inducer, because the MX induced altered enzyme protein pattern is consistent with the pleiotropic effects of PB on mouse cytochrome P450 enzymes. The PB-type hepatic pleiotropic response results in a CYP2B mediated enzyme activity and induction of other forms of cytochrome P450 like CYP1A and CYP3A (Lubet et al., 1990 and 1992). Recently, a 17bp sequence in the 5'portion of the PB inducible CYP gene has been identified that interacts with and binds to a PB inducible/activated protein in the nuclei of liver cells (He and Fulco, 1991). However, the mechanism by which CYP2B induction occurs, has not been resolved. The fact that in our chronic MX exposure study induction of CYP1A enzymes and proteins occurred at lower MX concentrations (MX 7-8 mg/kg) (Fig. 15) than induction of CYP2B (70-80 mg MX/kg/day) (Fig. 17) might indicate that CYP1A induction should not be viewed only as part of a pleiotropic response but might result from multiple interactions of MX and or MX metabolites with diverse CYP450 enzyme families.
Recovery of induced enzyme (CYP1A1 and CYP1A2) activities, occurred already at two weeks after cessation of chronic MX (7-8 mg/kg/day) exposure in adult rats of both sexes of (Fig. 16 A,B). CYP1A protein values reached control values within two weeks in male rats, while protein levels in the female decreased significantly over a two weeks period but reached control levels only between 2 and 8 weeks after cessation of MX exposure. This may be linked with the 4-8 times higher MX load in fat and organs of females (Suter-Eichenberger et al., 1998).

5.5.2 Developmental exposure to MX

Developmental induction of P450 genes has been described for the mouse embryo (Giachelli and Omiecinski, 1987) and, following 3-MC treatment, in near term rat fetuses (Crested et al., 1986). In liver of fetuses of dams exposed to 7-8 mg MX/kg/day, no enzyme activities were detected one day before birth. Maternal MX concentrations resulting from low dose exposure during pregnancy might not have attained levels sufficient for transplacental enzyme induction. However, both EROD and MROD were significantly induced in neonates (PN 1) by maternal exposure to 7-8 mg MX/kg/day (Fig. 19 A). At this stage, EROD activity predominates, with a 3-fold induction. In the case of MROD, no constitutive activity was present at birth, but significant, though very low activity was detected following MX exposure. During postnatal ontogeny, CYP1A1 activity (EROD) levels increased gradually, whereas CYP1A2 (MROD activity) was still significantly lower than CYP1A1 (Fig. 19 B). During this period, exposure to MX occurs via milk (Suter-Eichenberger et al., 1998). The data on neonates and the cross-fostering data on PN14 indicate that both pre- as well as postnatal exposure are effective in inducing CYP1A1 and CYP1A2; the magnitude of induction of the two enzymes through milk during the postnatal period appeared to be similar to the effect seen after combined pre- and postnatal exposure (Fig. 19 B). The quantitative difference between the two enzyme activities is reminiscent of observations by Crested et al. (1986) who reported that CYP1A1 protein reached maximal levels in newborns following 3-MC injection, whereas CYP1A2 became detectable only at the age of two weeks, but in the case of MX, CYP1A2 (MROD) appears to be inducible perinatally.

The P14 stage was studied with an extended dose-range. This analysis revealed that EROD as well as MROD activity can be significantly induced in offspring by the low
maternal dose of 2-3 mg MX/kg/day (1.7 and 1.9-fold, respectively). A 3.0 and 3.7-fold increase over control levels of EROD and MROD, respectively, was observed at this age following maternal exposure to 7-8 mg MX/kg/day (Fig. 18 A,B). Thus, the magnitude of induction of EROD is similar to that observed in neonates.

While fetal liver has been shown to be refractive to PB induction (Guenthner and Mannering, 1976), CYP2B protein was induced by MX at PN 14, consistent with results obtained with other compounds by Cresteil et al. (1986). When compared to the inductive effect of the model inducer PB, the data point to MX as a strong CYP2B inducer during early postnatal development (Fig. 20).

In contrast to the induction pattern in the parent generation, 14 day old rats exposed to the highest MX concentration (maternal dose: 70-80 mg MX/kg) showed a marked increase in CYP3A protein (Fig. 20). CYP3A, a member of the steroid-inducible cytochrome P450 subfamily (Cooper et al., 1993), can be induced by dexamethasone, PCN, PB and other chemicals (Arlotto et al., 1987). Corticosterone secretion by fetal adrenals appear to coincide with the development of enzyme activities, and the perinatal development of this enzyme appears to require corticosterone in vivo. Glucocorticoids in turn may cause precocious development of hepatic cytochrome P450 in neonatal rats that is far more responsive than the adult (Leakey and Fouts, 1979). The effectiveness of MX on CYP3A in the developing animal might possibly be influenced by the sensitivity to glucocorticoids. MX might therefore in addition act indirectly through increased endogenous glucocorticoid levels on developmental regulation of CYP3A.

Catalytic activities of CYP1A tend to result in adverse health outcomes because these enzymes can catalyze the insertion of oxygen atoms into substrates at positions sterically hindered from interaction with phase II enzymes, i.e., any reactive metabolites produced by CYP1A may be especially resistant to detoxification (Watkins, 1990). But production of harmful metabolites is not limited to the CYP1A family of enzymes. CYP3A are capable of activating aflatoxins and mycotoxins to carcinogenic metabolites (Watkins, 1990; Gonzales, 1989). MX has been identified as an inducer of toxifying enzymes metabolizing the pregenotoxicants benzo[a]pyrene and aminoanthracene. Thus synergistic effects of the enzyme inducer MX and pregenotoxicants seem possible (Mersch-Sundermann et al., 1996a).

In conclusion, chronic oral musk xylene affects cytochrome P450 microsomal enzymes in adult male and female LE rats at fat tissue levels exceeding human fat levels by a
factor of about 85 (males) to 400 (females) (Suter-Eichenberger et al., 1998). The developing animal is sensitive at an early age. It reacts to MX exposure with an induction of CYP450 enzymes whose pattern differs in part from the adult one. MX induced functional changes resulting from developmental exposure may not only be different from adult, but may possibly also be effective within a shorter time frame, i.e. within five weeks (3 weeks before and 2 weeks after birth) and at levels of exposure that are only about 50-fold higher than human fat levels. The LOAEL (lowest observed adverse effect level) of 2-3 mg MX/kg/day and a NOAEL (none observed adverse effect level) of 0.7-0.8 mg MX/kg/day for developmental toxicity of MX resulting from this study is two orders of magnitude lower than a recently published developmental NOAEL (Ford, 1998).

5.6 ACKNOWLEDGEMENTS

The study was supported by a grant of the Bundesamt für Umwelt, Wald und Landschaft (BUWAL). We thank Dr. Felix Waechter and Monique Simmonet, Novartis AG, Basel, Switzerland, Dr. Dietmar Benke and Claudia Michel, Zurich, for assistance in the analysis of Western blots. Our thanks also go to Vreni Haller and to Annette Jaccard for their technical assistance.
6 REPRODUCTIVE TOXICOLOGY

6.1 INTRODUCTION

"The rats at birth are bright red, ugly and helpless little creatures" (Small, 1899), but the physical and behavioral development during the first weeks of their lives is spectacular. These developmental processes can be influenced by environmental conditions but also by a multiplicity of adverse influences occurring during prenatal life. Rats have the advantage that their rather short life span of approximately two years permits the complete study of developmental effects over the entire range from conception to death. During the first three weeks of postnatal life the rat brain is most vulnerable because it is in the period of fastest growth (Davison and Dobbing, 1968). Therefore, this is the most interesting period for the study of physical and behavioral development.

