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8EHQ - 0798 - 13707

PDCN: 88960000187

Attention: TSCA 8(e) Coordinator

Re: Follow up; Styrene (CASRN 100-42-5); Two-Year Mouse Study;
TSCA Section 8EHQ-96-13707

Dear Sir or Madam:

Enclosed please find Volume I of the final report for a 104 week repeat dose inhalation combined toxicity/carcinogenicity study in mice conducted by Huntingdon Life Sciences Ltd. and sponsored by the Styrene Information and Research Center (SIRC).¹ This filing is intended to supplement previously-submitted information identified by the document tracking number 8EHQ-96-13707 under section 8(e) of the Toxic Substances Control Act (TSCA).

The executive summary (pp. 14-15) contained in Volume I summarizes the final results of data previously reported under section 8(e). Because the study identifies effects on certain end points in the mouse that have not been observed in styrene workers, or in the SIRC-sponsored chronic rat study, SIRC has already begun additional research intended to permit a more definitive interpretation of the chronic mouse data. Of particular interest is the relevance of the effects observed in the mouse to human health risk and whether the extrapolation of these effects to assess human health risk is based on sound science. Research already under way on the metabolism and mode of action of styrene is expected to be vital to this evaluation.

The final report is an extensive, ten-volume (3,400 pages) compilation. A complete table of contents and the main body of the report are contained in the enclosed

¹ Section 8(e) information pertaining to this study was previously submitted on SIRC's behalf by The Society of the Plastics Industry, Inc. under EPA No. 8EHQ-96-13707. SIRC, a former special purpose group of SPI, recently became an independently incorporated organization and is submitting supplemental information under the same docket number to facilitate the Environmental Protection Agency's ability to reference section 8(e) submissions associated with the mouse study. Moreover, it is appropriate to submit the final report as supplemental 8(e) information because the report is intended to further evaluate, interpret, and confirm prior submissions and finalizes information on this study that was submitted on a preliminary basis.



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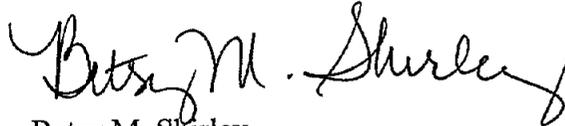
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The Styrene
Information
and Research
Center, Inc.
(SIRC)

Volume I. SIRC can provide the Agency with the appendices to the final report that are contained in Volumes 2-10 upon request. SIRC is also submitting the enclosed TSCA Health and Safety Study cover sheet to comply with the Agency's request that all section 8(e) data be voluntarily provided in this format.

If you have additional questions or comments, I can be contacted at 202-974-5319.

Sincerely yours,

A handwritten signature in black ink that reads "Betsy M. Shirley". The signature is written in a cursive style with a large initial "B" and a long, sweeping tail on the "y".

Betsy M. Shirley
Executive Director

Enclosures

TSCA HEALTH & SAFETY STUDY COVER SHEET - revised 6/25/96

TSCA CBI STATUS:

CHECK IF THIS PAGE CONTAINS CONFIDENTIAL BUSINESS INFORMATION (CBI)

Clearly mark the confidential information with bracketing and check the box in the appropriate section (Contains CBI).
Submit a sanitized cover sheet with CBI deleted. Mark the sanitized copy, "Public Display Copy" in the heading.

1.0 SUBMISSION TYPE <input type="checkbox"/> Contains CBI <input type="checkbox"/> 8(d) <input checked="" type="checkbox"/> 8(e) <input type="checkbox"/> FYI <input type="checkbox"/> 4 <input type="checkbox"/> OTHER: Specify _____ <input type="checkbox"/> Initial Submission <input checked="" type="checkbox"/> Follow-up Submission <input type="checkbox"/> Final Report Submission Previous EPA Submission Number or Title if update or follow-up: 8EHQ-96-13707 Docket Number, if any: #88-96-0000187 <input type="checkbox"/> continuation sheet attached																										
2.1 SUMMARY/ABSTRACT ATTACHED (may be required for 8(e); optional for §4, 8(d) & FYI) <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	2.2 SUBMITTER TRACKING NUMBER OR INTERNAL ID 8EHQ-96-13707	2.3 FOR EPA USE ONLY																								
3.0 CHEMICAL/TEST SUBSTANCE IDENTITY <input type="checkbox"/> Contains CBI <i>Reported Chemical Name (specify nomenclature if other than CAS name):</i> CAS# <u>100-42-5</u> Purity <u>98.8 to >99.5</u> <input checked="" type="checkbox"/> Single Ingredient <input type="checkbox"/> Commercial/Tech Grade <input type="checkbox"/> Mixture Trade Name: <u>STYRENE</u> Common Name: <u>STYRENE</u>																										
<table style="width:100%; border-collapse: collapse;"> <thead> <tr> <th style="width:30%;"></th> <th style="width:20%; text-align:center;"><u>CAS Number</u></th> <th style="width:30%; text-align:center;"><u>NAME</u></th> <th style="width:20%; text-align:center;"><u>% WEIGHT</u></th> </tr> </thead> <tbody> <tr> <td>Other chemical(s) present in tested mixture</td> <td style="text-align:center;">71-43-2</td> <td>Benzene</td> <td style="text-align:center;"><1-8 ppm</td> </tr> <tr> <td></td> <td style="text-align:center;">100-41-4</td> <td>Ethylbenzene</td> <td style="text-align:center;">149-280 ppm</td> </tr> <tr> <td></td> <td style="text-align:center;">25247-68-1</td> <td>Styrene Dimer</td> <td style="text-align:center;"><1-15 ppm</td> </tr> <tr> <td></td> <td style="text-align:center;">96-09-03</td> <td>Styrene Oxide</td> <td style="text-align:center;"><1-7 ppm</td> </tr> <tr> <td></td> <td style="text-align:center;">33394-60-4</td> <td>Tertiary Butyl Catechol</td> <td style="text-align:center;">4-34 ppm</td> </tr> </tbody> </table>				<u>CAS Number</u>	<u>NAME</u>	<u>% WEIGHT</u>	Other chemical(s) present in tested mixture	71-43-2	Benzene	<1-8 ppm		100-41-4	Ethylbenzene	149-280 ppm		25247-68-1	Styrene Dimer	<1-15 ppm		96-09-03	Styrene Oxide	<1-7 ppm		33394-60-4	Tertiary Butyl Catechol	4-34 ppm
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4.0 REPORT/STUDY TITLE <input type="checkbox"/> Contains CBI STYRENE: 104 WEEK REPEAT DOSE INHALATION COMBINED TOXICITY/CARCINOGENICITY STUDY IN MICE <input type="checkbox"/> continuation sheet attached																										
5.1 STUDY/TSCATS INDEXING TERMS [CHECK ONE] HEALTH EFFECTS (HE): <input checked="" type="checkbox"/> ENVIRONMENTAL EFFECTS (EE): _____ ENVIRONMENTAL FATE (EF): _____																										
5.2 STUDY/TSCATS INDEXING TERMS (see instructions for 4 digit codes) STUDY TYPE: <u>CTCA</u> SUBJECT ORGANISM (HE, EE only): <u>MICE</u> MAMM/ ROUTE OF EXPOSURE (HE only): <u>INHL</u> VEHICLE OF EXPOSURE (HE only): _____ Other: _____ Other: _____ Other: _____ Other: _____																										
6.0 REPORT/STUDY INFORMATION <input type="checkbox"/> Contains CBI <input checked="" type="checkbox"/> Study is GLP Laboratory <u>HUNTINDGON LIFE SCIENCES LTD.</u> Report/Study Date <u>MAY 28, 1998</u> Source of Data/Study Sponsor (if different than submitter) _____ Number of pages <u>3400</u> <input type="checkbox"/> continuation sheet attached																										
7.0 SUBMITTER INFORMATION <input type="checkbox"/> Contains CBI Submitter: <u>BETSY M. SHIRLEY</u> Title: <u>EXECUTIVE DIRECTOR</u> Phone: (202) 974-5319 Company Name: <u>THE STYRENE INFORMATION AND RESEARCH CENTER, INC.</u> Company Address: <u>1801 K STREET, N.W., SUITE 600K, WASHINGTON, D.C. 20006</u> Submitter Address (if different): _____ Technical Contact: <u>BETSY M. SHIRLEY, STYRENE INFORMATION AND RESEARCH CENTER</u> Phone: <u>(202)974-5319</u> <input type="checkbox"/> continuation sheet attached																										
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STYRENE

104 WEEK REPEAT DOSE INHALATION COMBINED TOXICITY/CARCINOGENICITY
STUDY IN MICE

Volume 1

Report

CONFIDENTIAL

SYI 7/963830

STYRENE

**104 WEEK REPEAT DOSE INHALATION COMBINED TOXICITY/CARCINOGENICITY
STUDY IN MICE
Volume 1**

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Report issued 28 May 1998

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid, Statutory Instrument No. 654.

