Styrene Information and Research Center (SIRC)
1300 Wilson Boulevard, Suite 120[illegible] Arlington, Virginia 2[illegible]

Note on CBI

AR 31093

January 13, 2000

2000 JAN 19 AM 11:17

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Confidential Business Information Center
Room G099
Office of Pollution Prevention and Toxics
United States Environmental Protection Agency
401 M Street, Mail Code 7407, SW
Washington, DC 20460

Contain NO CBI

Dear Sirs:

The US EPA National Center for Environmental Assessment is currently reviewing the health-related data on styrene in the process of updating the IRIS file. Attached are copies of final reports of several recently completed studies on styrene that have not yet been published in the open literature, but which we think will be useful in understanding the target organ and species specific toxicity of styrene. We are submitting these studies through the TSCATS system so they can be available for public review. No confidential business information is being claimed for these studies.

Studies submitted:

Sumner, S., 1999. Metabolism of Styrene in Human Volunteers. CIIT report (9-10-99) to the Styrene Information and Research Center, pp. i-iv, 1-1 to 1-17, 2-1 to 2-8, Fig.2-1 to 2-12, 3-1 to 3-4, fig 3-1 to 3-4b.

Green, T. 1999a. Styrene: a 10 day inhalation study to investigate effects on mouse and rat lung. Central Toxicology Laboratory Report No. CTL/R/1546 (June 21, 1999), pp. 1-34.

Green, T. 1999b. The role of cytochrome P-450 in styrene induced cell division in the mouse lung. Central Toxicology Laboratory Report No. CTL/R/1409 (June 21, 1999), pp. 1-26.

Green, T. 1999c. The effects of styrene on mouse lung following oral dosing. Central Toxicology Laboratory Report No. CTL/R/1410 (June 21, 1999), pp. 1-12.

Green, T. 1999d. Nasal and lung histoautoradiography in male CD rats and male CD1 mice following a 6 hour inhalation exposure to[^{14}C]styrene.

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Central Toxicology Laboratory Report No. CTL/R/1411 (October 5, 1999), pp. 1-64, photographic supplement 1-16.

Green, T. 1999e. The metabolism of styrene by rat, mouse and human nasal cytochrome P-450's. Central Toxicology Laboratory Report No. CTL/R/1412 (October 20, 1999), pp. 1-31.

Green, T. 1999f. The role of cytochrome P450 in styrene-induced nasal lesions in the mouse. Central Toxicology Laboratory Report No. CTL/R/1418 (August 9, 1999), pp. 1-19.

If you have any questions, you can contact me.

Sincerely,

George Cruzan, PhD, DABT
SIRC Science Consultant
ToxWorks, 39 Manners Road, Ringoes, NJ 08551-1717
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email: ToxWorks@aol.com

cc: Betsy Shirley, SIRC
IRIS Subgroup

MR 31093

MEMO FOR RECORD **2 FEB 2000**

TO: **Gwen Shepard**
TSCA Docket Coordinator

FROM: **Pam Measley**
Alt TSCA Docket Coordinator

Gwen,

I called Mr. George Cruzan, SIRC Science Consultant on 2 Feb 00 at 3:25pm and asked about this. He stated that it was suggested by a Mr. Gary Foureman at RTP, NC (919-541-1183), that this be sent to us to be proce..ed under the TSCATS system. He also that "STYRENE" was being reviewed under the "IRIS PROGRAM".

I also asked him should we be concerned about the "Confidentiality Claim" stated within each of the copies of this study. He said that this the lab's "standard statement". SIRC's cover letter (1st paragraph, last sentence) says that no CBI is being claimed.

02/03/2000
Pam
This document WAS probably placed in the outgoing mail box By mistake. I ~~did~~ not process this document, but after Reviewing it and Ed Gross Reviewing this document.
This is an 8d study. I have given the document to Bernice for processing
This first paragraph, 2nd sentence states "health-related data" on styrene. 1st page attached

A 06

Metabolism of Styrene in Human Volunteers

Report Prepared for the Styrene Information and Research Center (SIRC)

Submitted by the Chemical Industry Institute of Toxicology (CIIT)

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Executive Summary

In rats, mice, and humans, styrene is metabolized to styrene oxide (SO) and subsequently hydrolyzed to produce a number of products (e.g., mandelic acid, MA; phenylglyoxylic acid, PGA; and hippuric acid, HA). MA and PGA have been used as markers of exposure in humans. Mercapturic acids derived from SO conjugation with glutathione have been detected in urine of exposed rodents, and have recently been suggested to occur in urine from occupationally exposed workers. Several metabolites (e.g., phenylethanol, phenylaceturic acid) that could be derived from a mechanism other than oxidation of styrene to SO, have been detected in urine and there has been one report of low levels of vinylphenol (possibly derived from ring-epoxidation) in urine from exposed workers. Rats and mice metabolize SO to a similar extent via glutathione conjugation and epoxide hydrolase, but the production of metabolites that lead to ring-open compounds or to phenylaceturic acid (PAA) is different between species. It is possible that a greater toxicity for styrene in mice, as compared to rats, may arise from the production of these metabolites. Understanding the toxicokinetics of styrene in humans and its comparison with rodents is important for extrapolation of human health risks associated with potential styrene exposures.

Healthy male volunteers were exposed to 50 ppm uniformly labelled carbon-13 styrene [$^{13}C_8$]styrene for 2 hr. Blood and expired air were time-course collected during and following exposure. Urine was time-course collected following exposure. Blood samples were analyzed for the concentration of styrene and styrene oxide and pooled blood samples were analyzed for the presence of a styrene-oxide-derived Hb-adduct. Exhaled air was analyzed for the concentration of styrene. Styrene-derived urinary metabolites were characterized using both HPLC and NMR spectroscopy.

The total uptake of styrene was similar (~ 3.9 mmol) between the four subjects. The subjects exhaled 1-2% of the dose as styrene following exposure. The blood concentration of styrene increased during the 2 hr exposure and rapidly decreased following exposure termination. The peak styrene blood concentration (8 µmol/ml) was reached between 1 hr 45 min and 2 hr following exposure initiation. Peak ~ 2 hr blood levels of SO were more variable (2.7 nmol/L-13.4 nmol/L) between individuals as compared with peak styrene blood levels (7-9 µmol/L). For three subjects, the peak SO blood level was reduced by 50% within 2 hr following exposure termination. The fourth subject had similar SO levels immediately and 2 hr following exposure termination, and the value was reduced 50% by 4 hr following termination of exposure. Based on adduct analysis of pooled globin samples, an adduct increment of ~ 0.003 pmol/g globin/ppm · hr was estimated for these subjects.

The end of exposure urinary levels of styrene (0.28 µmol/L) were 25-fold lower than styrene blood levels and rapidly declined following exposure termination. The total styrene recovered in urine accounted for < 0.02% of the total uptake. Maximum urinary concentrations of MA (117-436 µg/ml; ~ 22% of uptake) were reached ~ 2 hr following exposure termination for all subjects. Peak concentrations (0.035 to 0.12 mg/ml; ~ 6% of uptake) of PGA were reached between 4 and 6 hr following exposure termination (depending on the subject). MA was detected in urine up to 23 hr following exposure, while PGA was detected in urine for up to 42 hr.

HA (0.11 mM) and PAA (0.09 mM) were quantitated in urine collected 4 hr following exposure initiation and detected in urine collected 7 hr, but not 23 hr, following exposure initiation. Metabolites derived from other pathways of styrene metabolism (such as glutathione conjugation with SO, ring epoxidation) were not detected in these studies.

The metabolites MA, PGA, and HA are derived from hydrolysis of SO, while the metabolite PAA is derived from a second pathway of metabolism (presumably via conversion of styrene to phenylacetaldehyde). Based on evaluation of urinary metabolites, the majority of styrene metabolism in humans occurs via hydrolysis to SO (>95%), while a smaller percentage of styrene metabolism (< 5%) occurs via conversion to phenylacetaldehyde. These observations are consistent with a low level of conversion of styrene to phenylacetaldehyde (3-5%) in styrene exposed rats (125 - 500 ppm for 6 hr), and in contrast to a significantly greater metabolism through this pathway (~ 12 - 20%) for styrene exposed mice (125 - 500 ppm for 6 hr).

Organization of Study

This study was originated by the Chemical Industry Institute of Toxicology and was funded by the Styrene Information and Research Center. The exposure of human volunteers, and measurement of styrene and styrene-derived metabolites in expired air, blood, and urine were conducted by Dr. Gunnar Johanson and Dr. Agneta Löf at the National Institute of Working Life (NIWL, Stockholm, Sweden). Measurement of a styrene oxide-derived hemoglobin (Hb) adduct in blood was conducted by Dr. Siv Osterman-Golkar (Arrhenius Laboratories, Stockholm, Sweden). Dr. Susan Sumner (CIIT, Research Triangle Park, USA) analyzed urine for the presence of [$^{13}C_8$]styrene-derived metabolites.

Part 1: Metabolism of Inhaled ^{13}C-Labeled Styrene in Human Volunteers

1 Methods and procedures

1.1 Staff

The following personell participated in the NIWL part of the project:
- Gunnar Johanson, senior researcher, project leader
- Agneta Löf, senior researcher
- Bengt Sjögren, senior laboratory physician
- Lena Ernstgård, medical laboratory technologist
- Elisabeth Gullstrand, laboratory engineer
- Karl Sigvardsson, laboratory engineer

1.2 Ethical permission

The study was approved by the Regional Ethical Committee at the Karolinska Institute, Stockholm (dnr 98-225)

1.3 Recruitment of volunteers

Four male volunteers were recruited by asking subjects via the telephone. The subjects were selected from the register within the toxicokinetic research group. The register included individuals that had earlier participated, or expressed active interest in participating in similar experiments. During the recruitment process the subjects were informed orally and in writing about the aims, procedures, and potential health hazards of the study. Exposures were carried out only after medical examination and signing of the written information.

1.4 Chemicals

- Acetonitrile, HPLC Ultra, JT Baker Deventer Holland
- Creatinine, >99%, Merck Darmstadt Germany
- 1-Decane-sulfonic acid, sodium salt, 98%, Aldrich Steinheim Germany
- Dimethylsulfoxide (DMSO), 99.8% for spectroscopy, Merck Darmstadt Germany
- Ethanol, 95% Finsprit, Kemetyl Sweden
- ß-Glucuronidase, type H1, from Helix Pomatia, Sigma St. Louis
- Hippuric acid, 99%, Sigma St Louis USA
- Mandelic acid (MA), DL-, 99%, Lancaster Morecambe England
- Methanol, Lichrosolv, Merck Darmstadt Germany
- n-Hexane, >98% Lichrosolv, Merck Darmstadt Germany
- Ortho-methylhippuric acid, 98%, Tokyo Chemical Industry
- Phenylglyoxylic acid, >99%, Fluka Switzerland
- 1-Phenylpropylene oxide (PPO), CAS no. 14212-54-5, 98%,Sigma,St.Louis
- Potassium dihydrogen phosphate, crist, extra pure, Merck Darmstadt Germany
- Sodium acetate trihydrate, p.a., Merck Darmstadt Germany
- Styrene oxide (SO), CAS no. 96-09-3, 98+%, density 1.05, Lancaster Morecambe England
- Styrene, $^{13}C_8$-labelled, as test material, stabilized with hydroquinone, >99 atom% ^{13}C, 98.5% chemical purity, Isotec, Miamisburg OH, item no. T83-84036, batch no. CL2073-2
- Styrene (ST), unlabelled (for standard curves), CAS no. 100-42-5, density 0.906, bp 145.2°C, mw 104.15, vapor pressure (20°C) 0.6 kPa, purity 99%, Lancaster, Morecambe, England

1.5 Health examination

The medical examination consisted of four parts:
- Questionnaire
- General physical examination
- Clinical blood analyses
- Urine strip test

The questionnaire contained questions regarding symptoms and previous diseases. Specific questions concern:
- Hepatic or biliary affections, such as hepatitis
- Kidney disease
- Gastrointestinal disease
- Joint or muscular affections
- Diabetes
- Goiter and other thyroid diseases
- Heart diseases, such as angina
- Hypertension
- Asthma or chronic bronchitis
- Allergy
- Other eye affections
- Eczema
- Other affections

The questionnaire also contained questions on present use of pharmaceutical drugs, including aspirin and other non-prescription drugs, alcohol and smoking habits, and previous exposure to organic solvents.

A general physical examination was performed with particular attention to any findings in the questionnaire.
A fasting venous blood sample was analyzed with respect to:
- Alanine aminotransferase
- Albumin
- Alkaline phosphatase
- Aspartate aminotransferase
- Calcium
- Cholesterol
- C-reactive protein
- Creatinine
- Erythrocyte count
- Fructose amine
- Glucose
- Glutamyl transpeptidase
- Hematocrit
- Hemoglobin
- Hemogram (MCV, MCH, MCHC)
- Hepatitis B (HbsAg)
- Hepatitis C
- HIV 1+2
- Iron
- Leukocyte count
- Total iron binding capacity
- Triglycerides
- Thrombocyte count
- Urate

Blood chemistry analyses were carried out by Calab, Stockholm, Sweden.

On the day of exposure, pre-exposure urine was examined for presence of glucose, leukocytes, albumin, and hemoglobin by strip testing (Ecur 4-test).

The volunteer would only be included in the study if considered healthy. All findings in the medical examination were treated as patient information. It was stored in a locked file and controlled by the physician. In case of disease the subject would be referred to a specialist or a hospital for further treatment.

1.6 Medical surveillance during exposure

During the day of experiment a physician was present within the institute building available at all times and reachable via a cellular telephone. The name and telephone number of the physician was written in the individual exposure protocol. It was the responsibility of the physician to decide which measures should be taken in case of medical complications.

1.7 Raw data management

Raw data were registered and linked to individual reports. These reports along with the chromatograms and other laboratory printouts were filed in folders marked "^{13}C-STYREN-98" and temporarily stored in room L 1-3014 for data management and reporting purposes. In the reporting all data allowing personal identification, such as last name, date of birth etc., were eliminated. Such information was only stored in the medical files.

After data management is completed all raw data will be stored in a locked space for 10 years.
Raw data were entered into Microsoft Excel 97 workbooks for further evaluation. The Excel documents were stored together with the raw data on MS-DOS formatted 3.5" disks and as paper printouts, both labeled "^{13}C-STYREN-98".

1.8 Exposure protocol

The exposure protocol contains the following information:

1) Name of study
2) Name of volunteer
3) Height and weight of volunteer
4) Date of exposure
5) Responsible physician during exposure, including telephone number
6) Time of exposure start
7) Nominal and actual time of events (exposure start, exposure end, capillary sample, venous blood sample, exhalation, urine sample)

1.9 Exposure climate conditions

Exposures were carried out inside an 20 m^3 stainless steel exposure chamber with 18-20 air changes per hour. Ambient air temperature was maintained at 18°C and relative humidity at 40%. Temperature and humidity were continuously recorded (Vaisala HMP 36) and logged (Squirrel Meter Logger 1200 Series, Grant) on a personal computer at 1-min intervals.

1.10 Physical workload and heart rate monitoring

During exposure the volunteer performed light physical exercise at a workload of 50W on a bicycle ergometer (Monark ergomedic 829E). Before the experiment the volunteer was instructed on how to use the bicycle. Heart rate was recorded by means of automatic analysis of the ECG-wave (Polar PE 3000). Physical data, including pedaling frequency, actual workload, and heart rate, were continuously recorded (Squirrel Meter Logger) at 1-min intervals on a personal computer.

1.11 Correction for molecular weight

No adjustments for differences in molecular weight, density, detector response, etc. between non-labeled and ^{13}C-labeled styrene and their metabolites were made. This was based on the assumption that, on a molar basis, labeled and non-labeled have the same flame ionization detector response (i.e. peak area units per moles) and density (i.e. moles per liter). Thus, amounts and concentrations of labeled substances were slightly erroneous when expressed on a gram basis but were expected to be correct when expressed on a molar basis, or when compared with the corresponding non-labeled substances on a gram basis.

1.12 Generation of styrene vapor

Styrene vapor was prepared in home-made polyester-laminated aluminum bags with a volume of approximately 150-180 L. The bags were filled with 130-160 L of clean laboratory air using a steel drum spirometer. The bags were then filled with 34-43 µL ^{13}C-styrene depending on the volume of air in the bag. The amount of styrene needed is calculated as:

A (µL) = Bag volume (L) x Target conc. (mg/ m³) / Density / 1000,

where the target concentration is set to 220 mg/ m³ and the density 0.906.

The bag was allowed to equilibrate for at least 15 min to allow evaporation and even distribution of styrene vapors. The air temperature in the laboratory was checked daily. Each bag was analyzed for styrene concentration prior to use. Prior to the experiments stability checks were performed by repeated volume measurement and gas chromatographic (GC) analysis of styrene from the same bag. The volume was constant for at least 1 h, whereas the concentration of styrene declined by 4.9% in 24 h. Bags were always used within 30 min.

1.13 Air sampling and analysis

During exposure the volunteer inspired at a voluntary rate via a face-tight silicone rubber mask (Hans Rudolph Inc., 8930 Series) connected to a inhalatory bag with styrene vapor in air via a unidirectional valve. Exhaled air was transferred from the face mask via a second valve and a heated (40°C) hose to a 7-L heated (40°C) glass mixing chamber. Exhaled air was directed from the chamber either to a gas chromatography injector loop or to an absorption tube for later automated thermal desorption and GC analysis. Sampling was performed every 30 min during exposure and at 8 occasions after exposure.

Pulmonary ventilation was continuously monitored by a respiratory meter (K.L. Engineering Haeger Gasanalysator AB, Model S-340) attached prior to the inhalatory valve. Data were logged on a personal computer at 1-min intervals.

Styrene-containing air in the inhalatory bags and in exhaled air was sucked via Teflon tubes to the GC injector loop by means of an air pump (Gast DDA-P101-BN, 15 L/min). GC analysis was carried out using the following equipment:

- Perkin Elmer GC, Model Auto System Gas Chromatograph
- Gas loop injector, volume 0.5 mL
- Flame ionization detector
- Chrompack capillary column CP-Sil 52CB, 25 m, ID 0.53 mm, coating 2 µm
- GC integrator Turbochrom v. 4.1

The analytical conditions were:

- Carrier gas nitrogen, column pressure 7.5 psi
- Loop 180°C, column isothermal 180°C, detector 250°C
- Retention time of styrene 1.95 min

Exhaled air was also adsorbed on stainless steel ATD tubes filled with 20/40 Mesh Tenax (Supelco) by means of a air pump. Sampling was done for 2.00 min at a flow rate of about 280-400 mL/min depending on the inner flow resistance of the ATD tubes. Each ATD tube was flow calibrated. Duplicate samples were collected. The tubes were capped at both ends with brass caps. The tubes were analyzed in the afternoon after the end of exposure. The equipment used for thermal desorption and GC analysis was:

- Perkin Elmer GC, Model Auto System Gas Chromatograph
- Perkin Elmer Automated Thermal Desorption system ATD-400
- Flame ionization detector

- Chrompack capillary column CP-Sil 8CB, 25 m, ID 0.32 mm, coating 2 µm
- GC integrator Turbochrom v. 4.1

The thermal desorption settings were:
- Desorption oven 250°C, desorb time 2 min, valve 225°C, trap –30°C, high 300°C, hold 2.0 min, line 200°C
- Pressure 9.6 psi
- Desorb flow 55.6 mL/min
- Outlet split 9.3 mL/min
- Inlet split 10 mL/min

The analytical conditions were:
- Carrier gas nitrogen, column pressure 6.0 psi
- Injector 200°C, column isothermal 80°C for 5 min, then 25°C/min to 250°C, then 250 isothermal for 10 min, detector 250°C
- Retention time of styrene 10 min

1.14 Air standards

Unlabeled material was used in the preparation of all standards. In other words, it was assumed that, on a molar basis, the response factors for unlabeled and ^{13}C-labeled styrene and styrene metabolites were the same in any of the detector systems (FID, UV, TIC) (see also section 1.11).

For air analyses, daily standards were prepared by injecting known volumes of styrene with appropriate Hamilton syringes or air-tight glass syringes in 5 L or 10 L Tedlar bags (SKC 232-series) filled with known volumes of clean air by means of a calibrated air pump (Aircheck 224-PCXR8, SKC). The following air standards were prepared:

For inhalatory bags and exhaled air during exposure
- 280 mg/ m^3 (3.20 L air + 1.00 µL ST)
- 168 mg/ m^3 (3.20 L air + 0.60 µL ST)
- 93.4 mg/ m^3 (4.80 L air + 0.50 µL ST)

For exhaled air during decay
- Standard bag with 1121 mg/m^3 ST (1.60 L air + 2.0 µL ST)
- 2.10 mg/m^3 ST (1.60 L air + 3.00 mL from standard bag)
- 1.05 mg/m^3 ST (1.60 L air + 1.50 mL from standard bag)
- 0.140 mg/m^3 ST (1.60 L air + 200 µL from standard bag)
- 0.035 mg/m^3 ST (1.60 L air + 50 µL from standard bag)

1.15 Venous blood sampling and storage

Venous blood was sampled prior to exposure and at five specified times after exposure in 3-4 heparinized Vaccutainer tubes (Venoject VT-100H) via a 0.8x40 mm stainless steel Venoject needle. Blood from one tube, taken prior to exposure, was used to make standards for the head-space gas chromatograhic analysis of styrene in blood. Integral blood was used for analysis of styrene oxide. One tube was centrifuged at 1400g x10 min (Hettich Universal 16A). The plasma was transferred to two 3-mL glass tubes (polyethylene cap) and one 3-mL polystyrene Ellerman tube (polyethylene cap) and stored at –70°C for later analysis of metabolites.

Preprinted adhesive paper labels were attached to each vial. Each label contained the following information:

- "^{13}C-STYREN-98"
- Name of subject
- Sample number

Sample boxes were labeled "^{13}C-styrene-98".

1.16 Capillary blood sampling

Prior to sampling the volunteer immersed his hand in warm water (38-40°C) for 1 min. After drying the hand with a clean towel, capillary blood was sampled at specified times. Pricking on digit 3 or 4 was done with blood lancets (Minilancet, CCS Clean Chemical Sweden AB). Blood was collected in two 100-µL heparinized glass capillary (Kebo AB) and immediately transferred to a 20-mL glass head-space vial. The vial was immediately sealed with a Teflon-lined rubber septum containing 10 µL dimethylsulfoxide. The samples were stored at room temperature until being analyzed by head-space gas chromatography later the same day. To minimize potential sampling bias, especially during capillary sampling, the same technician performed all blood sampling.

An empty head-space vial was left open at the site of blood sampling during the exposure to styrene. The vial was capped by the end of exposure and analyzed for styrene. This was done to assure that blood samples were not contaminated.

1.17 Urine sampling and storage

Urine was sampled prior to the start of exposure and at the nominal times 126, 240, 370, 540, 780, 1320, and 1440 min after the exposure start. Actual sampling times were recorded in the individual exposure protocol. The volunteer was instructed to completely void the bladder on each occasion and to note the actual sampling time on the bottle. Urine was collected in 500-mL glass bottles and immediately capped with polyethylene screw caps. Samples were processed within 5 min, except home samples (at 540, 780, and 1320 min) which were processed the following morning. The total sample volume was measured with a 200-mL or 500-mL glass cylinder. Aliquots of about 2 x 5 mL were stored at –20°C (freezer in laboratory L1-3064) in glass scintillation vials with screw caps covered with aluminum foil, for later analysis. In addition, aliquots of 2 x 2 mL urine were stored at –70°C (freezer outside laboratory L1-3079) in 3-mL glass tubes (polyethylene cap) for NMR analysis at CIIT.

As with the blood samples, preprinted labels were attached to each vial.

Sample boxes were labeled "^{13}C-STYREN-98".

1.18 Shipping of urine samples

Frozen urine samples (-70°C) were shipped to CIIT by World Courier AB, Märsta Sweden. Samples were kept frozen by means of carbon dioxide ice throughout the transport.

1.19 Analytical method for styrene in blood and urine

Capillary blood and urine samples were added to head-space vials as described previously.

Individual, daily standard curves (Figures 1-1 and 1-2) were prepared by adding different amounts of styrene to DMSO. The amounts used were 0.896, 4.48, 8.96, 22.4, 44.8, and 89.6 mg styrene per L DMSO. Pre-exposure samples of blood (200 µL) or urine (2 mL) were added to 20 mL glass head-space vials. Thereafter, 10 µL of styrene in DMSO was added to each vial and the vial was rapidly capped with a gas-tight Teflon lined rubber septum. Individual, daily standard curves for styrene in urine were prepared in the same way, except that 0.2 mL blood was replaced by 2 mL urine. The final concentration ranges of styrene were thus 0.430 – 43.0 umol/L blood and 0.043 – 4.30 umol/L urine. Spiked standard samples were prepared and analyzed in duplicate.

Analysis was carried out by head-space GC using the following equipment:
- GC Carlo Erba Fractovap 2350
- Automatic head-space sampler, Hewlett Packard 7694
- Flame ionization detector
- Chrompack capillary column WCOT, CP-Sil 8, 50 m, ID 0.53 mm, coating 2 um

The analytical conditions used were:
- Thermosetting of vials at 50°C for 20 min
- Carrier gas nitrogen
- Vial pressure 9.7 psi, pressurizing time 0.2 min, injection time 1 min
- Loop 65°C, transfer line 65°C, injector 50°C, column oven 50°C
- Column pressure 8.6 psi
- Retention time of styrene 17.68 min

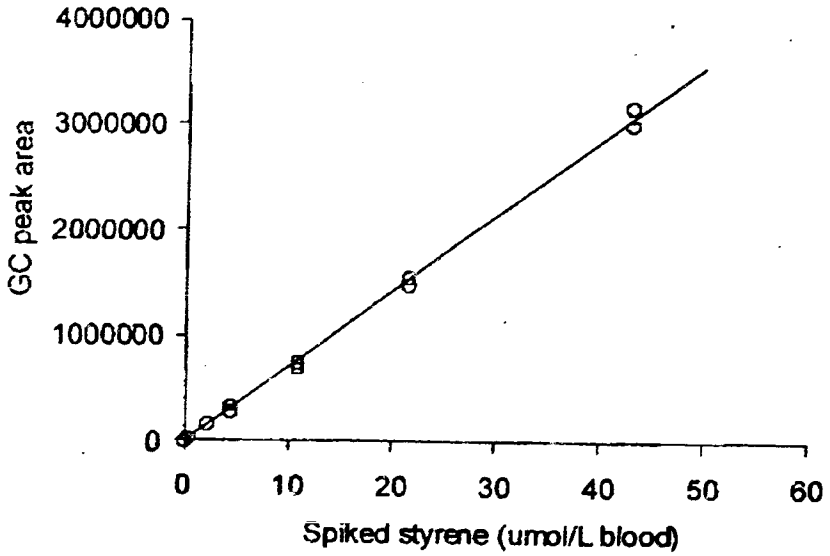

Figure 1-1. Standard curve for blood spiked with styrene. Response is expressed in GC-FID peak area units. Circles or squares represent actual measurements. The straight lines represent the best fit as obtained by linear regression forced through the origin.

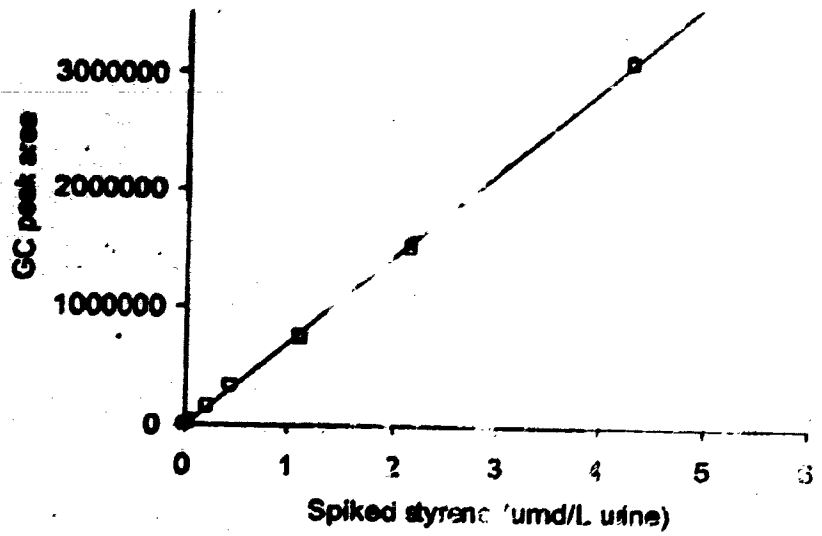

Figure 1-2. Standard curve for urine spiked with styrene. Response is expressed in GC-FID peak area units. Circles or squares represent actual measurements. The straight lines represent the best fits obtained by linear regression forced through the origin.