Rats weigh about 5 g at birth (postnatal day 1, PN1). They double their birth weight within four days and weigh about five to nine times their birth weight at postnatal day 21 (PN21). The normal growth rate is influenced by the nutrition of the mother during gestation and lactation (Smart and Dobbing, 1971a and 1971b), by maternal behavior and by the litter size (Seitz, 1954). Pups of smaller litter size weigh significantly more than pups of larger litters due to better maternal care and larger quantities of milk available. As behavior is depending on physical development of the animal, measurement of physical signs is important. Table 11 shows the timing of physical development of Sprague-Dawley rats (SIV-strain). Timing may vary slightly between different strains and sexes (Frank, 1976).
Table 11: Physical development of Sprague-Dawley rats (PN1=day of birth) (Alder, 1983)

It is useless offering pups inadequate stimuli prior to normal time of development, which they cannot perceive, because their corresponding sensory-organs are not functioning yet, or to offer a stimulus which they perceive but are not able to react to because their motor system is not sufficiently developed.

The only response of a pup to any test situation which can be observed is a movement, either an involuntary movement as a reflex, or a voluntary movement towards or away from the offered stimulus. Test procedures used in behavioral teratology are divided into two main groups depending on the period of life of the offspring when measurements are done. Figure 21 contains an overview of various procedures which can be applied in behavioral teratology.

Postnatal physical development

<table>
<thead>
<tr>
<th>1st period</th>
<th>Function of reflexes</th>
<th>sensory development</th>
<th>spontaneous movements</th>
<th>neuromuscular maturation</th>
<th>learning and memory (observations)</th>
<th>ultrasonic vocalization</th>
<th>birth to weaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd period</td>
<td>Function of reflexes</td>
<td>function of sensory organs</td>
<td>spontaneous movements</td>
<td>neuromuscular measurements</td>
<td>operant conditioning (tests)</td>
<td>social behavior</td>
<td>weaning through adulthood</td>
</tr>
<tr>
<td>non-behavioral</td>
<td>behavioral</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Fig. 21: Detection of prenatal CNS-damage with postnatal test procedures (Alder, 1983)
The aim of these postnatal tests was the detection of a possible defect due to prenatal MX exposure. It is known that changes in early behavior often move along with changes in physical development. A reduction in body weight and growth are the earliest indicators of some toxic action by the chemical (Alder, 1983).

6.2 MATERIAL AND METHODS

6.2.1 Animals
Female and male Long Evans rats (Møllegaard Breeding & Research Centre, Denmark) were bred in our colony. Groups of 3-4 animals of the same sex were housed in humidity and temperature controlled rooms (relative humidity 50%; 22 ±1°C) with a light/dark cycle (light on between 02.00-16.00) and allowed free access to food and water.

6.2.2 Food pellets containing musk xylene
The preparation of food pellets with different concentrations of musk xylene was done by Provimi Kliba AG, Kaiseraugst, Switzerland. Different amounts of musk xylene were dissolved in soya oil (Morga) to achieve the following MX concentrations in chow: 100 mg/kg and 1000 mg/kg. The control food pellets consisted of the same matrix (Provimi Kliba no. 343, including 1% of soya oil). Different charges of food pellets containing different concentrations of MX were analyzed. The concentrations corresponded to 90-100% of the nominal value (see 4.3.2).

6.2.3 Chemicals
Musk xylene (1-tert.-butyl-3,5-dimethyl-2,4,6-trinitro-benzol, MX) kindly provided by Ch.Grundschober, IFRA, Geneva, Switzerland was essentially 100% pure as determined by gas chromatography equipped with a $^{63}$Ni electron capture detector (ECD) (Suter-Eichenberger et al., 1998).
6.2.4 **MX-exposure schedule**

5-6 week old female and male rats were fed for a minimum of 10 weeks with either control pellets or food pellets containing 100 mg MX/kg or 1000 mg MX/kg food pellets (corresponding to a daily intake of 7-8 mg MX/kg or 70-80 mg MX/kg body weight) according to OECD Guideline 415 for testing of chemicals (one generation reproduction toxicity study). Receptive females were mated overnight with exposed male rats matched for MX diet and age. 2-3 sperm positive dams were kept together in cages and isolated one day before parturition at gestational day 22 (GD22). The day of birth (GD23) was defined as postnatal day 1 (PN1). Females were exposed to MX before and during pregnancy and lactation.

6.2.5 **Litter size**

To allow a more uniform litter size of all treatment groups, litters were adjusted to 8-10 animals by adding animals from other litters or removing animals randomly at postnatal day 2 (PN2).

6.2.6 **Physical development: Body weight**

Body weight was determined in all pups of each litter and treatment group on postnatal days 2, 4, 6, 9 and 14.

6.2.7 **Behavioral toxicology: Righting reflex**

Righting reflex was measured in all pups of all treatment groups on postnatal days 2 and 4. When an animal is placed on its back on a horizontal surface, it immediately turns over to rest in the normal position with all four feet on the ground. Surface righting is timed using a stopwatch from the moment the animal is released until it resumes a fully prone position. The observation period amounted to a maximum of 1 minute. The experiment was carried out in an empty room with standardized light intensity and a preheated table surface. All pups of one dam were removed together and adjusted to the room for several minutes before testing.
6.2.8 *Indices of fertility and pregnancy*
Parturition index (number of parturitions divided by the number of females confirmed pregnant) and gestation index (number of females with pups born alive divided by the number of females confirmed pregnant) of dams were determined. Body weight gain during pregnancy and body weight loss after parturition were obtained by measuring body weight of dams on gestational days 1 and 22 and on postnatal day 2.

6.2.9 *Indices of reproductive function*
To determine litter size, live birth index (number of pups born alive PN1 divided by the number of pups born), viability index (number of pups alive PN2 divided by the number of pups born alive) and 14-day survival index (number of pups alive PN14 divided by the number of pups kept at PN2) were evaluated, alive and dead offspring were counted and examined for gross malformations within 12 hours after birth. Sex in offspring was determined on postnatal day 2 and the cages inspected for dead or missing pups on postnatal days 2, 4, 6, 9 and 14.

6.2.10 *Statistical analysis*
Data were analyzed for statistical significance by analysis of variance followed by Scheffe's post-comparison test or Student's t-test. * p < 0.05 and ** p < 0.01 were considered as statistically significant.

