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INVESTIGATIONS ON THE POTENTIAL FOR CARCINOGENICITY

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STYRENE TOXICOLOGY INVESTIGATIONS ON THE POTENTIAL FOR CARCINOGENICITY
CONTENTS

SUMMARY . . . 1

SECTION 1. INTRODUCTION . . . 2

SECTION 2. PRESENTATION OF NEW RESULTS . . . 4
2.1. TOXICOKINETICS . . . 4
2.2. MACROMOLECULAR BINDING . . . 5

SECTION 3. COMPARATIVE EVALUATION OF DATA . . . 9
3.1. EVALUATION OF DATA IN RELATION TO BIOASSAYS . . . 9
3.2. EVALUATION OF MACROMOLECULAR BINDING DATA . . . 16
3.3. CONCLUSION . . . 18

APPENDIX: REVIEW OF THE TOXICOKINETICS, MACROMOLECULAR BINDING, CARCINOGENICITY AND MUTAGENICITY DATA FOR STYRENE AND STYRENE-7, 8-OXIDE . . . 19

APPENDIX A. TOXICOKINETICS . . . 20
A.1. METABOLISM . . . 20
A.2. EFFECTS OF STYRENE ON XENOBIOTIC METABOLISING ENZYMES. . . . 25
A.3. ELIMINATION . . . 29
A.4. TOXICOKINETICS . . . 29

APPENDIX B. MACROMOLECULAR BINDING . . . 32
B.1. HAEMOGLOBIN BINDING . . . 32
B.2. DNA BINDING . . . 33

APPENDIX C. CHRONIC TOXICITY/CARCINOGENICITY . . . 35
C.1. STYRENE EPIDEMIOLOGY STUDIES . . . 35
C.2. STYRENE LONG-TERM ANIMAL STUDIES . . . 38
C.3. STYRENE OXIDE LONG-TERM ANIMAL STUDIES . . . 44

APPENDIX D. MUTAGENICITY . . . 47
D.1. MUTAGENICITY STUDIES . . . 47
D.2. CYTOGENETIC STUDIES . . . 48

BIBLIOGRAPHY . . . 51

MEMBERS OF THE TASK FORCE . . . 60

MEMBERS OF THE ECETOC SCIENTIFIC COMMITTEE . . . 61

STYRENE TOXICOLOGY INVESTIGATIONS ON THE POTENTIAL FOR CARCINOGENICITY

SUMMARY

At present there is no clear evidence that styrene is carcinogenic in laboratory animals or in man. The absence of an exposure related oncogenic response in several animal bioassays or appropriate epidemiology investigations precludes the use of quantitative risk assessment procedures. Concern has been expressed, however, that a putative carcinogenic potential exists as a result of the formation and presence in tissues of styrene 7,8-oxide, an intermediate metabolite of styrene..

To address these concerns new toxicokinetic data on styrene and the metabolic occurrence of styrene oxide have been generated and are presented together with information on macromolecular binding in vivo. The toxicokinetic parameters for styrene and styrene oxide permit a quantitative description of the major pathways of styrene and styrene oxide in the rat and mouse under various conditions of exposure.

Based on experimental data a physiologically based pharamcokinetic (PB-PK) model for styrene and styrene oxide has been developed, validated and used to calculate the body burden of styrene and styrene oxide for man in relation to animals in terms of AUC_{SO} (AUC_{SO} is the area under the concentration time curve for styrene oxide in blood). This approach referred to as "semiquantitative margin of safety" ("SMS") provides the opportunity to assess the putative hazard of styrene to man in relation to specific conditions in laboratory animals. Comparison of predicted and measured AUC_{so} values in animals with that in man exposed to an anticipated workplace concentration of 50ppm styrene shows AUC_{SO} values up to 400 times lower in man than in exposed animals in which no treatment related oncogenic response was observed.

In addition, the results of DNA binding studies showed that styrene metabolism in the rat or the mouse does not lead to the production of biologically significant DNA binding intermediates. Studies with styrene oxide failed to find any evidence of DNA binding while investigation with styrene showed only minute levels of binding considered too low to cause an increase in the incidence of cancer.

It can be concluded that the carcinogenic potential of styrene, if one exists at all, must be so low that occupational or environmental exposure to styrene is unlikely to present any genotoxic or carcinogenic hazard for man.