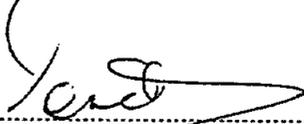
Good Laboratory Practice, The United Kingdom GLP Compliance Programme, Department of Health & Social Security 1989, and subsequently the United Kingdom Good Laboratory Practice Regulations 1997 Statutory Instrument No. 654.

EC Council Directive, 87/18 EEC of 18 December 1986, (No. L 15/29).

Good Laboratory Practice in the testing of Chemicals OECD, ISBN 92-64-12367-9, Paris 1982, subsequently republished OECD Environment Monograph No. 45, 1992.

United States Environmental Protection Agency, (TSCA), Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).


.....
Derek W Coombs, B.Sc.,
Study Director,
Huntingdon Life Sciences Ltd.

22 May 1998
.....
Date

QUALITY ASSURANCE STATEMENT

This report has been audited by Huntingdon Life Sciences Quality Assurance Department (Huntingdon). The methods, practices and procedures reported herein are an accurate description of those employed at Huntingdon during the course of the study. Observations and results presented in this final report form a true and accurate representation of the raw data generated during the conduct of the study at Huntingdon.

Inspections were made by the Quality Assurance Department of various phases of the study as conducted at Huntingdon and described in this report. The dates on which the inspections were made and the dates on which findings were reported to the Study Director and to Management, Huntingdon Life Sciences are given below.

Phase of Study	Date of Inspection	Date of Reporting
Protocol Review	15 August 1994	15 August 1994
Ophthalmoscopy	23 August 1994	23 August 1994
Study preparation	1 September 1994	2 September 1994
Animal husbandry	14 - 15 September 1994	16 September 1994
Test substance control		
Test atmosphere generation		
Aerosol analysis		
Clinical signs		
Data audit		
Serology sampling	25 October 1994	26 October 1994
Animal husbandry	2 November 1994	3 November 1994
Test substance control		
Test atmosphere generation		
Aerosol analysis		
Clinical signs		
Data audit		
Blood sampling	29 November 1994	29 November 1994
Animal husbandry	31 January 1995	1 February 1995
Test substance control		
Test atmosphere generation		
Aerosol analysis		
Clinical signs		
Data audit		
Serology sampling	14 February 1995	14 February 1995

Phase of Study	Date of Inspection	Date of Reporting
Blood sampling	28 February 1995	28 February 1995
Animal husbandry }	2 May 1995	2 May 1995
Test substance control }		
Test atmosphere generation }		
Aerosol analysis }		
Clinical signs }		
Data audit }		
Animal husbandry }	31 July - 3 August 1995	3 August 1995
Test substance control }		
Test atmosphere generation }		
Aerosol analysis }		
Clinical signs }		
Data audit }		
Serology sampling }		
Styrene oxide determination }		
Ophthalmoscopy }	29 August - 1 September 1995	1 September 1995
Blood sampling }		
Blood sampling }	6 September 1995	7 September 1995
Urinalysis }		
Terminal procedures }		
Animal husbandry }	23 October 1995	24 October 1995
Test substance control }		
Test atmosphere generation }		
Aerosol analysis }		
Clinical signs }		
Data audit }		
Serology sampling	23 November 1995	24 November 1995
Serology sampling	16 January 1996	16 January 1996
Animal husbandry }	23 January 1996	23 January 1996
Test substance control }		
Test atmosphere generation }		
Aerosol analysis }		
Clinical signs }		
Data audit }		
Room air analysis	6 February 1996	6 February 1996

Phase of Study	Date of Inspection	Date of Reporting
Blood sampling	27 February 1996	28 February 1996
Urinalysis } Blood sampling } Terminal procedures }	5 March 1996	5 March 1996
Animal husbandry } Test substance control } Test atmosphere generation } Aerosol analysis } Clinical signs } Data audit }	23 April 1996	23 April 1996
Terminal procedures	15 July 1996	15 July 1996
Animal husbandry } Test substance control } Test atmosphere generation } Aerosol analysis } Clinical signs } Data audit }	22 July 1996	23 July 1996
Room air analysis	8 August 1996	8 August 1996
Ophthalmoscopy	23 August 1996	28 August 1996
Blood sampling } Urinalysis } Terminal procedures }	2 - 3 September 1996	3 September 1996
Process inspections		
Clinical pathology Haematology	8 August - 9 September 1994	9 September 1994
Clinical chemistry/urinalysis	3 - 5 October 1995	5 October 1995
Automated systems	4 - 7 December 1995	8 December 1995
Haematology	2 - 6 February 1996	7 February 1996
Clinical chemistry/urinalysis	4 - 26 April 1996	26 April 1996
Automated systems	10 - 11 July 1996	18 July 1996

Phase of Study	Date of Inspection	Date of Reporting
Slide production	24 August - 4 September 1995	6 September 1995
Slide production	20 - 21 February 1996	23 February 1996
Slide production	17 - 18 June 1996	20 June 1996
Slide production	12 - 13 September 1996	13 September 1996
Slide reading	5 October 1995	5 October 1995
Slide reading	26 March 1996	27 March 1996
Slide reading	25 June 1996	28 June 1998
Slide reading	10 September 1996	11 September 1996
Date of reporting audit findings of the study and final report to Study Director and Management		22 May 1998



.....
 Kevin P. de-Salis, B.A. (Hons.), C.Biol., M.I.Biol., Dip.R.Q.A.,
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 Huntingdon Life Sciences Ltd.

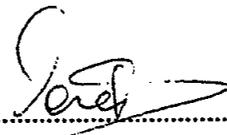


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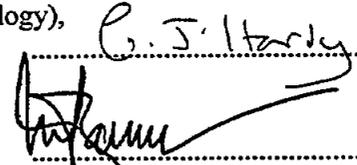
RESPONSIBLE PERSONNEL

STUDY MANAGEMENT

Derek W. Coombs, B.Sc.,
Study Director.


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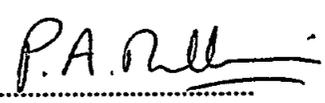
OPHTHALMOSCOPY

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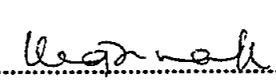

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PATHOLOGY

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MASS SPECTROMETRY

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SUMMARY

This study was performed to assess the chronic toxicity and carcinogenic potential of styrene, administered by whole-body inhalation exposure to CD-1 mice over a period of 2 years.

Exposure was carried out for 6 hours a day, 5 days a week at exposure levels of 0, 20, 40, 80 or 160 ppm. The initial group size was 70 mice of each sex, of which 10 mice of each sex were allocated for sacrifice following 52 and 78 weeks of exposure. Due to increased mortality in female control mice terminal sacrifice took place during Week 98. Male mice were sacrificed as scheduled following 104 weeks of exposure.

Samples of blood were removed from mice immediately after a typical exposure (Week 74) and analysed for styrene monomer and styrene oxide. The results obtained were as follows:

Dose (ppm)	Styrene monomer (ng/g)		Styrene oxide (ng/g)	
	Male	Female	Male	Female
0	0	0	0	0
20	69	30	BLQ	BLQ
40	177	106	2.5	1.4
80	654	527	11.8	6.2
160	1461	1743	33.5	20.1

During the first week of exposure increased activity by mice exposed to 160 ppm was evident, compared with control mice. This activity was most obvious at the start of exposures and was not notable during the remainder of the study.

Two female mice exposed to 160 ppm styrene died (one found dead and the other dying in transit to the necropsy area) during the first two weeks of exposure. These deaths were considered treatment-related. Discoloured areas of the liver surface were seen in one mouse at necropsy. Microscopic examination revealed hepatocyte necrosis in both which was considered a factor contributory to death. There were no other effects of styrene exposure on survival in males or females.

Statistical analysis of the bodyweight gain data showed that during the first 13 weeks of the study mice of both sexes exposed at 160, 80 or 40 ppm gained statistically significantly less weight than control mice. This effect was most apparent in male mice and at the 160 ppm exposure concentration.

Week	Weight gain as % of control							
	20 ppm		40 ppm		80 ppm		160 ppm	
	Male	Female	Male	Female	Male	female	Male	Female
53	100	100	92	97	77	100	69	97
104 (97)	92	(100)	92	(85)	77	(92)	69	(85)

Reduced food consumption in mice of both sexes exposed to 160 ppm styrene and males only exposed at 80 ppm styrene was evident during the first 13 weeks of the study. For the remainder of the study food consumption of groups exposed at 160 ppm (both sexes) and 80 ppm (males only) was reduced compared with controls. Lower food conversion efficiency was evident during the first 8 weeks of the study, especially in male mice exposed to 80 and 160 ppm styrene. Increased water consumption was recorded for mice exposed to 160 ppm styrene during the first 4 weeks of the study. This was considered a non-specific reaction commonly seen in inhalation studies performed with materials of an irritant nature.

No treatment-related effects on ophthalmic parameters, haematology, blood biochemistry, urinalysis and organ weight were evident during the study. Serological analyses of blood samples removed at 8 week intervals from sentinel mice did not reveal the presence of positive titres for any of the range of pathogenic organisms tested.