6.3 **RESULTS**
6.3.1 *Indices of fertility and pregnancy*
As shown in Table 12 treating dams with 70-80 mg MX/kg/day (1000 mg MX/kg) had a significant effect on body weight gain during pregnancy as well as on body weight loss after parturition compared to the control group. Body weight and age of dams were slightly increased at gestational day 1 (GD1). Parturition and gestation indices however, remained uninfluenced, whereas dams treated with 7-8 mg MX/kg/day (100 mg MX/kg) showed higher values than the appropriate control group.
Table 12: Indices of fertility and pregnancy of dams exposed to MX before, during and after pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Maternal MX dose (mg/kg)</th>
<th>Maternal MX dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7-8</td>
</tr>
<tr>
<td>Parturition index (%)</td>
<td>87.0 (n=23)</td>
<td>94.4 (n=18)</td>
</tr>
<tr>
<td>Gestation index (%)</td>
<td>82.6 (n=23)</td>
<td>88.9 (n=18)</td>
</tr>
<tr>
<td>Body weight GD1 (g)</td>
<td>262.9±18.6 (n=23)</td>
<td>267.1±17.8 (n=18)</td>
</tr>
<tr>
<td>Age of dams (weeks)</td>
<td>19.1±2.9 (n=23)</td>
<td>20.8±3.9 (n=18)</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>117.6±17.4 (n=18)</td>
<td>115±13.7 (n=13)</td>
</tr>
<tr>
<td>Body weight loss (g)</td>
<td>65.4±13.7 (n=12)</td>
<td>67.9±10.8 (n=9)</td>
</tr>
</tbody>
</table>

Table 12: Indices of fertility and pregnancy of dams exposed to MX before, during and after pregnancy

- Dams were fed without or with 7-8 respectively 70-80 mg MX kg/day
- Parturition index = (number of parturitions / number of females confirmed pregnant) X 100
- Gestation index = (number of females with pups born alive / number of females confirmed pregnant) X 100
- Body weight of dams on GD1
- Age of dams on mating date
- Body weight gain of dams during pregnancy from GD1 to GD22
- Body weight loss of dams during parturition from GD22 to PN2
- Values are means ±S.D., number of animals in brackets
- Asterisks indicate statistical significance from control compared by Student's t-test
  * p < 0.05; ** p < 0.01

6.3.2 Reproductive outcomes

MX treatment had no effect on live birth index (Tab. 13). Viability index was affected by MX at the high concentration (72 % compared to 92 % of control group), 14-day survival index was decreased in both treatment groups, 13 % at the lower dose (tendency not significant) and 48 % at the higher MX dose (significant decreased). Litter size was also significantly decreased at the high MX maternal dose, whereas sex distribution of offspring remained unaffected in all groups at postnatal day 2. No gross external malformations were found in male and female offspring.
<table>
<thead>
<tr>
<th>Maternal MX dose (mg/kg)</th>
<th>Maternal MX dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7-8</td>
</tr>
<tr>
<td>0</td>
<td>70-80</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Live birth index (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>99±4</th>
<th>96±10</th>
<th>98±6</th>
<th>98±4</th>
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<tbody>
<tr>
<td>(n=18)</td>
<td>(n=15)</td>
<td>(n=19)</td>
<td>(n=19)</td>
<td></td>
</tr>
<tr>
<td>Viability index (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90±13</td>
<td>85±20</td>
<td>93±9</td>
<td>73±25**</td>
</tr>
<tr>
<td>(n=18)</td>
<td>(n=15)</td>
<td>(n=19)</td>
<td>(n=19)</td>
<td></td>
</tr>
<tr>
<td>14-day survival index (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>81±23</td>
<td>68±43</td>
<td>87±23</td>
<td>39±44**</td>
</tr>
<tr>
<td>(n=17)</td>
<td>(n=15)</td>
<td>(n=19)</td>
<td>(n=19)</td>
<td></td>
</tr>
<tr>
<td>Litter size&lt;sup&gt;e,g&lt;/sup&gt;</td>
<td>9.9±2.8</td>
<td>9.7±2.2</td>
<td>10.9±2.3</td>
<td>8.1±2.0**</td>
</tr>
<tr>
<td>(n=19)</td>
<td>(n=16)</td>
<td>(n=19)</td>
<td>(n=19)</td>
<td></td>
</tr>
<tr>
<td>Male pups on PN2 (%)&lt;sup&gt;f,g&lt;/sup&gt;</td>
<td>43.1±22.0</td>
<td>55.9±17.2</td>
<td>51.3±15.5</td>
<td>47.8±20.5</td>
</tr>
<tr>
<td>(n=19)</td>
<td>(n=15)</td>
<td>(n=19)</td>
<td>(n=18)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 13: Indices of reproductive function of offspring from dams exposed to MX before, during and after pregnancy<sup>a</sup>**

<sup>a</sup> Dams were fed without or with 7-8 respectively 70-80 mg MX/kg/day

<sup>b</sup> Live birth index = (number of pups born alive PN1 / number of pups born) X 100

<sup>c</sup> Viability index = (number of pups alive PN2 / number of pups born alive) X 100

<sup>d</sup> 14-day survival index = (number of pups alive PN14 / number of pups kept at PN2) X 100

<sup>e</sup> Litter size includes pups born alive and / or dead on PN1

<sup>f</sup> Male pups in % of total litter size on PN2

<sup>g</sup> Values are means ±S.D., number of litters in brackets

Asterisk indicates statistical significance from control compared by Student’s t-test

**<sup>**p < 0.01**</sup>

6.3.3 Postnatal growth and behavioral toxicology

Effects of in utero and lactational MX exposure on weight gain in male and female offspring are shown in Figure 22. From days 1 through 14, the high MX dose decreased mean body weight by 12.7 to 37.6 % as compared to control group. Male pups seemed to be more affected than female pups.
Body weight increase in male offspring

![Graph showing body weight increase in male offspring](image)

Body weight increase in female offspring

![Graph showing body weight increase in female offspring](image)

**Fig. 22 A,B:** Body weight increase in male and female offspring of dams treated without or with 7-8 respectively 70-80 mg MX/kg/day

Righting reflex, present on the first day of life, was measured in male and female offspring on postnatal day 2 and 4 (Fig. 23). Female offspring treated with 7-8 mg MX/kg/day showed a significant increase (163 %) in reflex behavior on postnatal day 2. 2 day old male offspring in both treatment groups had increased time latencies that were not statistically significant.
6.4 DISCUSSION

6.4.1 Indices of fertility and pregnancy

Treatment of dams with 70-80 mg MX/kg/day had a significant effect on body weight gain during pregnancy as well as on body weight loss after parturition. Factors influencing body weight before and after birth are food/water intake before and during pregnancy, health condition of the pregnant dam and litter size. Both, the decreased weight gain and weight loss of the dams are clear signs of maternal toxicity at this MX dose, although the treated dams did not seem to suffer from health problems, as neither parturition nor gestation indices were altered. Altered developmental outcome observed in the offspring might therefore at least in part be due to maternal toxicity. At 70-80 mg MX/kg/day, body weight at gestational day 1 was slightly increased as compared to the control group. Litter size was significantly decreased in the higher MX treated dams at postnatal day 1, a fact that might explain the lower body weight gain during pregnancy and the observed lower weight loss at parturition in this treatment group. Whether the decreased litter size is the result of an increased prenatal resorption rate of embryos or is due to a decline in fecundity of the dam, is not known.
Noteworthy is the significant difference of parturition and gestation indices between the two control groups, that might be connected to seasonal breeding problems or else be of unknown origin such as stress conditions due to changes in animal caretakers. According to our experience pregnant dams are very sensitive to stress.