1.20 Analytical method for styrene oxide in blood

2 mL venous blood was pipetted with a glass syringe to a glass vial standing in ice and containing 50 µL internal standard (1 µg/mL phenylpropylenoxide in ethanol). 3 mL n-hexane was added and the vial was sealed with a Teflon-lined screw cap. The vial was vigorously shaken (Vortex mixer) for about 30 sec and then centrifuged for 3 min at 3800 rpm (2400 g, Hettich Universal 16A). The hexane phase was transferred with a glass Pasteur pipette to a second screw-cap glass vial and the extraction procedure was repeated with 3 mL n-hexane. The combined hexane extracts were concentrated to approximately 250 µL by evaporation under nitrogen at room temperature. Duplicate blood samples were prepared, one of which was stored at –70°C (freezer outside laboratory L1-3079) for future analysis.

Standard curves of 0-12.5 ng/mL blood were prepared by adding 0, 0.5, 2, 4, 6, 16, or 25 µL of a freshly prepared stock solution of 1 µg/mL styrene oxide in ethanol (10 µL styrene oxide added to 10 mL ethanol) to glass vials containing 2 mL blood sampled prior to styrene exposure. Additions of styrene oxide were performed with Hamilton syringes of appropriate sizes.

Styrene oxide in the n-hexane blood extracts was determined by GC-MS using the following equipment:

- Hewlett Packard GC, HP-6890
- Mass selective detector HP-5973
- On column injector
- Autosampler HP-6890
- Capillary column HP5 19091J-433 from ChromTech (5% phenyl methyl siloxane), 30 m, ID 0.25 mm, coating 0.25 um
- Software for GC integrator and MS analysis: HP Chem Station

The following conditions were used:

- On column injector 70°C initially, 20°C/min to 200°C
- Oven 50°C isothermal for 10 min, then 10°C/min to 180 °C, then isothermal 4 in, then 20°C/min to 220°C, then post-run 240°C isothermal for 5 min
- Carrier gas helium, pressure 82.5 kPa, column flow 1.5 mL/min

- Injection volume 5 µL
- Retention times styrene oxide approximately 14.7 min at m/u 128.1 (^{13}C-SO) or 120.1 (non-labeled), phenylpropylene oxide approximately 15.8 min at m/u 134.1.
- Electron ionization at 70 eV

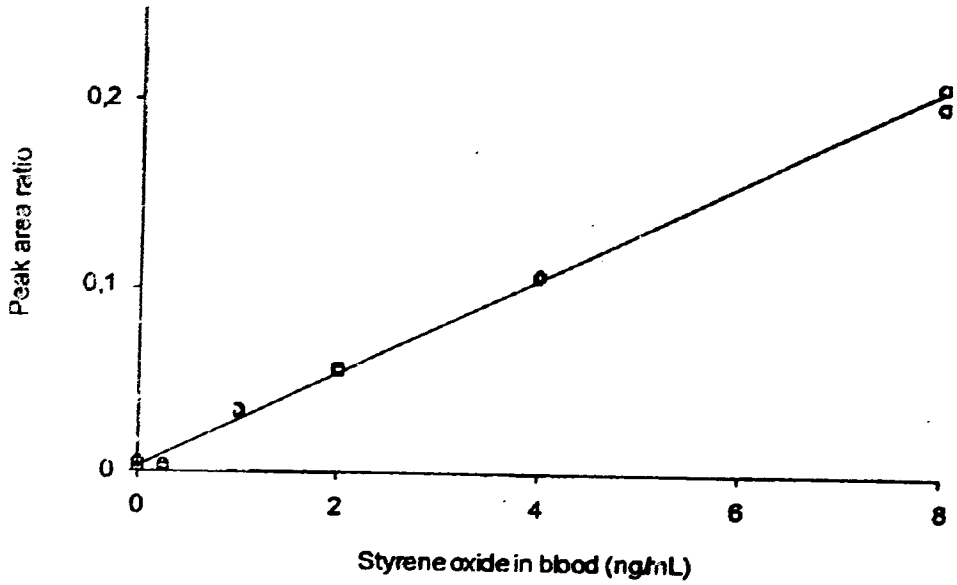

Figure 1-3. Standard curve for blood spiked with styrene oxide. Response is expressed as the styrene oxide / phenylpropylene oxide GC-MS-SIM peak area ratio or peak height ratio. Circles or squares represent actual measurements. The straight lines represent the best fit as obtained by linear regression forced through the origin.

Quantitation was performed by comparing the sample with the standard curve with respect to styrene oxide / phenylpropylene oxide single ion monitoring peak area ratios or peak height ratios. The standard curve was obtained by linear regression.

1.21 Analytical method for mandelic acid in urine

Mandelic acid was analyzed according to Ogata and Taguchi (Int Arch Occup Environ Health 1988;61:131-140) with minor modifications. 100 µL urine, 100 µL internal standard (1 mg/mL ortho-methylhippuric acid) and 9.80 mL distilled water were added to a polystyrene tube and sealed with a polyethylene cap (Kebo AB, Sweden). After mixing for 5 min (Mixer 440, Swelab Instrument, Stockholm) the sample was centrifuged for 5 min at 2000 rpm (Hettich Universal 16A). A standard solution was prepared by mixing equal volumes of aqueous solutions of creatinine (4 mg/mL), hippuric acid (4 mg/mL), phenylglyoxylic acid (4 mg/mL), and mandelic acid (4 mg/mL). Standard solutions were prepared by serial 1:1 dilutions with distilled water. Urine standards were prepared by adding 100 µL control urine (collected prior to exposure), 400 µL standard solution, 100 µL internal standard and 9.40 mL distilled water. The final standards correspond to 0.5, 1.0, 2.0, and 4.0 mg of each additive per mL urine. Creatinine was added for identification purposes only.

The following equipment was used:
- HPLC, Hewlett Packard 1050
- Autosampler
- Diode array detector
- ODS column, Hichrom, Chrompack, 4.6 mm x 150 mm
- Integrator HP Chemstation v. 5.02

The analytical conditions for mandelic acid were:

- Injection volume 10 µL
- Mobile phases A=20 mM potassium dihydrogenphosphate and 3 mmol/L sodium 1-decanesulfonate adjusted to pH 3.3, B=acetonitrile
- Flow rate 0.75 mL/min
- Isocratic A:B = 85:15 for 7 min, reconditioning by A:B = 60:40 for 3 min
- Wavelength 210 nm

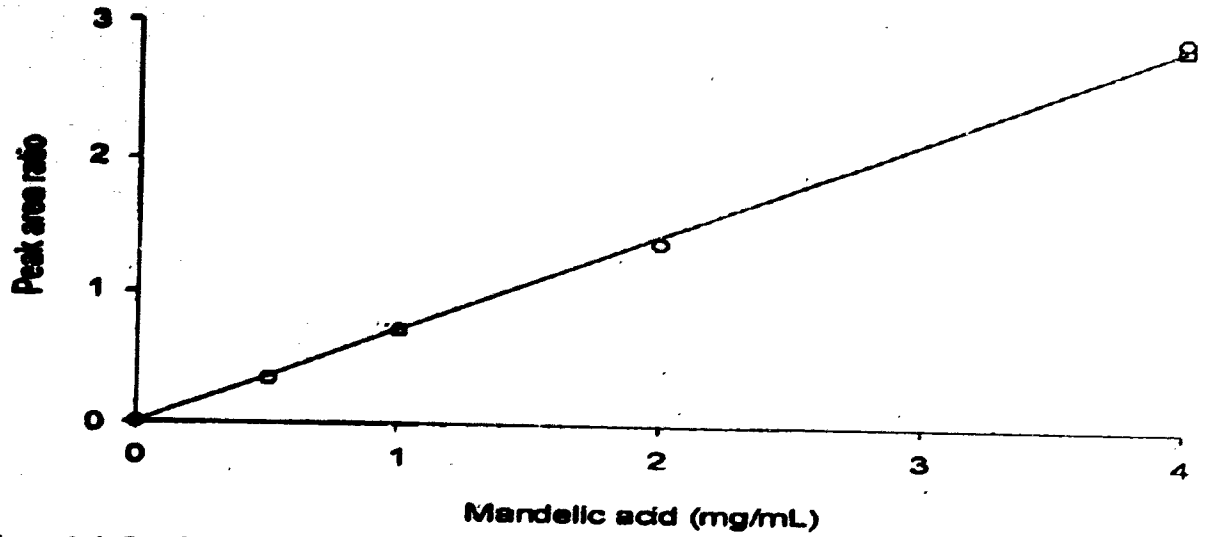

Figure 1-4. Standard curve for urine spiked with mandelic acid. Response is expressed as the mandelic acid / ortho-methylhippuric acid HPLC-UV peak area ratio. Circles or squares represent actual measurements. The straight lines represent the best fit as obtained by linear regression forced through the origin.

Peak identification was made by retention time and visual comparison of the spectrum in the UV-VIS range (190 – 600 nm) with a spiked sample. The detection limit of mandelic acid depends on the presence of interfering peaks and varies between urine samples. In most cases, no interfering peaks were present. The detection limit was then determined by the minimum detectable peak of approximately 10 peak area units, corresponding to approximately 20 µg mandelic acid per mL urine.

1.22 Analytical method for phenylglyoxylic acid in urine

Phenylglyoxylic acid was analyzed according to Ogata and Taguchi (Int Arch Occup Environ Health 1987;59:263-272) with minor modifications. 1 mL urine, 1 mL methanol, and 500 µL internal standard (ortho-methylhippuric acid) were added to a polystyrene tube. The sample was sealed, mixed, and centrifuged as described for mandelic acid in the previous section. Standard solutions were prepared as described for mandelic acid. The standards were prepared so as to correspond to final concentrations of phenylglyoxylic acid of 0.25, 0.5, 0.1, and 0.2 mg/mL urine.

In addition, phenylglyoxylic acid was analyzed after incubating 100 µL urine, 100 µL ß-glucuronidase solution, and 800 µL acetate buffer (0.2 M sodium acetate adjusted to pH 5.0) overnight (approximately 16 h) at 37°C in a shaking water bath (RCS 20, Lauda, Germany). The ß-glucuronidase solution was prepared by adding 0.26 g of glucuronidase to 2.6 mL acetate buffer. The solution corresponded to 10,000 international units per sample.

The HPLC equipment used was the same as described for mandelic acid.

The analytical conditions for phenylglyoxylic acid were:

- Injection volume 10 µL
- Mobile phases: A=5 mM potassium dihydrogenphosphate adjusted to pH 2.5, B=acetonitrile
- Flow rate 0.8 mL/min
- Isocratic A:B = 90:10 for 17 min, reconditioning by A:B = 60:40 for 2 min
- Wavelengths 254 and 225 nm

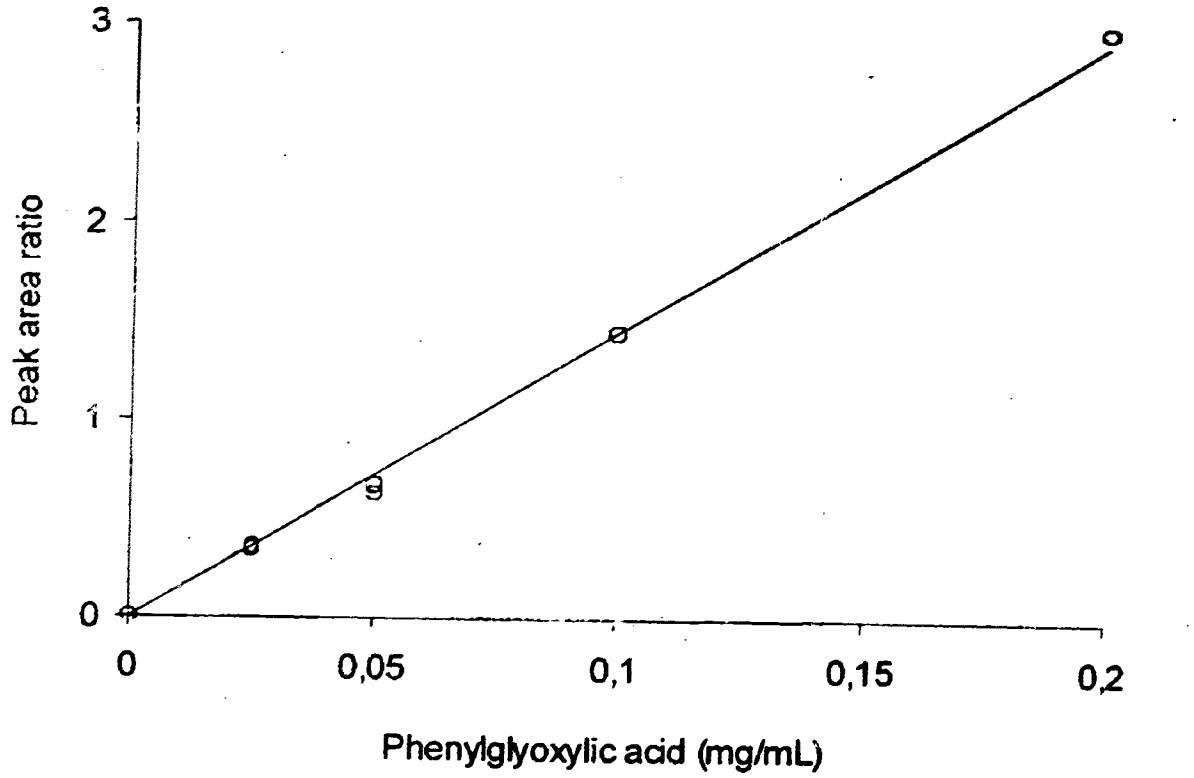

Figure 1-5. Standard curve for urine spiked with phenylglyoxylic acid. Response is expressed as the phenylglyoxylic acid / ortho-methylhippuric acid HPLC-UV peak area ratio. Circles or squares represent actual measurements. The straight lines represent the best fit as obtained by linear regression forced through the origin.

Peak identification of phenylglyoxylic acid was made as described for mandelic acid. The smallest detected peak corresponds to approximately 5 µg phenylglyoxylic acid per mL urine.

2 Results

2.1 Exposure conditions

The first exposure experiment (numbered 1) was canceled after about one hour of exposure due to progressive illness, diagnosed as common cold. The following four experiments (numbered 2-5) were successful with one minor problem. Thus, in one experiment (no. 3) the exposure was not proper during the first 28 min due to a leak on the inhalatory side. Due to this problem, subject no. 3 was exposed for 127 and not 120 min. This means that the subject was slightly less exposed than would be the case had there been no problem with leakage. Although subject no. 3 had a longer exposure (127 min) than other subjects (120 min), expressing the data (Tables 1-1 and 1-2) as 120 min for subject no. 3 is a good

approximation. The percentage calculations for subject no. 3 may be in slight error because the actual uptake for subject no. 3 may be in question due to a leakage during the first 28 min of the 127 min exposure.

Table 1-1. Physiologic measurements during the experiment.

Subject no.	Ventilation, L/min (BTPS) 0-30 min	30-60 min	60-90 min	90-120 min	Breathing frequency, min^{-1}
2	22.1	24.3	23.5	23.5	18.6
3	21.4	17.9	24.2	22.2	18.5
4	19.4	18.1	23.2	22.8	20.9
5	18.2	20.6	17.6	20.3	18.8
Mean	20.3	20.2	22.1	22.2	19.2
CV%	9.0%	14.7%	13.9%	6.2%	6.0%

2.2 Styrene exposure and uptake

Table 1-2. Exposure to, uptake, and post-exposure exhalation of ^{13}C-styrene.

Subject no.	Inhaled conc. mg/m^3	Total inhaled		Uptake during exposures			Exhaled after exp		
		mg	mmol	umol	mg	% of inhaled	umol	mg	% of uptake
2	234	651	6.25	4321	450	68.1%	32.0	3.34	0.7%
3	233	647	6.21	4481	467	73.2%	30.6	3.18	0.7%
4	228	605	5.81	3789	395	65.3%	68.7	7.16	1.8%
5	217	524	5.03	2916	304	59.5%	65.2	6.79	2.2%
Mean	228	607	5.83	3877	404	66.5%	49.1	5.12	1.4%
CV%	3.4%	9.7%	9.7%	18.2%	18.2%	8.5%	42%	42%	57%

2.3 Styrene in blood and urine

The time course of styrene in blood is presented in Table 1-3 and Figure 1-5 and that of styrene in urine in Table 1-4 and Figure 1-6. Subject no. 3 was not properly exposed in the beginning of the experiment. This is reflected in rather low styrene levels in the first 4, and especially in the first 2, blood samples (Table 1-3). For ease of comparison, the post-exposure sampling times in subject no. 3 have been adjusted for the difference in exposure duration (127 min instead of 120 min), i.e. the true sampling time for 126 min was 127 + 6 = 133 min.

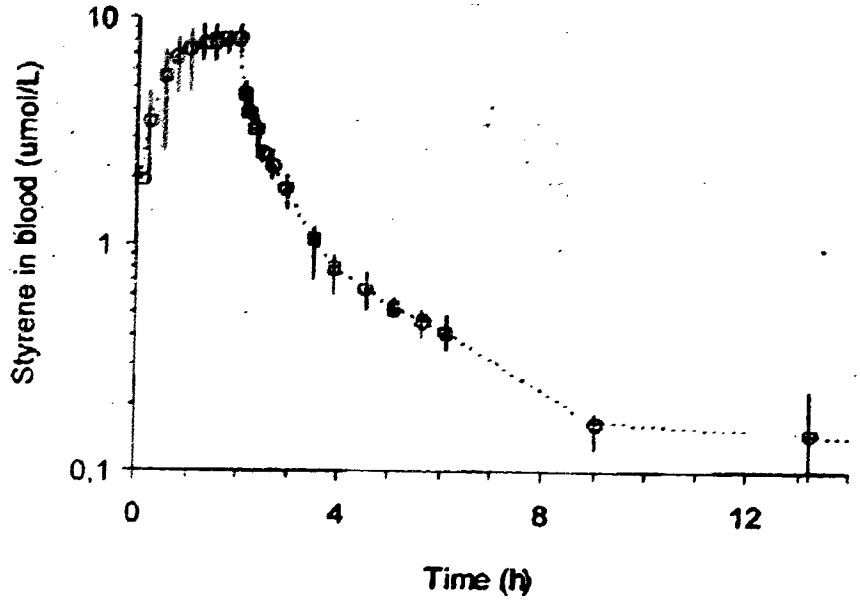

Figure 1-6. Semi-logarithmic presentation of average levels of styrene in blood in 4 volunteers exposed via nose and mouth to 50 ppm styrene for 2 h during light physical exercise (50 W). Only data from day of exposure are shown. Circles or squares represent actual measurements. The straight lines represent the best fit as obtained by linear regression forced through the origin. Horizontal and vertical bars indicate the ranges in sampling time (from exposure start) and styrene concentrations, respectively.

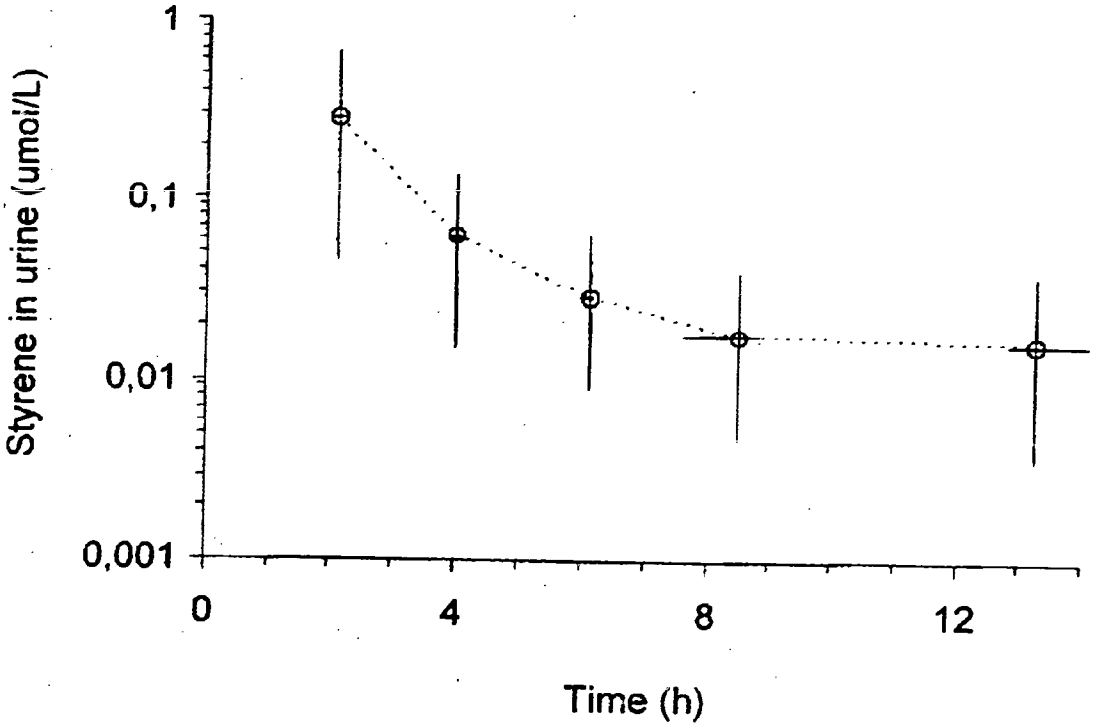

Figure 1-7. Semi-logarithmic presentation of average levels of styrene in urine in 4 volunteers exposed via nose and mouth to 50 ppm styrene for 2 h during light physical exercise (50 W). Only data from

day of exposure are shown. Circles or squares represent actual measurements. The straight lines represent the best fit as obtained by linear regression forced through the origin.

Table 1-3. Styrene in blood (umol/L) in 4 volunteers exposed via nose and mouth to 50 ppm styrene for 2 h during light physical exercise (50 W).

Time, min	Subject 2	Subject 3	Subject 4	Subject 5	Average	St Dev
0	n.d.	n.d.	n.d.	n.d.	-	-
5.03	n.a.	0.41	3.14	2.28	1.94	1.40
15.0	5.20	0.72	5.09	3.20	3.55	2.10
30.6	7.00	3.40	5.70	5.92	5.50	1.51
44.6	8.27	4.27	6.91	7.55	6.77	1.76
60.1	8.75	6.10	6.86	7.53	7.31	1.12
75.0	9.18	6.44	8.70	6.64	7.74	1.40
90.1	8.44	7.80	8.22	6.83	7.82	0.71
105	9.27	7.02	8.62	7.01	7.98	1.15
119	8.57	6.99	8.37	8.09	8.01	0.70
126	5.31	4.57	4.37	4.43	4.67	0.43
130	4.33	3.37	3.97	3.94	3.90	0.40
139	3.33	n.a.	3.00	3.38	3.24	0.21
146	2.93	2.27	2.55	2.53	2.57	0.27
158	2.52	1.92	2.36	2.21	2.25	0.26
176	1.92	1.42	1.86	1.92	1.78	0.24
209	1.19	0.87	1.01	1.17	1.06	0.15
233	0.90	0.67	0.79	0.77	0.78	0.09
270	0.59	0.65	0.61	0.71	0.64	0.05
304	0.45	0.57	0.50	0.56	0.52	0.06
338	0.43	0.49	0.40	0.55	0.47	0.07
365	0.40	0.43	0.37	0.43	0.41	0.03
542	0.25	0.11	0.21	0.08	0.17	0.08
792	0.16	0.13	0.21	0.09	0.15	0.05
1332	0.11	0.05	0.11	0.08	0.09	0.03
1416	0.09	0.07	0.12	n.d.	0.09	0.03

n.d.= not detected
n.a. = not analyzed

Table 1-4. Styrene in urine (umol/L) in 4 volunteers exposed via nose and mouth to 50 ppm styrene for 2 h during light physical exercise (50 W).

Time, h	Subject 2	Subject 3	Subject 4	Subject 5	Average	St Dev
0	n.d.	n.d.	n.d.	n.d.	-	-
2.1	0.371	0.236	0.256	0.257	0.280	0.061
4.0	0.071	0.055	0.046	0.072	0.061	0.013
6.1	0.027	0.019	0.033	0.035	0.028	0.007
8.5	0.019	0.015	0.013	0.022	0.017	0.004
13.3	0.013	0.012	0.022	0.017	0.016	0.005
21.9	0.014	0.007	0.019	0.013	0.013	0.005
23.6	0.006	0.006	0.009	0.007	0.007	0.001
41.9	n.a.	n.a.	0.006	n.a.	0.006	-
51.2	n.a.	n.a.	0.007	n.a.	0.007	-

n.a. = not analyzed

2.4 Styrene oxide in blood

Results of the GC-MS measurements of styrene oxide in venous blood are presented in Figure 1-8 and Table 1-5. The samples from subject 3 were analyzed in October 1998 and the remaining samples in January 1999. In these analyses peak height appeared to be more reliable than peak areas since at low levels peak areas tended to be more sensitive to base line fluctuations.

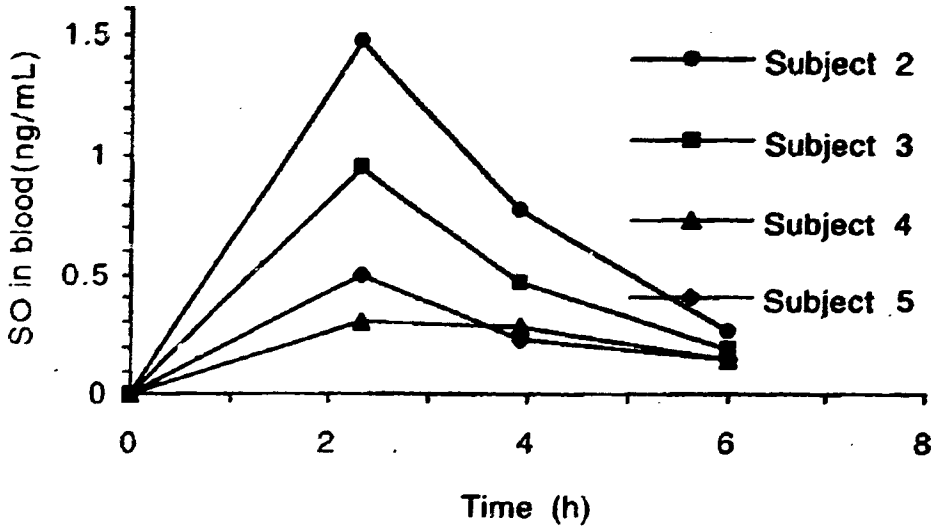

Figure 1-8. Styrene oxide in venous blood.

Table 1-5. Styrene oxide in venous blood.

Average time from start of exposure (h)	SO in venous blood (ng/mL)					
	Subject 2	Subject 3	Subject 4	Subject 5	Mean	St Dev
0	n.d.	n.d.	n.d.	n.d.	-	-
2.3	1.46	0.95	0.30	0.50	0.80	0.52
3.9	0.77	0.47	0.28	0.23	0.44	0.25
6.0	0.26	0.19	0.14	0.15	0.18	0.06
23.2	n.d.	n.d.	n.d.	n.d.	-	-

n.d.= not detected

2.5 Mandelic acid and phenylglyoxylic acid in urine

Results for mandelic acid and phenylglyoxylic acid are presented in Figures 1-9 and 1-10 and Tables 1-6 and 1-7, respectively. The average 48-h recoveries of mandelic acid and phenylglyoxylic acid were 546 (range 266 – 1180) umol and 160 (range 104 – 206) umol, respectively.

Treatment of the urine samples with ß-glucuronidase overnight did not increase the yield of phenylglyoxylic acid. On the contrary, slightly reduced concentrations were seen in most samples, the average decrease being 19%, as compared to pretreatment values (data not shown). This suggests that conjugation is of negligible importance.

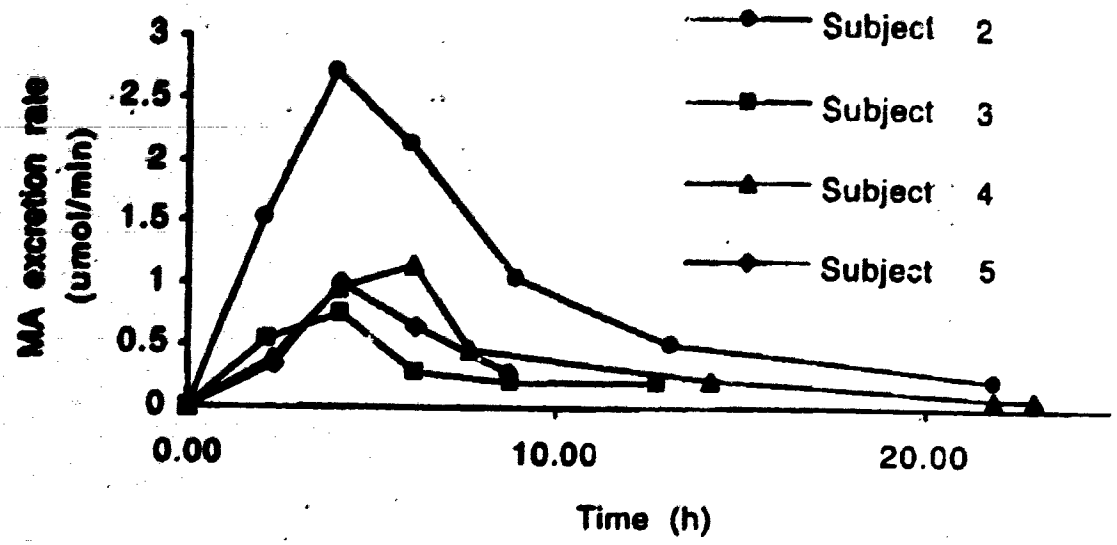

Figure 1-9. Mandelic acid excretion in urine from 4 volunteers exposed via nose and mouth to 50 ppm styrene for 2 h during light physical exercise (50 W).