Macroscopic examination at necropsy following 97 weeks of exposure (female mice) and 104 weeks of exposure (male mice), revealed an increase in lung masses in treated groups compared with controls. The increases in both sexes were not dose-related.

Statistical analysis of the data by Fisher's Exact Test showed that there was a significant increase in the incidence of pulmonary bronchiolar-alveolar adenomas in males receiving 160, 80 or 40 ppm and in females receiving 160, 40 or 20 ppm styrene. This effect showed no clear dose-relationship. The incidence of bronchiolar-alveolar carcinomas was also significantly increased in females receiving 160 ppm. The results of the analysis of the combined tumour data (adenoma and/or carcinoma) was similar to that of benign tumours alone.

Further analysis by 'time-to-tumour' statistical methods confirmed the findings from the Fisher's Exact Test but also showed that, for benign and malignant tumours combined, the incidence was significantly increased in the 20 ppm dose group for both sexes.

Analysis of the size (diameter) of lung tumours revealed an increase in the percentage of small tumours in mice receiving styrene, particularly in males, and this was reflected by a decrease in the mean size of tumours in treated males.

There was no apparent difference in the morphological characteristics of lung tumours between control and treated mice.

Non-neoplastic treatment-related histopathological lesions were seen in the lungs of mice from all groups receiving styrene. At the 52 and 78 week kills the changes comprised decreased eosinophilia of the epithelial cells in the terminal bronchioles and bronchiolar epithelial hyperplasia which also extended into alveolar ducts. At the terminal kill, there was also an increased incidence of foci or areas of bronchiolar-alveolar hyperplasia in all groups treated with styrene when compared with the controls. Bronchiolar epithelial hyperplasia was focal in distribution in the lower dosage groups but, during the latter stages of the study, the lesion became more widespread and the epithelium often showed folding, with a consequent papillary appearance at the higher dosage levels. There was no evidence of inflammatory lesions in the affected areas.

Treatment-related changes were also present in the nasal passages in male and female mice from all dosage groups. These changes were mainly confined to the olfactory epithelium and underlying structures and were located predominantly in the dorso-median airway, but extended to the dorso-lateral and median airways in the higher dose groups at later stages of the study. The major findings were respiratory metaplasia of the olfactory epithelium and changes to the underlying Bowman's glands, including dilatation, respiratory metaplasia, hyperplasia, eosinophilic material/debris and cholesterol clefts. The lesions showed progression with time. At 52 weeks, respiratory metaplasia was not seen in the olfactory epithelium in mice receiving 20 ppm, but by 78 weeks it was present in a proportion of mice from all groups receiving styrene. Bowman's gland changes were present in all groups receiving styrene at 52 weeks. In addition to these changes, atrophy of the olfactory nerve fibres was seen in mice receiving 40, 80 or 160 ppm. These mice included one mouse treated at 40 ppm killed at 52 weeks. Focal loss of bone from the turbinate were also seen more frequently as the study progressed. In some mice with marked changes, there was a reduction in width of the lamina propria in the affected area and this was associated with a loss of Bowman's glands and olfactory nerve fibres.

INTRODUCTION

This study, carried out in the Department of Inhalation Toxicology at Huntingdon Life Sciences Ltd, Huntingdon Research Centre, England, was designed to assess the effects of exposure of mice to the vapour of styrene over a period of 104 weeks. Exposures were carried out on 5 days of each week for 6 hours per day.

The initial group size was 70 males and 70 females. Interim kills of 10 male and 10 female mice from each group were scheduled following 52 and 78 weeks of exposure. Therefore, the group size from Week 53 onwards was 60 males and 60 females, and 50 males and 50 females from Week 79 onwards.

Due to increased mortality of female mice in Group 1 (Air control) terminal sacrifice of surviving females was performed during Week 98 of the study. Performance of scheduled terminal (Week 104) clinical and laboratory investigations was rescheduled for female mice during Weeks 96 and 98. Mortality of male mice was such that termination remained as scheduled for Week 105.

This report provides details of the methodology and presents all the results obtained during the study. A total of 10 long-term animal studies have been performed with styrene to date, and two additional long-term studies have been conducted on a mixture containing 70% styrene and 30% β -nitrostyrene. Nine of these studies have been reviewed (McConnell and Swenberg, 1993, 1994).

Although most of these studies have deficiencies in design or conduct, the available data are nevertheless substantial and complex. Six of the nine studies showed no statistically significant increase in tumours of any type, while the remaining three have major flaws or deficiencies which preclude definitive conclusions. A long term rat study, sponsored by SIRC, showed no statistically significant increase in tumours of any type.

The purpose of this study was to conduct a state-of-the-art chronic toxicity/oncogenicity inhalation bioassay in mice as a follow up to the previous chronic toxicity/oncogenicity inhalation bioassay in the rat. The inhalation route was chosen because it is the predominant potential route of human exposure.

The exposure levels on this study were 160, 80, 40, 20 and 0 ppm.

Twenty-five male and 25 female mice were included as sentinel animals for periodic (8-week intervals) blood sampling. The blood was analysed using ELISA methodology to establish the serological status of the population. The mice were housed singly and distributed among all the groups, but were not exposed in chambers.

The study was conducted in compliance with the principles of Good Laboratory Practice as set forth in:

Good Laboratory Practice, The United Kingdom GLP Compliance Programme, Department of Health & Social Security 1989, and subsequently the United Kingdom Good Laboratory Practice Regulations 1997 Statutory Instrument No. 654.

EC Council Directive, 87/18 EEC of 18 December 1986, (No. L 15/29).

Good Laboratory Practice in the testing of Chemicals OECD, ISBN 92-64-12367-9, Paris 1982, subsequently republished OECD Environment Monograph No. 45, 1992.

United States Environmental Protection Agency, (TSCA), Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Ministry of International Trade and Industry, Directive 31 March 1984, Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI.

This study complied with US EPA TSCA guideline Federal Register, 40 CFR 789.3320.

The study protocol and all amendments are presented in Appendix 78.

RELEVANT STUDY DATES

Protocol approved by:

Study Director:	11 August 1994
HRC Management:	11 August 1994
Study Sponsor:	18 August 1994

Animals arrived at Huntingdon Life Sciences,
Huntingdon Research Centre:

18 August 1994

Health check (Haematology, serology, necropsy):

19 August 1994

Exposures commenced:

5 September 1994

Ophthalmoscopy:

23,24 August 1994
29,30 August 1995
2 July 1996 (female only)
23 August 1996 (male only)

Haematology:

29 November 1994
28 February 1995
1 September 1995
27 February 1996
4 July 1996 (female only)
27 August 1996 (male only)

Biochemistry and urinalysis:

4 - 8 September 1995
4,5 March 1996
15,16 July 1996 (female only)
2,3 September 1996 (male only)

Styrene/styrene oxide in blood:
(Repeated)

23,24 November 1995
18 January 1996/1,2 February 1996

Interim kill
(after completion of 52 weeks of exposure): 4 - 8 September 1995

Interim kill
(after completion of 78 weeks of exposure): 4,5 March 1996

Terminal kill
(after completion of 97 weeks of exposure): 15 - 18 July 1996 (female only)
(after completion of 104 weeks of exposure): 2, 3 and 5 September 1996 (male only)

ANIMALS AND MANAGEMENT

ANIMALS

Seven hundred and ninety (395 male and 395 female) mice of the CD-1 strain, aged approximately 4 weeks were obtained from Charles River (USA), Portage, Michigan, USA on 18 August 1994.

All of the mice were examined upon receipt. On the day following arrival (19 August 1994) 10 male and 10 female mice were selected at random, tail marked 751 - 760 (male) and 761 - 770 (female) and designated as the health check group.

HEALTH CHECK

Blood samples for haematological and serological investigations were collected from the orbital sinus of the 10 male and 10 female mice assigned to the health check group (Nos. 751 - 770). The haematological parameters measured are detailed in the **LABORATORY INVESTIGATIONS** section of this report. These mice were then killed and examined macroscopically. No abnormalities were found that indicated ill health in the batch of animals supplied. Lungs, liver, kidneys, spleen and heart were preserved in buffered 10% formalin.

Low white cell numbers were recorded for these health check mice. No indications of disease were present, and the other haematological parameters measured were considered normal. It was concluded that a likely explanation for these low white cell values was the relatively high depth of anaesthesia, necessary in order to remove up to 0.6 ml of blood for both haematological and serological analyses. In order to check this supposition and to confirm the adequate health status of the mice on study, additional blood was removed from 5 male and 5 female reserve group mice (temporarily numbered 771 to 780 for this purpose) during Week -2 of the study (26 August 1994). Total white blood cell and differential counts only were analysed, and the samples were removed using the minimum level of anaesthesia necessary. The results obtained were considered normal for the age and strain of mice used.