### 6.4.2 Reproductive outcome

MX treatment had a significant effect on reproductive outcome. Live birth index remained uninfluenced, but viability index was significantly decreased in the highest dosed MX group, pointing to a marked developmental impact during early period. From postnatal day 2 to 14 offspring survival was significantly decreased in the highest treatment group, while treatment with the lower dose of MX showed a tendency of decreased survival without reaching statistical significance. The cause of death at present remains unknown. Increased mortality followed significantly diminished body weight increase. Preliminary histological data of a few animals showed fat tissue atrophy and some liver- (subacute congested liver), kidney- (diapedetic bleeding of kidney), heart- (cardiac insufficiency) and lung- (dilatation of pulmonary alveoli) alterations. To evaluate the possible cause of increased mortality, profound histological, morphological and functional analysis are needed to be done. A cross-fostering procedure where control mothers would nurse the pups of the treated mothers and vice versa might be valuable in elucidating possible causes of postnatal mortality increase. This would help to identify the most sensitive pre- or postnatal period to developmental impact by MX. Additionally it would be important to study more thoroughly maternal toxicity or neurotoxicity. Our observations revealed that treated dams often needed more time to retrieve their offspring. They more often dispersed their pups over the whole cage, leaving them isolated for a longer time period than control dams. Studying maternal behavior would help to elucidate whether maternal toxicity, or milk toxicity (MX and metabolites) would be causative factor(s) to increased pup mortality. In addition, the pup’s ability to suck should be monitored. An efficient milk production was guaranteed by maintaining a litter size of 8 to 10 animals during lactation period. In many cases we had to adjust litter size in order to guarantee sufficient milk production. This points to a very important problem in developmental toxicology, namely the possibility of maternally mediated effects on the conceptus (Hood, 1989). Khera (1984
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and 1985) proposed that increased resorption rates, low fetal body weight and a number of common malformations and variations in rodents and rabbits occurred as a secondary result of maternal toxicity and not via direct effects on the offspring. The defects were rarely seen at doses that were apparently not maternally toxic, were species-specific in some cases and often did not appear to be dose-related. Khera’s proposal has been criticized by Kavlock et al. (1985) and other groups as showing a possible association but not proving a cause-and-effect relationship. Therefore it was suggested that when nonspecific endpoints (such as fetal weight) are altered, the fetus is likely to have been affected directly. But this is not a definitive statement. Additionally species or strain differences can play a role in the outcome as well (Hood, 1989). Our study revealed a dose-dependent impairment in offspring without any apparently visible maternal toxicity.

6.4.3 Postnatal growth and behavioral toxicology

As mentioned before, body weight increase was significantly diminished after treatment with 70-80 mg MX/kg/day until postnatal day 14. It is unknown whether this body weight reduction would be reversible or would remain until adulthood. For that reason body weight measurement should continue for a longer time period. Righting reflex determination present on the first day of life revealed a heterogeneous picture after MX treatment. In general, no significant changes were obtained. But there is a general problem in behavioral tests in immature animals. Measured changes in pups are often subtle and subject to a great variation dependent on a variety of environmental influences. Standardizations of such tests, including sometimes also cross-fostering procedure, are mandatory (Alder, 1983; Zbinden, 1981).

The question arises whether it is better to perform just a few significant tests on certain days, or a whole test battery. Since it is not known which physiological and behavioral functions are affected by a given chemical, it is certainly more informative to conduct a large variety of test procedures. The performance of a number of tests may itself be a stressful procedure for a very young animal. Dams and pups may be stressed by permanent disturbances, leading to changes in nursing and fostering. Beside testing for righting reflex, other behavioral tests were realized (homing test, open field, ultrasound) (Altman and Sudarshan, 1975; Alder et al., 1977; Alder, 1983) but have not been
evaluated, since either methodical problems arised or the data sets obtained were incomplete.

For the first time we demonstrated that chronic oral MX treatment had significant effects on reproductive outcome at a dosage where adult first generation animals remained without any visible signs of toxicity, unless the effects on pregnant dams. 70-80 mg MX/kg/day initiated increased neonatal mortality followed by a significant decrease in body weight. Further studies must be done to evaluate possible mechanisms in developmental impact by MX in offspring of treated dams, taking also into account maternal health status and species and strain specificities.
7 GENERAL DISCUSSION

It is becoming increasingly apparent that the toxicity of a foreign compound and its mode of expression are dependent on many variables. Apart from large variations in susceptibility between species related to differences in the metabolism and disposition of a compound, within the same species many factors may be involved. The genetic constitution of a particular organism is known to be a major factor in conferring susceptibility to toxicity in some cases. The age of the animal and certain characteristics of its organ system may also be important internal factors. External factors such as chemical characteristics, the dose of the compound or the manner in which it is given, the diet of the animal are also important for the eventual toxic response. There are also many ways in which an organism may respond to a toxic compound. Toxic responses may be the all-or-none type such as the death of the organism (LD$_{50}$), or they may be graded responses and manifest themselves by various ways like pathological changes (development of a tumour, destruction of tissue), biochemical changes (enzyme induction, enzyme inhibition), physiological changes (blood pressure, temperature) or changes in normal status (body weight, food and water intake, organ weight) (Timbrell, 1991).

In the same way toxicity of MX depends on many variables. Factors which influence toxicity in different species especially in rat, mouse and human will be discussed.

7.1 FACTORS INFLUENCING ABSORPTION

Due to its octanol/water partition coefficient (log P, 5.20; Helbling et al., 1994), MX has a lipophilic character. From the observed pattern of MX distribution in organs of mice and rats (Kokot-Helbling et al., 1995c; Suter-Eichenberger et al., 1998) and its chemical characteristics (lipophilic, non-ionized ) it can be concluded that MX distributes passively through membranes across a concentration gradient. The site of entry of a compound may be also important in the final toxic effect. In our studies we gave MX orally to rats. Compounds like MX are absorbed by passive diffusion anywhere in the gastrointestinal tract in contrast to ionizable substances. Their absorption rate depends partially on the pH of the gastrointestinal tract which is known to vary between species.
No data on absorption rates and bioavailability of MX in different species are available, but due to its physico-chemical properties there should be no considerable differences, concerning the parent compound. In contrast, marked differences will exist in the absorption of metabolites formed by nitro reductases in anaerobic intestinal bacteria, due to known species differences in the quality and quantity of intestinal bacteria (Rowland, 1986, see 7.3). Gastrointestinal absorption is influenced by water solubility, particle size, presence of food or gut motility. Administering MX, a nearly water unsoluble substance in food pellets containing 1 % of soya oil, will facilitate absorption by dissolving it in the food. Absorption from the gastrointestinal tract is of particular importance because compounds so absorbed are transported via the hepatic-portal vascular system directly to the liver (Timbrell, 1991). Extensive metabolism in the liver may alter the structure of the compound, making it more or less toxic. But also the enzymatic potency of gut bacteria may contribute to its toxicity (see 7.3).