Table 1-6. Mandelic acid excretion in urine (umol/min).

Sample no.	Average mid-sampling time (h)	Subject 2	Subject 3	Subject 4	Subject 5	Average	St Dev
0	0	-	-	-	-		
1	1.1	1.520	0.541	0.391	0.337	0.697	0.555
2	3.1	2.704	0.743	0.955	1.004	1.352	0.909
3	5.1	2.144	0.276	1.133	0.649	1.051	0.809
4	7.3	1.038	0.187	0.470	0.288	0.496	0.380
5	10.9	0.502	0.194	0.215	n.d.	0.304	0.172
6	17.6	0.213	n.d.	0.071	n.d.	0.142	0.100
7	22.7	n.d.	n.d.	0.073	n.d.	0.073	-
8	30.2	n.d.	n.d.	n.d.	n.d.	-	-
9	41.6	n.d.	n.d.	n.d.	n.d.	-	-

n.d.= not detected

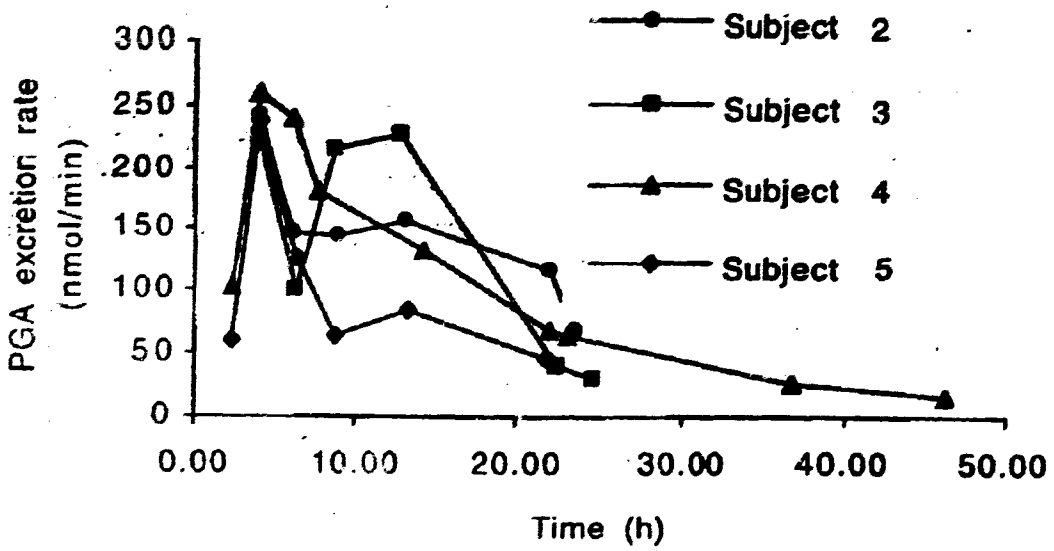

Figure 1-10. Phenylglyoxylic acid excretion in urine from 4 volunteers exposed via nose and mouth to 50 ppm styrene for 2 h during light physical exercise (50 W).

Table 1-7. Phenylglyoxylic acid excretion in urine (nmol/min).

Sample no.	Average mid-sampling time (h)	Subject 2	Subject 3	Subject 4	Suoject 5	Average	St Dev
0	0	-	-	-	-	-	-
1	1.1	n.d.	n.d.	102	60	81	30
2	3.1	242	225	261	238	242	15
3	5.1	146	101	239	126	153	60
4	7.3	145	215	181	64	151	65
5	10.9	156	228	132	83	150	60
6	17.6	117	39	69	45	68	35
7	22.7	68	30	64	n.d.	54	21
8	30.2	n.d.	n.d.	26	n.d.	26	-
9	41.6	n.d.	n.d.	15	n.d.	15	-

n.d.= not detected

Part 2: Urinary Metabolites of Inhaled ^{13}C-Labelled Styrene in Human Volunteers

2.1 Staff

The following personnel participated in the portion of the study conducted at the Chemical Industry Institute of Toxicology:

Susan Sumner, Scientist II
Timothy Moore, Senior Research Assistant
Carla Williams, Research Assistant

2.2 Standards and storage of urine

Mandelic acid (lot 07114MT), phenylglyoxylic acid (lot 02817AZ), hippuric acid (lot 08823KW), phenylaceturic acid (lot 04421DZ), and deuterium oxide (lot 07917LQ) were obtained from Aldrich Chemical Co. Urine samples were stored at ~ – 80 °C after receipt at CIIT.

2.3 Preparation of samples for NMR analysis

Samples were prepared for NMR analysis by adding 100 µL D_2O to 400 µL urine, for each time-point for subjects 2 and 3 and for selected time-points (control, # 2, and #5) for subjects 1, 4, and 5. Dioxane was added (up to 1 µL) to some samples for use as an internal reference (66.6 ppm). The 100 µL and 400 µL aliquots were delivered with calibrated pipettes. The dioxane was delivered with a 1 or 5 µL syringe set to deliver ~ 0.8 - 1.0 µL. The actual amount added was determined by weight.

Concentrated samples were prepared for selected time-points (control, # 2, #4, and #7) for participants 2, 3, 4, and 5. The concentrated samples were prepared by adding 10 ml methanol to 3 ml urine, vortexing to precipitate protein, centrifuging to separate the protein pellet, and reducing the transferred supernatant with $N_{2(g)}$. The procedure was repeated using 5 ml methanol and the dried $N_{2(g)}$ supernatant was resuspended in D_2O.

2.4 NMR methods for detection of ^{13}C-styrene derived metabolites in urine

NMR spectra were acquired with a 5-mm dual proton-multinuclear probe on a Varian VXR-300 spectrometer (Palo Alto, CA). Proton decoupled carbon-13 NMR spectra were acquired in the double precision mode with a relaxation delay of 10 s and a 60° pulse width. Spectra were referenced to urea at 162.5 ppm. Spectra were acquired using a similar number of transients so that comparisons of concentration could be made based on signal-to-noise (S/N) (with consideration of total urine volume) of metabolites in the various spectra.

2.5 Metabolites previously detected in urine from rats, mice, or humans

The major metabolic route for styrene involves conversion to styrene oxide (SO) via cytochrome P-450 2E1.[1] Other cytochrome P450 isozymes are also believed to be involved in the in vivo metabolism of styrene.[2] Metabolites in the urine of rats administered styrene have been investigated following a variety of dosing regimens (Figure 2-1; reviewed by Bond, 1989[3]; reviewed by Sumner and Fennell, 1994[4]). In rats, mice, and humans, SO undergoes hydrolysis to styrene glycol, and conversion to mandelic acid (MA) and its further metabolites (e.g., phenylglyoxylic acid, PGA, hippurate, HA). SO conjugation with glutathione results in the excretion of metabolites such as *N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)cysteine, *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-cysteine, and *N*-acetyl-*S*-

(phenacyl)cysteine in urine of rats and mice. Glutathione conjugates of SO have also been described in urine of styrene exposed humans.[5] Phenylacetic acid (PAA) and phenylaceturic acid (PAA') have been detected in styrene exposed rats[6,7] and mice[7]. However, these metabolites have not been shown in urine of rats exposed to styrene oxide.[6] The urinary excretion of phenylethanols from styrene exposed rats, and the excretion of phenylethanols from phenylacetic acid exposed rats has lead to the suggestion that phenylacetaldehyde-derived metabolites from styrene exposures do not involve conversion of styrene to styrene oxide.[6] Another hypothesis is that the production of phenylacetaldehyde requires conversion of styrene to 7,8-styrene oxide simultaneously with epoxide rearrangement.[6] 3,4-Ring epoxidation for styrene has been suggested after detection of low levels (0.1% of 100 mg/kg gavage dose) of 4-vinylphenol in the urine of styrene exposed rats and workers.[2,3] Metabolites derived from 3,4-ring epoxidation have also been described for styrene exposed rats and mice.[7]

2.6 Metabolites detected in the present study in urine from humans exposed via face mask for 2 hr to 50 ppm uniformly labelled ^{13}C-styrene.

Signals detected in the ^{13}C-NMR spectra of urine from uniformly labelled [^{13}C]styrene exposed humans were assigned to the ^{13}C-labelled carbons of styrene-derived metabolites based on expected chemical shifts for previously identified metabolites (Figure 2-1) in urine of styrene exposed rats, mice, or humans (reviewed by Sumner and Fennell, 1994[4]). In addition, the chemical shift positions were compared with those previously reported for metabolites in urine from rats and mice exposed to [7,8-^{13}C]styrene.[7]

The ^{13}C NMR spectra of control urine (i.e., pre-exposure urine) collected from humans prior to exposure (Figure 2-2) contains signals from endogenous compounds that are assigned to urea, hippurate, and creatinine. Signals from endogenous compounds appear as singlets in the ^{13}C NMR spectra, since they arise for molecules that contain naturally abundant (1.1%) ^{13}C-nuclei. ^{13}C NMR spectra were also acquired for control urine (Figure 2-3) after protein precipitation and concentration. Additional signals appeared in the concentrated urine that are also attributed to endogenous compounds.

Signals in the ^{13}C NMR spectra of urine from a human exposed to uniformly labelled [^{13}C]styrene (Figures 2-4 to 2-8) are recognized as metabolites of [^{13}C]-styrene due to multiplet patterns that arise from carbon-carbon coupling between enriched ^{13}C-nuclei. The coupling patterns do not arise for endogenous compounds that contain natural abundant (1.1%) ^{13}C-nuclei. The chemical shifts (center of multiplet pattern) and coupling constants (J_{cc}) for styrene-derived metabolites in urine from exposed humans are summarized in Table 2-1.

[^{13}C]Styrene-derived signals labelled MA (Figures 2-4 and 2-5) have the greatest intensity (i.e., highest concentration) for urine samples collected at early time-points (~ 4 hr and ~ 6 hr following exposure initiation). A reduced signal to noise (i.e., less intense peaks) occurs for samples collected at later time-points (≥ 9 hr following exposure initiation). The chemical shift positions for the C7 and C8 carbons (75 and 179 ppm; Table 2-1) are consistent with those assigned to mandelic acid (MA) in urine from [7, 8-^{13}C]styrene exposed rats and mice,

and the carbon-carbon coupling constants (J_{cc}, Table 2-1) are consistent with multiple splitting from uniformly labelled [^{13}C]styrene. A multiplet at 140 ppm is assigned to the C1 carbon of MA, and a group of peaks are present near 126-128 ppm which are consistent with the other ring carbons of MA. The presence of MA is further confirmed by comparison to shifts obtained for a standard of MA spiked in control (pre-exposure) urine (Table 2-1). ^{13}C NMR spectra (Figures 2-6 to 2-8) of urine from subjects 1, 4, and 5 also show a greater concentration of MA at the ~ 4 hr time-point as compared with the ~ 13 hr time-point.

In addition to the presence of peaks for MA, other signals derived from the labelled carbons of styrene are apparent in some spectra acquired directly for urine. However, in many cases the presence of additional signals is difficult to distinguish from background, and urine samples were concentrated (results below) for further analysis. The presence of PGA is apparent for the direct urine of subjects 3, 4 & 5 (Figures 2-5, 2-7, and 2-8; ~4 hr) based on signals located at 173 and 197 ppm (C8 and C7 carbons, respectively), consistent with those detected in urine from rats and mice exposed to [^{13}C]styrene. Multiplets are also present for the labelled C1 carbon of PGA (~ 135 ppm) and for the other labelled ring carbons (126-132 pm). These chemical shifts are consistent with those obtained for a synthetic standard of PGA in control urine (Table 2-1). Similar to MA, the signals for PGA are more intense at the earlier time-points (~ 4 hr) as compared with the later time-points (~ 13 hr). Additional peaks arise for urine analyzed prior to concentration (e.g., Figure 2-7, top trace, expanded regions for ~ 4 hr spectrum) near 42 ppm (broad doublet of doublets, J_{cc}~ 48,49 Hz with long range coupling) and 174 ppm (broad doublet J_{cc}~ 49 Hz with long range coupling) that have patterns consistent with derivation from the labelled carbons of styrene. These shifts are consistent with the presence of phenylacetic acid/phenylaceturic acid (PAA), and its presence is confirmed in spectra obtained for concentrated urine samples (below). Peaks at 170 ppm (broad doublet J_{cc}~ 66 Hz with long range coupling) are present that are consistent with assignment to hippuric acid (see below).

^{13}C NMR spectra of concentrated urine (Figures 2-9 to 2-11) clearly shows the signals for both MA and PGA for the ~ 4 hr spectra (Figure 2-10). The signals for MA are apparent but greatly reduced in the ~ 23 hr spectra (Figure 2-11). The presence of PAA is confirmed in the spectra of concentrated urine by distinct multiplets centered at 42 and 174 ppm (e.g., Figure 2-9 for expanded regions). In addition, these shifts are consistent with those obtained (Table 2-1) for a standard of PAA in control urine.

Uniformly labelled [^{13}C]styrene-derived carbons are also present at 170 ppm (e.g., Figure 2-10, subject 4) consistent with signals detected in urine from [7,8-^{13}C]styrene exposed rats and mice and assigned to further metabolic products of MA (e.g., hippuric acid, HA). The direct coupling (J_{cc}=66 Hz) is consistent with splitting from the C1 carbon and the broad peaks are consistent with long-range splitting from other ring carbons. A standard of HA spiked in control urine gives rise to shifts for the CO carbon at 170 ppm (Table 2-1). A broad doublet is also detected at 175 ppm with a direct coupling (J_{cc}=66 Hz) consistent with splitting of a CO carbon by the C1 carbon and the broad peaks are consistent with long-range splitting from other ring carbons. An assignment was not made for this metabolite.

NMR spectra were evaluated for all other known styrene metabolites (Figure 2-1). However, other metabolites such as derivatives of glutathione conjugates of SO or products that could be produced via ring epoxidation could not be confirmed either directly in urine or for

concentrated urine samples (~ 4 hr, ~7 hr, and ~ 23 hr after exposure initiation). Signals for endogenous compounds are present in concentrated urine (Figure 2-3 for controls) in regions (e.g., 50-70 ppm) where other styrene-derived metabolites would be expected to arise. Previous investigators have predicted that workers with MA acid excretion of 200 mg/mL would excrete >2 mg/mL mercapturic acid (summarized in reference 5). Using this relationship, SO-derived mercapturic acid levels in our study could be as high as 3.2 mg/L (i.e., MA ~ 2 mM; ~320 mg/L). Assuming a SO-derived mercapturic acid molecular weight of ~ 270 mg/mmol, this relationship would equate to ~ 0.01 mM. The NMR method used for this study enabled quantitation of styrene-derived metabolites to 0.1 mM.

2.7 Quantitation

Quantitation of metabolites via NMR was complicated by the low signal to noise for styrene derived metabolite in urine from these subjects. However, an estimate of the concentrations of PAA and HA were made based on comparison of integral values for the labelled carbons with that of dioxane added as an internal standard. This estimate was made for several time-points (~ 4 hr, ~ 7hr, ~ 23 hr) for concentrated urine samples.

To determine the validity of using this method, MA and PGA were also quantitated against the dioxane internal standard and compared with the results generated by HPLC (part 1 of report). The concentration of MA was calculated based on comparing the integral for the methine carbon of MA (75 ppm) with that of dioxane (66 ppm):

$$[MA] = \{[dioxane]^*(integral\ MA)^*(1.1/99)\}/(integral\ dioxane/4).$$

The integral for dioxane is divided by 4, since it arises from 4 equivalent carbons, and the ratio of 1.1/99 accounts for the 99% ^{13}C-label on MA relative to the naturally abundant ^{13}C carbons in dioxane.

The concentration of PGA was based on comparison of the relative integral for the PGA carbonyl carbons to that of the MA carbonyl carbon.

$$[PGA]=\{[MA]^*(integral\ MA_{co})\}/integral\ PGA_{co}.$$

Overall, quantitative results by NMR are in close agreement with those obtained by the other analytical methods, where the average and standard deviations for [MA] and [PGA] for subjects 2-5 are not different from those determined by HPLC (Table 2-2). However, comparison of individual time-points for each subject does show more variability. This variability may arise due to procedures used in sample preparations.

Estimates for the HA and PAA concentrations were obtained in a similar manner (Table 2-2). MA, PGA, and HA are derived from the same pathway of SO metabolism (after hydrolysis of SO). PAA is derived from a second pathway of metabolism (following conversion of styrene to phenylacedaldehyde). The metabolites produced following hydrolysis of SO (MA, PGA, HA) account for the major percentage (~95 %) of the total assigned excreted metabolites of styrene for the urine samples (#2) collected at ~ 4 hr. PAA accounts for only a small percentage (~ 5 %) of the described metabolites.

C 03

Table 2-1. Chemical shifts and coupling constants for styrene derived metabolites in urine from exposed humans and comparison of chemical shifts with those obtained for standards and for styrene exposed rats and mice.

Metabolite identification[c]	SUBJECTS 1-5[a] Chemical shift (ppm)[d]	SUBJECTS 1-5[a] Carbon-carbon coupling (J_{cc})[e]	RAT/MOUSE[b] Chemical shift (ppm)[d]	RAT/MOUSE[b] Carbon-carbon coupling (J_{cc})[e]	PROPOSED SHIFTS Predicted shift[f]	PROPOSED SHIFTS Standard shift[g]
MA						
C7	74.7	48, 52 (d,d,bl)	74.9	54 (d)	72	74.4
C8	179.2	54 (d, bl)	179.1	54 (d)	175	178.6
C1	140.3	m	na[h]		138	139.8
ring	126-128	m	na		127-128	127-129
PGA						
C7	196.8	~66, 50 (d,d)	196.6	66 (d)	188	196.6
C8	172.7	~66, 13 (d, l)	172.6	66 (d)	166	172.7
C1	135.0	m	na		133	134.9
ring	126-133	m	na		129-133	128-132
HA[i]						
C7	170.1	66 (d, bl)	170.01-170.1	s	168	170.2

[a] Subjects were exposed to 50 ppm uniformly labelled [^{13}C]styrene for 2 hr. Styrene derived metabolite contain complex splitting patterns from direct and long-range carbon-carbon coupling.

[b] Rats and mice were exposed to 500 ppm [7,8-^{13}C]styrene for 6 hr. Styrene derived metabolites give rise to doublets produced from coupling between the adjacent ^{13}C-labelled carbons. (Sumner et al., unpublished results, 1999).

[c] MA, mandelic acid; PGA, phenylglyoxylic acid; HA, hippuric acid; PAA, phenylacetic acid/phenyl aceturic acid

[d] Chemical shifts for the center of multiplet patterns. Small differences in chemical shifts between subjects or between humans and rodents are expected due to differences in urine composition (e.g., pH).

[e] Carbon-carbon coupling constants measured in the 1D spectrum: d=doublet; d,d=doublet of doublets; s=singlet; b=broad; l=long-range splitting; m, multiplet. Differences in coupling constants between subjects are expected due to differences in line width. Differences between rodent and human data are expected to differences in position of ^{13}C-labels.

[f] Shifts predicted through the STN International Data Base.

[g] Chemical shifts for standards spiked in pre-exposure control urine.

[h] Not applicable because styrene was labelled only at the C7 and C8.

[i] These shifts are consistent with hippuric acid. Partially resolved singlets were present in the urine form exposed rats and mice, consistent with the presence of several compounds possessing only the C7 labelled carbon from the administered [7,8-^{13}C]styrene compounds. This degree of resolution was not obtained in the ^{13}C-NMR spectra of metabolites in human urine due to the multiple splitting patterns derived form the uniformly labelled ^{13}C-styrene.

C1	ov[j]		na		134	131.9
ring	ov		na		128-132	127-128
PAA[k]						
C7	42.1	48, 49 (d,d,bl)	42.0	48 (d)	41-43	42.2
C8	173.9	49 (d, bl)	174.08	48 (d)	173-178	174.8
C1	ov		na		135	135.4
ring	ov		na		127-129	126-129
Unknown						
C7	175.1	66 (d, bl)	nd			
C1	ov					
ring	ov					

[j] The ring carbon resonances overlap those from the metabolites (MA) with higher signal intensity.

[k] These shifts are consistent with phenylacetic acid and phenylaceturic acid. Large doublets with resolved shoulder peaks are present in the urine form exposed rats and mice, consistent with the presence of both compounds. This degree of resolution was not obtained in the ^{13}C-NMR spectra of metabolites in human urine due to the multiple splitting patterns derived form the uniformly labelled ^{13}C-styrene.

Table 2-2. Comparison of MA and PGA concentrations determined by NMR and HPLC and estimates for the HA and PAA concentrations for urine collected at ~ 4, 7, or 23 hr.

	NMR (mM)[a]	HPLC (mM)[b]	% Total[c]
4 hr			
MA (n=4)	1.4 ± 0.51	1.9 ± 1.1	73 ± 8.6
PGA (n=4)	0.36 ± 0.24	0.36 ± 0.24	18 ± 7.6
PAA (n=4)	0.09 ± 0.04	nd	4.5 ± 1.6
HA (n=4)	0.11 ± 0.06	nd	5.7 ± 0.69
7 hr			
MA (n=4)	0.28 ± 0.12	0.38 ± 0.22	68 ± 8.9
PGA (n=4)	0.13 ±0.05	0.13 ± 0.07	32 ± 8.9
PAA (n=3)	cd	nd	
HA (n=3)	cd	nd	
23 hr			
MA (n=2)	0.19 ± 0.04	0.19	

[a] cd, detected but not integrated due to low signal-to-noise

[b] nd, did not quantitate PAA or HA by HPLC

[c] based on NMR results

Figure Captions

Figure 2-1. Proposed metabolism of styrene. The scheme is proposed from metabolites detected in urine, tissues, or blood of styrene exposed rats and mice, exposed workers, or exposed volunteers (reviewed by Bond, 1989[3] ; reviewed by Sumner and Fennell, 1994[4]).

Figure 2-2. ^{13}C-NMR spectra of urine collected from subjects 1-5 (bottom to top) prior to exposure. Urine samples were prepared by adding an aliquot of dioxane (<1 µL) and D_2O (100 µL) to 400 µL of urine.

Figure 2-3. ^{13}C-NMR spectra of urine collected from subjects 1-5 (bottom to top) prior to exposure. Urine samples were concentrated after protein precipitation and resuspended in D_2O.

Figure 2-4. ^{13}C-NMR spectra of urine collected from subject 2 prior to exposure (bottom trace) and at approximately 2, 4, 6, 9, 13, 22, 24, 37, and 46 hr (bottom to top) following initiation of exposure to 50 ppm ^{13}C-styrene for 2 hr. Urine samples were prepared by adding an aliquot of dioxane (<1 µL) and D_2O (100 µL) to 400 µL of urine.

Figure 2-5. ^{13}C-NMR spectra of urine collected from subject 3 prior to exposure (bottom trace) and at approximately 2, 4, 6, 9, 13, 22, 25, 37 and 46 hr (bottom to top) following initiation of exposure to 50 ppm ^{13}C-styrene for 2 hr. Urine samples were prepared by adding an aliquot of dioxane (<1 µL) and D_2O (100 µL) to 400 µL of urine.

Figures 2-6 thru 2-8. ^{13}C-NMR spectra of urine collected from subjects 1, 4, and 5 prior to exposure (bottom trace) and at approximately 4 and 13 hr (bottom to top) following initiation of exposure to 50 ppm ^{13}C-styrene for 2 hr. Urine samples were prepared by adding an aliquot of dioxane (<1 µL) and D_2O (100 µL) to 400 µL of urine.

Figure 2-9. ^{13}C-NMR spectra of concentrated urine from Subject 4. The urine was collected ~ 4 hr following exposure initiation, concentrated after protein precipitation, and resuspended in D_2O.

Figure 2-10. ^{13}C-NMR spectra of urine collected from subjects 2-5 (bottom to top) at approximately 4 hr following initiation of exposure to 50 ppm ^{13}C-styrene for 2 hr. Urine samples were concentrated after protein precipitation and resuspended in D_2O.

Figure 2-11. ^{13}C-NMR spectra of urine collected from subjects 2-5 (bottom to top) at approximately 23 hr following initiation of exposure to 50 ppm ^{13}C-styrene for 2 hr. Urine samples were concentrated after protein precipitation and resuspended in D_2O.

Figure 2-12. ^{13}C-NMR spectra of urine collected from subjects 2-5 (bottom to top) at approximately 7 hr following initiation of exposure to 50 ppm ^{13}C-styrene for 2 hr. Urine samples were concentrated after protein precipitation and resuspended in D_2O.

C 07

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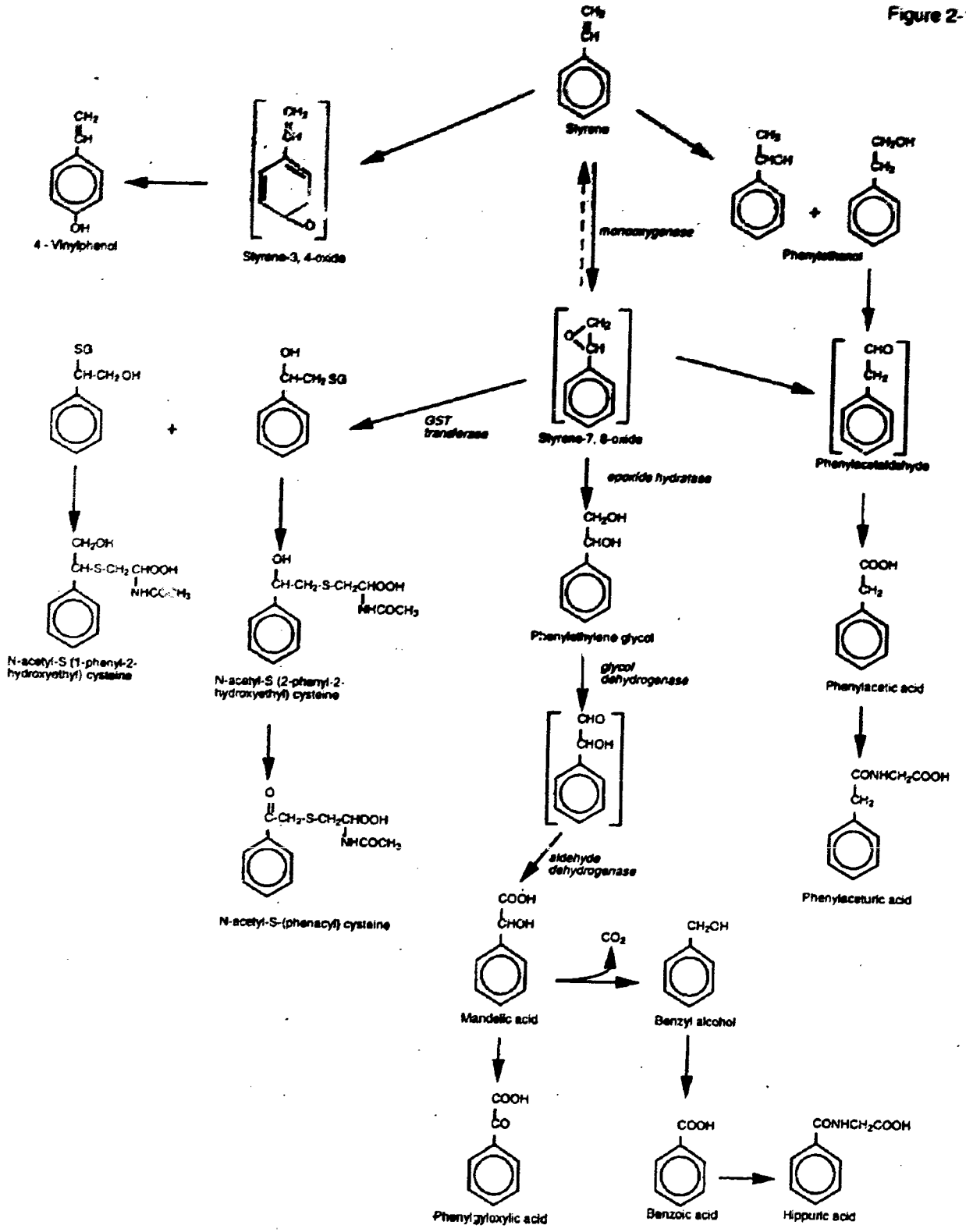
7. Sumner et al., unpublished results.