The blood samples for serology were run into proprietary vials (Microtainer® Ltd) and the serum separated by centrifugation and frozen. The frozen serum samples were packed in solid carbon dioxide and despatched to:

J.R. Needham,
The Microbiology Laboratories,
56 Northumberland Road,
North Harrow,
Middlesex,
HA2 7RE.

The serum samples were screened, using the ELISA technique, for the following:

- GD-VIII;
- Mycoplasma pulmonis;
- Mouse hepatitis virus;
- Minute virus of mice;
- Polyoma virus;
- Pneumonia virus of mice;
- Sendai virus;
- Reovirus 3.

Prior to the start of exposures, on 2 September 1994 the pre-exposure serology, haematology, macroscopic pathology finding at health check necropsy, clinical observations and pre-exposure ophthalmoscopy data were reviewed by a Veterinary Officer. There was no evidence of disease or any condition which may have affected the conduct or outcome of the study.

There were no adverse findings and all mice were considered satisfactory for use on the study.

In addition, at 8-week intervals throughout the study, samples of blood were removed from 5 male and 5 female Sentinel mice for serological evaluation using the ELISA technique. The mice sampled at each interval were selected such that a rotation every 40 weeks occurred (at least for the first half of the study), equivalent to every fifth sampling event. However, due to unscheduled deaths this procedure was modified as appropriate during the second half of the study.

The Sentinel mice were housed exactly as the other mice on study, were distributed throughout all groups and remained on the cage racks during the 2-year investigation.

ALLOCATION

Allocation took place on 19 August 1994. Individual bodyweights were processed using a computer program which selected 770 mice (385 male and 385 female) for allocation to 7 groups such that the group mean bodyweights were approximately equalised. These animals were uniquely identified by numbers tattooed on the tail on 22, 23, 25 and 26 August 1994.

The identification of individual mice in the 7 groups together with the group designation and group mean bodyweights at allocation were as follows:

Group Designation	Mouse identification		Group mean bodyweight (g)	
	Male	Female	Male	Female
1 (Air control)	1 - 70	351 - 420	21.63	19.23
2 (20 ppm styrene)	71 - 140	421 - 490	21.57	19.17
3 (40 ppm styrene)	141 - 210	491 - 560	21.66	19.21
4 (80 ppm styrene)	211 - 280	561 - 630	21.64	19.21
5 (160 ppm styrene)	281 - 350	631 - 700	21.70	19.24
Sentinel	701 - 725	726 - 750	21.64	19.24
Health check	751 - 760	761 - 770		
(Reserves used for second health check bleed)	771 - 775	776 - 780		
Reserve (at allocation)	A - J	K - T	21.70	19.10

All allocated mice were subjected to pre-dose ophthalmic examination 23 and 24 August 1994 . Twelve mice (2 male and 10 female) were found to have unacceptable eye lesions and were removed from the study on the advice of the Veterinary Officer performing the ophthalmic examination. Mice from the reserve group replaced these rejected animals .

The rejected mice, together with the eye lesion(s) present and the reserve mice that were used as replacements were as follows:

Rejected mouse	Eye lesion	Replacement, reserve mouse*
143M	Anterior cortical opacity of the lens -right eye	A
232M	Nuclear cataract-right eye	E
371F	Central corneal opacity - left eye	K
375F	Diffuse posterior polar opacity - both eyes	L
385F	Diffuse posterior polar opacity - left eye	N
	Hyaloid remnant - right eye	
410F	Retinal disruption - both eyes	O
419F	Retinal disruption- right eye	S
517F	Cataract - right eye	T
430F	Retinal disruption - both eyes	M
457F	Diffuse posterior polar opacity - left eye	Q
636F	Cataract - right eye	P
+668F	Traumatic damage - left eye	R

* Due to identification procedures taking 4 days to complete it was not possible to finish tattooing by the time that pre-exposure ophthalmic examinations were due to be performed. As a result reserve mice were identified A to T on the tail during this examination. Each replacement was tail tattooed with the number of the mouse that it replaced.

+ Mouse NAD at ophthalmoscopic examination. Damage to the eye noted during examination on 25 August 1994.

The rejected mice were removed from the study and replacement animals allocated on 25 August 1994.

The remaining reserve animals were killed on the first day of exposure (5 September 1994).

The mice were approximately 6 weeks old at the start of exposures.

ACCOMMODATION

The mice were housed singly in suspended solid floored polypropylene cages with stainless steel mesh tops. Each cage was 15 cm wide, 33 cm long and 13 cm high. Hardwood chips (supplied by R.S. Supplies, Finedon, Northants, England) were provided as bedding. The bedding was changed twice each week. Clean cages were introduced at approximately 2-week intervals throughout the study. The mice were housed and exposed in a single room (Room 9, Building Y11; Schematic presented as Figure 1) and, additionally, after the start of the exposure period, each group was positioned on an individual cage battery (rack). Each test group battery was in a separate ventilated cabinet within the holding room in order to avoid the possibility of inhalation of test material from the fur of mice in other groups. Air entering the holding/exposure room was extracted separately through each of the ventilated cabinets.

Control mice were housed on racks in the same room as the test groups, but within a separate ventilated cabinet.

Cages and racks were cleaned using a tunnel washing machine with wash and rinse sections, manufactured by Wincanton Engineering, Sherbourne, Dorset, DT9 3ND, England. The detergent used was Cachel® obtained from Modular Systems Hygiene Services Ltd, England (Appendix 80).

The temperature and relative humidity of the holding room were recorded continuously using a Kent Clearspan TH105 chart recorder.

Holding room temperature and relative humidity remained within protocol defined limits, $21 \pm 3^{\circ}\text{C}$ and $55 \pm 15\%$ respectively for the majority of the time (Appendix 2). Details of any deviation from set limits are also presented in Appendix 2.

Lighting was controlled to give 12 hours light (07.30 - 19.30 hours) and 12 hours dark per 24 hours.

DIET

While in their cages, all mice had access to a weighed quantity of standard quality-controlled laboratory mouse food (SDS Rat and Mouse No. 1 SQC modified maintenance diet, Special Diets Services, Witham, Essex). The actual diet batches used during the study are presented in Appendix 4.

Analyses were made on all batches of diet used to establish levels of basic nutrients and of specified substances and micro-organisms likely to have been present in the feed components and which, if in excess of specified amounts, might have an undesirable effect on the test system. The information available did not indicate that any non-nutrient substance likely to influence the effect of the test compound could reasonably be expected to be present in the diet (see Appendix 3).

The analytical data have been lodged in Huntingdon Life Sciences Archives.

Tap water was available from moulded polypropylene or polycarbonate water bottles at all times while the mice were in their cages. The water bottles were rinsed and refilled daily and thoroughly cleaned at twice weekly intervals during the study.

Results of the routine physical and chemical analyses of water at source (sampling point, Grafham Final Water) as conducted by the supplier, Anglian Water Services Ltd, have been made available to Huntingdon Life Sciences. Additionally, levels of specified substances known to be present from time to time in local water and which, if in excess of the maxima recommended for (human) drinking water might have an undesirable effect on the test system, are determined in the tap water at approximately 6-monthly intervals. A list of the principal parameters measured is given in Appendix 3. The information available did not indicate that any substance likely to influence the effect of the test substance could reasonably be expected to be present in the drinking water.

The analytical data have been lodged in Huntingdon Life Sciences Archives.

TEST SUBSTANCE ADMINISTRATION AND EXPOSURE LEVELS**TEST SUBSTANCE**

Name: Styrene monomer.
 Chemical name: Phenylethylene or vinyl benzene.
 CAS number: 100-42-5
 Received from: Shell Chemicals UK Ltd,
 Carrington Works,
 Urmston,
 Manchester,
 M31 4AJ,
 ENGLAND.

Receipt, batch and usage:

The liquid styrene monomer was supplied in batches of 3 x 10 kg drums, each assigned a batch number by the Supplier (the initial batch was composed of 6 x 5 litre drums). Full details are presented in Appendix 5. Styrene from a total of 45 drums was used in this study.

Batch and purity analysis:

At the start and finish of use of each drum aliquots of styrene were taken for analysis. The aliquots were analysed by gas chromatography-mass spectrometry. Details of the methods and results are presented in Appendix 6.

Upon receipt an aliquot from each of the drums in each batch was removed, combined and analysed for benzene, ethylbenzene, styrene oxide, styrene dimer and tertiary butyl catechol (TBC). The range of values over the study period are given below:

Styrene monomer:	98.8 to >99.5 %
Benzene:	<1 to 8 ppm
Ethylbenzene:	149 to 280 ppm
Styrene dimer:	<1 to 15 ppm
Styrene oxide:	<1 to 7ppm
TBC:	4 to 34 ppm

Storage:

All drums were stored at 4°C except during use.

Stability:

The test substance supplied was stabilised at source with TBC.

Safety Data Sheet:

Appendix 7.

ADMINISTRATION

Styrene vapour was administered to mice by whole-body exposure for 6 hours a day, 5 days a week, for 104 consecutive weeks. The first exposure was on 5 September 1994 and the final exposure on 17 July 1996 (female mice) and 4 September 1996 (male mice).