In reality, MX contamination in man is mainly the result of absorption via skin (see 3.5.2). It is also absorbed by the gastrointestinal tract (fish, food etc.) and by the lungs (inhalation). Absorption of MX has been assessed in human, rat and hairless guinea pig skin using the in vitro SAM system (Skin Absorption Model) (Bronaugh and Stewart, 1985 and 1986) and in vivo studies (Kokot-Helbling, 1995b; Hood et al., 1996; Ford, 1998). There appears to be relatively good agreement between the extent of absorption observed in vitro and that observed in vivo in the same species, if omitting MX amounts in the so-called „skin reservoir“. The skin is known to exhibit reservoir characteristics, whereby compounds are rapidly absorbed from the surface into the tissue but slowly released into the systemic circulation (Vickers, 1963). Thus a depot of the chemical is accumulating in the skin, resulting in continued local exposure even after skin contact has ceased. To quantify the absorption of MX from human skin into the receptor fluid beyond 24 hours (in vitro), studies were continued for an additional 6 days. The fact that 24-hours skin levels (78 % of total in skin amount was found in the stratum corneum) were reduced by nearly 75 % at the end of day 7 suggests that most of the absorbed MX in skin at 24 hours may, within a week, be systemically absorbed in vivo (Hood et al., 1996). Also other authors support the importance of the skin reservoir as a main source for systemic absorption. A linear relationship has been shown between the amount of a chemical present in rat stratum corneum at 30 minutes after application in vivo, and the total amount absorbed (excreted and in epidermis and dermis) in 4 days (Rougier et al.,
indicating that measurement of the amount of chemical in the stratum corneum allows the prediction of total systemic absorption (Hotchkiss, 1995). As shown by Hood et al. (1996), absorption through rat skin in vitro (50 %) was about two times higher than through human skin in vitro (22 %). Other authors have also reported that in general human skin is less permeable to xenobiotics than is the skin of most laboratory animals (Bartek et al., 1972). These marked differences in absorption suggest that care should be taken relying on animal data to provide an accurate model for human absorption. The extent of percutaneous absorption depends upon a number of factors concerning the physico-chemical nature of the compound, the vehicle of application, the surface area and region of application, the time of skin contact, occlusion of the skin surface, the extent of skin hydratation, skin temperature and the degree of skin barrier compromisation by disease or physical damage and the dose and concentration applied. For many compounds absorption linearly increases up to a maximum level, but is non-linear thereafter. Hence the relationship between the applied dose and the extent of absorption is both compound and species specific. On the other hand care must be taken by extrapolating data without knowledge of dose effects (Hotchkiss, 1998). In the same way it should be noted that the epidermis has significant metabolic activity. So substances can be metabolised as they are absorbed and before they reach the peripheral circulation (see 7.3).

In general, the dose of a compound, the dose interval and the manner in which it is given lead to different toxic responses dependent on the tissue concentration. In our studies, chronic oral MX exposure of adult male and female rats revealed no dose related effects on general hepatic parameters such as absolute and relative liver weights and microsomal protein levels. Preliminary liver histology in animals exposed to the highest MX dose of 70-80 mg/kg/day disclosed no hepatocellular changes. In contrast, MX 100 mg/kg given intraperitoneally for just 5 consecutive days to male adult Wistar rats, produced a marked increase in relative liver weight and microsomal protein levels (Iwata et al., 1993a). Also mice showed an analogue change in general hepatic parameters (Maekawa et al., 1990; Lehman-McKeeman et al., 1997b). Thus, acute short term single high doses of MX appear to lead to significant changes in liver weight parameters, while continuous dietary intake at comparable dose levels over 10 to 17 weeks up to 70-80 mg MX/kg/day is not effective in inducing general hepatic changes in mice and rats. These data reveal, that environmental and human risk assessment should be
rather based on results obtained by long term low dose studies than on acute short term single high dose studies. Additional differences in the site of absorption may be neglected by comparing internal tissue concentrations.

7.2 FACTORS INFLUENCING DISTRIBUTION

The distribution may vary between species because of differences in factors such as proportion and distribution of body fat, rates of metabolism and excretion and hence elimination and the presence of specific uptake systems in organs (Timbrell, 1991). Kokot-Helbling (1995c) found that in BALB/c mice the distribution of MX in the investigated tissues (muscle, adipose tissue, brain, kidney, liver) was proportional to their lipid content after a single oral application of MX (170 mg/kg body weight). In rats treated with the same dose this relation was also found for blood, liver, and muscle, while the content in the kidney was slightly elevated. The adipose tissue contained an unproportional, high level of MX. This was explained with the model of the adipose tissue as a "deep compartment", from where MX is more slowly deliberated than it is metabolised in the liver. Compared with mice the adipose tissue of rats is poorly supplied with blood. This situation of a "deep compartment" was assumed to be only relevant for single applications, as after repeated applications MX would not be transferred from the blood into the adipose tissue against a concentration gradient but into the lipids of other tissues.

We studied the distribution of MX in adult female and male rats fed with MX (7-8 mg/kg/day) during 10 weeks (see chapter 4). It revealed that the adipose tissue contained even after chronic exposure to MX an over proportional level of MX. Additionally, it should be noted that significant concentrations were also present in the ovary and in the adrenal gland of both sexes. Like MX, chlorphentermine is found to accumulate also in fatty tissues and the adrenal gland after multiple doses. The accumulation of chlorphentermine resulted in a disturbance of lipid metabolism and a dramatic accumulation of phospholipids, especially in the adrenals (Timbrell, 1991). Preliminary data from our studies showed, that next to over proportional levels of MX in the adrenal gland of adult rats of both sexes, HDL concentrations measured in blood samples were also significantly increased in both sexes, pointing to a possible disturbance of lipid metabolism similar to chlorphentermine. The relative tissue
distribution appeared to be similar in adult male and female rats, however, absolute tissue levels of females exceeded up to 6.8 times those of males in all tissue studied. Immature rats (PN1 liver, PN14 adipose tissue) in contrast showed no sex difference in MX tissue concentrations, but a dose dependent accumulation, with relatively higher concentrations in the upper dose range, suggesting an enhanced accumulation at higher doses. In man, there are indications that MX levels in adipose tissue samples from women tend to be somewhat higher than those of men (Rimkus et al., 1994), while no gender differences were observed in human blood levels after a single oral dose of MX (Kokot-Helbling, 1995a). Also other authors found no correlation between sex and MX concentrations in human blood samples in a biological monitoring of a general population from Germany (Käfferlein et al., 1997; Angerer and Käfferlein, 1997). Thus, differences in MX accumulation seems to be species dependent.

In humans the elimination half-life amounted to be about 60-94 days, about 50 times longer than the half-life in rats (1.6 days) (Kokot-Helbling, 1995a and 1995c), which has to be taken into consideration. The facts that only the free compound will distribute into tissues and the high affinity of MX for proteins in humans may be responsible for the accumulation of MX in target organs and the restricted distribution in humans. The half-life may be also extended. In rats, no data are available concerning MX binding to plasma proteins.