Figure 2-1


Styrene
4 - Vinylphenol
Styrene-3, 4-oxide
monooxygenase
Phenylethanol
GST transferase
Styrene-7, 8-oxide
epoxide hydratase
Phenylacetaldehyde
N-acetyl-S (1-phenyl-2-hydroxyethyl) cysteine
N-acetyl-S (2-phenyl-2-hydroxyethyl) cysteine
Phenylethylene glycol
glycol dehydrogenase
Phenylacetic acid
N-acetyl-S-(phenacyl) cysteine
aldehyde dehydrogenase
Phenylaceturic acid
Mandelic acid
Benzyl alcohol
Phenylglyoxylic acid
Benzoic acid
Hippuric acid

C 09

Figure 2-2

Control Urine

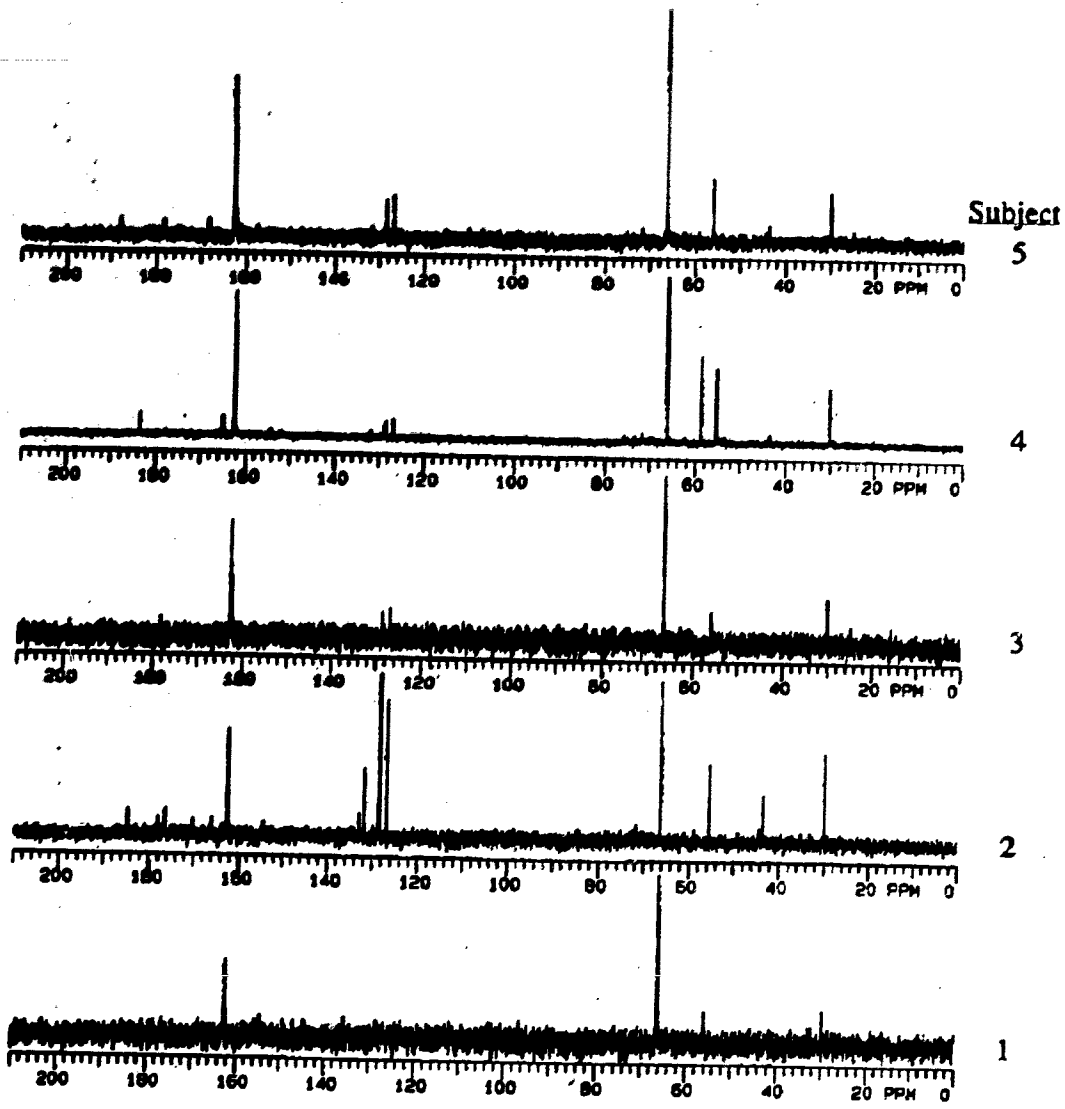
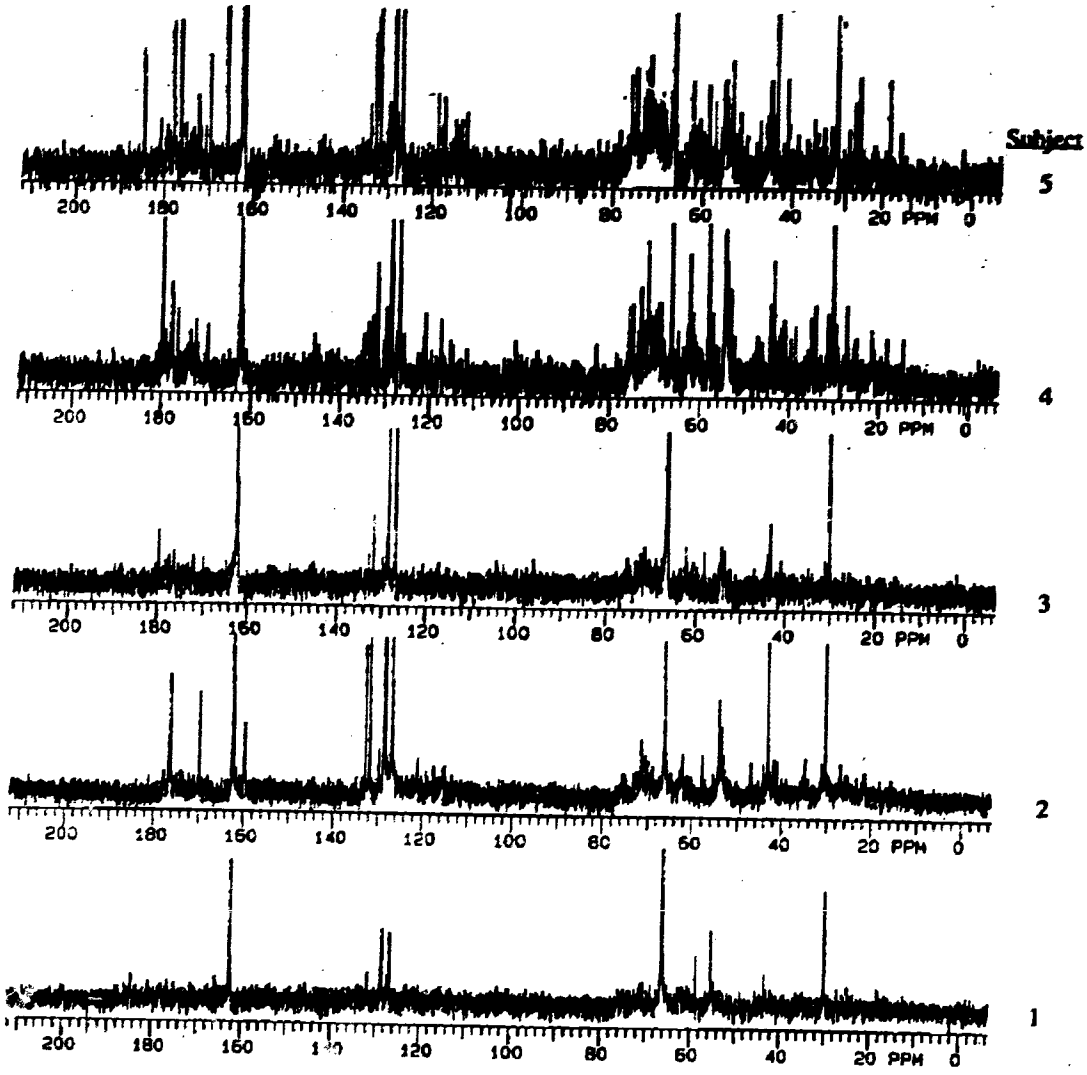


Figure 2-3

Concentrated Controls



C 11

Figure 2-4A

Subject 2

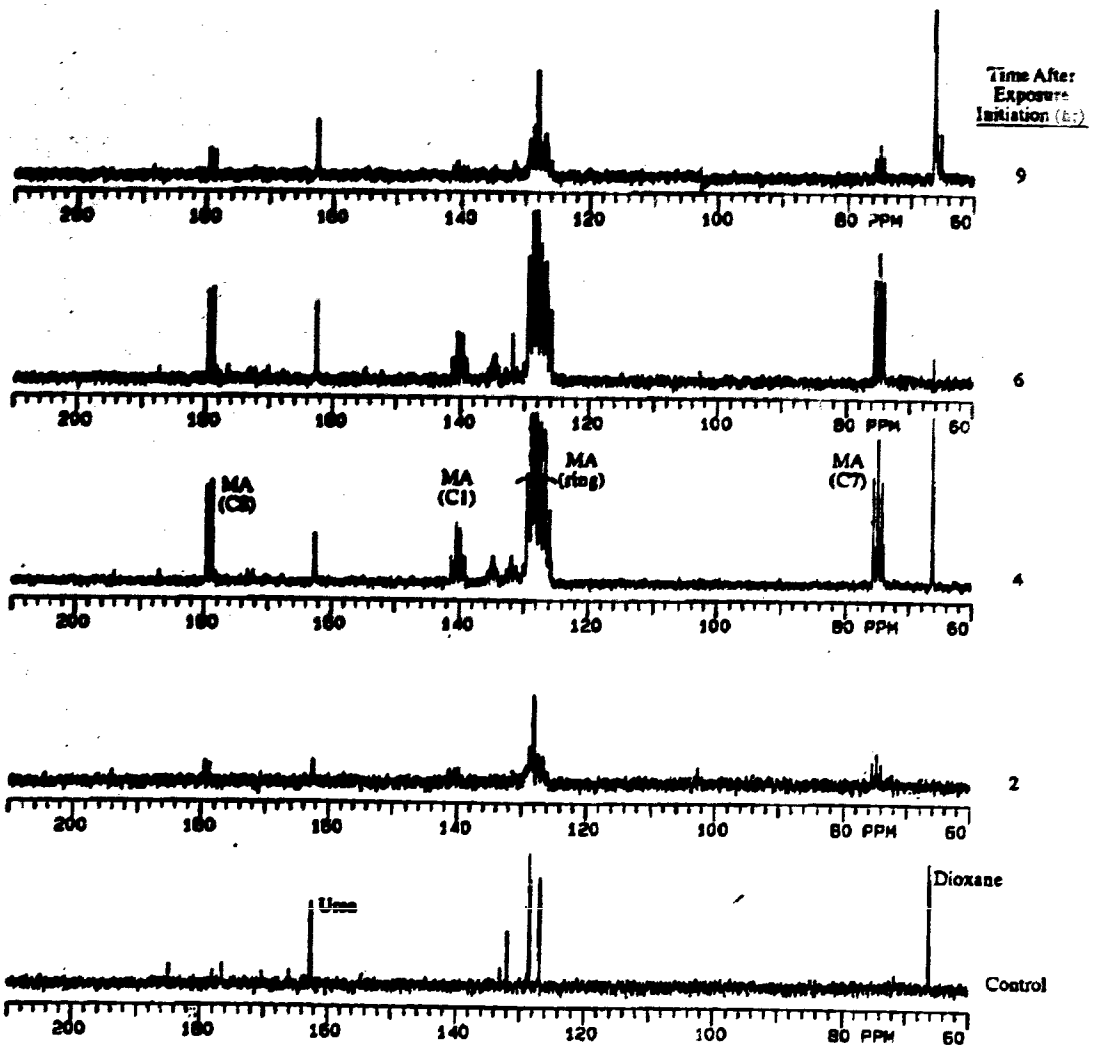
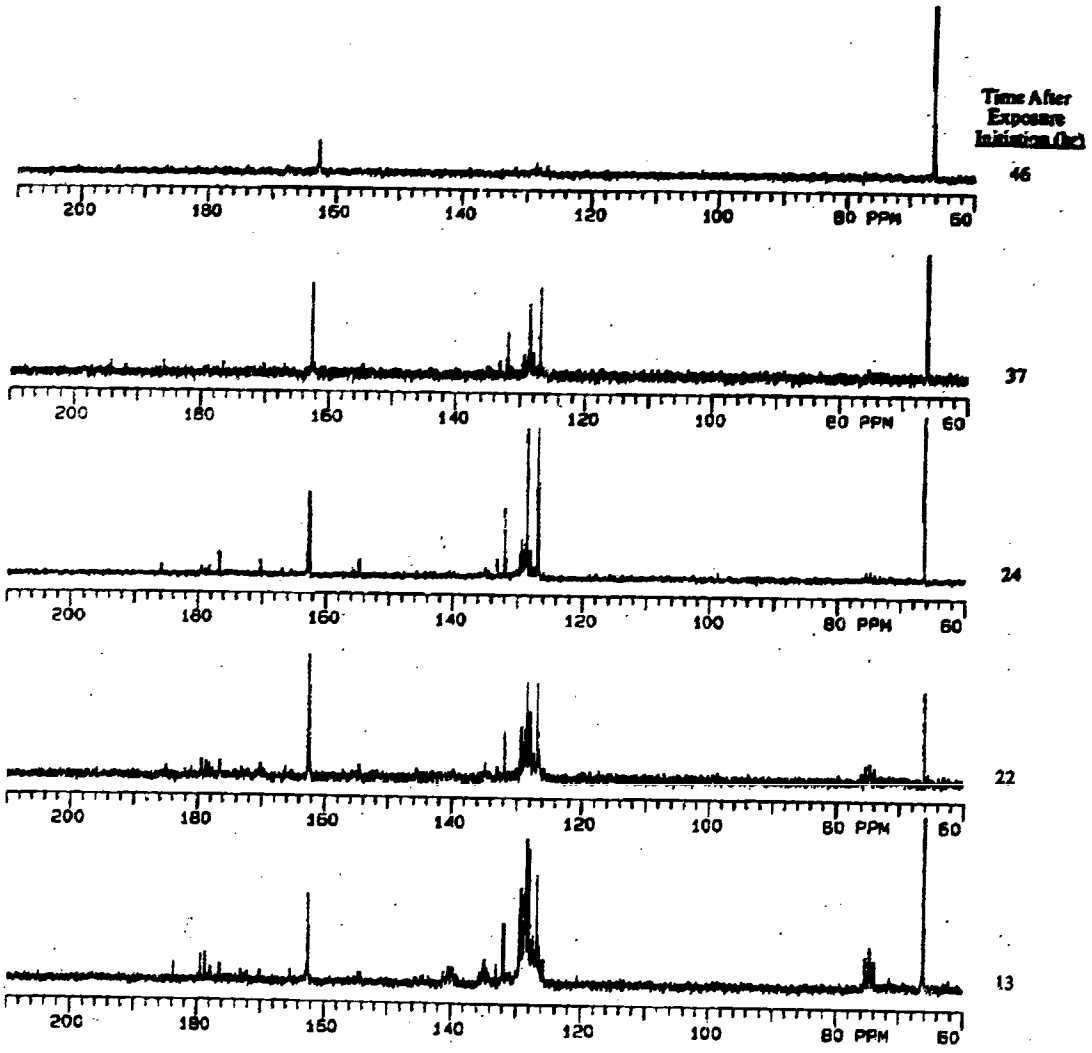


Figure 2-4B

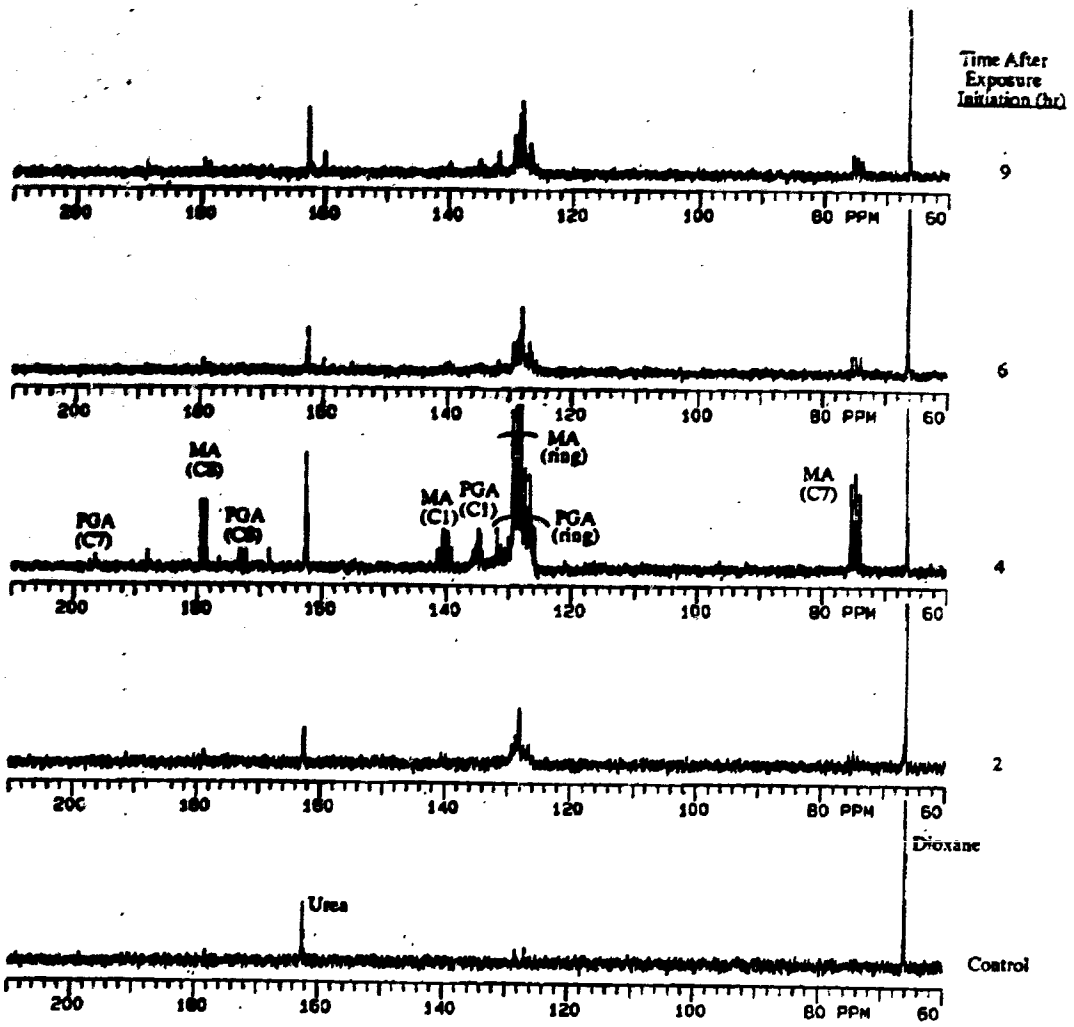
Subject 2



C 13

Figure 2-5A

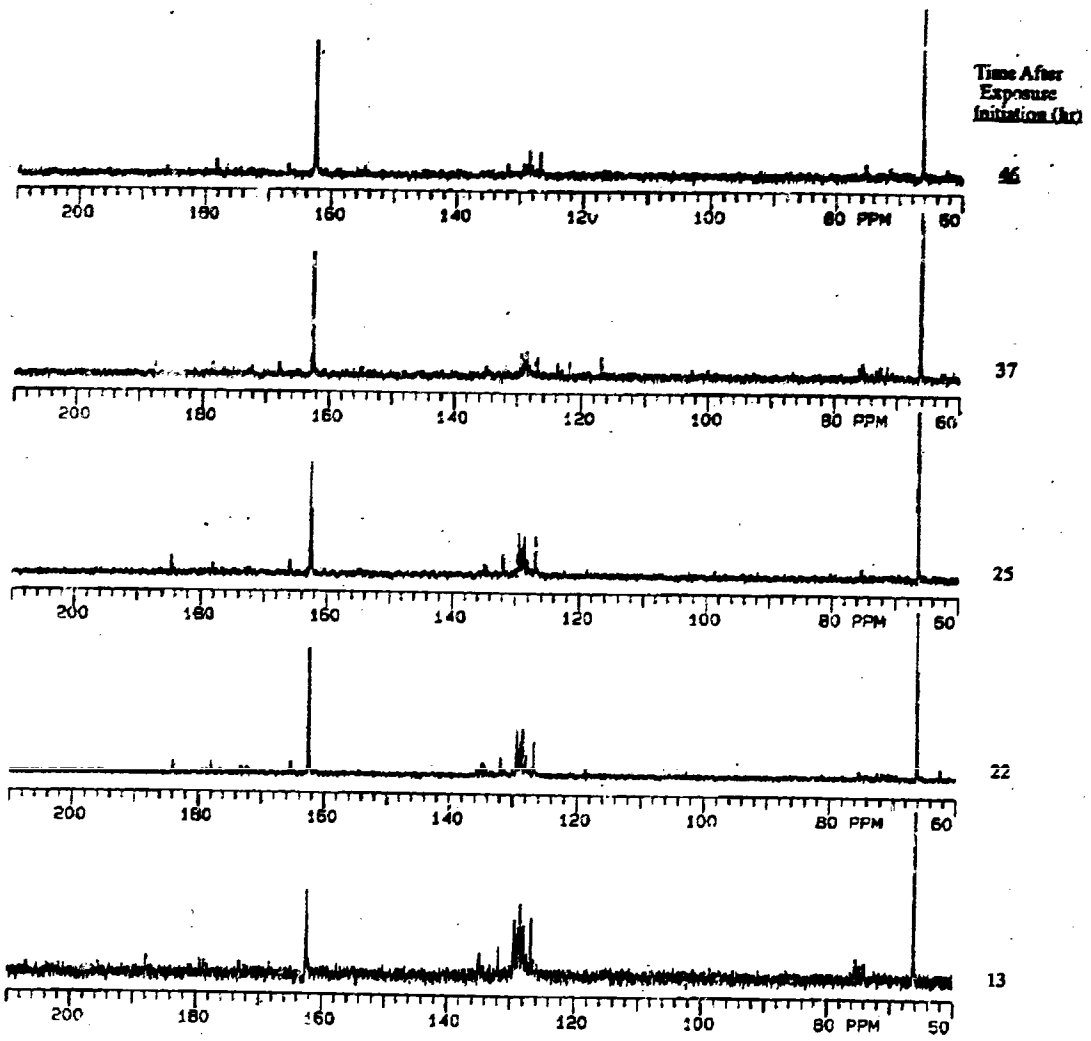
Subject 3



C 14

Figure 2-5B

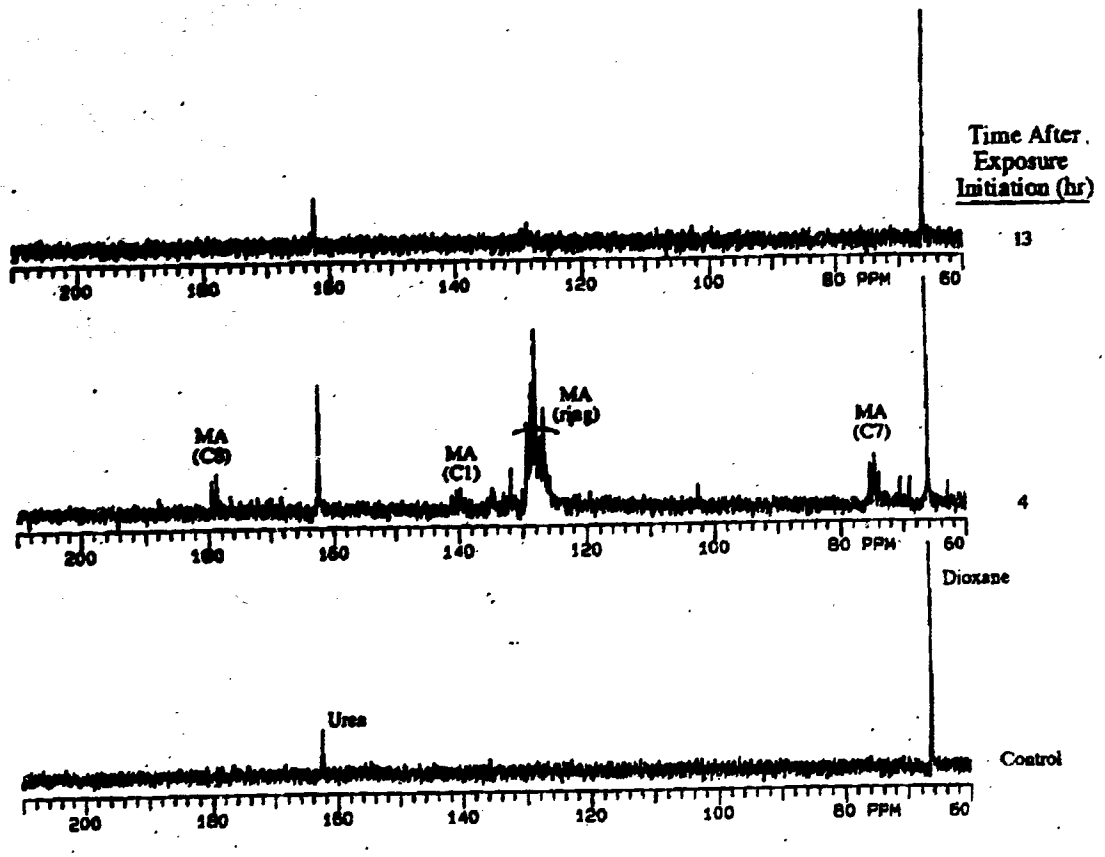
Subject 3



D 01

Figure 2-6

Subject 1



D 02

Figure 2-7

Subject 4

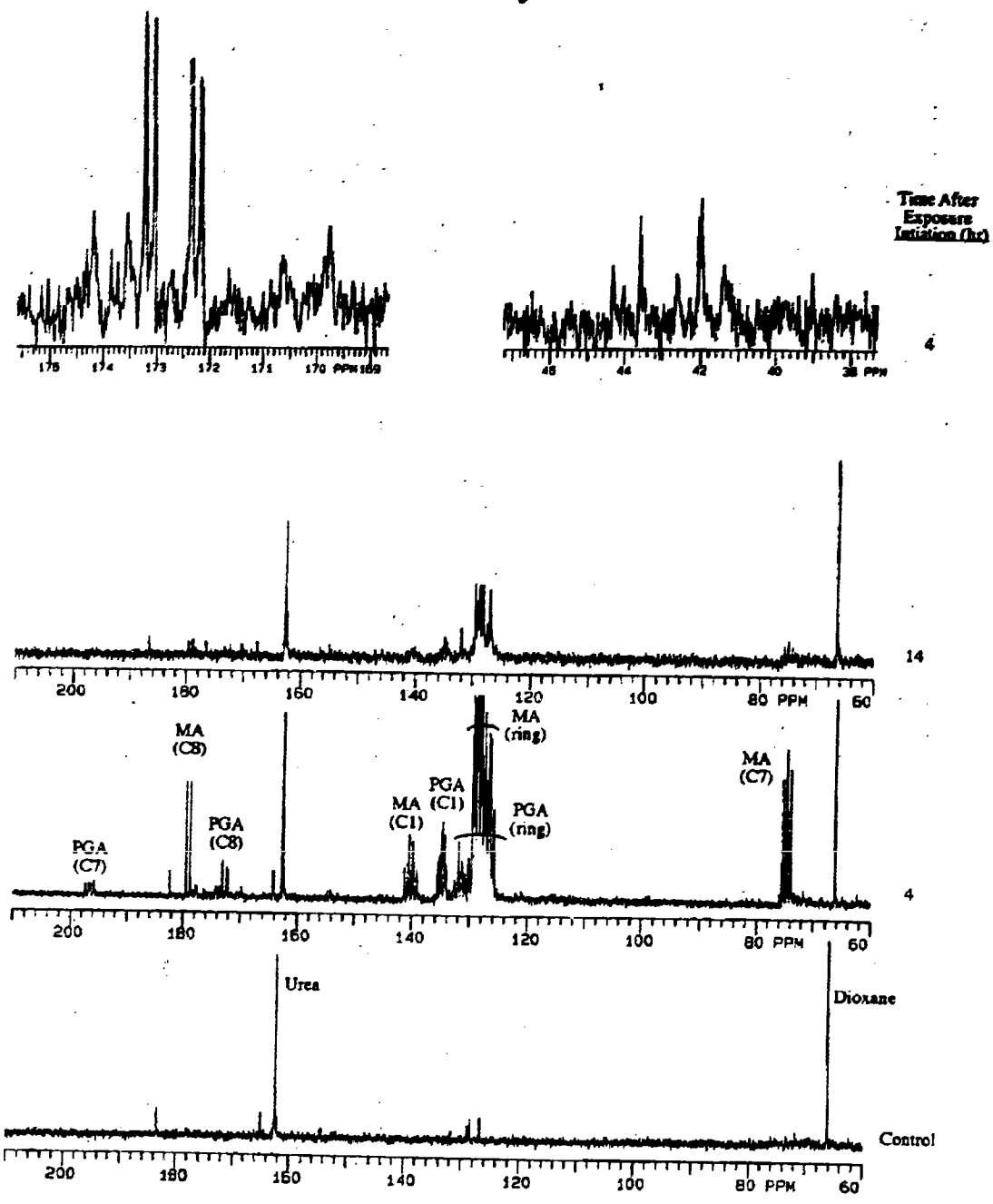
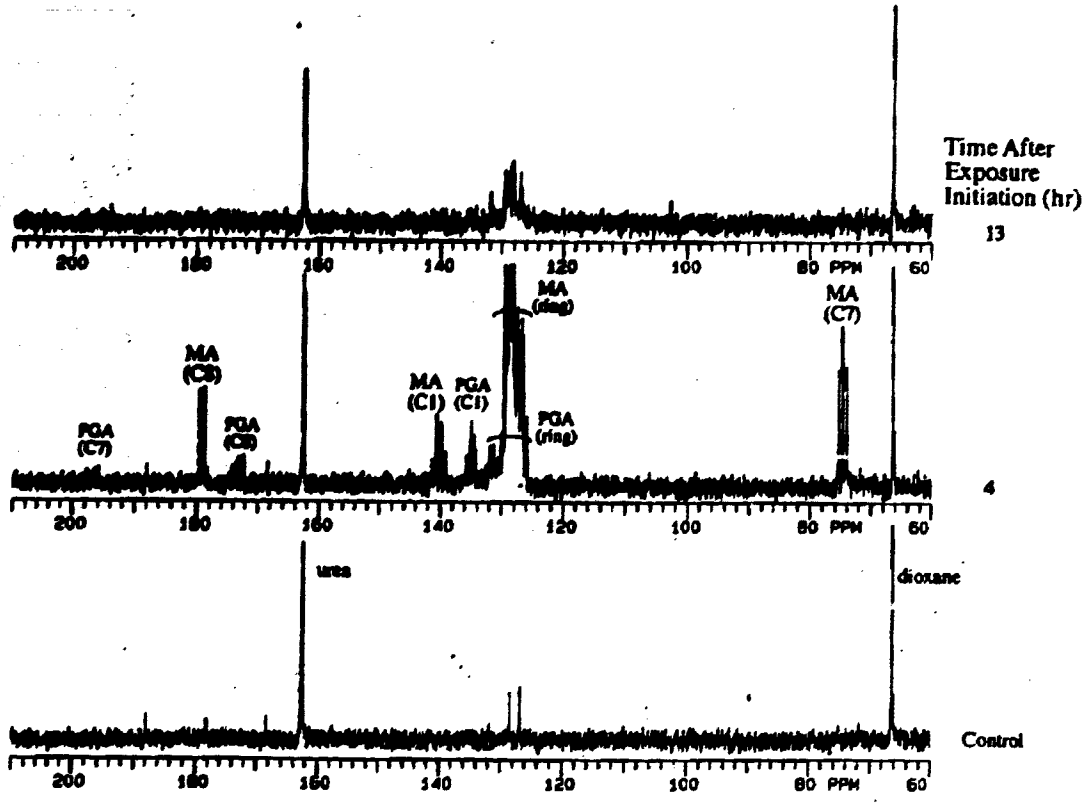