Control mice received only air in an identical chamber to that used for the test groups.

Exposures on 2 to 4 September 1996 inclusive (Week 105), were performed during the terminal necropsy of surviving male mice.

EXPOSURE LEVELS

The target concentration for each group was as follows:

Group	Designation	Concentration (ppm)
1	Air control	0
2	Low dose	20
3	Low inter dose	40
4	High inter dose	80
5	High dose	160

The dose levels were chosen based on data generated in a 13-week toxicity study performed at Huntingdon Life Sciences (SYI 6/942537).

JUSTIFICATION FOR EXPOSURE LEVELS

In the 13-week toxicity study (SYI 6/942537) two of 10 female mice exposed to 200 ppm styrene died during the first week of exposures. Both showed marked necrosis of olfactory epithelium, and mouse 93 ♀ also showed minimal hyperplasia of transitional epithelium in the nasal passages. The liver of both mice showed marked centrilobular hepatocyte necrosis and congestion of centrilobular sinusoids, and hepatic necrosis was considered to be the factor contributory to death for these two mice. Increased sorbitol dehydrogenase activity and total bile acids were evident after one week of exposures in a sub-group of mice also exposed at 200 ppm, included specifically to assess any effects on the liver. Reduced bodyweight gain was evident in mice exposed at 200 ppm.

At termination following 13 weeks of treatment macroscopic liver changes, including irregularities in the surface topography and adhesions, were seen in female mice exposed to 200 ppm. Microscopic liver changes typified by hepatocyte loss and associated inflammatory changes were also seen at termination in mice exposed at 200 ppm, and to a lesser extent in occasional female mice exposed to 150 ppm.

Microscopic changes in the nasal passages, including atrophy of the olfactory epithelium and olfactory nerve fibres, together with dilatation, hypertrophy and hyperplasia of Bowman's glands were seen at all levels of exposure, 50, 100, 150 and 200 ppm. In general mice exposed to 50 ppm were less affected. These changes were typical of the known irritant nature of styrene vapour.

Based upon the findings in the 13-week study the upper exposure concentration was fixed at 160 ppm and the lower at 20 ppm, with intermediates of 40 and 80 ppm. At the selected High dose of 160 ppm evidence of liver involvement was expected, together with reduced bodyweight gain, at least over the initial few weeks of the study, and 160 ppm was regarded as the maximum tolerable dose.

EXPOSURE SYSTEM AND PROCEDURE

INHALATION CHAMBERS (Figure 2)

The inhalation chambers used have a nominal internal volume of approximately 2.43 m³. The body of a chamber has the approximate dimensions 1.34 m wide × 1.02 m deep and 1.45 m high. Upper (inlet) and lower (outlet) pyramidal figures are attached to the body. The lower pyramidal figure slopes down to the rear of the chamber. The chambers are of all-welded 316 stainless steel construction and are fitted at the front with hinged glass panelled doors for access. Door seals are effected using rubber moulded strips. A canister air distribution plenum is mounted in the upper pyramidal figure. A square tubular extract plenum, perforated on its lower surface, is located at the centre of the lower pyramidal figure 5 cm below the base of the figure. A 5 cm diameter drain is located at the rear centre of the lower figure.

Four sample ports are located in the back wall of the chamber. The sample ports in the back wall of the chamber connect with the stainless steel tubing of the sampling system. These extend into the chamber space for different distances in order to obtain samples from 4 different points within the chamber. The ends of these probes are fitted with 60 µm stainless steel filters to prevent ingress of animal fur. A fifth port in the rear wall is used to monitor the pressure within the chamber, and a sixth port in the side of the extract ducting immediately downstream of the chamber is used for monitoring of chamber temperature and humidity with a wet/dry thermistor probe.

During operation, filtered temperature and humidity controlled air was supplied to the chambers by an air handling unit separate from that supplying conditioned air to the room. The volume flow of air to the chambers was monitored using Venturi nozzles⁽¹⁾ and recorded by a computerised monitoring system connected to flow sensors mounted in the air supply ducts. Air (and the test vapour) entered the chamber at the apex of the upper pyramidal figure through a tangentially mounted duct and was extracted through the square plenum in the chamber base. Pressure within each exposure chamber was maintained at approximately 1 - 10 mm water below that in the room by adjustment of the inlet and extract fan settings. The chamber internal pressure was monitored using a Magnehelic gauge⁽²⁾ attached to a port located in the rear wall of each chamber.

During exposures, the animals were held in stainless steel mesh cages. A cage had 10 compartments each measuring 20 × 20 cm (maximum dimensions) and was 20 cm high. Each compartment held a single mouse and each animal had access to water during exposure from a water bottle mounted in each individual compartment. Cages were evenly distributed on 5 levels within a chamber and cage positions were altered according to a pre-arranged plan on a weekly basis.

(1) Type VN 32/P/B. Tekflo Ltd, Albany Road, Granby Industrial Estate, Weymouth, Dorset, England

(2) Dwyer Instrument Inc, Michigan City, Indiana, USA

During the first week of exposures all chambers were cleaned daily using a water spray. However, due to overnight retention of residual water on the framework/mesh of the cages chamber relative humidity was high at the start of the following exposure. With Sponsor agreement the cleaning regimen was changed so that washing of the chambers only took place post-exposure on the Friday of each week. This allowed the chambers to dry out over each week-end period. This regimen was considered not to affect the integrity of the study.

VAPOUR GENERATION SYSTEM (Figure 3)

The vapour generation system for Groups 4 (80 ppm) and 5 (160 ppm) comprised reservoirs of liquid styrene, liquid delivery lines containing particulate filters, metering valves and vapourisers immersed in a water bath maintained at a temperature of $60 \pm 1^\circ\text{C}$. Liquid was forced from the reservoir (under pressure with nitrogen) through the metering valves into the vaporisers where evaporation took place. The vapour was mixed with diluent air at 80 litres/minute and carried to the air entry points on the inhalation chambers. The liquid reservoir, liquid and vapour transport lines and metering valves were all constructed from stainless steel.

The upper curved surface of each liquid reservoir⁽³⁾ was fitted with a central, "O" - ring seal filler cap, a 3-way valve to allow pressurisation with nitrogen gas and pressure release following exposures and a safety "pop-off" valve set to operate at 5 psig above operating pressure. Each reservoir was mounted on an electronic load cell⁽⁴⁾ and the weight of the contents displayed continuously. The reservoirs were tared empty on the load cells prior to use on the study.

Liquid was transported from the base of each reservoir to a metering valve⁽⁵⁾. Particulate filters (stainless steel, $7 \mu\text{m}$ pore size⁽⁵⁾) were incorporated into the liquid lines between the manifold and each valve in an attempt to protect the valves by minimising the passage of any particulates present. The liquid passed by the valves was led to the generator through tubing approximately 3 mm in diameter.

The vapourisers used to generate at the Group 2 (20 ppm) and Group 3 (40 ppm) levels were supplied with liquid styrene contained in glass barrelled syringes fitted with PTFE plungers mounted on infusion pumps (Precidor® Type 5003). Each syringe was connected with the vapourisers using 1/16 inch PTFE tubing. The syringes were weighed when filled and again at the end of each exposure in order to calculate nominal usage from daily weight loss.

The all-glass vapourisers consisted of a glass body with an air-inlet at the base immediately below an internal fritted glass disc through which the air⁽⁶⁾ passed. The test liquid dripped on the centre of the disc from a capillary tube connecting with the test liquid supply. The resultant vapour produced was swept out of the vapouriser at the top through stainless steel delivery lines connected with the air inlet duct of each chamber.

⁽³⁾ Newson Gale Ltd, 51 Norsey Road, Billericay, Essex, CM11 1BG, England

⁽⁴⁾ Huntleigh Industrial Controls Ltd, Load Cell Division, Portman Moor Industrial Estate, East Moors, Cardiff, South Glamorgan, CF22 2HB

⁽⁵⁾ Nupro Co, Willoughby, Ohio 44094, USA

⁽⁶⁾ Compressed air source, particulate and carbon filtration, dew point approximately 2°C

Toggle valves in the liquid delivery lines allowed any one or all 4 liquid supply lines to be shut down quickly. All connections in the liquid delivery system were either welded or made with compression fittings.

The vapour generation system was housed in an extract cabinet to minimise contamination of the work environment. A schematic diagram of the room layout is presented in Figure 1.

At intervals of approximately 12 days the reservoirs were filled with styrene from one of the supply drums using nitrogen under pressure; the amount of liquid in each reservoir was indicated by the load cell display. At each fill event each reservoir was filled to a arbitrary target weight determined by the optimal minimum residence time of use for the substance under test. A series of stainless steel particulate filters (60 μm , 15 μm , 2 μm and 0.5 μm) were installed in the liquid supply line prior to entry into the reservoir filling system in order to protect the fine control needle valve from any foreign particulate material. The weight of each reservoir was recorded.