7.3 FACTORS INFLUENCING METABOLISM

It is well-established that nitroreduction to primary amines is a major metabolic pathway for a variety of nitroaromatic compounds, and the role of anaerobic intestinal bacteria in this reductive metabolism is recognized (Rickert, 1987). Intestinal bacteria play an obligatory role in the metabolic activation and genetic toxicity of the hepatocarcinogen, 2,6-dinitrotoluene (2,6-DNT) (Mirsalis et al., 1982; Rickert et al., 1984) and the hepatic genotoxicity of 2-nitrotoluene (2-NT) (Doolittle et al., 1983). In Fischer-344 rats 2,6-DNT is rapidly absorbed after oral administration. The first step is oxidation at the methylgroup to yield 2,6-dinitrobenzyl alcohol. Conjugation with glucuronic acid renders the 2,6-dinitrobenzyl alcohol suitable for excretion in to the bile. Intestinal microflora hydrolyze the excreted 2,6-dinitrobenzyl glucuronides and reduce one of the nitro groups. The aminonitrobenzyl alcohols are reabsorbed for final
activation in the liver. N-and C-hydroxylation by hepatic cytochrome P450 form two metabolites: 2-hydroxylamino-6-nitrobenzyl alcohol and 2-amino-5-hydroxy-6-nitrobenzyl alcohol. Sulfate conjugation of 2-hydroxylamino-6-nitrobenzyl alcohol has been implicated in the final activation step of 2,6-DNT, which is expected to be sufficiently unstable to decompose to an electrophilic nitrenium ion which could then react with critical cellular macromolecules to cause hepatocarcinogenicity (Kedderis et al., 1984). 2-NT is suggested to be bioactivated in a manner similar to 2,6-DNT (Doolittle et al., 1983; DeBethizy and Rickert, 1984; Chism et al., 1984). In male B6C3F1 mice and Wistar rats, MX is metabolized in vivo by nitroreduction catalyzed by intestinal flora that yields 2 aromatic monoamine metabolites, o-NH₂-and p-NH₂-MX (see 3.6.2.3.4 Fig. 7), reflecting the location of the amine substitution relative to the t-butyl function on MX (Minegishi et al., 1991; Lehman-McKeeman et al., 1997b). But in contrast, despite the formation of amine metabolites, MX does not show any genotoxic potential up to doses of 5000 mg/kg in an in vivo-in vitro UDS assay (Api et al., 1995a). These high doses should produce significant levels of amine metabolites (Minegishi et al., 1991). The negative results of the UDS assays indicate that amine metabolites of MX may not generate genotoxic intermediates. Recent studies revealed that the amine metabolites and not the parent MX are responsible for a pleiotropic response similar to that of PB-type enzyme inducers (Lubet et al., 1992; Lehman-McKeeman et al., 1997a).

Similar to PB-type enzyme inducers, a 7 day acute MX administration by oral gavage up to doses of 200 mg/kg in mice produced liver weight increase, hepatocellular hypertrophy, small increases in microsomal activity for O-dealkylation of 7-ethoxy and 7-methoxyresorufin and N-demethylation of erythromycin, small increases in CYP1A and CYP3A protein levels and strongly induced CYP2B protein levels (Lehman-McKeeman et al., 1997a). The fact that in our chronic MX exposure study induction of CYP1A enzymes and proteins occurred at lower MX concentrations (MX 7-8 mg/kg/day) than induction of CYP2B (70-80 mg/kg/day) may indicate that CYP1A induction should not be viewed only as part of a pleiotropic response but may result from multiple interactions of MX and/or MX metabolites with diverse CYP450 enzyme families (Hostetler et al., 1988). Additionally, MX effects on hepatic metabolism may also be modulated by the dose or the manner in which it is given and the treatment period.
Many arylamines, particularly heterocyclic arylamines, are well-known carcinogens that are activated by N-oxidation, catalyzed primarily by CYP1A2 (Butler et al., 1989). Although we observed a significant increase in EROD/MROD enzyme activity and CYP1A, namely CYP1A2 protein levels in adult and 14 day old rats, MX was negative in a battery of genotoxicity tests (Api et al., 1995a), but the isoenzyme induction increased metabolism (= toxification) of several procarcinogens, among them benzo[a]-pyrene and 2-aminoanthracene (Mersch-Sundermann et al., 1996a). Also Minegishi et al. (1991) could not detect any nitroso and hydroxylamine intermediates, which have been assumed to proceed by reduction of amine-to nitrogroups or by N-oxidation of amine groups. Thus, the question about the mechanism of MX-induced mouse liver tumors (Maekawa et al., 1990) can not be answered at the moment, genotoxic and non-genotoxic mechanism may be involved. This is in contrast to other authors (Lehman-McKeeman et al., 1997a, 1997b and 1997c), who classified MX as a non-genotoxic chemical, that may cause mouse liver tumours in a manner analogous to that of PB.

Until now, studies focused on possible toxic effects of the parent compound MX. Due to recent findings (Lehman-McKeeman et al., 1997c), which identified an amine metabolite of MX (p-NH2-MX), formed by nitro reductases in anaerobic intestinal bacteria, to be responsible for the increase in liver weight and CYP2B protein levels, it is important that further studies will differ between effects of MX and its metabolites. Also internal tissue concentrations of MX metabolites from experimental studies and man should be available in the near future to relate possible toxic effects to target tissue concentrations. There are numerous differences in the gut microflora between individuals and animal species which makes extrapolation of metabolic and toxicological data from laboratory animals to man difficult. Humans have much lower bacterial nitroreductase activity than rat, mouse, and hamster, implying that certain nitro compounds such as nitrobenzenes and dinitrotoluenes would be more potent in rodents than in man. Also the differences in the distribution pattern of the gut flora between animals and man should be considered in relation to the main sites of absorption of drugs namely the duodenum and proximal small intestine. There are numerous differences between the adult and infant gut floras. It is likely that the drug metabolising activity of the flora also exhibits developmental changes (Rowland, 1986). Finally, diet-induced changes in the species composition of the gut microflora in rodents have been demonstrated. The major non-fermentable carbohydrate component present in many
dietary fibres is cellulose which, when added to a purified rodent diet at 0-40 %, caused a marked concentration-dependent decrease in total numbers of microorganisms, accompanied by a decrease in the activities of several microbial enzymes including azo-, nitro- and nitrate reductases (Mallett et al., 1983; Rowland et al., 1985). In conclusion, there is a need for improvements in the methods used for studying bacterial metabolism, particularly to provide data of direct relevance to man. Several alternative approaches are under investigation. Rats, mice and hamsters fed a purified, fibre-free diet exhibit a greater similarity to man in terms of their caecal nitrate and nitro reductase activities than animals given stock laboratory diets (Rowland et al., 1983). Utilizing germfree rats contaminated with human faecal organisms allows in vivo metabolic and toxicological studies to be performed with animals whose caecal microflora possesses many microbial and enzymatic properties characteristic for the human gut flora (Rowland, 1986). Next to different capacities of intestinal nitroreduction in rodents and man, also significant distinctions in hepatic cytochrome P450 activities (see 3.6.2.3.1-3) must be noted comparing experimental and human data.

Considering the main uptake route of MX over the skin in humans, skin metabolism may also play a role in MX metabolism. MX has been shown to be metabolized extensively after systemic administration in rats (Minegishi et al., 1991), but no metabolites of MX formed by the skin were found, suggesting that MX remains unmetabolized in human skin and biotransformation may occur predominantly in systemic organs (Hood et al., 1996). Next to high esterase-, aldehyde- and alcoholdehydrogenase activities, spectral evidence for cutaneous cytochrome P450 has been obtained. The most important cytochrome P450 families for the metabolism of foreign compounds (CYP450 1-3) have been also found in rat and human skin, although they do not necessarily indicate that catalytic activity is present (Hotchkiss, 1992). In rats, metabolic activity in skin is generally about 2 % of that in liver (Pannatier et al., 1978). The considerable mass of tissue with its extensive surface area must be taken into account. Studies in freshly excised human and rodent skin in the in vitro SAM system and cultured epidermal keratinocytes, suggest that the skin is able to metabolize certain fragrance chemicals, but not others, during absorption (Beckley-Kartey and Hotchkiss, 1997; Nasseri-Sina et al., 1997). The extent to which such metabolism occurs depends on the chemical itself and the precise nature of the enzyme systems involved.
7.4 HUMAN RISK ASSESSMENT: NOAEL(C), LOAEL(C) AND SAFETY FACTOR

For a risk assessment of environmental compounds, sufficient human data are seldom available. Sometimes body burden concentrations were measured, but dose-response relationships are obtained only from experimental studies. Thus, risk assessment is often based on dose-response relationships and NOAEL(C) (see 3.8 and 3.8.1) from experimental studies. These results can be compared with estimated daily uptake rates or internal plasma-or organ concentrations in humans, considering pharmacokinetic differences and susceptibility. With increasing difference between concentrations occurring in humans and experimental dose levels, extrapolations become more uncertain. Effects, which originate from high concentration levels, may be not relevant to human risk assessment. Additionally, the organism is able to assimilate moderate substance load. The last two reflections plead for experiments with chronic low dose exposure which reflects more the environmental situation.