SECTION 1. INTRODUCTION

Styrene is an important chemical of wide industrial use, particularly in the manufacture of polymers and reinforced plastics. Styrene is a naturally occurring as well as a man-made chemical. Entry into the atmosphere occurs via automobile exhaust, as a result of smoking, during production, storage and processing of styrene, and to a minor extent during or after use of the final product. Environmental and occupational exposures to styrene occur predominantly via inhalation. The toxicity of styrene has been reviewed (Fielder, 1981; WHO, 1983; BUA, 1990; Vainio, 1991). Irritation of the mucous membranes and effects on the nervous system are the most commonly reported occupational findings.

In recent years the discussion about a possible carcinogenic potential of styrene has become controversial. At present, there is no clear evidence that styrene is carcinogenic in man or laboratory animals; the available data are inadequate to reach definitive conclusions. Limitations and deficiencies exist in many of the long-term animal studies and human epidemiology studies involving styrene. The toxicological significance of styrene-7,8-oxide (styrene oxide) as an intermediate metabolite of styrene has become the focus of recent concern. Styrene oxide is mutagenic in *in vitro* prokaryotic and eukaryotic systems (Appendix D.1). In long-term gavage studies it causes increased tumour incidences in the forestomach of rodents at high doses but no treatment related systemic tumours were detected (Appendix C.3). The significance of these tumours is highly uncertain with respect to risk estimation for man where styrene oxide occurs only at low concentrations as an intermediate metabolite.

Knowledge of the kinetics of formation and degradation of styrene oxide together with definitive data on the macromolecular binding of styrene and styrene oxide are important prerequisits for understanding tissue dosimetry. The use of such data in a physiologically-based pharmacokinetic model (PB-PK) provides a better understanding of the internal dosimetry of styrene oxide in laboratory animals and man allowing a more meaningful evaluation of the carcinogenic potential of styrene than was formerly possible.

The objective of this document is to present new information on metabolism, toxicokinetics and macromolecular binding as generated in the ECETOC research programme which substantially contribute to the evaluation of the carcinogenic potential of styrene.

The following studies were performed:

- Study on the kinetics of styrene and styrene oxide in rats and mice.

Dr. J. G. Filser (1992). Forschungszentrum fuer Umwelt und Gesundheit GmbH, Institut fuer Toxikologie, 8000 Muenchen.

- Investigation of the adduct formation between styrene or styrene-7,8-oxide and deoxyribonucleic acid (DNA) in rats, in mice and *in vitro*.
  Prof. Dr. W. K. Lutz (1992). Eidgenoessische Technische Hochschule und Institut fuer Toxikologie, Universitaet Zuerich.

- Investigation of the adduct formation between styrene or styrene metabolites and haemoglobin or blood proteins in rats and mice (*in vitro* and *in vivo*).
  Dr. S. Osterman-Golkar (1992). Dep. of Radiobiology, University Stockholm.

The above reports are available from ECETOC.

Data from these studies are used to examine the putative hazard of styrene for man in relation to specific conditions used in laboratory animal studies.

SECTION 2. PRESENTATION OF NEW RESULTS

2.1. TOXICOKINETICS

Concern about the carcinogenic potential of styrene has been focused on the occurrence of styrene oxide as an intermediate metabolite of styrene (Appendix A). Previous studies suggested that the rat is a good animal model for understanding the toxicokinetics of styrene in man, at least for inhalation exposure concentrations up to 80ppm. Anderson and Ramsey (1983) developed a (PB-PK) model which describes the non-linear behaviour of styrene in rats following higher exposures (Appendix A.4). Toxicokinetic data for styrene oxide and its relation to styrene had not however been determined. Although scattered analytical data on styrene oxide in blood are available, these are inadequate for making predictions regarding body burden of styrene oxide in rats or man exposed to styrene. Furthermore kinetic data for styrene in mice (inhalation) are not available. The studies of Filser (1992) were performed in the ECETOC research program to fill these data gaps period.

The results of these studies show that at steady state the rate of metabolism of inhaled styrene in rats and mice increases linearly with exposure concentration up to about 260ppm. Below this concentration, there is little bioaccumulation of inhaled styrene. At concentrations below 260ppm transport to the metabolising enzymes is the rate limiting step for metabolism rather than their enzymatic capacity. Kinetic behaviour of styrene is strongly influenced by physiological parameters such as blood flow and especially alveolar ventilation rate.