Syringes were filled by aspiration from the supply drum each day.

PROCEDURE

A detailed operating procedure was available to all staff within the exposure room throughout the study. This was updated as necessary. A copy of all procedure documents produced during the study have been retained with the raw data.

The mice were loaded into the exposure chambers. The time taken to load the animals was 'on average' between 35 and 45 minutes. When all the mice had been loaded into the chamber the chamber doors were sealed.

The temperature of the water bath was checked and the air supply to the generator was turned on and adjusted to the desired flow rate. The Low and Low Intermediate dose syringes were filled, weighed and their weight recorded, prior to mounting on the infusion pumps.

Each reservoir was pressurised with nitrogen gas, the weights recorded prior to the toggle valves being opened. Simultaneously with opening the toggle valves in the liquid feed line the infusion pumps were switched on and the chamber environment monitoring program was started. Liquid flowed from the reservoirs through the control valves and from the syringes to the generators where the vapour was produced. Any adjustments of the liquid flows during the study were made on the basis of the concentrations of styrene measured within the inhalation chambers⁽⁷⁾.

In general, exposures commenced approximately 08.30 - 09.00 on each day of exposure.

- (7) Control valve settings were established during initial trials which preceded the start of animal exposures. Following the introduction of animals into the inhalation chambers, some minor adjustments to the control valve settings were necessary. Thereafter, minimal adjustments only were usually required in order to maintain target concentrations.

Following daily exposures, the toggle valves were closed and the weight of each reservoir again recorded. In addition the infusion pumps were turned off, the syringes removed and their weights recorded.

The vaporisers were cleared of residual liquid by continuing to pass air through them for approximately 25 minutes. The air supplies were then closed down. The mice were unloaded from the exposure chambers and returned to their holding cages. The air control mice were removed towards the end of the 25 minute clearance period. As each group was unloaded the chamber door was shut, and the mice on the cage battery placed in an extract cabinet. The unloading procedure occupied a similar time to loading.

On days when blood samples were removed for styrene and styrene oxide analyses during Weeks 64 and when repeated during Week 74, staggered exposure took place, with a planned 20 minutes between each group. This allowed time for removal of blood from the 10 mice of each sex sampled in each group.

Maintenance of the vapour generation system during the study was largely confined to periodic changing of the filters in the liquid supply lines and cleaning of solenoids in the chamber sampling system.

During handling of the test material, and during operations involving the animals, protective garments, vinyl gloves and respiratory protection (either combined gas and particle filter⁽⁸⁾ or particulate filter⁽⁹⁾) were worn.

CHAMBER MONITORING DURING EXPOSURE

Information on the environment within each inhalation chamber was collected at intervals of 30 minutes during each 6-hour exposure period. The data collection sequence and display were controlled by a microprocessor. All information was displayed on a monitor as it was collected. A hard copy of the data for each chamber was printed immediately after collection. At the same time, the data were stored on a floppy disk. At the end of each exposure the data were reformatted and presented as separate records for each inhalation chamber used.

A Tulip compact 2 computer and screen connecting with an NEC pinwriter P6 plus was used to monitor and record the system performance during exposure. The displayed data were printed and stored on disk simultaneously.

The monitoring system was organised by a program designed for the purpose. The program was divided into three basic sections of operation;

- an initial pre-exposure setting up phase;
- the exposure monitoring phase and
- a post-exposure data collation.

⁽⁸⁾ Type FC6, Siebe Gorman Co Ltd, Gwent, Wales

⁽⁹⁾ Type 8810, 3M, UK Plc, Berkshire, England

Pre-exposure checks

In the initial phase, prompted by the program screen display, study identification, dates, times and other relevant study details together with barometric pressure, airflow transducer calibration, gas chromatograph calibration and actual chamber airflow data sets were entered and stored.

Exposure monitoring phase

Coincident with the start of generation by opening the liquid supply to the vapour generators, the monitoring phase was started. Each chamber's environment was monitored over a 6-minute cycle, during which airflow, temperature, and analysed concentration were recorded. During this cycle, calculation of relative humidity together with conversion of the analysed absolute concentration data to ppm was made. The data obtained during each cycle were displayed on the screen, printed as hard copy and stored on disk.

This cycle of monitoring took place in the following sequence, High dose, High-inter dose, Low-inter dose, Low dose and Air control and continued throughout the six-hour exposure period. A total of twelve cycles were recorded.

Nominal concentration

Due to the relatively low weight loss from each reservoir over each 6-minute cycle, and the consequent inability of the load cells used to consistently quantify the amounts used the weight of each reservoir was recorded at the start and finish of each exposure. The reported nominal concentration was calculated manually from the total weight loss of each reservoir over each exposure period divided by the total air volume passing through the chamber over the same period. Factors to account for liquid density, temperature and daily barometric pressure were incorporated into calculation of the nominal in terms of ppm.

Post-exposure phase

At the end of 6 hours, the individual cycle data collected for each chamber during exposure were collated, summarised and printed as part of the program function. The mean values, together with standard deviation were calculated for each parameter recorded. These data were printed and stored on disk. The chamber t₉₉ equilibration time when operated under the conditions described in this report was approximately 17 minutes. As a consequence the first data set produced for each chamber (i.e. the first 30 minutes of generation) was excluded from calculation of mean data.

At each sampling, the information collected from each chamber was as follows:

Temperature	(°C)
Relative humidity	(%)
Airflow	(litres per minute)

and in addition, for the chambers containing styrene vapour

Analysed concentration	(ppm)
------------------------	-------

The monitoring systems are described in detail below:

Temperature and relative humidity - Temperature and relative humidity measurements were based on the wet and dry bulb principle using probes manufactured within Huntingdon Life Sciences Ltd, Huntingdon Research Centre.

Two matched platinum resistance probes, in thermally isolating fittings, were mounted projecting into the exhaust duct at the rear of each chamber. One probe was covered by a close-fitting wick which connected with a water reservoir attached to the device. Relative humidity was calculated from dry and wet probe values using psychrometric data stored in a look-up table in the memory of the microprocessor.

Airflow - The volume flow of air to the inhalation chambers was measured using brass-bodied Venturi nozzles and associated differential pressure transducers and flow transmitters⁽⁸⁾. The Venturi nozzles were installed in the air supply duct work leading to each chamber, in accordance with recommendations for such devices.

Each nozzle used was supplied with a calibration of pressure against airflow. Mathematical descriptions of the calibrations were derived and incorporated into the system program to enable flows to be calculated from the signals provided by the transmitters. The integrated operation of the flow measuring system was checked by applying a range of pressure signals to the transducers and comparing the flows indicated by the system with the calibration data supplied by the manufacturer. Correction factors for the installation (to allow for actual, compared with calibration, conditions) were applied.

Concentration of styrene - The concentrations of styrene in air within the 4 inhalation chambers used to expose the animals were measured using a gas chromatograph (Appendix 8)⁽⁹⁾. Samples were also removed from the Air control chamber. One instrument was used.

The gas chromatograph was located in the laboratory adjacent to the cabinet in which the generation system was housed.

The instrument was connected with a selected sampling port by programmed switching of banks of valves under the control of the microprocessor. Gas sampling lines were 0.6 cm diameter stainless steel tubing. A secondary calibration loop circuit was used for calibration of the gas chromatograph and for daily checking of the standard response.

Two standard vapour mixtures were used daily to confirm the calibration during the study period of animal exposures. Previous to the start of animal exposures 4 standard calibration mixtures were used to obtain the standard response factor. Four standards were also used if considered necessary during the study in order to check any deviation from the acceptable standard area response (see below).

- ⁽⁸⁾ Venturi nozzles, Type VN 32/P/B, Transducers, Type TR 7, Transmitters, Type TR 2, all supplied by Tekflo Ltd, Albany Road, Granby Industrial Estate, Weymouth, Dorset, England.
- ⁽⁹⁾ Shimadzu GC-14A, Dyson Instruments, Hetton Lyons Industrial Estate, Hetton, Houghton le Spring, Durham, DH5 ORH

Before the start of animal exposures, the operating conditions for the gas chromatograph were identified and the instrument calibrated using prepared calibration gas mixtures. The criterion used for acceptance of satisfactory operation of the analysis system was that the values on any day should not deviate from the initial data by more than ± 2 standard deviations about the mean, calculated for each calibration standard in the original weighted regression. If the above was exceeded, 4 standard concentrations of styrene vapour were used each day until such time as the response returned within the original limits, or until sufficient data was accumulated to allow calculation of the new response factor and limit of acceptance.

The operating conditions for the gas chromatograph are presented in Appendix 8.

Mathematical descriptions (regression analysis) of the initial calibration were derived and incorporated into the system program to enable concentrations to be calculated from the signals provided by the chromatograph. The accumulated calibration data were reviewed at intervals during the study and, if necessary, the regression data incorporated into the program were revised. Details of such reviews are retained with the raw data.