Depending upon the endpoint studied, several dose-response curves may be generated for one substance. The curves may have different steepness, especially in reproductive studies. Furthermore, dose-response curves are related to different stages of development (Marquardt, 1994).

Another important aspect in risk assessment are possible effects of metabolites. This question has not yet been systematically addressed in studies on nitro musks, but recent studies revealed an amine metabolite of MX to be responsible for some hepatic effects (see 3.6.2.3.4).

7.4.1 Determination of human safety factors

There are two possible bases for the calculation of human safety factors

A: Daily intake of humans compared to daily dosage in the laboratory animal

B: Comparison of tissue concentrations in humans vs laboratory animal model

For the comparison of longterm effects in different species with differences in metabolism, tissue concentrations provide a more reliable basis. In the future all such comparisons should be based on tissue levels.
7.4.1.1 Safety factors calculated on the basis of results from our developmental LE rat model studies

<table>
<thead>
<tr>
<th>Musk xylene</th>
<th>NOAEL/LOAEL from daily intake</th>
<th>NOAEC/LOAEC from tissue/fat concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>human rat Endpoint: enzyme induction Endpoint: enzyme induction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NOAEL LOAEL</td>
<td>NOAEC LOAEC</td>
</tr>
<tr>
<td></td>
<td>human rat Endpoint: 14-day survival index Endpoint: 14-day survival index</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NOAEL LOAEL</td>
<td>NOAEC</td>
</tr>
<tr>
<td>daily</td>
<td>0.014 mg/kg 0.7-0.8 mg/kg 2-3 mg/kg s.f. = 50 s.f. = 179</td>
<td></td>
</tr>
<tr>
<td>intake</td>
<td>systemic</td>
<td>fat Endpoint: enzyme induction conc.</td>
</tr>
<tr>
<td></td>
<td>0.014 mg/kg 0.7-0.8 mg/kg 2-3 mg/kg s.f. = 50 s.f. = 179</td>
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<tr>
<td></td>
<td>human rat Endpoint: 14-day survival index Endpoint: 14-day survival index</td>
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<td>intake</td>
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<td>intake</td>
<td>systemic</td>
<td>fat Endpoint: enzyme induction conc.</td>
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<tr>
<td></td>
<td>0.014 mg/kg 0.7-0.8 mg/kg 2-3 mg/kg s.f. = 50 s.f. = 179</td>
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</tbody>
</table>

**Table 14:** Human safety factors calculated on the basis of results from our developmental rat studies.

**NOAEL:** No observed adverse effect level; **LOAEL:** Lowest observed adverse effect level; **NOAEC:** No observed adverse effect concentration; **LOAEC:** Lowest observed adverse effect concentration; **s.f.:** safety factor

a Systemic human MX intake was estimated indirectly by Ford (1998) from dermal exposure (= 0.18 mg/kg/day). Animal daily intake calculated by measuring daily food uptake of the parent generation (see chapter 4, 5 and 6).
b MX concentrations were analysed in human adipose tissue and milk samples (Helbich, 1995; Rimkus, 1998) and in adipose tissue of 14 day old rats from dams treated with different amounts of MX (see chapter 4).
c Microsomal enzyme induction (EROD/MROD) determined in 14 day old rats from dams treated with different amounts of MX (see chapter 5).
d Determination of 14-day survival index and body weight increase of 2-14 day old rats from dams treated with different amounts of MX (see chapter 6).
7.4.1.2 Safety factors calculated on the basis of results from other experimental studies

<table>
<thead>
<tr>
<th>Musk xylene</th>
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<tr>
<td><strong>NOAEL</strong></td>
<td></td>
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<tr>
<td>from daily intake</td>
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<tr>
<td>human</td>
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<tr>
<td>daily</td>
<td></td>
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<tr>
<td>intake</td>
<td></td>
</tr>
<tr>
<td>0.014 mg/kg</td>
<td></td>
</tr>
<tr>
<td>systemic</td>
<td></td>
</tr>
</tbody>
</table>

Table 15: Human safety factors calculated on the basis of results from other experimental studies

- *Human and rat MX intake (dermal and systemic) calculated by Ford (1998); respectively by Ford (1990 and 1998)*
- *Liver weight increase determined in a 90-day dermal toxicity study (Ford et al. 1990, see 3.6.2.1)*
- *Embryo-fetal development from dams treated orally (gavage) on days 7 through 17 of gestation (Ford, 1998 unpublished data, endpoints not mentioned, see 3.7)*

7.4.1.3 Comparison of different safety factors

Safety factors based on our developmental rat model tend to be lower when tissue fat values rather than intake levels are compared. Safety factors for embryo-fetal development on the basis of NOAEL showed remarkable differences between our studies and the literature (Ford, 1998). Our safety factor based on LOAEL (5357, Tab. 14) is even lower than the NOAEL published by Ford, which shows that the experimental study design is of great importance. Unfortunately, Ford did not mention the endpoints used in his study.

In general, safety factors of 500-1000 x are recommended for developmental studies (Rees et al., 1990). The safety factors based on our developmental study remain below this value.

7.5 ENVIRONMENTAL RISK ASSESSMENT (ERA)

A PEC/PNEC ratio for aquatic ERA was published to be at or below 0.1 for MX (Tas et al., 1997). In 1998, Gatermann et al., detected for the first time amine metabolites of
MX in the effluents of wastewater, indicating a high metabolic activity of microorganisms in filter-beds of sewage purification plants (see 3.4.2). MX amine metabolites were identified to be responsible for some hepatic effects in mice (Lehmann-McKeeman et al., 1997c). Since ERA for aquatic organisms do not include possible effects of amine metabolites in the aquatic environment, further investigations must be done to gain sufficient toxicological data for environmental risk assessment.