At exposure concentrations of styrene above 300ppm the rate of metabolism at steady state is limited by biochemical parameters of the metabolizing enzymes (V_{max} and Km_{app}). Saturation of metabolism (V_{max}) is reached at about 600ppm in rats and 900ppm in mice (rats: 224μmol/hr×kg; mice: 625μmol/hr×kg). Above 300ppm, bioaccumulation increases rapidly with increasing styrene exposure. At levels above 2,000ppm, bioaccumulation reaches its maximum.

The concentration of styrene oxide was measured in the blood of rats and mice following exposure to styrene or styrene oxide via different dose routes (oral, intraperitoneal and inhalation). At steady state following exposures to styrene between 20 and 800ppm, styrene oxide was detected in the blood of both species. In rats, blood concentrations of styrene oxide correlate linearly with the rate of metabolism of styrene. This indicates that the rate-limiting step is the formation of styrene oxide rather than its detoxification. In contrast, such a clear correlation is not found in mice. At exposure concentrations up to 260ppm similar

styrene oxide kinetics are seen in both species. A sharp increase of styrene oxide levels occurs in mice after exposure to styrene concentrations higher than 260ppm.

The latter observation is interpreted as follows: at exposure concentrations of styrene below 260ppm the relatively higher metabolic rate (styrene to styrene oxide) in mice is compensated by a high elimination rate of styrene oxide. This leads to comparable amounts of styrene oxide in rats and mice. At exposure concentrations above 260ppm, greater amounts of styrene oxide become systemically available in mice than in rats. In mice, levels up to 7,500ng/ml were reached during exposure to 800ppm styrene, while in rats blood concentrations of styrene oxide did not exceed 460ng/ml. This may indicate that certain styrene oxide metabolic pathways are overwhelmed in the mouse at styrene concentrations greater than 260ppm.

Concentrations of styrene oxide in blood after intraperitoneal or intravenous administration of styrene oxide to rats and mice confirmed the results of the inhalation experiment with styrene, indicating that biotransformation of styrene oxide is faster in mice than in rats at low styrene exposure concentrations.

Concentrations of styrene oxide in blood were also measured after oral administration of styrene oxide to rats and mice. In both species a large variation of blood/plasma styrene oxide concentrations was found. Despite this variation it could be concluded that styrene oxide in blood after oral administration represented <5% of that seen after i.p. administration of the same dose. This difference may, at least partly, be due to the fast hydrolysis of orally administered styrene oxide at the acidic pH in the stomach.

Based on the new experimental data, a physiologically-based pharmacokinetic model (PB-PK) has been developed and verified by Filser and Nolan (in press). Body burden or tissue levels in rats or mice can now be predicted for various conditions of exposure to styrene or styrene oxide. This model can also be used to predict the styrene oxide body burden for man exposed to styrene. Such calculated/predicted results have been verified by experimental data.

2.2. MACROMOLECULAR BINDING

A review of published data concerning DNA and haemoglobin adduct formation is given in Appendix B.

2.2.1. HAEMOGLOBIN BINDING

As part of the ECETOC research programme the study conducted by Osterman-Golkar (1992) investigated the possible adduct formation between styrene or styrene oxide and haemoglobin or other blood proteins in rats and mice following intraperitoneal (i.p.) administration of styrene or styrene oxide and to determine the feasibility of *in vivo* monitoring of styrene exposure by quantification of blood protein adducts. A summary of results is presented below.

The experiments show that haemoglobin adducts are formed and can be detected following i.p. administration of styrene or styrene oxide to rodents. Methods were developed for the determination of an N-terminal valine adduct (hydroxyphenethylvaline); this adduct was found to be stable in contrast to adducts involving carboxyl groups which were found to be unstable. The amount of N-terminal valine adducts, as a measure of *in vivo* dose was determined in mice and rats administered styrene oxide or styrene at doses ranging up to about 250mg/kg body weight.

In mice treated over a dose range of 50 to 250mg styrene oxide/kgbw the dose response curve for styrene oxide binding to N-terminal valine is non-linear with a disproportionate increase in binding being seen at the higher dose levels. This effect is consistent with toxicokinetic data which shows that at high systemic SO exposures the detoxification system in the mouse becomes overwhelmed thereby compromising the clearance mechanism. In mice dosed with styrene the level of binding was considerably lower than that seen in mice treated with an equivalent dose of the oxide, e.g. the level of binding measured in mice treated with styrene (dose level approximately 200mg/kgbw) was only about 5% of that measured in mice receiving an equivalent dose of the oxide.