Concentration of styrene in room air - Room air was collected into Tedlar® gas bags at quarterly intervals throughout the study and the styrene content (if any) measured. These samples were collected approximately mid-way through exposure on the day.

Concentration of styrene oxide - Once a month towards the end of exposure a sample was removed from each chamber using a gas tight syringe. Each sample was injected onto the column of a gas chromatograph, separate from the instrument used for styrene monomer analysis. The concentration of styrene oxide, if present, was determined using external standards. The conditions of analysis are presented in Appendix 9.

OBSERVATIONS

CLINICAL SIGNS

All cages were checked at least twice daily, once in the morning and again in the afternoon, for dead or moribund animals. These checks normally coincided with the transfer of the animals to and from the inhalation exposure chambers on exposure days (Monday to Friday), approximately 08.30 and 16.30 hours. On Saturdays and Sundays, a similar check was made except that the final check was carried out at approximately 1600 hours.

All signs of ill health, together with any behavioural changes or response to treatment, were recorded for individual animals. An individual animal record was maintained on the basis of:

- any observation, considered to be of possible importance, made at any time during the study.
- a careful external examination of individual animals including palpation for masses made at weekly intervals, at weekends, throughout the study.

BODYWEIGHT

The weight of each mouse, main and sentinel groups, was recorded at the time of allocation of animals to the experimental groups, once weekly during the first 13 weeks of exposure, and every 4 weeks thereafter. In addition, the weight of each animal was recorded at necropsy.

The bodyweights of animals which were either known, or appeared, to be losing weight were monitored at other times as requested by the Study Director.

FOOD CONSUMPTION

The weight of food consumed by each mouse, main and sentinel groups, was recorded on a weekly basis throughout the study.

WATER CONSUMPTION

The water consumption of each main group mouse was measured at intervals during the study. The bottles were weighed each day and the weight loss recorded. Measurement was performed daily for seven days during Weeks -1 (pre-dose), 1, 4, 12, 25, 51, 77, 96 (surviving female mice only) and 103 (surviving male mice only).

OPHTHALMIC EXAMINATION

The eyes of all mice allocated to the study were examined using a Keeler indirect ophthalmoscope. Examinations were performed pre-exposure, and on surviving main group mice during Weeks 52, 96 (female mice only) and 104 (male mice only) of exposure.

Prior to examination, drops of a tropicamide ophthalmic solution were administered to the eyes of all mice for dilation of the pupil.

LABORATORY INVESTIGATIONS

Sampling schedule:

The day following receipt, 10 male and 10 female mice, taken at random, were assigned as the health check group and had blood removed for haematological analyses. In addition, during the pre-dose acclimation period reserve group mice were bled to confirm white blood cell parameters.

During the study blood samples for haematological analysis were collected during Weeks 13, 26, 52, 78, 96 (female mice only) and 104 (male mice only) of the study. Urine samples were collected overnight prior to blood samples for biochemical analysis, which were removed immediately before sacrifice during weeks 53, 79, 98 (female mice only) and 105 (male mice only).

Ten male and ten female mice in each group were sampled for haematological, biochemical and urinalysis investigations. Where necessary, due to unscheduled deaths, replacement mice were bled. Up to and including Weeks 52 and 78 the mice sampled included the animals scheduled for interim kill during Week 53 and 79.

Sampling methodology:

Urinalysis: The mice were placed into individual metabolism cages after exposure. Urine was collected overnight for approximately 16 hours (1700 to 0900 hours) during which time all mice were deprived of food and water. At the end of the urine collection period the mice were returned to their holding cages and allowed free access to water only for at least 1 hour prior to further procedures on the day.

Haematology: All mice were allowed access to food and water overnight prior to removal of blood samples.

Biochemistry: Following access to water only after overnight urine collection, approximately 0.6 ml of blood were removed for analysis of blood biochemistry parameters. The mice were allowed access to food and water prior to sacrifice on the day of blood removal.

Blood samples were removed from the orbital sinus while each mouse was lightly anaesthetised by ether vapour.

Haematology

EDTA anticoagulant was used. The parameters measured, together with the methods and units used, were as follows:

Bayer Technicon H1E haematology analyser:

	Units
Packed cell volume (PCV)	%
Haemoglobin (Hb)	g/dl
Red cell count (RBC)	$\times 10^{12}/l$
Mean corpuscular haemoglobin concentration (MCHC) (Hb \times 100/PCV)	g/dl
Mean corpuscular volume (MCV): (PCV \times 10/RBC)	fl
Mean corpuscular haemoglobin (MCH): (Hb \times 10/RBC)	pg
Total white cell count (WBC Total)	$\times 10^9/l$

Differential white cell count (Diff): Preparation of blood smear, stained with modified Wright's stain. Standard microscopy, counting 100 cells:

Neutrophils	(N))
Lymphocytes	(L))
Eosinophils	(E)) $\times 10^9/l$
Basophils	(B))
Monocytes	(M))

Cell morphology -the most common morphological changes (anisocytosis, micro/macrocytosis, variation in colour, hypo/hyperchromasia, left shift, atypical/blast cells) was recorded as follows:

-	=	No abnormalities detected
+	=	slight
++	=	moderate
+++	=	marked

In the case of atypical/blast cells, or other abnormalities, confirmation or a written description from a blood film will be made.

Reticulocyte count (Retic) - microscopy of blood smear
(Dacie, J.V. and Lewis, S.M., *Practical Haematology*, 1966, 3rd Ed., p 28) %

Platelet count (Plts) $\times 10^9/l$

Biochemistry

The blood was placed into proprietary collection vials containing lithium heparin anticoagulant. The vials were centrifuged at 3200 `g' for 3 minutes and the plasma analysed for the parameters listed below:

The following parameters were analysed with a Roche Cobas centrifugal analyser, using the appropriate test kit

	Units
Creatine phosphokinase (CPK), also known as `creatine kinase' using BCL test kit Reaction temperature 30°C	mU/ml

The following parameters were analysed with an Hitachi 737 Clinical Chemistry Analyser:

Total Protein	g/dl
Albumin (Alb)	g/dl
Globulin (Glob) - By subtraction, total Protein (g/dl) minus albumin (g/dl)	g/dl
Urea nitrogen	mg/dl
Alkaline phosphatase (AP) - reaction temperature 30°C	mU/ml
Total bilirubin	mg/dl
Creatinine	mg/dl
Sodium (Na)	mEq/l
Potassium (K)	mEq/l
Calcium (Ca)	mEq/l
Inorganic Phosphorus (P)	mEq/l
Chloride (Cl)	mEq/l
Cholesterol (Chol)	mg/dl
Glucose-hexokinase mediated	mg/dl

Units

Glutamic-pyruvic transaminase (GPT), also known as 'alanine aminotransferase'
 Reaction temperature 30°C mU/ml

Glutamic-oxaloacetic transaminase (GOT), also known as 'aspartate aminotransferase'
 Reaction temperature 30°C mU/ml

Urinalysis

The following estimations were performed using the appropriate methodology, as described below:

Volume ml

SG

Colour and appearance (visual assessment only)

pH - by pH meter

Protein - Roche Cobas centrifugal analyser using modified method of
 Macart, M. and Gerbaut, L. *Clin.Chim. Acta.*, 1984, 141, 77 mg/dl

Qualitative tests:

Total reducing substances (TRS) - Clinitest®

Glucose)

Ketones)

Bile pigments) - Multistix®

Urobilinogen)

Haem pigments)

Clinitest® and Multistix® are diagnostic reagents obtained from Ames Company, Stoke Poges, England, and are used as qualitative indicators of analyte concentration. Results are reported according to the following convention:

Tr	=	"trace" of analyte
+	=	"small amount" of analyte
++	=	"moderate amount" of analyte
+++	=	"large amount" of analyte
++++	=	"very large amount" of analyte

For haem pigments this degree of differentiation is not possible and the results are reported as negative (O) or positive (+) only.

Microscopy: For microscopic examination, a portion of the urine sample was centrifuged at approximately 1500 g for 10 minutes and the resulting deposit spread on a microscope slide. The deposit was examined for the presence of the following:

Epithelial cells	E
Polymorphonuclear leucocytes	P
Mononuclear leucocytes	M
Erythrocytes	R
Organisms	O
Renal tubule casts	C
Other abnormal constituents	A

The grading of cell frequency in the centrifuge deposit is as follows:

- 0 = none found in any field examined
- 1 = few found in some fields examined
- 2 = few in all fields examined
- 3 = many in all fields examined

Styrene monomer/styrene oxide in blood

Blood was removed from the orbital sinus of 10 male and 10 female mice (scheduled for interim sacrifice following 78 weeks exposure) from each group during Week 64 of the study.

The blood samples were run into tubes prepared by the Department of Metabolism and Pharmacokinetics, who performed the analyses. Analyses were performed as a separate study, Huntingdon Life Sciences Study No. SYI/7A. The blood samples were analysed for styrene monomer and styrene oxide.