Figure 2-8

Subject 5



D 04

Figure 2-9

Subject 4

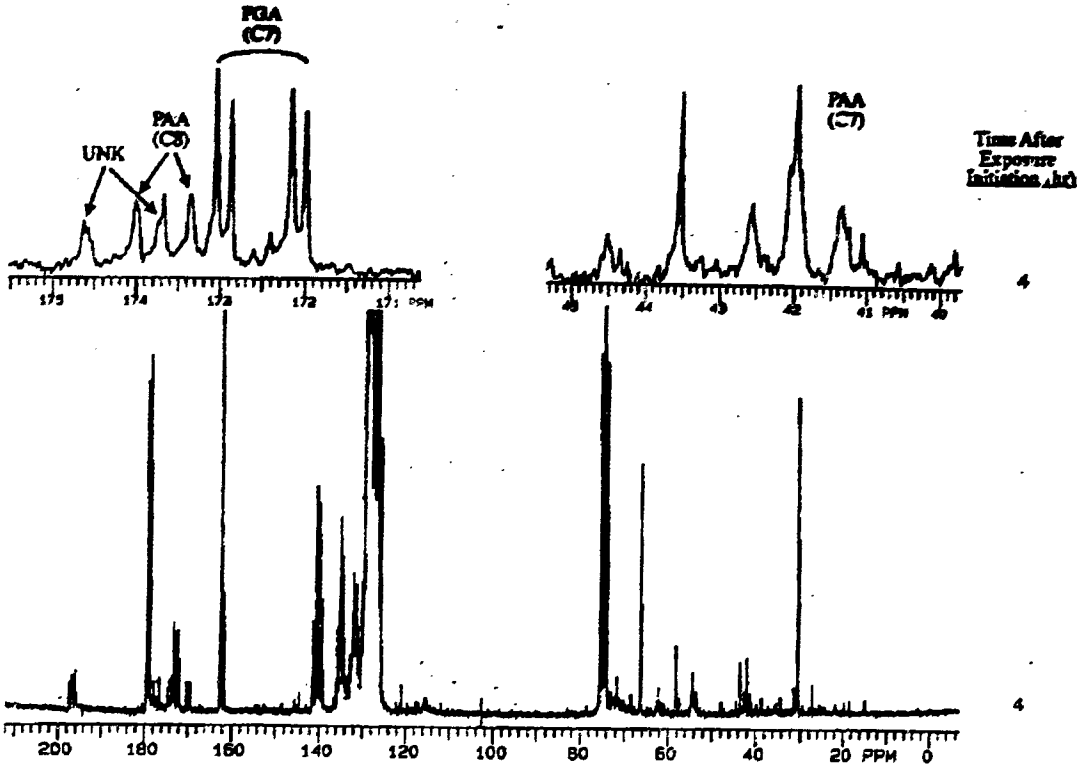


Figure 2-10

Concentrated Urine: 4hr Time Point

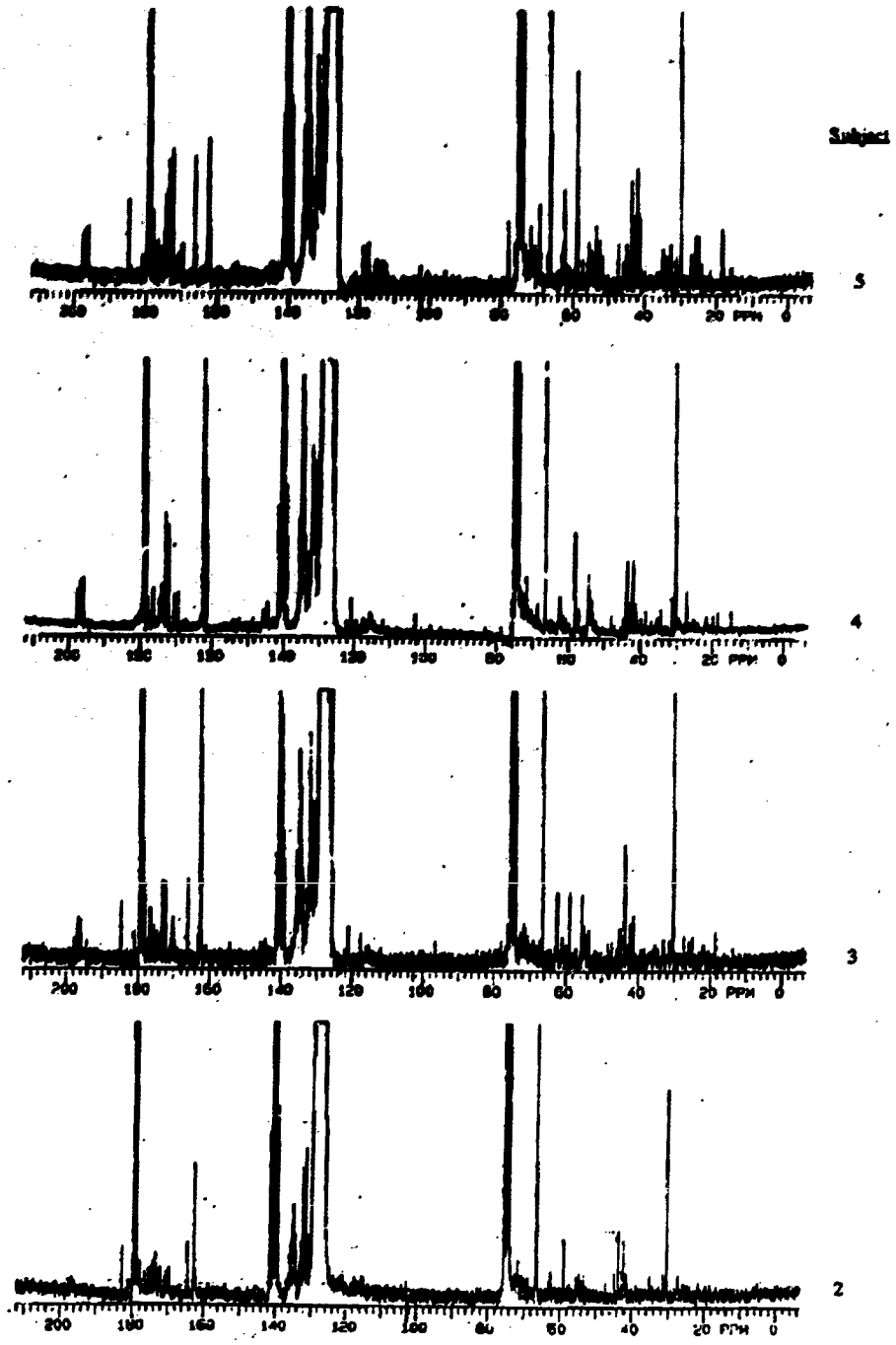
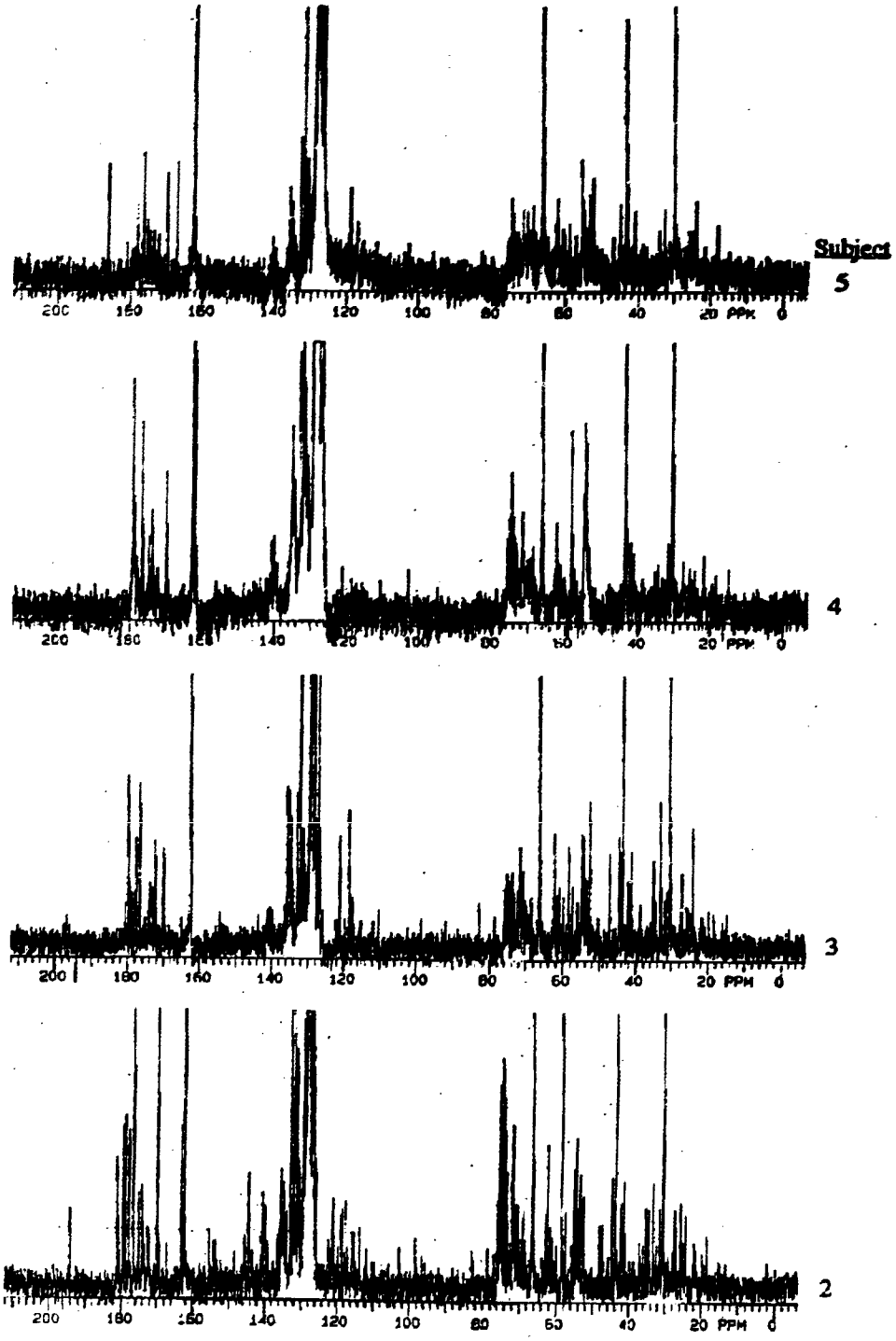


Figure 2-11

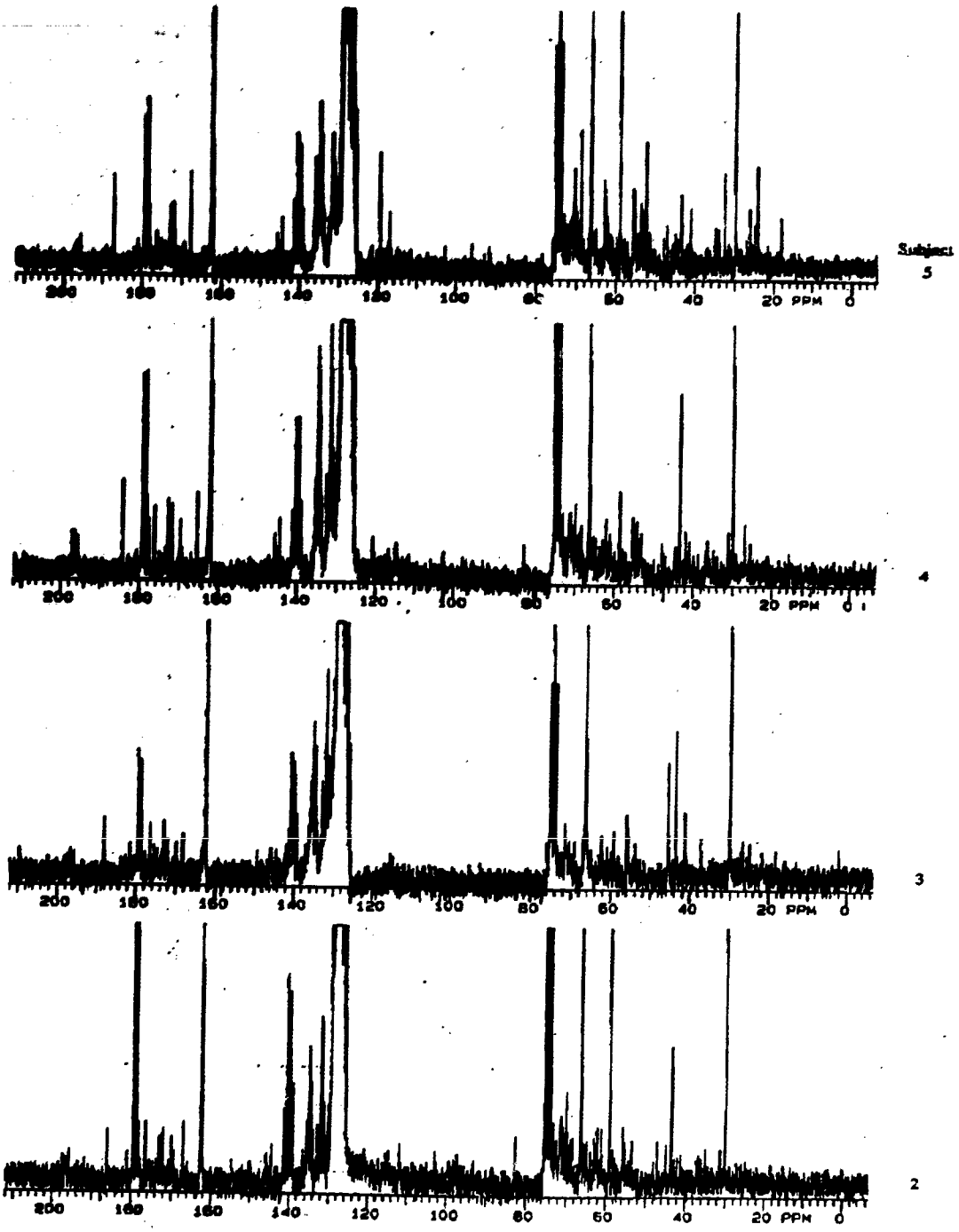
Concentrated Urine: 23hr Time Point



D 07

Figure 2-12

Concentrated Urine: 7hr Time Point



Part 3: Hemoglobin Adducts in ^{13}C-Styrene-Exposed Volunteers

3.1 Staff

The following personnel participated in the portion of the study conducted at the Department of Molecular Genome Research, Arrhenius Laboratories for Natural Sciences, Stockholm University:

S. Osterman-Golkar

H. Licea Pérez

3.2 Chemicals

Pentafluorophenyl isothiocyanate (PFPITC, >97%) from Fluka (Buchs, Switzerland) was purified on a Sep-Pak silica cartridge before use (Törnqvist et al., 1988). *m*-Chloroperbenzoic acid was obtained from Fluka (Buchs, Switzerland).

3.3 Synthesis and characterization of the pentafluorophenylthiohydantoin derivative of hydroxy[$^{13}C_8$]phenethylvaline

m-Chloroperbenzoic acid, 2.9 mmol, was added to a solution of 150 µl (1.3 mmol) ^{13}C-styrene in 2.5 ml dichloromethane. The reaction mixture was stirred for 5 h at room temperature during which time a precipitate formed. After cooling to 0 °C, the reaction mixture was kept at this temperature for 2 h. The precipitate was filtered off using a 0.8 cm^2 DynaGard syringe filter (Microgon Inc., Laguna Hills, CA). A 500 µl portion of the filtrate was reacted overnight with 1 ml 0.1 M ^{14}C-L-valine (1 µCi/mmol) in the presence of triethylamine at 50 °C and with continous shaking. n-Propanol, 2.5 ml and 15 µl PFPITC was added to the water phase for preparation of pentafluorophenylthiohydantoin (PFPTH) derivatives (see below). The derivatives were purified by HPLC using a C18 column (Kromasil 100-5C18, 250 x 4.6 mm, Berks, UK). A 30 min gradient from 50 - 100 % acetonitrile was used and 1 ml fractions were collected. The fractions containing radioactivity (21-25) were pooled, acetonitrile was evaporated under nitrogen and the PFPTH derivatives were extracted into toluene. The product was characterized (Figures 3-1, 3-2a and 3-2b) by GC-MS and gas chromatography/tandem mass spectrometry (GC/MS-MS)[1].

3.4 Quantification of hemoglobin adducts

Globin was precipitated according to Mowrer et al. (1986). The derivatizations were carried out essentially as described by Törnqvist et al. (1988) with some modifications. Two samples of 300 mg globin per individual per time point (before exposure and one day after exposure) were dissolved in 5 ml formamide in 10 ml test tubes with a teflon-lined caps, then 160 µl 1 M NaOH and 60 µl PFPITC were added to each sample. The reactions were carried out at room temperature overnight and then completed at 45 °C for 1.5 h. The PFPTH derivative of hydroxyphenethyl[$^2H_8$]valine, 3.9 pmol, was added as internal standard (Licea Pérez et al., 1997). PFPTH derivatives of hydroxyphenethylvaline were extracted with 4 x 3 ml of diethyl ether. The ether extracts were evaporated under nitrogen. The extracts were

pooled to give one sample per individual and time point. The samples were dissolved in 2 ml toluene and were washed twice with water, twice with freshly prepared 0.1 M sodium carbonate and twice again with water. After evaporation of the toluene the samples were redissolved in 2 ml methanol/water (60:40) and extracted with 2 ml hexane. The hexane phase was transferred to a new test tube and was evaporated under nitrogen. The samples were dissolved in 50 µl toluene for analysis by GC/MS-MS.

The GC/MS-MS analyses were carried out using a Finnigan TSQ-700 instrument in the negative ions chemical ionisation (NICI) mode. The technical details for GC were as follows: column; DB5-MS (30 m, 0.32 mm i.d., 1.0 µm phase thickness), retention gap; methyl deactivated fused silica (CHROMPACK; 2 m, 0.53 mm i.d.). Helium was used as carrier gas at constant gas pressure 5.6 psi, GC temperature program; 1 min at 100 °C, 20 °C/min to 240 °C for 7 min,10 °C/min to 320 °C for 8 min and finally isothermal, 320 °C for 5 min, injector; VARIAN 1093 SPI (septum-equipped programmable), injector temperature program; 100-320 °C, 157 °C/min for 1.4 min and then isothermal at 320 °C for 16 min. The operating procedures for the mass spectrometer were: methane as reagent gas, ion source pressure 4.8 torr (640 Pa), ion source temperature; 120 °C, ionisation energy; 70 eV. Argon was used as collision gas, pressure; 1 mTorr (0.14 Pa), collision energy; 15 eV. Samples of 2 µl were injected on column. The retention times of the two peaks of the internal standard were about 15:13 min and 15:29 min, respectively; the retention times of the PFPTH derivatives of the analytes were 15:15 min and 15:32 min, respectively. The daughter ions m/z 140, 319 and 390 of the parent ion 432 ($[M-20]^-$) of the analyte and 147, 325, and 384 of the parent ion 431 ($[M-21]^-$) of the internal standard, respectively, were recorded. The quantifications were based on peak areas and assuming equal response of the analyte and the internal standard.

3.5 Results and discussion

The fragmentation pattern of the PFPTH derivatives of hydroxy[$^{13}C_8$]phenethylvaline is shown in Figures 3-2a and 3-2b. Two characteristic major fragments, m/z 432 and m/z 319, were formed representing $[M - HF]^-$ and $[M - OH^{13}C^{13}C_6H_5 - HF]^-$, respectively. Figure 3-3a shows the fragmentation of hydroxyphenethyl($^2H_8$)valine with the corresponding fragments m/z 431 ($[M-^2HF]^-$) and m/z 325 ($[M - CHCC_6H_5 - ^2HF]^-$). The fragments 432 of the analyte and 431 of the internal standard, respectively, were chosen as parent ions in the MS-MS-analysis. Daughter ions specific to the analyte (Figure 3-2b) and internal standard (Figure 3-3b), respectively, were recorded for adduct quantification.

The samples from each individual were analyzed. However, only trace amounts of hydroxy[$^{13}C_8$]phenethylvaline-PFPTHs were seen in the chromatograms. Therefore, pooled samples were prepared of the samples collected prior to and after exposure, respectively. The level of hydroxy[$^{13}C_8$]phenethylvaline in the pooled sample was estimated as 0.3 pmol/g globin (corrected for background; Figures 3-4a and 3-4b) corresponding to an adduct increment of about 0.003 pmol/g globin per ppm x h.

Prolonged exposure to reactive chemicals results in steady state levels of hemoglobin adducts. The steady state resulting from occupational exposure corresponds to about 9 weeks of exposure during working hours, i.e. 360 hours of exposure. Christakopoulos et al.

(1993) reported an average hemoglobin adduct level of about 18 pmol/g globin (corrected for a methodological background) in reinforced plastic workers exposed to an average styrene workplace concentration of about 75 ppm. The adduct increment per ppm x h is 0.001 pmol/g globin (28/(360 x 75) as estimated from Christakopoulos' data. A similar estimation based on data from Vodika (1999, unpublished results) gives 0.0003 pmol/g globin/ppm x h.

The uncertainty associated with the estimation of exposure is considerable in studies evaluating hemoglobin adducts in occupationally exposed workers. Adducts accumulate over the lifespan of the erythrocyte (~ 120 days in humans). However, it is generally only feasible to make a few point estimates of styrene concentrations during the course of the "window" in which adducts accumulate. Therefore, while the adduct measurement can be conducted with reasonable certainty, the exposure estimate assumes that the pattern of exposure follows that of the point measurements. Thus, the estimates of the relationship between exposure and adducts (adducts increment per hr) obtained from occupational exposure will have a high degree of uncertainty.

3.6 References

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3.7 Figure Captions

Figure 3-1. Ion chromatogram from the GC/MS-MS analysis of the synthetic PFPTH derivative of hydroxy[$^{13}C_8$]phenethylvaline.

Figure 3-2. a) Negative ion chemical ionization spectrum of the PFPTH derivative of hydroxy[$^{13}C_8$]phenethylvaline and b) collision induced daughter ion spectrum of m/z 432 of the PFPTH derivative of hydroxy[$^{13}C_8$]phenethylvaline.

Figure 3-3 a) Negative ion chemical ionization spectrum of the PFPTH derivative of hydroxyphenethyl[$^{2}H_8$]valine and b) collision induced daughter ion spectrum of m/z 431 of the PFPTH derivative of hydroxyphenethyl[$^{2}H_8$]valine.

Figure 3-4. Ion chromatogram from the GC/MS-MS analysis of a) globin obtained one day after exposure to [$^{13}C_8$]styrene (upper trace, analyte; lower trace, internal standard) and b) globin obtained before the exposure.