Similar studies in the rat also indicated a deviation from linearity with again comparatively more binding being seen with increasing dose. In the rat however, this deviation was much smaller as compared with the mouse; again supporting the toxicokinetic data that had shown that the rat is more efficient at clearing SO from the body than is the mouse. The levels of binding measured in the rat were also lower as compared with the mouse, i.e. at an i.p. dose of approx. 50mg/kgbw the level of N-terminal valine binding was approximately 3 times higher in the mouse as compared with rat.

With further refinement and verification by the inhalation dose route, measurement of haemoglobin adducts may provide the basis for the development of a suitable biological monitoring method.

2.2.2. DNA BINDING

In long-term gavage studies styrene oxide has caused an increased incidence of tumours in the forestomach of rodents at high doses, but no increase in systemic tumours (Appendix C.3). Genotoxic and non-genotoxic mechanisms may be involved in the development of the tumours. To assist in the evaluation of the genotoxic potential of styrene oxide, DNA binding experiments were conducted *in vivo*. A summary of the results generated for ECETOC by Lutz (1992) is presented below.

In vitro experiments showed that DNA adducts were formed to a small but measurable extent during a 24-hour incubation of tritiated styrene oxide with calf thymus DNA. This is consistent with the *in vitro* mutagenic potential of styrene oxide (Barale, 1991).

To evaluate the genotoxic potential of styrene oxide *in vivo*, radiolabelled styrene oxide was administered by gavage in corn oil to male CD rats at levels of 1.65 and 240mg/kgbw. After 4 or 24 hours, DNA from forestomach, glandular stomach and liver was isolated, purified and its radioactivity determined. At 4-hours the radioactivity in DNA samples from the forestomach and the liver at both dose levels was below the limit of detection. Expressed in units of the Covalent Binding Index, [CBI = μmol adduct per mol DNA nucleotide/mmol chemical administered per kg body weight; Lutz, 1986], the DNA-binding potency in the forestomach and liver was below 2.6 and 2.0, respectively. In the glandular stomach at 4 hours and in most 24 hour samples, DNA was slightly radiolabelled. Enzymatic degradation of the DNA and separation of the nucleotides showed that the tritium represented biosynthetic incorporation of radio-label into newly synthesized DNA rather than covalently bound radiolabel. The limit of detection for DNA adducts in the glandular stomach was at a CBI <1.0.

Radiolabelled styrene oxide was administered by i.p. injection to male B6C3F1 mice. Liver DNA was analysed after 2 hours. No radioactivity was detectable at a limit of detection of CBI <0.6.

Although forestomach tumours were observed in bioassays (Maltoni *et al*, 1979), the *in vivo* studies with styrene oxide in rats did not demonstrate detectable DNA adducts in the forestomach at a limit of detection of CBI <2.6. This corresponds to an ability to detect 7 adducts per 10^8 DNA nucleotides in the low dose experiment. Hence a purely genotoxic mechanism of tumorigenic action of styrene oxide in the forestomach is unlikely.

Radiolabelled styrene was also administered by inhalation in a closed chamber to male and female CD rats and B6C3F1 mice. The metabolised dose was between 20 and

39mg/kgbw in rats and between 70 and 110mg/kgbw in mice. Exposure time was between 5 and 9 hours and peak concentrations in the chamber were at 200 and 400ppm, for rats and mice, respectively. DNA from the liver and the lung (rats only) was purified and analyzed for nucleotide adducts. In mouse liver DNA a small but detectable amount of radioactivity eluted with similar retention times as adducts prepared from calf thymus DNA and styrene oxide *in vitro*. The DNA-binding potency averaged at 0.1CBI units.

In rat liver DNA, no adducts were observed at a limit of detection of 0.1 to 0.2CBI units. Two DNA samples from rat lung available at high yield allowed a lower limit of detection. Adduct-related radioactivity was detectable at the level of 0.07CBI units.

The results of these experiments indicate that styrene metabolism in the rat and mouse does not result in the production of potent DNA binding intermediates. This conclusion is supported by the studies with styrene oxide in which no adduct formation was detected in these species.

SECTION 3. COMPARATIVE EVALUATION OF DATA

3.1. EVALUATION OF DATA IN RELATION TO BIOASSAYS

The assessment of hazard from exposure to chemicals focuses on dose-related adverse effects seen in animal bioassays or epidemiological investigations. Quantitative carcinogenicity risk assessment procedures require an exposure related oncogenic response in either laboratory animals or in human beings. Such approaches are not possible with styrene using the carcinogenicity data currently available. Eleven long-term animal studies with styrene to date have collectively shown no clear evidence of carcinogenicity related to styrene (Appendix C.2). Likewise the available epidemiology information shows no convincing evidence of cancer in workers occupationally exposed to styrene. Hence, there is no basis to conclude that styrene is carcinogenic.