Due to time constraints removal of the samples was performed on 2 occasions, 23 and 24 November 1995, Week 64. Five male and 5 female mice from each group were sampled on each day. Collection from each group was staggered by 20 minutes in order to allow sufficient time for removal of blood, and in order to remove samples at approximately the same time post exposure for each group. Exposure on these 2 days were staggered as necessary in order to comply with the sampling procedure.

Analysis of these samples indicated the apparent presence of styrene in the blood samples from the control (Group 1) mice. As an appreciable storage time was involved prior to eventual analysis it was presumed that contamination had occurred in some way during storage, although the mechanism was unknown.

Therefore, in order to check the method validity and to confirm/deny the presence of styrene in air control mice, an additional set of samples was removed from 3 male and 2 female Air control group mice on 18 January 1996 (Week 72) and analysed immediately. No styrene was found in these samples.

With Sponsor approval the procedure was repeated on 1 and 2 February 1996 (Week 74) using the same number of mice and employing the exact procedure and methodology used during Week 64. The mice re-bled 18 January 1996 were not bled at this time. Analysis of this second set of samples was performed within 2 days of removal. Styrene was not detected in Air control samples.

Details of methodology and results are presented in Appendix 10. The investigations are also reported separately in Huntingdon Life Sciences Report No. SYI 7A/982611.

The data from the first set of samples are not presented in this report. The results are stored with the raw data from the study.

Venous blood smears

Venous blood smears were prepared, where practicable, from all mice sacrificed at the interim and terminal kills, and from all mice that were sacrificed for humane reasons, mass size or due to moribund condition throughout the study. Samples were not taken from any mouse found dead.

The smears were air dried, fixed in methanol and stained with modified Wright's stain and stored. Examination of these prepared smears was not performed.

TERMINAL STUDIES

Interim kill

Following 52 and 78 weeks exposure, interim kills took place during Weeks 53 and 79. Originally the first 10 male and 10 female mice from each group were scheduled for sacrifice at week 53 and the next 10 male and 10 female mice in sequence from each group were scheduled for sacrifice during week 79. However, in accordance with a protocol directive which stated that any mouse that died or was sacrificed was not to be replaced, unscheduled deaths reduced the number of mice sacrificed at both interim kills to the following:

Group	Interim kill Week 53	Interim kill Week 79
1 (Air Control)	10 male + 10 female	8 male + 8 female
2 (20 ppm styrene)	10 male + 10 female	10 male + 9 female
3 (40 ppm styrene)	9 male + 9 female	10 male + 8 female
4 (80 ppm styrene)	10 male + 10 female	8 male + 9 female
5 (160 ppm styrene)	10 male + 10 female	6 male + 10 female

Terminal kill

According to a directive agreed with the Sponsor contained in a protocol amendment if survival of Group 1 (Air control) fell below 50% the termination of all groups of the affected sex would take place. Therefore, after 97 weeks of exposure, all surviving female mice were killed. This decision was reached in consultation with the Sponsor as a result of lower survival in female Air control group mice.

Male mice were killed following 104 weeks of exposure.

The kill took place on weekdays: 15, 16, 17 and 18 July 1996, Week 98 (surviving female mice), and 2, 3 and 5 September 1996, Week 105 (surviving male mice). Where possible, equal numbers of mice from each group were sacrificed each day and all mice were exposed up until the day prior to which they were killed.

Any mouse found dead or sacrificed following 104 weeks of exposure was classified as a terminal kill animal and processed as such.

Surviving sentinel mice used for health screening purposes during the in-life phase of the study were killed and discarded after the last animal of the main study was killed.

Sacrifice

With the exception of mice from which urine and blood for blood biochemistry analyses was being collected, animals were allowed access to food and water overnight prior to sacrifice.

The mice were killed by intraperitoneal injection of Expiral® (pentobarbitone sodium). Each mouse was exsanguinated by severing of the brachial arteries.

At interim and terminal kills, a sample of bone marrow was aspirated from the femur of each mouse. A smear was prepared, air dried, fixed in methanol, stained with modified Wright's stain and stored for future possible examination, should histopathological abnormalities be found that would indicate this course of action.

Macroscopic pathology and organ weight analysis

All superficial tissues, including the urogenital orifices and tail, ear pinna, external auditory meatus, and eyes, were examined visually and by palpation for distortion, swelling or other evidence of tumour formation; similar attention was given to the mammary tracts and the subcutaneous structures. The external nares, buccal cavity and tongue were examined and the cranial roof removed to allow observation of the brain, pituitary gland and cranial nerves. After ventral mid-line incision and skin reflection, all subcutaneous tissues were examined including regional lymph nodes, mammary and thyroid/parathyroid glands. The condition of the thoracic viscera was noted, with due attention to the thymus, lymph nodes and heart.

The abdominal viscera was examined before and after removal; the urinary bladder was briefly distended with fixative, opened and examined under low-power magnification.

The stomach and caecum were incised and examined and, in addition portions of duodenum, jejunum, ileum, colon and oesophagus were incised and examined. The lungs were removed and all pleural surfaces examined under suitable illumination. The liver was sectioned at intervals of a few millimetres; the kidneys were incised and examined. Any abnormalities in the appearance and size of the gonads, adrenals, uterus, intraabdominal lymph nodes and accessory reproductive organs were recorded. Any lesions suggestive of neoplasia were noted, including details of location size and multiplicity. Any evidence of adhesion or possible invasion to adjacent structures was noted.

The weights of the lungs, liver, kidneys, testes, ovaries, adrenals, brain and spleen of all mice killed during the interim or terminal sacrifice were recorded. Organ weights from mice found dead or killed *in extremis* were recorded at the pathologists discretion but were not included in statistical analyses.

Macroscopic examination of all animals at scheduled sacrifice took place under the supervision of a member of the Royal College of Pathologists.

The pathologist was also available for consultation during macroscopic examination of animals found dead or sacrificed in moribund condition.

Fixation of tissues/organs

Preservation of organs/tissues was in 10% neutral buffered formalin with the exception of the eyes, which were preserved in Davidson's fixative. The lungs were infused via the trachea and the nasal passages flushed with 10% neutral buffered formalin prior to immersion in fixative.

Preserved organs/tissues

The following were preserved from interim kill and terminal kill mice and also from all unscheduled deaths:

adrenals*(2)	kidneys*(2)	spinal column (spinal cord
alimentary tract*	gall bladder ^{(1)*}	from cervical, thoracic
(oesophagus ⁽¹⁾ , stomach ⁽²⁾ ,	larynx ⁽²⁾ and pharynx*(1)	and lumbar levels) ^(2LS+3TS)
duodenum ⁽¹⁾ , jejunum ⁽¹⁾ ,	liver*(5)	spleen*(1)
ileum ⁽¹⁾ , caecum ⁽¹⁾ , colon ⁽¹⁾ ,	^(a) lungs* (carina ⁽¹⁾ , all lobes	sternum (for bone and
rectum ⁽¹⁾)	and mainstem bronchi ⁽⁴⁾	marrow) ^{(1)*}
aorta*(1)	lymph nodes* (cervical	testes*(2) (with
bone marrow (smear)	and mesenteric) ⁽²⁾	epididymides) ⁽²⁾
brain* (medullary, cerebellar	mammary gland*(1)	thymus* (where present) ⁽¹⁾
and cortical sections) ⁽³⁾	ovaries*(2)	thyroid*(2) (with
eyes*(2)	pancreas*(1)	parathyroid) ⁽²⁾
femur* (with joint) ⁽¹⁾	pituitary*(1)	tongue
gall bladder*	prostate*(1)	trachea*(1)
Harderian gland*(2)	salivary gland*(1)	urinary bladder*(1)
head (*nasal cavity, ^(b)	sciatic nerve*(1)	uterus* (corpus and cervix) ⁽³⁺²⁾
paranasal sinuses, oral	skeletal muscle(1)	vagina
cavity,* nasopharynx, middle	seminal vesicles*(1)	other macroscopically abnormal
ear, teeth, lachrymal gland	skin*(1)	entities
and Zymbal's gland) ⁽³⁾		(including masses or tumours
heart*(1)		with their regional lymph
		nodes)*

() Number of sections examined histologically per organ/tissue

^(a) Lungs with carina (4+1) or trachea (with carina) (1+1)

^(b) Three levels of nasal passages were examined after Young J.T. (1981), *Histopathological Examination of the Rat Nasal Cavity. Fundamental and Applied Toxicology*, 1 : 309 - 312

LS Longitudinal section

TS Transverse sections

Microscopic examination

Light microscope examination was performed on 4 µm thick sections, stained with haematoxylin and eosin. The tissues/organs examined were those identified with * in the Preserved organs/tissues list.

Examination of the above organs/tissues at terminal kill was confined to mice in Group 1 (Air control) and 5 (160 ppm styrene), with the exception of nasal turbinates, lungs, liver, kidneys and macroscopic abnormalities, which were also examined from mice in Groups 2 (20 ppm styrene), 3 (40 ppm styrene) and 4 (80 ppm styrene).