3.8 Comments on the study

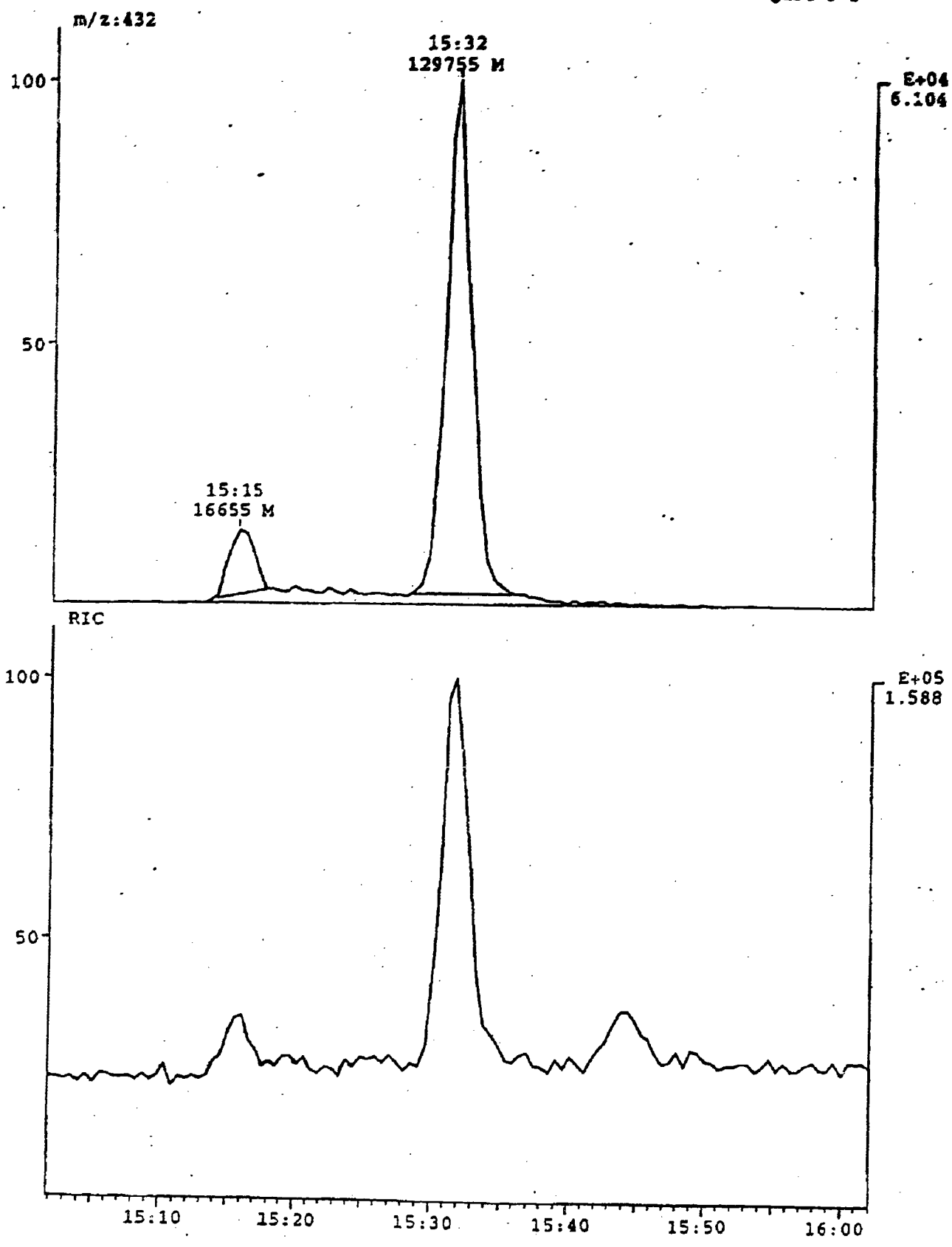
We have shown that it is possible to measure hemoglobin adducts in a pooled sample from all the exposed individuals. If differences between individuals are of interest it would also be possible to pool samples taken at the different time points from each of the individuals. However, such an extension of the project cannot be performed without financial support.

It would also be of interest to use the samples for an analysis of cysteine adducts with the approach used by Rappaport and collaborators.

[1]Abbreviations: GC/MS-MS; tandem mass spectrometry; PFPITC, pentafluorophenyl isothiocynate; PFPTH, pentafluorophenylthiohydantoin

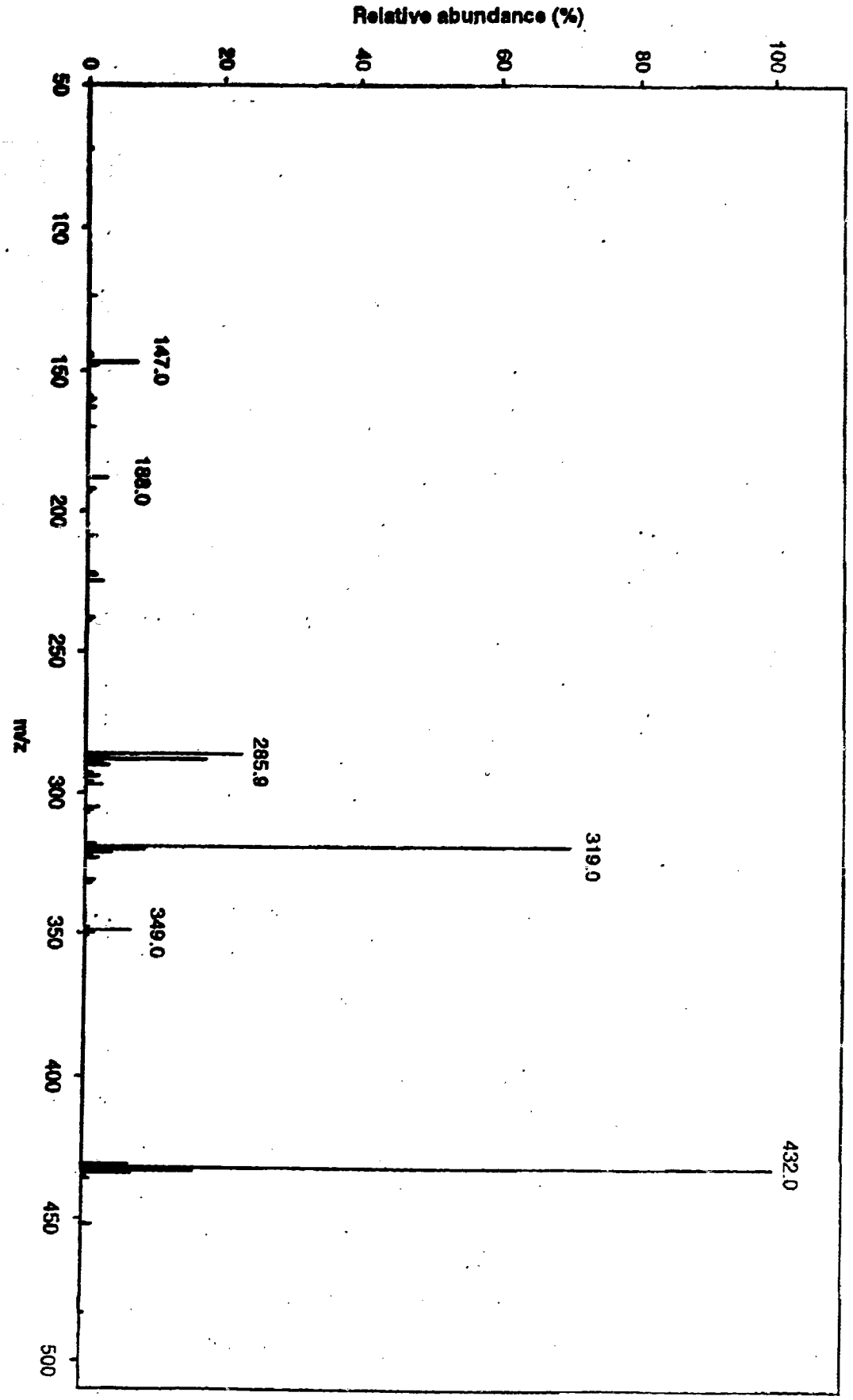
D 12

Figure 3-1



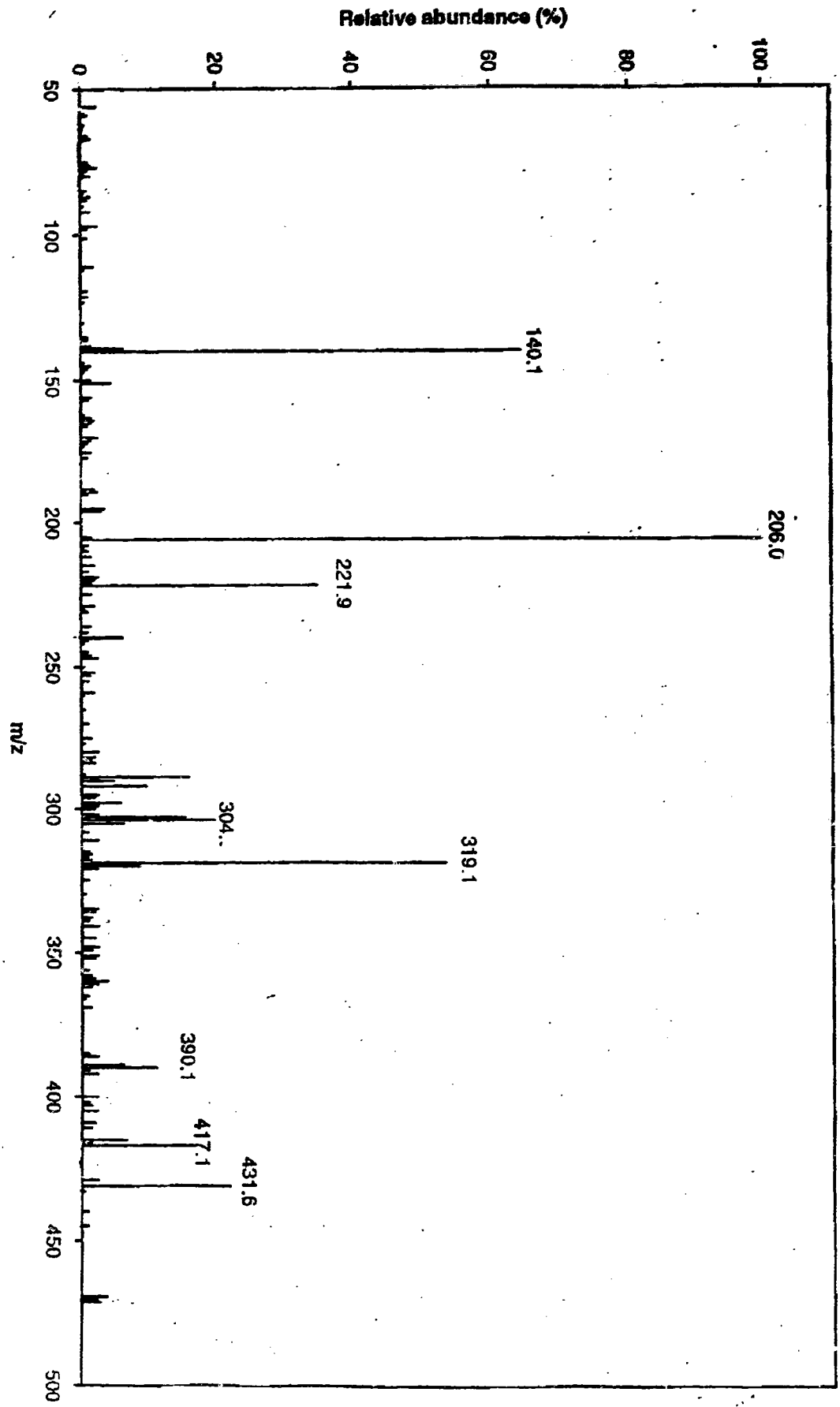
D 13

Figure 3-2a



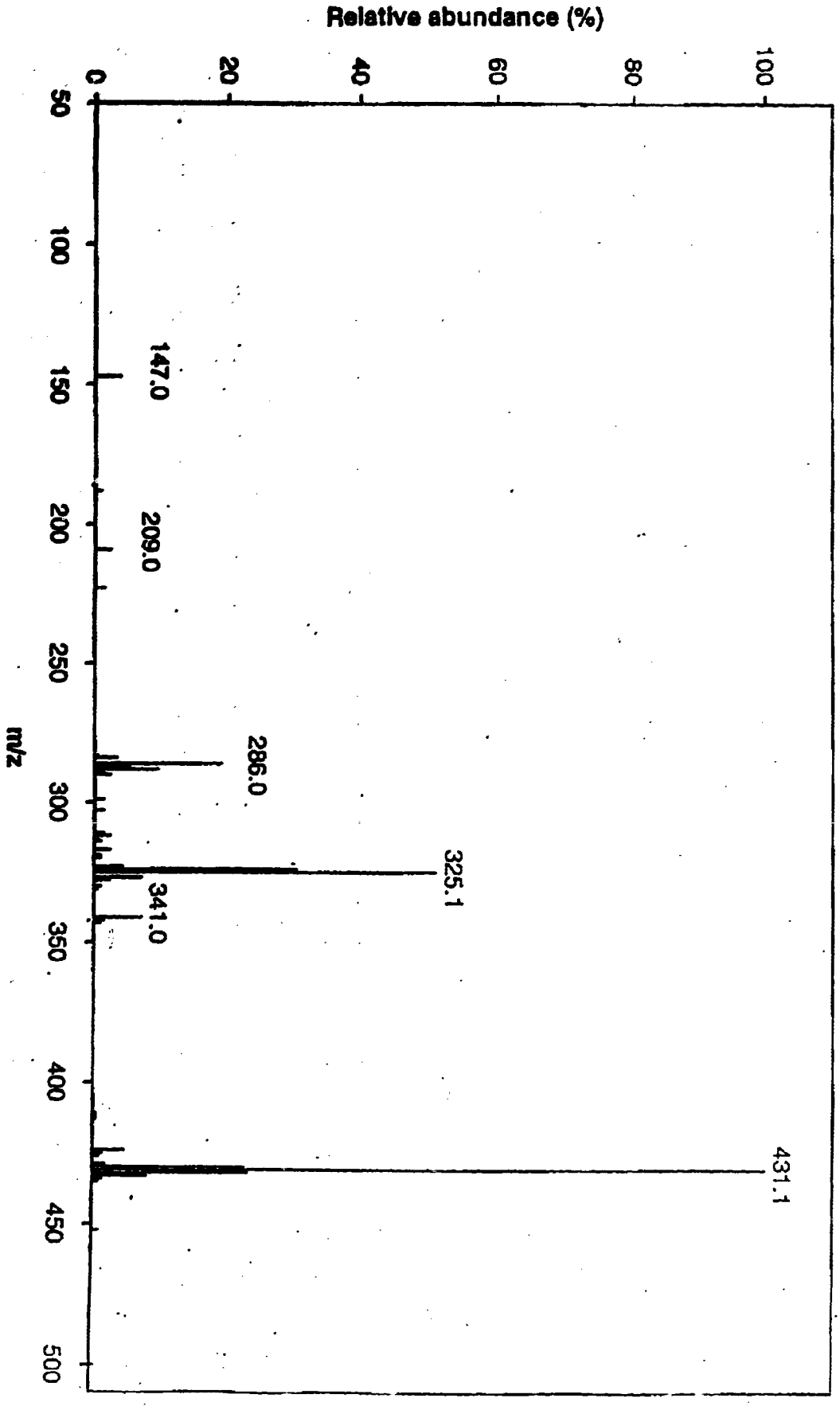
D 14

Figure 3-2b



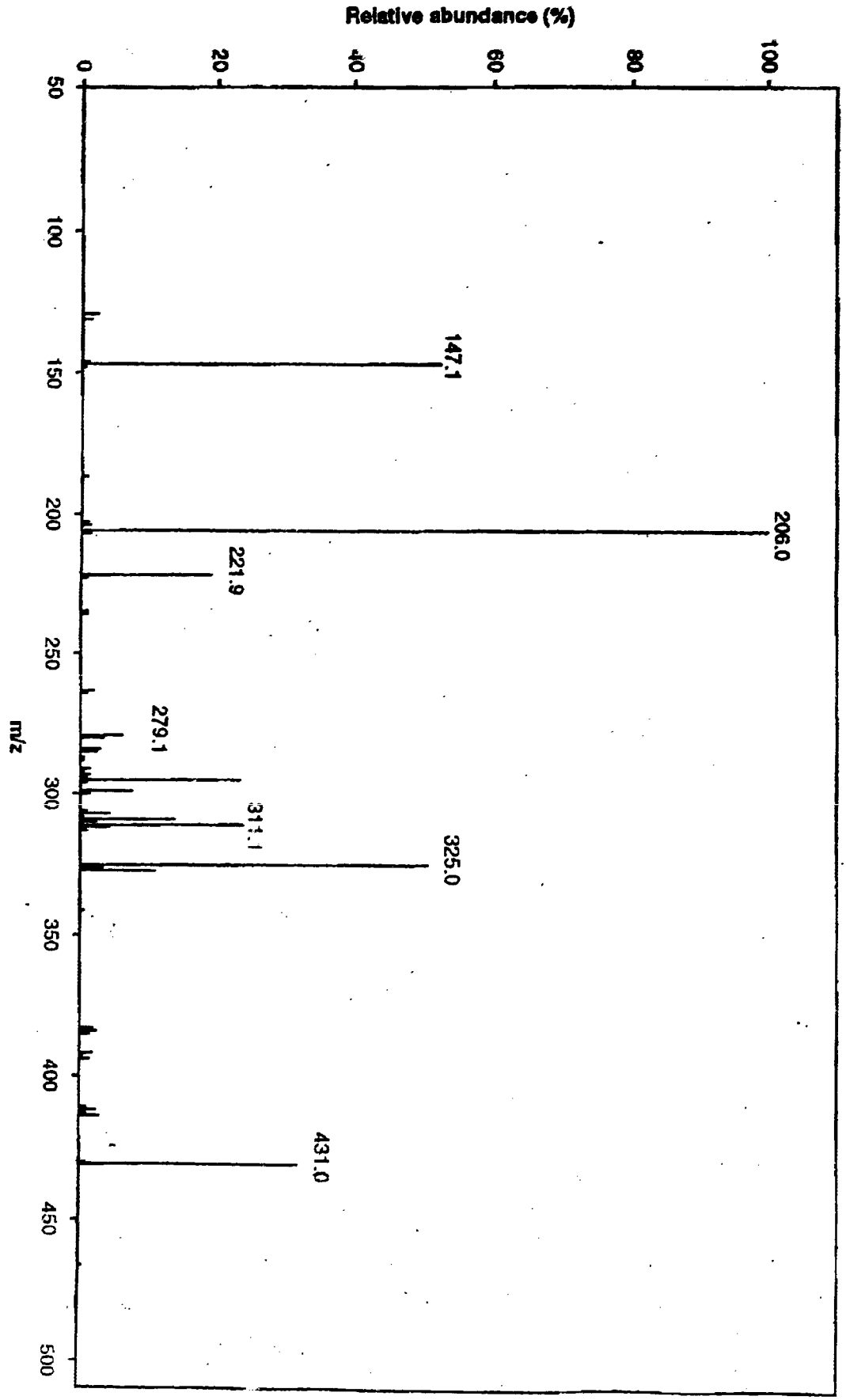
E 01

Figure 3-3a



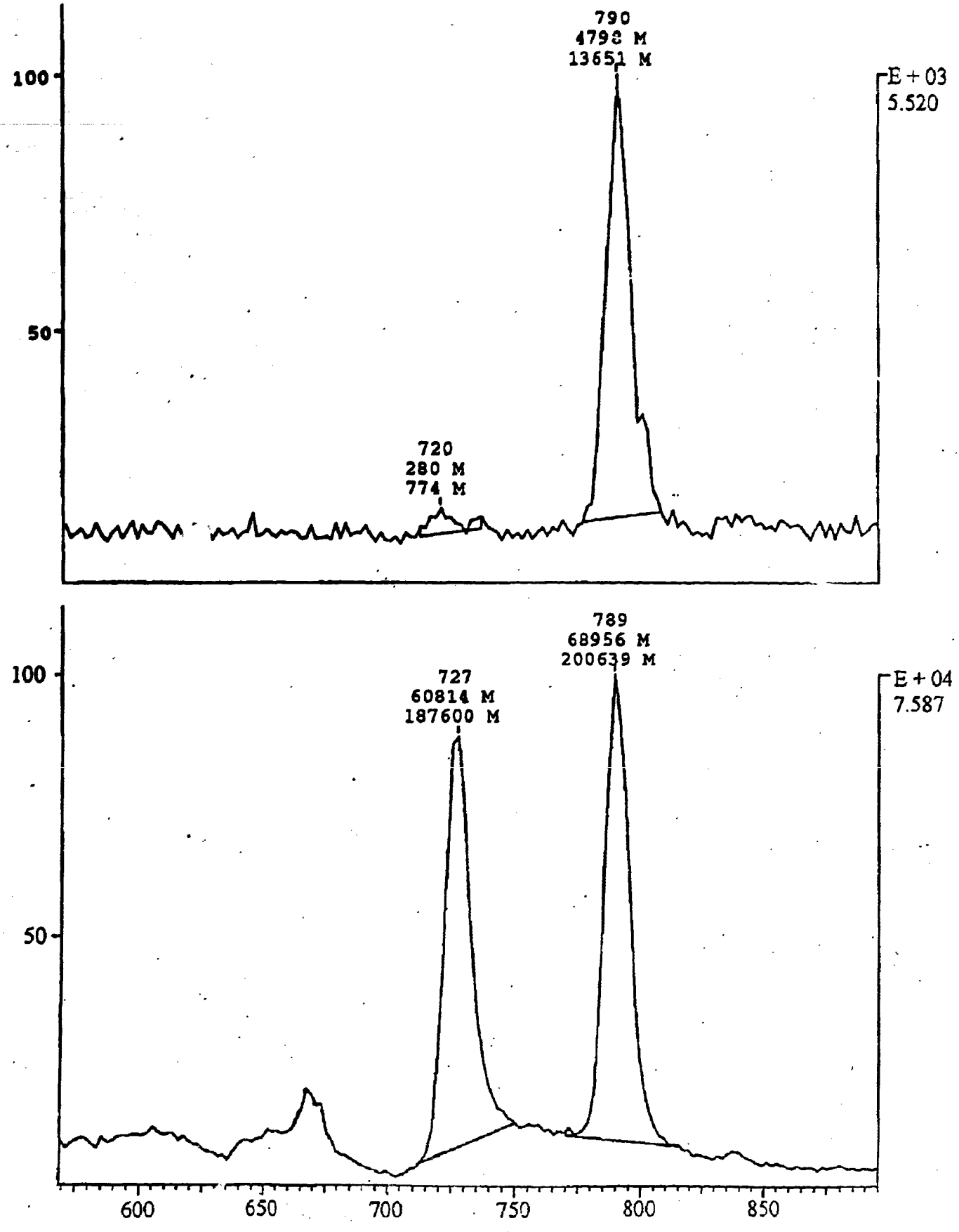
E 02

Figure 3-3b



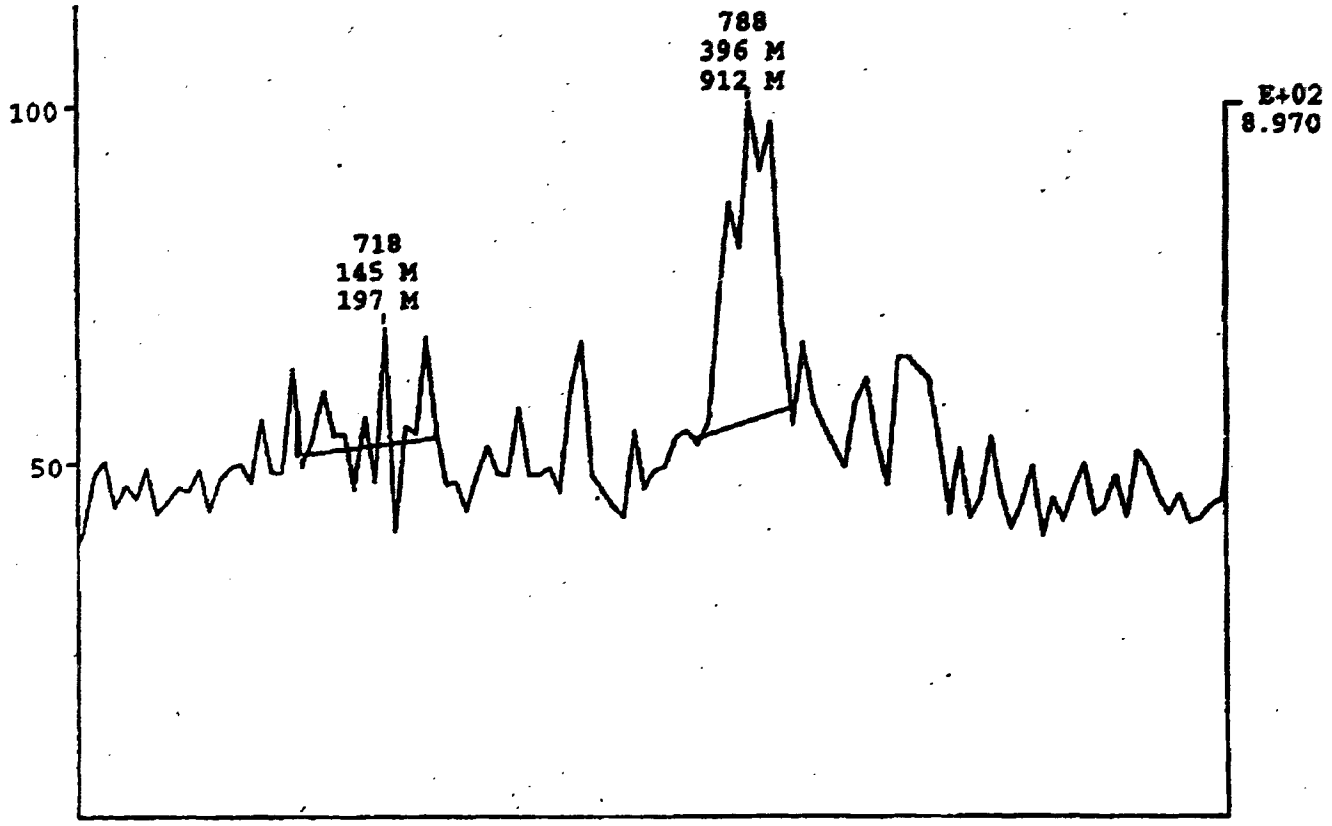
E 03

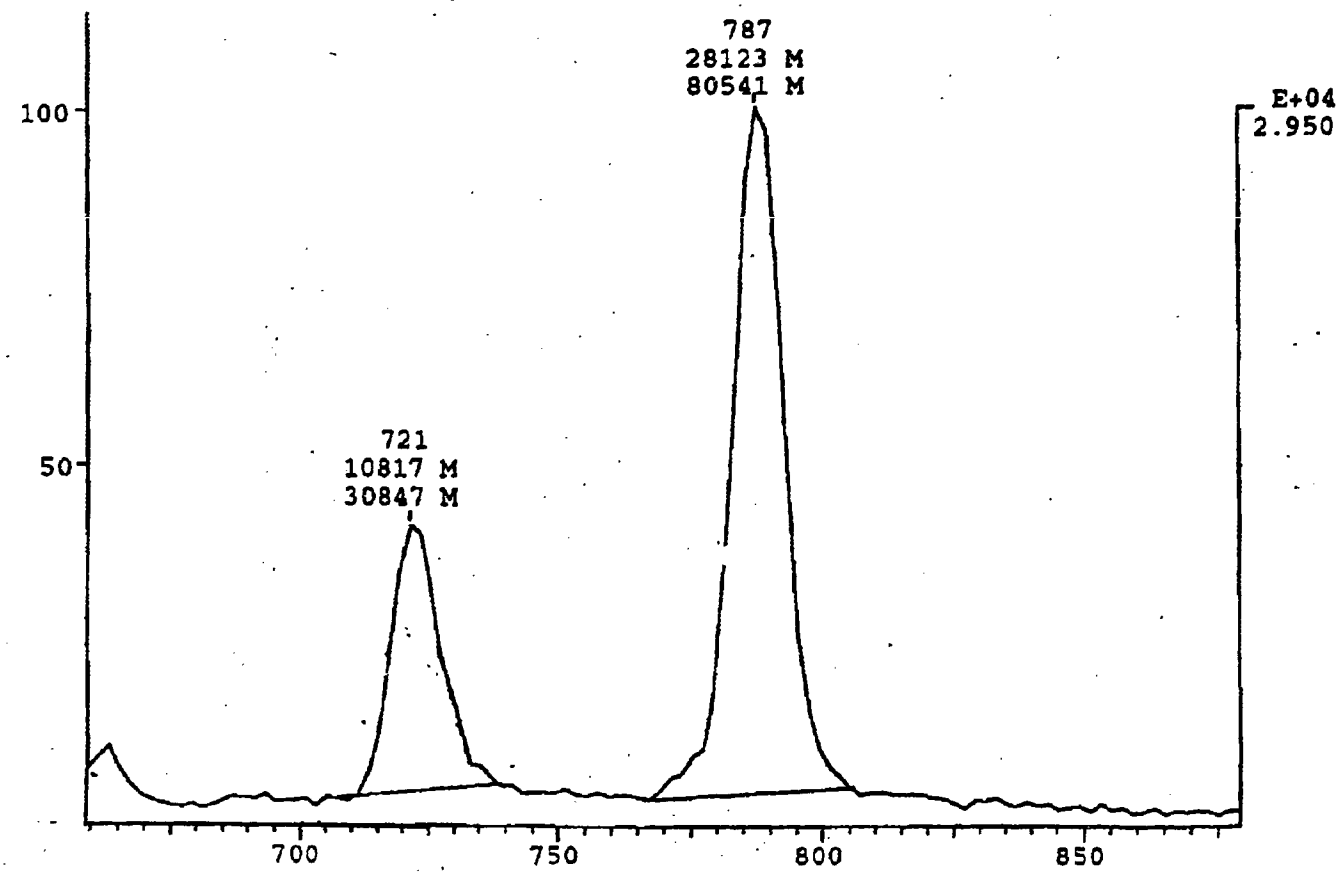
Figure 3-4a



E 04

Figure 3-4b





F 01

CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

REPORT NO: CTL/R/1346

STYRENE: A 10 DAY INHALATION STUDY TO INVESTIGATE EFFECTS ON MOUSE AND RAT LUNG

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AUTHOR

T Green

DATE OF ISSUE

June 21ST 1999

STATEMENT OF DATA CONFIDENTIALITY CLAIM

THIS DOCUMENT CONTAINS INFORMATION CONFIDENTIAL AND TRADE SECRET TO CEFIC.