7.6 CONCLUSIONS

MX contamination found in various samples of the environment and human tissues derives from perfumes, cosmetics, pharmaceutics, detergents and other household products. Due to their physical and chemical properties, nitro musks are little biodegradable, lipophilic and have a high bioaccumulation potential. Detergents reach the environment via waste water and purification plants and bioaccumulate in aquatic food chains. But in contrast to other substances with similar characteristics (e.g. organohalogenated compounds), the main human contamination route is thought to occur by way of percutaneous absorption and not by food intake. Recently, some lipophilic sunscreen agents from cosmetic products were also analysed for the first time in human milk samples (Hany and Nagel, 1995), further demonstrating that human skin is not a strong barrier to lipophilic compounds from cosmetics. The highest MX absorption in humans results from body lotion because it is applied to large areas of the body and remains on the skin for relatively long periods of time (Rimkus, 1998). Inhalative uptake is also possible but seems to be quantitatively negligible. In contrast to the voluntary human exposure from cosmetics, involuntary exposure of terrestrial wild life to these compounds is occurring by the oral route. In addition, mammalian pups and human babies are ingesting synthetic musks present in the milk. We chose to introduce this exposure scenario for investigations on effects in early life with our LE rat model, administering MX orally dissolved in food pellets. A study conducted by Ford et al. (1990) revealed similarities between results of oral and dermal chronic administration of MA, supporting oral uptake studies as an alternative to dermal absorption studies. Reprotoxicity including developmental toxicity is presently considered to be one of the critical issues for environmental chemicals. Our results revealed qualitative and quantitative differences in pharmacokinetic parameters of adult rats and their offspring.
depending on the investigated endpoint. Rats exposed to MX in adulthood exhibited a marked sex difference in MX levels of several organs, which was not present in their offspring. The load in offspring results from a combination of exposure via placental transfer and milk, reaching higher MX tissue concentrations than found in similarly treated male adult rats. The bioaccumulation potential, the sex difference in adult rats and the consequences of developmental exposure rise questions about possible consequences for animal and human health that require further investigations.

All studies clearly showed, that depending on treatment period, developmental state, dose and mode of application of MX, considerable qualitative and quantitative differences of a defined toxic response resulted. In our chronic MX exposure study, induction of CYP1A enzymes and proteins occurred at lower MX concentrations than induction of CYP2B without increase in liver weight and without any histopathological liver changes. This contrasts with reports by other authors which classified MX as a PB-like inducer due to a primary induction of CYP2B protein and increased weight and histopathological changes in liver. Our data indicate that CYP1A induction should not be viewed only as part of a pleiotropic response. The effects of MX on CYP enzymes may result from multiple interactions of MX and/or MX amine metabolites with diverse CYP450 enzyme families. Also our finding of a different enzyme induction pattern in developmental rats (additionally increased CYP3A protein levels) requires further investigations. A special sensitivity of the developing organism is also indicated by a significant increase in mortality of 14 day old offspring (60 %) in the absence of toxic signs in the dam. It should be further clarified whether the increased pup mortality is due to direct developmental toxicity of MX.

Recent studies demonstrate that an amine metabolite ($p$-NH$_2$-MX), and not the parent compound MX is accountable for some hepatic effects in mice. Whether this is also true for other toxic effects and other species, must be elucidated in further experiments. No data on amine metabolites are so far available for human tissues.

Until now studies on adverse human health effects have primarily focused on photosensitivity and dermal reactions. Due to the scarcity of additional toxicological data on MX and its amine metabolites, it is not yet possible to assess human health risk. In 1995, IARC concluded that MX is not classifiable as carcinogenic to humans (group 3). Further investigations are needed to obtain sufficient toxicological data for risk assessment, with consideration of possible pharmacokinetic species differences.
In conclusion, compounds exhibiting persistence in the environment and bioaccumulation represent a potential problem for the environment and for humans. This was demonstrated in the past, e.g. for organohalogenated compounds and should be recognized in the present for synthetic musk fragrances and other groups of chemicals (e.g. UV-filter substances and plastic materials).

Recent analyses of musk fragrance residues in environmental and human samples show that nitro musk concentrations are decreasing and polycyclic nitro musk concentrations are increasing. This suggests that the perfume industry attempts to overcome "the problem nitro musks" by replacing them by polycyclic musks. However, polycyclic musks also exhibit a bioaccumulation potential and toxicological data concerning these compounds are hardly available today.


Bundestagsdrucksache 13/487 vom 13.02.1995


Chou, I., Dick, D., Bronaugh, R.L.: Skin reservoir effects and bioavailability of dermally applied chemicals in hairless guinea pigs. Toxicologist 14, 183 (1994)


De Boer, J.: Chlorobiphenyls in bound and non-bound lipids of fishes; comparison of different extraction methods. Chemosphere 17 (9), 1803-1810 (1988)


Doolittle, D.J., Sherrill, J.M., Butterworth, B.E.: Influence of intestinal bacteria, sex of the animal, and position of the nitro group on the hepatic genotoxicity of nitrotoluenne isomers in vivo. Cancer Res. 43, 2836-2842 (1983)


Kokot-Helbling, K.S.: Toxicokinetics and distribution of MX in BALB/c mice and Wistar rats. In: Critical kinetic aspects of lipophilic compounds, Diss ETH No. 11152, 84-93 (1995c)


Michnovicz, J.J., Bradlow, H.L.: Induction of estradiol metabolism by dietary indole-3-carbinol in humans, JNCI 82, 947 (1990)


Müller, U.: Jahresbericht Kantonales Laboratorium Bern (1993)


Parker, R.D., Buehler, E.V., Newmann, E.A.: Phototoxicity, photoallergy, and contact sensitization of nitro musk perfume raw materials. Contact Dermatitis 14, 103-109 (1986)


Romann, E.: Jahresbericht Kantonales Laboratorium Zürich. 43 (1993)


Small, W.S.: Notes on the psychic development of the young white rat. Amer. J. Psychol. 11, 80-100 (1899)


Walbaum, H.: Das natürliche Moschusaroma. J. Prakt. Chem. 73, 488 (1906)


CURRICULUM VITAE

REGINA SUTER-EICHENBERGER

Date of Birth: September 3rd, 1965
Citizenship: Beinwil am See and Beromünster, Switzerland
Civilian status: married to Peter M. Suter

1972 – 1977 Primary school in Burg/Menziken (AG)
1977 – 1981 Bezirksschule in Menziken (AG)
1981 – 1985 Alte Kantonsschule Aarau, typus B
1985 – 1991 ETH Zurich, Student of Pharmacy
1991 – 1994 Practical work in different pharmacies
1994 – 1999 Ph. D. student at the Institute of Pharmacology, University of Zurich
LIST OF PUBLICATIONS

ORIGINAL PAPERS

Suter-Eichenberger, R., Altorfer, H., Lichtensteiger, W., Schlumpf, M.:
Bioaccumulation of musk xylene (MX) in developing and adult rats of both sexes.
Chemosphere 36 (13), 2747-2762 (1998)

Suter-Eichenberger, R., Boelsterli, U.A., Conscience-Egli, M., Lichtensteiger, W.,
Schlumpf, M.:
CYP 450 enzyme induction by chronic oral musk xylene in adult and developing rat.
Toxicology letters, in press

ABSTRACTS

Suter, R., Boelsterli, U., Lichtensteiger, W., Schlumpf, M.: Chronic oral musk xylene
specifically induces cytochrome P450 1A in Long Evans rats. USGEB 95, A76/S16-21
(1995)

Suter, R., Boelsterli, U., Altorfer, H., Lichtensteiger, W., Schlumpf, M.: Chronic oral
musk xylene (MX) specifically induces cytochrome P450 1A in Long Evans rats.
USGEB 96, A84/P01 (1996)

musk xylene (MX) induces cytochrome P450 1A in Long Evans rats. EUROTOX 96,
Poster session 3R. Food/Air/Water/Soil (P3R-342), 95 (1996)

Schlumpf, M., Suter-Eichenberger, R., Conscience, M., Lichtensteiger, W.: Synthetic
musks fragrances: bioaccumulation and induction of CYP 450 liver enzymes in
developing and adult rats. European Association of Poisons Centres and Clinical
Toxicologists (EAPCCT), March 98

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