Despite this, there is some concern about the putative carcinogenic potential of styrene since styrene oxide is an intermediate metabolite of styrene and is positive in *in vitro* mutagenicity assays. Styrene oxide has also been shown to cause tumours of the forestomach in rodents when given at high oral doses by gavage (Appendix C.3). The neoplasias were associated with chronic irritation of the forestomach and there was no treatment-related systemic oncogenic effect. Moreover, there was no evidence of carcinogenicity at the site of application when styrene oxide was applied repeatedly to the skin of rodents.

The oncogenic response to styrene oxide in the rodent forestomach after gavage treatment is inappropriate as a basis for making a quantitative risk assessment for human beings exposed to styrene by inhalation. The relevance of rodent forestomach tumours for human risk is quite controversial.

Although conventional approaches to quantitative risk assessment are not possible for styrene, biochemical/mechanistic data are useful in clarifying the role of styrene oxide as intermediate metabolite and may help to answer concerns about its occurrence upon styrene exposure. Such considerations require an understanding of numerous factors, including species differences in the relative rates of formation and degradation of styrene oxide in various organs and tissues. The physiologically-based pharmacokinetic (PB-PK) model developed by Filser and Nolan (in press) and validated with the above data (section 2) provides a useful tool for these comparisons. The subsequent evaluation is therefore based on the experimental data and the PB-PK model comparing the internal styrene oxide dosimetry (AUC_{SO}) in the blood of laboratory animals and man under various exposure conditions to styrene and styrene oxide. Thus a perspective on the differences in tissue

styrene oxide levels in man occupationally exposed to styrene and in rodents showing no or minimal responses following exposure to styrene or styrene oxide is provided. In using this approach it is assumed that human or animal tissues show similar biological responses with comparable doses of styrene oxide. Since the area under the blood concentration time curve (AUC) is the integral of the toxicokinetic behaviour of a chemical, this parameter rather than peak blood levels has been used as surrogate for tissue dose. These AUC_{SO} values will then be used to examine the putative hazard of styrene for man. In this document this will be referred to as a "semiquantitative margin of safety" ("SMS").

This basic concept of a qualitative approach to risk assessment has recently been adopted by the "Committee on Carcinogenicity" in the Department of Health (HSE; 1991).

3.1.1. STYRENE OXIDE DOSIMETRY (AUC_{SO}) IN MAN EXPOSED TO STYRENE

Studies reported by Lof *et al* (1986a) indicated that maximum styrene oxide blood levels were of the order of 3.0×10^{-5}mmol/l when measured in human volunteers exposed to 70ppm styrene. This value is comparable with the maximum predicted styrene oxide blood concentration of 2.8×10^{-5}mol/l derived by using the PB-PK model (Figure 1). The close agreement between the measured and predicted values indicates that the PB-PK model is a reliable tool for estimating blood styrene oxide concentrations in man exposed to styrene.

The predicted blood styrene oxide concentrations in human beings exposed for 8 hours to 10, 20, 50, 70 or 100ppm styrene are shown in Figure 1, and the resulting AUC_{SO} values in man at these styrene exposure concentrations are compiled in Table 1. The predicted AUC_{SO} values increase linearly over a styrene exposure concentration range of 10 to 100ppm, as indicated by an approximate 10-fold increase in AUC_{SO} values with the 10-fold increase in styrene exposure concentration. National occupational exposure standards for styrene currently range from 10ppm to 100ppm. For the purpose of estimating the "SMS" values, a mid-range value of 50ppm has been used for man.

3.1.2. STYRENE OXIDE DOSIMETRY (AUC_{SO}) IN RATS AND MICE FOLLOWING ORAL ADMINISTRATION OF STYRENE OXIDE

In the bioassay reported by Lijinsky (1986) male and female Fischer 344 rats were given 275 or 550mg/kgbw, and B6C3F1 mice were given 375 or 750mg/kgbw styrene oxide in corn oil, 3 times per week, for up to 104 weeks. In mice, the high dose exceeded the maximum tolerated dose (MTD), as indicated by high levels of mortality. Consistent with the results of other styrene oxide bioassays (Appendix C.3), a high, dose-related