It should not be disclosed in any form to an outside party, nor should information contained herein be used by a registration authority to support registration of this product or any other product without the written permission of the Styrene Steering Committee, a CEFIC Sector Group.

I, the undersigned declare that this report constitutes a true record of the actions taken and the results obtained in the above study.

Dr T Green
(Study Director) ..... Date 21.06.99

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

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Mr G O'Connor	Analyst

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1 SUMMARY

Male and female CD-1 mice were exposed to 0, 40 and 160 ppm styrene, 6 hours/day for five days. Following a two day break the exposures were repeated for a further five consecutive days. Male and female CD rats were exposed to 0 and 500 ppm styrene using the same protocol. Groups of five animals were killed 17 hours after 1, 5, 6 and 10 exposures. Each animal was fitted with a minipump containing 5-bromo-2-deoxyuridine 3 days before sacrifice. Lungs were removed for histopathological examination and for quantitation of cell division rates. In mice killed after a single exposure there was a treatment related focal loss of apical cytoplasm of the non-ciliated cells , principally in the terminal bronchioles. At subsequent time points (after 5, 6 and 10 exposures) focal crowding of the non-ciliated cells was seen throughout the bronchiolar tree. A dose dependant increase in cell labelling indices was measured at the end of each 5 day exposure period with the largest effects (3-4 fold) in the terminal bronchioles after the first 5 days. There were no morphological changes or increases in cell division in the alveoli at any of the dose levels or time points. In rats exposed to 500 ppm there were neither morphological changes nor increases in cell division at any of the time points. In conclusion, the study has identified a number of degenerative changes in the non-ciliated cells of the bronchiolar epithelium, followed by increased cell division and cell crowding, in the mouse lung following exposure to styrene. These responses were not seen in the rat at significantly higher dose levels.

2 INTRODUCTION

Male and female CD-1 mice exposed by inhalation to 20, 40, 80 and 160 ppm styrene for up to 104 weeks had significantly increased incidences of alveolar/bronchiolar adenomas and adenocarcinomas (SIRC, 1998). These increases were not seen in Sprague Dawley rats exposed to up to 1000 ppm styrene (Cruzan et al. 1998).

The mode of action of styrene as a mouse lung carcinogen is unknown. Styrene is metabolised by cytochromes P-450 enzymes to styrene oxide, a weak electrophile which is potentially reactive with biological macromolecules (Bond, 1989). A number of mouse specific lung carcinogens of this type are known and are believed to act through mechanisms involving cytotoxicity and increases in cell division (Foster et al., 1992, 1994; Green et al., 1994). The high background tumour incidences in CD-1 mouse lung, 34% in male mice in the current study, implies that this strain would be particularly sensitive to chemicals which cause pulmonary toxicity and increases in cell division.

In order to investigate the mode of action of styrene as a mouse lung carcinogen and thereby provide a basis for understanding the likely response in humans exposed to this chemical, the effects of styrene on lung morphology and cell turnover have been studied in mice and rats. The protocol and dose levels used were taken from those used in the cancer bioassay. The animals were exposed 6 hours/day for 5 days followed by a two day break and exposure for a further 5 days. Mice were exposed to 0, 40 and 160 ppm and rats to 0 and 500 ppm. A number of time points were chosen to ensure that all potential lesions were detected in the study. For example, chemically induced damage to the lung Clara cell, a common target for a large number of chemicals, frequently occurs after a single exposure, but resolves on continued exposure (Foster et al. 1992). To ensure that lesions of this type were detected in this study, animals were killed after 1, 5, 6 and 10 exposures.

3. MATERIALS AND METHODS

3.1 Chemicals

Styrene (99%), stabilised with 10-15 ppm 4-tert-butylcatechol, was supplied by Sigma-Aldrich Co. Ltd, Poole, Dorset, UK. and assigned CTL reference number Y00115/003/001 and stored at 4°C. 5-Bromo-2'-deoxyuridine (BrdU, 99%) was obtained from the same supplier and assigned CTL reference number Y08952/001/003.

3.2 Animals

Male and female CD rats (125-150g bodyweight) and male and female CD-1 mice (22-27g bodyweight) were supplied by Charles River UK, Margate, Kent. Prior to the study the animals were housed in stainless steel cages in rooms equipped with a 12 hour light-dark cycle. The environment of the animal room was controlled with a temperature being maintained within a target range of 19-23 °C, a relative humidity within a target range of 40-70%, and between 25-30 air changes per hour. All conditions were monitored by the Honeywell site monitoring system and any excursions outside these ranges were noted by means of an external alarm and recorded.

Certified diet (CT1 diet, Special Diet Services, Witham, Essex, UK) and mains water were supplied *ad libitum* except during the exposure periods.

Animals were randomly assigned to treatment groups by a method based on individual bodyweights. Individual animals were identified by ear punching. All animals were observed to ensure they were normal before the start of the study and any showing adverse signs either before or during the study were killed.

3.3 Study design

Chambers of approximately 3.4m^3 capacity were used for the exposures (Doe and Tinston, 1981). Conditioned air was supplied to the chambers at a flow rate sufficient to give at least 12 changes per hour. Temperature, relative humidity and air flow were measured in the chambers every 30 minutes. Atmospheres were generated by allowing styrene to evaporate at 55°C into a stream of air at a controlled rate. The concentration of styrene in the chamber

was measured by gas chromatography at hourly intervals throughout the experiment. A Hewlett Packard HP5890 gas chromatograph fitted with a CPSIL5CB column (25m x 530µm operated at 120°C) and flame ionsiation detector was used.

Male and female mice (5/sex/group) were exposed whole body to 0, 40 and 160 ppm styrene, 6 hours/day for 5 days. Following a 2 day break, the mice were exposed for a further 5 days (6hr/day). Male and female rats (5/sex/group) were exposed to 0 and 500 ppm styrene using the same protocol. Control mice were exposed to air only, but were otherwise treated in a similar manner to the test animals. Bodyweights and clinical signs were recorded daily immediately before exposure. When minipumps were fitted, bodyweights were recorded before and after surgery.

Food and water were withheld during exposure and provided *ad libitum* post exposure. Groups of 5 mice and 5 rats of each sex were killed 17 hours after 1, 5, 6 or 10 exposures. Three days before sacrifice each animal was fitted with an osmotic minipump (Alzet 7 day) containing 200µl 5-bromo-2'-deoxyuridine (BrdU, 15 mg/l in 0.9% saline). The animals were killed with an overdose of halothane, exsanguinated, the lungs immediately excised, weighed and infused and fixed via the trachea with 10% neutral buffered formalin. The lungs were embedded in paraffin wax, for the rat, the left lobe was embedded in one block and the right lobes in another, for the mouse, one of the right lobes was removed and embedded in a single block together with the remainder of the lung. Sections (5-7µm) were cut and stained with haematoxylin and eosin. The quantitation of cell replication was undertaken as described by Foster et al. (1994). Areas for evaluation of labelling indices were randomly selected from each area of the lung.

In a repeat of part of the above study, groups of 5 mice were given a single 6 hour exposure to 0, 40 and 160 ppm styrene and killed 17 hours after the end of exposure. The mice were fitted with BrdU minipumps 3 days before sacrifice as described above. All other procedures post termination were as described above.

4 RESULTS

The atmosphere concentrations measured over the duration of the 10 day study were 39.5±1.8, 157±9.7 and 490±33.6 ppm and for the single repeat exposure 40.5±2.4, 161±3.1,

489±25.9. Temperature and humidity within the exposure chambers were within acceptable limits (19-23°C; 40-70% respectively). Bodyweight gains and clinical signs in animals exposed to styrene were normal and comparable to those in the control groups.

4.1 Lung pathology

There were no treatment-related findings present macroscopically following exposure of either mice or rats to styrene.

4.1.1 Mouse

In mice killed 17 hours after a single exposure, there was a treatment-related focal loss of apical cytoplasm from non-ciliated cells with the major affects being localised to the terminal bronchiolar area. The magnitude of the effect varied between individual animals but females were slightly more severely affected than were male mice. There was no obvious dose-response in this reaction with similar incidences and severities being found for the 40 and the 160 ppm groups (Appendix 1). Repeating the single exposures with a second group of mice confirmed the variability seen in the first experiment.

In mice exposed to styrene for 5 days, the major effect observed was focal crowding of non-ciliated cells in the bronchiolar epithelium throughout the bronchiolar tree but particularly in the terminal bronchiolar regions. Once again there was little indication of a dose-response and little difference in the sexes (Appendix 1). This response did not show the variability seen with the cytotoxicity after a single exposure.

In mice killed 17 hours after the first exposure of the second week (5 days exposure + 2 days rest +1 day exposure), focal crowding of non-ciliated cells was again the dominant feature with the terminal areas showing greatest affect. There was no obvious differences in the response between the sexes and little evidence of a dose response.

Focal crowding of non-ciliated cells was also evident throughout the bronchiolar tree and was particularly obvious in the terminal bronchiolar area in mice killed at the end of the study (5 days exposure + 2 days rest + 5 days exposure). There was no dose response in the effect, no

progression in severity from earlier time points and no evidence of a sex difference in response (Appendix 1).

4.1.2 Rat

There were no treatment related effects in the lungs of rats exposed to styrene and killed after 1, 5, 6 or 10 exposures (Appendix 1).

4.2 Cell replication

4.2.1 Mouse

Two hundred to two hundred and fifty cells were counted in each region of the lung. No significant changes in the labelling indices in control mice were seen throughout the duration of the study (Table 1).

There was no evidence of an increase in cell replication rates in any of the three compartments of the lungs of male or female mice killed after a single exposure to either 40 or 160 ppm styrene, nor were there any increases in the alveolar region of the lungs of male or female mice at any of the time points or dose levels used in the study (Table 1). There was however, a marked dose dependant increase in the terminal bronchioles of male and female mice after 5 exposures to styrene. At the 160 ppm dose level the labelling index was 3-4 fold that measured in unexposed mice. A weaker, although still significant response, was seen in the large bronchioles after 5 exposures to styrene. There was no evidence of a significant sex difference.

Following the first exposure of the second week (after a 2 day break), labelling indices had returned to control levels in all regions of the lung. At the end of the study significant increases in labelling indices were seen in the large bronchioles, but not in the terminal bronchioles, at the highest dose level. The labelling indices (mean ±S.D) for each region of the mouse lung are given in Table 1.

4.2.2 Rat

Two hundred to two hundred and fifty cells were counted in each region of the lung. No significant changes in labelling indices in control rats were seen throughout the duration of the study (Table 2).

The labelling indices were not increased in any of the regions of the lungs of rats exposed to 500 ppm styrene at any of the time points. In female rats, labelling indices were significantly reduced in large bronchioles and alveoli after 5 exposures and in the terminal bronchioles and alveoli after 6 exposures (Table 2).

5 DISCUSSION

Exposure of mice of both sexes to styrene at 40 and 160 ppm induced a statistically significant dose dependant increase in labelling indices in the bronchiolar epithelium of mice killed after 5 daily 6 hour exposures while not showing any effect on the alveoli. The increases were dependant upon continued exposure to styrene, having resolved after a 2 day break in the middle of the study only to reoccur at the end of the second week after a further 5 daily exposures. The terminal bronchioles were the most sensitive region of the lungs after 5 exposures, whereas increased labelling indices were only seen in the large bronchioles at the end of the study. The transient elevation in labelling indices is seen as a prerequisite to the appearance of increased numbers of non-ciliated cells (described as focal crowding in the tables) which was seen from day 5 until the end of the study.

The morphological changes seen in the lungs of mice killed after a single exposure to styrene consisted of a low incidence of focal loss of apical cytoplasm from non-ciliated cells. The magnitude of the response was difficult to quantify after infusion fixation and varied between individual animals. The variability is believed to reflect differences either between inidividual animals, or in the time taken for the lesion to develop (and resolve) at different

dose levels. Repeating the single exposures confirmed the variability seen in the main study. It is probable that a more consistent lesion would have been seen if lungs had been taken at earlier and more frequent time points.

Focal loss of apical cytoplasm was not a dominant features in the lungs of mice killed at later time points. After 5 or more exposures, focal crowding of non-ciliated cells was evident throughout the bronchiolar tree, particularly in the terminal regions. This response was very consistent between animals, supporting the view that the initial cytotoxicity had occurred in all animals and had probably reached a maximum between days 1 and 5 of the study. The proliferation of this cell type, which is one of the major sites for metabolism in the lung, may be analogous to the type of change encountered in the liver with compounds that induce their own metabolism and may indicate an increased metabolism of styrene by these cells. If the focal crowding was merely a response to the cytotoxicity seen after a single exposure then regeneration of cells to numbers similar to those seen in controls would be expected rather than that seen in the current situation where numbers elevated over those seen in controls were observed.

These studies suggest that the precursor of the lung tumours observed in mice exposed to styrene lies in the Clara cell population, the lack of morphological change or increases in labelling indices in the alveolar region apparently ruling out a role for alveolar type II cells. They also provide the basis of a mechanism of action, sustained hyperplasia of the Clara cells potentially increasing the risk of fixing either pre-existing or styrene-induced mutational events occurring in the hyperplastic population. It is also worthwhile noting that the Clara cell loss and subsequent cell proliferation seen in mice exposed to styrene differs from the responses normally seen with other Clara cell toxicants (Foster et al. 1992; Odum et al. 1992). Typically these involve cell vacuolation and repair, rather than sustained hyperplasia.

In contrast to the hyperplasia and elevated labelling indices seen in the bronchioles of mice, no morphological effects were observed in rat lung following exposure to styrene and, in those cases where effects on labelling index were seen, significant decreases were observed. These differences in response are consistent with the lack of carcinogenicity of styrene in the rat lung in the cancer bioassay.

In conclusion, this study has demonstrated a major difference between rats and mice in their response to styrene. The cytotoxicity and increased cell division seen in the mouse lung at dose levels as low as 40 ppm were not seen in the rat lung at a dose level of 500 ppm. Identification of the mouse lung Clara cell as the primary target for styrene is also consistent with the observed mouse-rat differences in response to this chemical. Not only does this cell type occur in greater numbers in the mouse lung than in other species (Smith et al. 1979; Plopper et al. 1980a,b), it is also known to have a higher metabolic capacity, particularly for cytochromes P-450 CYP2E1 and CYP2F2, two of the cytochrome P-450 isoforms reported to be responsible for the metabolism of styrene to its oxide (Guengerich et al. 1991; Buckpitt et al. 1995; Carlson 1997; Green et al. 1997). The relative number and known metabolic capacities of mouse and human Clara cells would also suggest that the responses seen in the mouse lung are unlikely to occur in human lung.

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TABLE 1: LABELLING INDICES IN MOUSE LUNGS FOLLOWING EXPOSURE TO STYRENE FOR UP TO 10 DAYS

	MALE			FEMALE		
Days Exposure	Large Bronchioles.	Terminal Bronchioles	Alveoli	Large Bronchioles	Terminal Bronchioles	Alveoli
Day 1						
0 ppm	7.4 ± 2.4	7.9 ± 2.8	7.6 ± 1.7	3.4 ± 1.5	9.5 ± 2.4	4.3 ± 1.9
40 ppm	8.0 ± 3.0	9.9 ± 4.3	4.7 ± 1.3	4.9 ± 1.9	6.7 ± 2.6	4.6 ± 1.9
160 ppm	7.3 ± 2.3	8.3 ± 1.6	3.7 ± 1.6	6.1 ± 3.0	10.1 ± 4.3	3.4 ± 1.3
Day 5						
0 ppm	8.5 ± 2.3	10.3 ± 2.6	4.9 ± 1.2	5.6 ± 1.1	7.3 ± 1.7	5.2 ± 2.1
40 ppm	10.8 ± 3.7	22.0 ± 5.3**	5.4 ± 1.1	9.8 ± 4.9*	16.3 ± 5.7**	5.0 ± 2.5
160 ppm	15.3 ± 3.1**	36.1 ± 11.9**	5.4 ± 1.4	11.3 ± 5.2*	29.6 ± 10.0**	5.4 ± 1.1
Day 6						
0 ppm	5.6 ± 2.0	9.1 ± 2.4	3.7 ± 1.0	3.7 ± 1.3	6.8 ± 3.7	2.0 ± 1.0
40 ppm	4.2 ± 0.7	6.5 ± 3.2	2.6 ± 0.8	3.9 ± 1.0	6.4 ± 1.8	2.4 ± 0.6
160 ppm	4.8 ± 1.6	9.5 ± 3.1	2.1 ± 1.1	4.8 ± 0.9	6.7 ± 1.5	3.0 ± 0.9
Day 10						
0 ppm	6.2 ± 2.2	8.1 ± 1.8	4.1 ± 1.1	3.8 ± 1.1	8.3 ± 1.9	3.0 ± 0.6
40 ppm	6.3 ± 2.0	8.8 ± 3.0	2.5 ± 1.1	6.3 ± 3.9	7.5 ± 1.8	2.5 ± 0.9
160 ppm	9.8 ± 2.5*	10.9 ± 3.8	2.3 ± 0.9	11.1 ± 3.5**	8.8 ± 2.8	3.0 ± 1.6

Statistical significance: * $p<0.01$; ** $p<0.001$.

TABLE 2: LABELLING INDICES IN RAT LUNGS FOLLOWING EXPOSURE TO STYRENE FOR UP TO 10 DAYS

	MALE			FEMALE		
Days Exposure	Large Bronchioles.	Terminal Bronchioles	Alveoli	Large Bronchioles	Terminal Bronchioles	Alveoli
Day 1						
0 ppm	12.0 ± 6.7	11.0 ± 3.9	14.1 ± 3.2	7.0 ± 1.8	6.4 ± 3.8	9.4 ± 3.1
500 ppm	15.2 ± 6.0	14.3 ± 8.6	15.2 ± 9.6	8.2 ± 3.1	6.1 ± 2.8	8.5 ± 3.1
Day 5						
0 ppm	10.1 ± 3.8	12.2 ± 3.0	8.6 ± 4.3	10.5 ± 4.8	9.1 ± 6.1	9.7 ± 5.3
500 ppm	10.2 ± 5.7	7.7 ± 4.9	6.9 ± 2.5	4.3 ± 1.5*	4.7 ± 1.4	4.9 ± 1.6*
Day 6						
0 ppm	11.2 ± 5.3	5.8 ± 1.6	6.9 ± 3.2	5.3 ± 2.1	7.7 ± 3.2	9.4 ± 5.0
500 ppm	13.4 ± 2.2	5.8 ± 1.9	7.7 ± 1.7	5.7 ± 3.0	4.3 ± 1.0*	3.8 ± 0.9*
Day 10						
0 ppm	14.0 ± 4.0	9.8 ± 4.0	8.2 ± 3.5	8.9 ± 5.3	6.9 ± 2.2	5.8 ± 3.2
500 ppm	9.9 ± 3.5	8.5 ± 3.5	7.6 ± 2.4	5.6 ± 1.9	6.5 ± 3.4	8.2 ± 2.5

Statistical significance: * $p<0.01$; ** $p<0.001$.

APPENDIX 1

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY: 1

STUDY NO: MM0189 MALE MICE

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 40 ppm	GROUP 3 160 ppm	GROUP 4 0 ppm	GROUP 5 40 ppm	GROUP 6 160 ppm
ANIMALS COMPLETED	5	5	5	5	5	5
LUNG						
EXAMINED	5	5	5	5	5	5
NO ABNORMALITIES DETECTED	5	4	4	5	3	5
Intra-bronchiolar cell debris (TOTAL)	0	0	0	0	1	0
marked	0	0	0	0	1	0
Bronchiolar cell necrosis (TOTAL)	0	1	0	0	0	0
minimal	0	1	0	0	0	0
Desquamated cells - alveolar duct (TOTAL)	0	0	0	0	1	0
moderate	0	0	0	0	1	0
Loss of apical cytoplasm from non-ciliated cells	0	0	0	0	1	0
Cell debris - alveolar duct region	0	0	1	0	0	0

APPENDIX 1 (CONT'D)

PATHOLOGY - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 1

STUDY NO: MM0189 FEMALE MICE

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 40 ppm	GROUP 3 160 ppm	GROUP 4 0 ppm	GROUP 5 40 ppm	GROUP 6 160 ppm
ANIMALS COMPLETED	5	5	5	5	5	5
LUNG						
EXAMINED	5	5	5	5	5	5
NO ABNORMALITIES DETECTED	5	0	0	5	1	1
Granulom/ta	0	0	0	0	0	1
Intra-bronchiolar cell debris (TOTAL)	0	0	0	0	3	0
minimal	0	0	0	0	2	0
slight	0	0	0	0	1	0
Bronchiolar cell necrosis (TOTAL)	0	1	0	0	2	0
minimal	0	0	0	0	2	0
slight	0	1	0	0	0	0
Clara cell exfoliation (TOTAL)	0	1	0	0	1	0
minimal	0	0	0	0	1	0
slight	0	1	0	0	0	0
Loss of apical cytoplasm from non-ciliated cells	0	4 *	5 **	0	2	3

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 5

STUDY NO: MM0189 MALE MICE

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 40 ppm	GROUP 3 160 ppm
ANIMALS COMPLETED	5	5	5
LUNG			
EXAMINED	5	5	5
NO ABNORMALITIES DETECTED	5	4	2
Focal crowding of non-ciliated cells (TOTAL)	0	1	3
minimal	0	1	3

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 5

STUDY NO: MM0189 FEMALE MICE

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 40 ppm	GROUP 3 160 ppm
ANIMALS COMPLETED	5	5	5
LUNG			
EXAMINED	5	5	5
NO ABNORMALITIES DETECTED	5	2	2
Focal crowding of non-ciliated cells (TOTAL)	0	3	3
minimal	0	3	3
Clara cell exfoliation (TOTAL)	0	0	1
minimal	0	0	1

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 6

STUDY NO: MM0189 MALE MICE

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 40 ppm	GROUP 3 160 ppm
ANIMALS COMPLETED	5	5	5
LUNG			
EXAMINED	5	5	5
NO ABNORMALITIES DETECTED	5	3	2
Focal crowding of non-ciliated cells (TOTAL)	0	2	3
minimal	0	1	1
slight	0	1	2

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 6

STUDY NO: MM0189 FEMALE MICE

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 40 ppm	GROUP 3 160 ppm
ANIMALS COMPLETED	5	5	5
LUNG			
EXAMINED	5	5	5
NO ABNORMALITIES DETECTED	5	3	3
Focal crowding of non-ciliated cells (TOTAL)	0	2	2
minimal	0	2	0
slight	0	0	2

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 10

STUDY NO: MM0189 MALE MICE

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 40 ppm	GROUP 3 160 ppm
ANIMALS COMPLETED	5	5	5
LUNG			
EXAMINED	5	5	5
NO ABNORMALITIES DETECTED	5	5	2
Focal crowding of non-ciliated cells (TOTAL)	0	0	3
slight	0	0	2
moderate	0	0	1

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 10

STUDY NO: MM0189 FEMALE MICE

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 40 ppm	GROUP 3 160 ppm	
ANIMALS COMPLETED	5	5	5	
LUNG				
EXAMINED	5	5	5	
NO ABNORMALITIES DETECTED	4	4	0	
Perivascular oedema (TOTAL)	1	0	0	
moderate	1	0	0	
Focal crowding of non-ciliated cells (TOTAL)	0	1	5	**
minimal	0	1	0	
slight	0	0	4	*
moderate	0	0	1	
Loss of apical cytoplasm from non-ciliated cells	0	1	0	

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 1

STUDY NO: MR0190 MALE RATS

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS(P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 500 ppm
LUNG ANIMALS COMPLETED	5	5
EXAMINED	5	5
NO ABNORMALITIES DETECTED	4	4
Haemorrhage with pneumonitis (TOTAL)	0	1
minimal	0	1
Crystal invoked alveolitis (TOTAL)	1	1
minimal	1	0
moderate	0	1

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 1

STUDY NO: MR0190 FEMALE RATS

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS(* P < 0.1 ** P < 0.01, ONE SIDED

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 500 ppm
ANIMALS COMPLETED	5	5
LUNG		
EXAMINED	5	5
NO ABNORMALITIES DETECTED	5	5

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENT - EXPOSURE DAY 5

STUDY NO: MR0190 MALE RATS

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS(* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 500 ppm
ANIIMALS COMPLETED	4	5
LUNG		
EXAMINED	4	5
NO ABNORMALITIES DETECTED	3	3
Perivascular neutrophil infiltration (TOTAL)	0	1
slight	0	1
Crystal invoked alveolitis (TOTAL)	0	1
minimal	0	1
Subpleural inflammatory foci (TOTAL)	1	0
minimal	1	0

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 5

STUDY NO: MR0190 FEMALE RATS

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS(* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 500 ppm
ANIMALS COMPLETED	5	5
LUNG		
EXAMINED	5	5
NO ABNORMALITIES DETECTED	4	5
Alveolitis (TOTAL)	1	0
minimal	1	0

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 6

STUDY NO: MR0190 MALE RATS

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL		GROUP 1 0 ppm	GROUP 2 500 ppm
LUNG	ANIMALS COMPLETED	5	4
EXAMINED		5	4
NO ABNORMALITIES DETECTED		5	3
Haemorrhage with pneumonitis (TOTAL)		0	1
slight		0	1

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 6

STUDY NO: MR0190 FEMALE RATS

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 500 ppm
ANIMALS COMPLETED	5	5
LUNG		
EXAMINED	5	5
NO ABNORMALITIES DETECTED	5	4
Haemorrhage with pneumonitis (TOTAL)	0	1
minimal	0	1

APPENDIX 1 (CON'TD)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 10

STUDY NO: MR0190 MALE RATS

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL SEX: MALES	GROUP 1 0 ppm	GROUP 2 500 ppm
ANIMALS COMPLETED	5	4
LUNG		
EXAMINED	5	4
NO ABNORMALITIES DETECTED	5	3
Haemorrhage with pneumonitis (TOTAL)	0	1
minimal	0	1

APPENDIX 1 (CONT'D)

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE - EXPOSURE DAY 10

STUDY NO: MR0190 FEMALE RATS

FISHER'S EXACT TEST:- GROUP 1 COMPARED WITH ALL OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	GROUP 1 0 ppm	GROUP 2 500 ppm
ANIMALS COMPLETED	5	5
LUNG		
EXAMINED	5	5
NO ABNORMALITIES DETECTED	3	4
Alveolitis (TOTAL)	1	0
minimal	1	0
Haemorrhage with pneumonitis (TOTAL)	1	0
minimal	1	0
Crystal invoked alveolitis (TOTAL)	0	1
minimal	0	1

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CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

REPORT NO: CTL/R/1409

THE ROLE OF CYTOCHROME P-450 IN STYRENE INDUCED CELL DIVISION IN THE MOUSE LUNG

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REPORT NO: CTL/R/1409

THE ROLE OF CYTOCHROME P-450 IN STYRENE INDUCED CELL DIVISION IN THE MOUSE LUNG

AUTHOR

Dr T Green

DATE OF ISSUE

June 21st 1999

STATEMENT OF DATA CONFIDENTIALITY CLAIM

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I, the undersigned declare that this report constitutes a true record of the actions taken and the results obtained in the above study.

Dr T Green
(Study Director) ..... Date 21.06.99.

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

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1. SUMMARY

Male CD-1 mice exposed by inhalation to 40 and 160 ppm styrene, 6 hours/day, for 4 days had significant increases in cell replication rates in the bronchiolar, but not alveolar, epithelium of the lung from day 3 onwards. There were no increases in cell replication rates when mice similarly exposed to styrene for 3 consecutive days were treated before and during the exposure period with 5-phenyl-1-pentyne, a cytochrome P-450 inhibitor. This experiment demonstrates that the increases in cell replication rates seen in mice exposed to styrene are caused by cytochrome P-450 derived metabolites of styrene.

2. INTRODUCTION

Male and female CD-1 mice exposed to 0, 20, 40, 160 ppm styrene 6 hr/day, 5 days/week for 104 weeks had increased incidences of lung tumours, predominantly benign adenomas, at dose levels of 20 ppm and above (SIRC, 1998). Styrene was not carcinogenic in the rat at dose levels of up to 1000 ppm (Cruzan et al. 1998). Acutely, there is evidence of pulmonary toxicity and increased cell division in mice, but not rats, exposed to styrene for up to 10 days (Green 1999). The species difference in response in the bioassay, and in the acute study, suggests that styrene requires metabolic activation to a more reactive form which is responsible for the observed toxicity. Styrene is known to be metabolised by cytochromes P-450, principally CYP2E1 and CYP2F2, to styrene oxide, a relatively weak electrophile which has some known reactivity with biological macromolecules (Guengerich et al. 1991; Carlson, 1997; Bond, 1989). If the premise that styrene is metabolically activated is correct, inhibiting the pulmonary cytochromes P-450 responsible should prevent or reduce the toxicity and cell division seen after exposure to styrene. In this report, pulmonary cell replication has been compared in mice exposed to styrene with that in mice exposed to styrene and treated with a cytochrome P-450 inhibitor. Preliminary studies were undertaken to establish the minimum dosing period required to induce a significant increase in cell replication, and to select a suitable in vivo inhibitor of cytochrome P-450 over that period.

3. MATERIALS AND METHODS

3.1 Chemicals

Styrene (99%), stabilised with 10-15 ppm 4-tert-butylcatechol, was supplied by Sigma-Aldrich Co. Ltd, Poole, Dorset, UK. and assigned CTL reference numbers Y00115/003/004-008 and Y00115/003/010-011 and stored at 4°C. 5-Phenyl-1-pentyne was obtained from Lancaster Chemical Co. UK. 5-Bromo-2'-deoxyuridine (BrdU, 99%) and diethyldithiocarbamic acid disodium salt were obtained from Sigma-Aldrich Co. Ltd, Poole, Dorset, UK.

3.2 Animals

Male CD-1 mice, 22-27g body weight, were supplied by Charles River, Margate, Kent. Prior to the study the animals were housed in stainless steel cages in rooms equipped with a 12 hour light-dark cycle. The environment of the animal room was controlled to provide conditions suitable for CD-1 mice with a temperature being maintained within a target range of 19-23 °C, a relative humidity within a target range of 40-70%, and between 25-30 air changes per hour. All conditions were monitored by the Honeywell site monitoring system and any excursions outside these ranges were noted by means of an external alarm and recorded.

Certified diet (CT1 diet, Special Diet Services, Witham, Essex, UK) and mains water were supplied *ad libitum* except during the exposure periods.

Animals were randomly assigned to treatment groups by a method based on individual bodyweights. Individual animals were identified by ear punching. All animals were observed to ensure they were normal before the start of the study and any showing adverse signs either before or during the study were killed.

3.3 Exposures

Chambers of approximately 3.4m^3 capacity were used for the exposures (Doe and Tinston, 1981). Conditioned air was supplied to the chambers at a flow rate sufficient to give at least 12 changes per hour. Temperature, relative humidity and air flow were measured in the chambers every 30 minutes. Atmospheres were generated by allowing styrene to evaporate at 55°C into a stream of air at a controlled rate. The concentration of styrene in the chamber was measured by gas chromatography at hourly intervals throughout the experiment. A Hewlett Packard HP5890 gas chromatograph fitted with a CPSIL5CB column (25m x 530µm operated at 120°C) and flame ionsiation detector was used.

Mice were exposed whole body to 0, 40 and 160 ppm styrene, 6 hours/day for the duration of the study. Control mice were exposed to air only, but otherwise treated in a similar manner to the test animals. Body weights and clinical signs were recorded daily immediately before exposure. When minipumps were fitted, bodyweights were recorded before and after surgery.

3.4 Quantitation of cell replication

Three days prior to sacrifice, mice from the control and treatment groups were surgically implanted with Alzet 7 day mini-pumps containing 200µl 5-bromo-2-deoxyuridine (BrdU) at a concentration of 15 mg/ml in 0.9% saline. Minipumps were fitted in the afternoon (post exposure) in order to allow the animals to recover prior to exposure the following day.

At the end of the experiments, mice were killed by exsanguination under terminal anaesthesia with halothane. The lungs were immediately excised, infused and fixed via the trachea with 10% neutral buffered formalin. The lungs were embedded in paraffin wax, 5-7µm sections cut, and BrdU positive nuclei visualised and quantified as described by Foster et al. (1994). Areas for evaluation of labelling indices were randomly selected from each area of the lung.

3.5 4-Day inhalation study

It was recognised that inhibiting pulmonary cytochromes P-450 continuously for long periods would be difficult and might have an adverse effect on the health of the mice. Previous studies had established that increased cell replication occurred after 5 days of exposure to styrene (6 hr/day). This study was undertaken to determine if significant increases in pulmonary cell replication occurred earlier than this.

Mice were exposed to 0, 40 and 160 ppm styrene, 6 hours/day, for up to 4 days. Groups of mice (10 per dose level) were killed immediately after the first exposure and at 18 hours following 1, 2, 3 and 4 exposures. BrdU minipumps were implanted in all animals 3 days prior to sacrifice. Pulmonary cell replication was measured as described above.

3.6 Inhibition of pulmonary cytochrome P450 - time course and dose setting

Groups of 3 mice were given daily oral doses of either 35, 50, 100 or 200 mg/kg 5-phenyl-1-pentyne as a solution in corn oil (10 ml/kg) and killed at intervals up to 72 hours after dosing. Further groups of 4 mice were given a single oral doses 1g/kg diethyldithiocarbamate (DEDTC) as a solution in corn oil (10 ml/kg) and killed 24 and 72 hours after dosing. The lungs were removed, washed in 0.15%KCl, weighed and homogenised in 9 volumes of SET. The homogenate was centrifuged at 3000rpm for 10 min, the pellet discarded, the supernatant

further centrifuged at 12500rpm for 15 mins and the pellet again discarded. The resultant supernatant centifuged at 100000rpm for 12 mins. The pellet (microsomal fraction) was resuspended in 9 volumes of buffer and centrifuged for a final time at 100000rpm for 12mins. The washed microsomal fraction (pellet) was resuspended in SET, at a concentration of 1mg original tissue per ml of buffer.

The metabolism of styrene to styrene oxide was measured in sealed glass tubes containing 0.25mg microsomal protein, 5mM $MgCl_2$, 2mM NADPH and 0.1mM trichloropropene oxide, 0.1M Potassium phosphate buffer, pH 7.4 in a final volume was 1ml. The tubes were allowed to equilibrate at 37°C for 2 minutes in a shaking water bath and the reaction was started by the addition of 1mM Styrene in DMSO. The reaction was terminated after 10 minutes by extraction with 2ml Hexane on a rotator for 25 minutes. The tubes were spun at 2000rpm for 10 minutes and the hexane layer removed for analysis. Styrene oxide was measured by high performance liquid chromatography using a modification of the method described by Carlson (1997). The R- and S enantiomers of styrene oxide were resolved on a Chirex ®-1-naPh 3,5-din column (250x4.6mm) fitted with a Chirex ®-NAPH guard column (30x4.6mm). The mobile phase was 0.02% propan-2-ol in n-hexane at a flow rate of 0.5ml/min. UV detection was at 219nm and standards of R- and S-styrene oxide were prepared in n-hexane.

In a second experiment, groups of 3 mice were given a single oral dose of 100 or 200 mg/kg 5-phenyl-1-pentyne, daily for 3 days. The mice were killed 24 hours after the third dose, the lungs were removed, microsomes were prepared, and styrene metabolism measured as above.

3.7 Cytochrome P-450 inhibition and cell repication

Groups of mice (20 per dose group) were exposed to 0, 40 and 160 ppm styrene, 6 hours/day, for 3 days. Twenty four hours before the start of the experiment half of the mice in each group were given a single oral dose of 200 mg/kg 5-phenyl-1-pentyne in corn oil (10 ml/kg) and the remainder were given corn oil alone (10 ml/kg). These doses was repeated immediately after exposure on each of the 3 days. Immediately after the first exposure all of the mice were fitted with BrdU minipumps. The mice were killed 18 hours after the third exposure. The study schedule is shown in Appendix 1.

4. RESULTS

4.1 4-Day inhalation study

The mean atmospheric concentrations of styrene over the 4 day study were 39.9±1.3 and 161±3.7 ppm. Temperature and humidity within the exposure chambers were within acceptable limits (19-23°C; 40-70% respectively). Bodyweight gains and clinical signs in animals exposed to styrene were normal and comparable to those in the control group.

Five hundred cells were counted for each compartment of the lung. Statistically significant increases in pulmonary cell replication were seen in mice after 3 and 4 days of exposure to either 40 or 160 ppm styrene (Figure 1, Table 1). The increases were seen in the epithelium of both terminal and large bronchioles, but not in the alveolar region. There was no evidence of a dose response.

Morphological changes were also noted in this study. Immediately after 6 hours of exposure to 40 ppm styrene there was evidence of necrosis and loss of cells, believed to be Clara cells, from large bronchioles. Clara cells in the terminal bronchioles were not overtly affected. 18 hours after the first exposure necrosis and sloughing of cells was minimal. At the later time points, after 2-4 exposures, there were some changes in the appearance of Clara cells and their organisation in the epithelium (Table 2).

4.2 Inhibition of cytochrome P-450

5-Phenyl-1-pentyne was found to be an excellent inhibitor of the cytochrome P-450 catalysed metabolism of styrene to styrene oxide. 24 hours after single dose of 100 mg/kg 5-phenyl-1-pentyne, styrene metabolism was reduced to 10% of control for the R isomer and 28 % for the S isomer. Activity recovered slowly and was still reduced to 52 (R)-74 (S)% of control 48 hours after dosing (Figure 2). When mice were given oral doses of 200 mg/kg 5-phenyl-1-pentyne, daily for 3 days, styrene metabolism in the lung was reduced to 5 % (R) and 18% (S) of control over the 3 day period (Figure 3).

A single dose of 1g/kg diethyldithiocarbamate also inhibited the pulmonary metabolism of styrene at 24 hours (16% for R and 23% for S) and 72 hours (63% for R and 68% for S).

Although both inhibitors could potentially have been used for this study, 5-phenyl-1-pentyne was selected based on the lower dose levels required and a lack of overt toxicity over the study period.

4.3 The effect of cytochrome P-450 inhibition on pulmonary cell replication

The mean atmospheric concentrations of styrene over the 3 day study were 38.0±2.2 and 157±7.2 ppm. Temperature and humidity within the exposure chambers were within acceptable limits (19-23°C; 40-70% respectively). Body weight gains and clinical signs in animals exposed to styrene were normal and comparable to those in the control group.

Five hundred cells were counted for each compartment of the lung. In the absence of 5-phenyl-1-pentyne, exposure to both 40 and 160 ppm styrene for 3 days caused an increase in the labelling index of bronchiolar epithelium with no effect in the alveolar region (Figure 4). Treatment of control mice with 5-phenyl-1-pentyne also caused a small increase in the labelling index in the absence of styrene. Mice given 5-phenyl-1-pentyne and exposed to styrene showed no increase in labelling index over that seen in unexposed mice dosed with 5-phenyl-1-pentyne alone (Figure 4, Table 3). Thus, it was concluded that inhibiting the cytochrome P-450 catalysed metabolism of styrene to styrene oxide with 5-phenyl-1-pentyne also inhibited the increases in cell division seen in mice exposed to styrene alone.

5. DISCUSSION

Since styrene is a species (mouse) and target organ (lung) specific carcinogen and toxin it is reasonable to assume that the specificity arises from the distribution and activity of enzymes involved in the metabolism of this chemical. It would be difficult to rationalise this level of specificity if styrene itself were the biologically active moiety. Styrene is metabolised by a number of isoforms of cytochrome P-450, the two major ones being CYP2E1 and CYP2F2 (Guengerich et al. 1991; Carlson, 1997). All known metabolites are believed to involve the cytochromes P-450 enzymes in their formation. The major products are the two enantiomeric forms of styrene oxide, an electrophile which has some reactivity with

biological macromolecules although not necessarily with DNA, at least in vivo. It follows therefore that inhibiting cytochrome P-450 activity in vivo should ameliorate styrene induced pulmonary toxicity.

In a previous study (Green et al 1999) in which mice were exposed to 40 and 160 ppm styrene, 6 hours/day, for 1, 5, 6 and 10 days, and in the 4 day study described in this report, styrene caused some pulmonary toxicity and a clear increase in cell division rates in the bronchiolar epithelium. The nature of the dose response seen in these studies is not entirely understood, the effects at 40 ppm being equal to or even greater than those at 160 ppm. This may reflect metabolic saturation or the possibility that the time course for the development of the lesion may be dose dependant. The region of the lung affected was time dependant, the terminal bronchioles showing an increase in cell division only at 5 days whereas a response was seen in the larger bronchioles at both 5 and 10 days. The morphological changes were even more variable, not only between dose levels but also between individual animals. However, in both the previous study (Green, 1999), and in this study, cell division was a consistent and quantifiable endpoint and was therefore chosen to test the hypothesis that the effects of styrene on the mouse lung were caused by cytochrome P-450 derived metabolites of styrene.

Blocking cytochrome P-450 activity in vivo for prolonged periods is difficult and involves the use of chemical inhibitors which may themselves be toxic. Increases in cell division were first noted after 5 days of exposure (Green, 1999), a period considered too long for an inhibitor study. Consequently, a 4 day styrene inhalation study was conducted with sacrifices after 1, 2, 3 and 4 exposures. As a result of this study it was concluded that an inhibitor study needed to be of 3 days duration, the earliest time at which cell division was significantly increased.

5-phenyl-1-pentyne is a known inhibitor of CYP2F2 and also has some inhibitory effect on CYP2E1 as well (Carlson 1997, Green, 1999). Equally important, this chemical was not overtly toxic in mice at dose levels which had a maximum inhibitory effect on pulmonary styrene metabolism. Using the dosing regime selected from the preliminary studies, styrene metabolism could be reduced to approximately 20% of control values throughout the duration of a 3 day study. However, if styrene oxide is, as seems likely, responsible for the

adverse effects in the mouse lung, the impact of an 80% reduction in cytochromes P-450 is potentially much greater than might be expected. Neither the epoxide hydrolases or the glutathione S-transferases responsible for the detoxification of styrene oxide would be affected by this treatment and hence the balance of activating and deactivating enzymes will be markedly changed in favour of detoxification. Overall, these effects were sufficient to protect mice treated with 5-phenyl-1-pentyne from styrene induced pulmonary toxicity. It can be concluded therefore that the increases in cell division seen in the mouse lung following exposure to styrene are caused by metabolites of styrene formed by CYP2F2 and CYP2E1.

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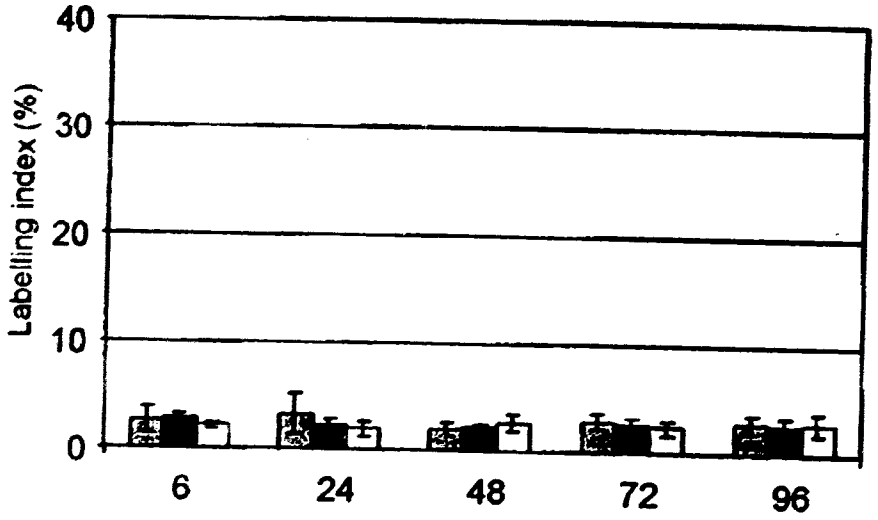
FIGURE 1 - PULMONARY LABELLING INDICES IN MICE EXPOSED TO STYRENE DAILY FOR UP TO 4 DAYS

Male CD-1 mice were exposed to 0 ■, 40 ■ and 160 □ ppm styrene, 6 hours/day, for up to 4 days.

Alveoli



Terminal Bronchioles

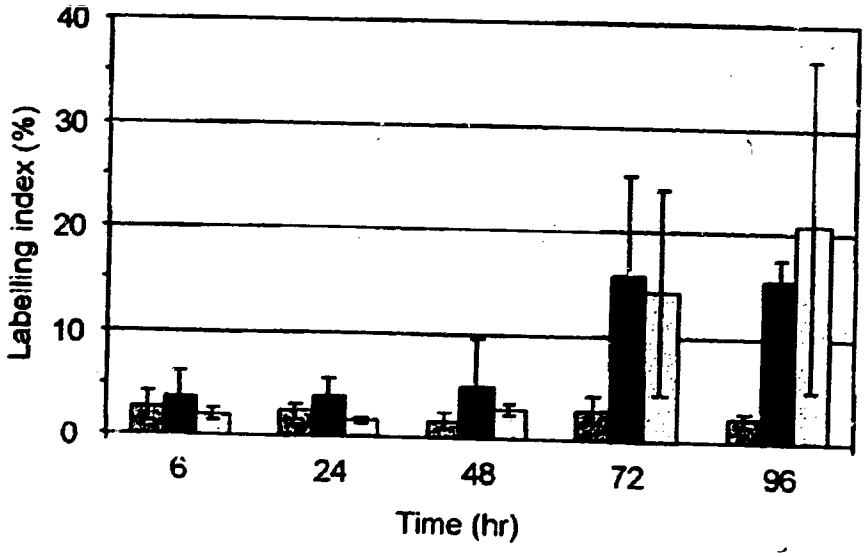


FIGURE 1 - PULMONARY LABELLING INDICES IN MICE EXPOSED TO STYRENE DAILY FOR UP TO 4 DAYS - CONTINUED

Large Bronchioles

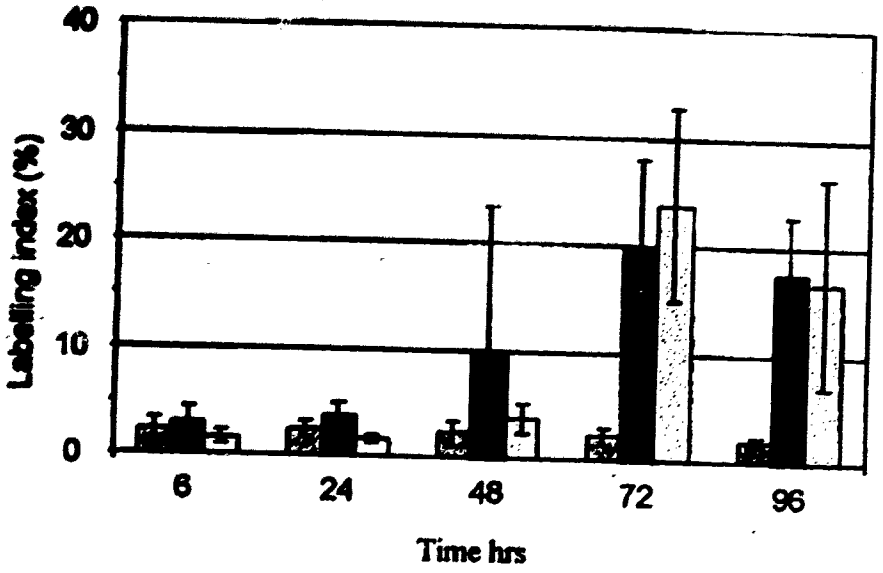


FIGURE 2 - INHIBITION OF PULMONARY STYRENE METABOLISM FOLLOWING A SINGLE ORAL DOSE OF 100 MG/KG 5-PHENYL-1-PENTYNE

Mice were given a single oral dose of 100 mg/kg 5-phenyl-1-pentyne and killed at the intervals shown. Styrene metabolism was measured in pulmonary microsomal fractions.

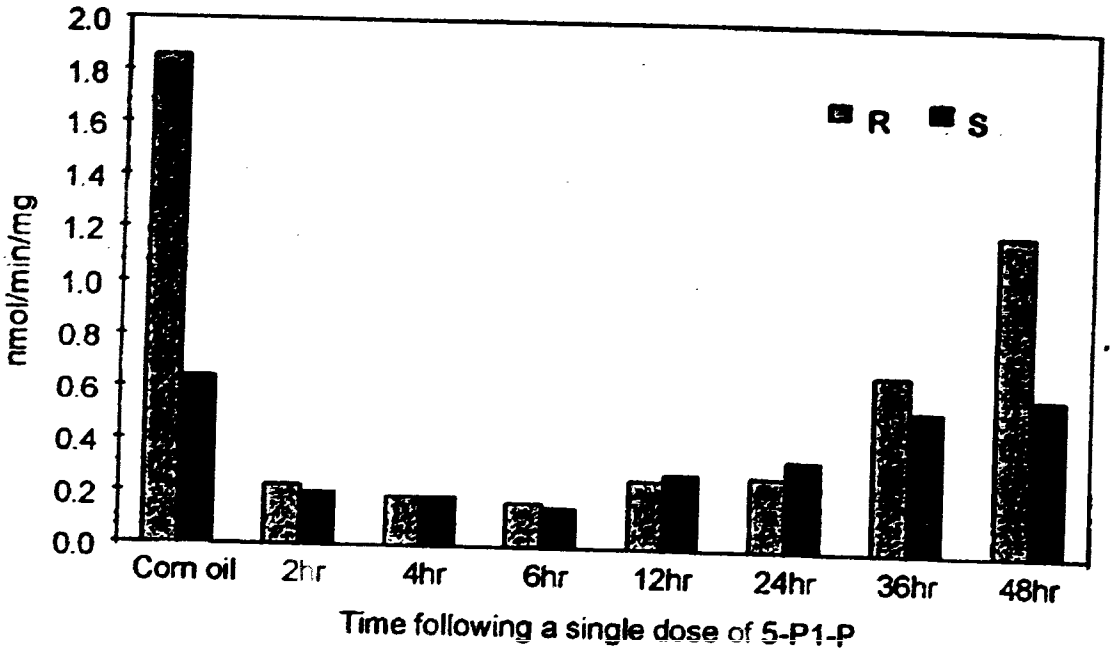


FIGURE 3 - INHIBITION OF PULMONARY STYRENE METABOLISM FOLLOWING SINGLE ORAL DOSES OF 5-PHENYL-1-PENTYNE, DAILY FOR 3 DAYS

Mice were given three single daily doses of 5-phenyl-1-pentyne and killed 24 hours after the final dose. Styrene metabolism was measured in pulmonary microsomal fractions

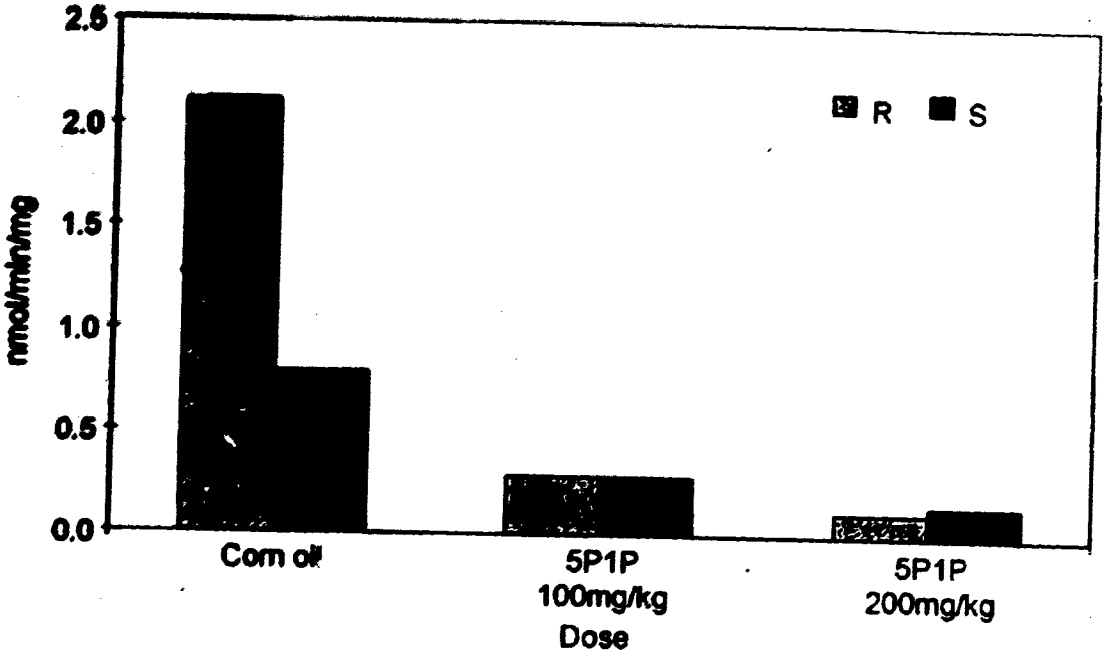
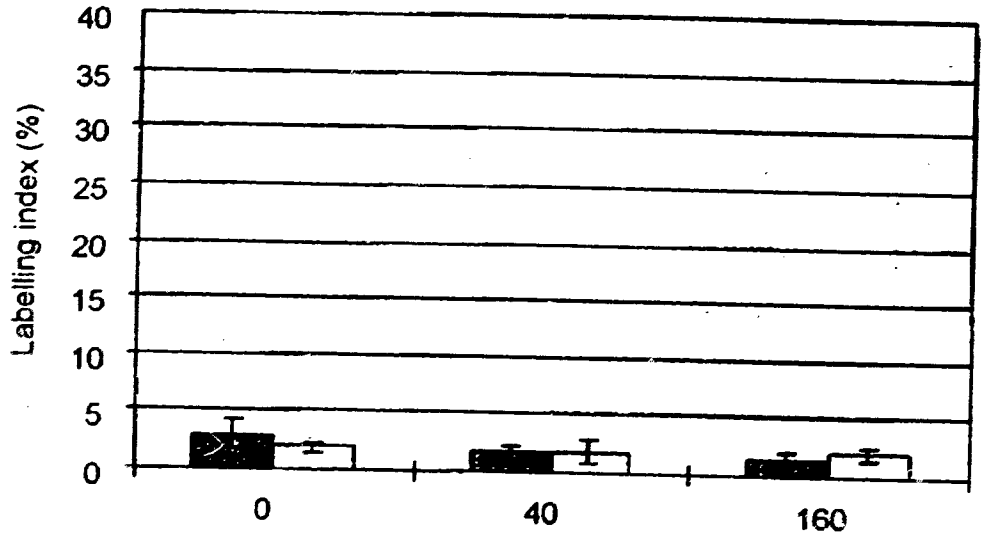


FIGURE 4 - THE EFFECT OF 5-PHENYL-1-PENTYNE ON STYRENE INDUCED CELL LABELLING INDICES IN MOUSE LUNG

Male CD-1 mice were exposed to 0, 40 and 160 ppm styrene, 6 hrs/day, for 3 days. 24 hrs before the start of the first exposure and immediately after each exposure one group of mice at each dose level was given single oral doses of 200 mg/kg 5-phenyl-1-pentyne.

■ control, □ 5-phenyl-1-pentyne

Alveoli



Terminal bronchioles

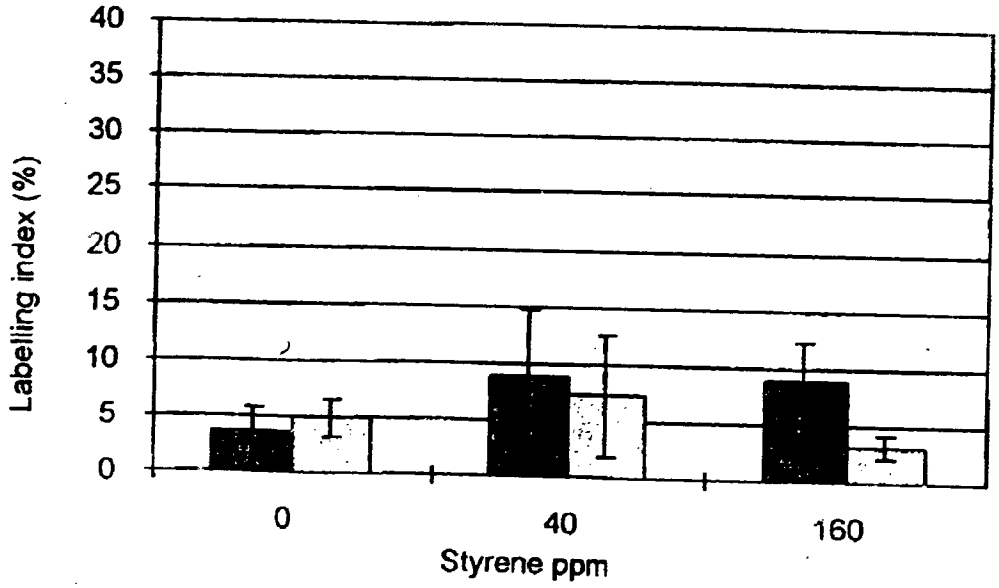


FIGURE 4 - THE EFFECT OF 5-PHENYL-1-PENTYNE ON STYRENE INDUCED CELL LABELLING INDICES IN MOUSE LUNG - CONTINUED

Large bronchioles

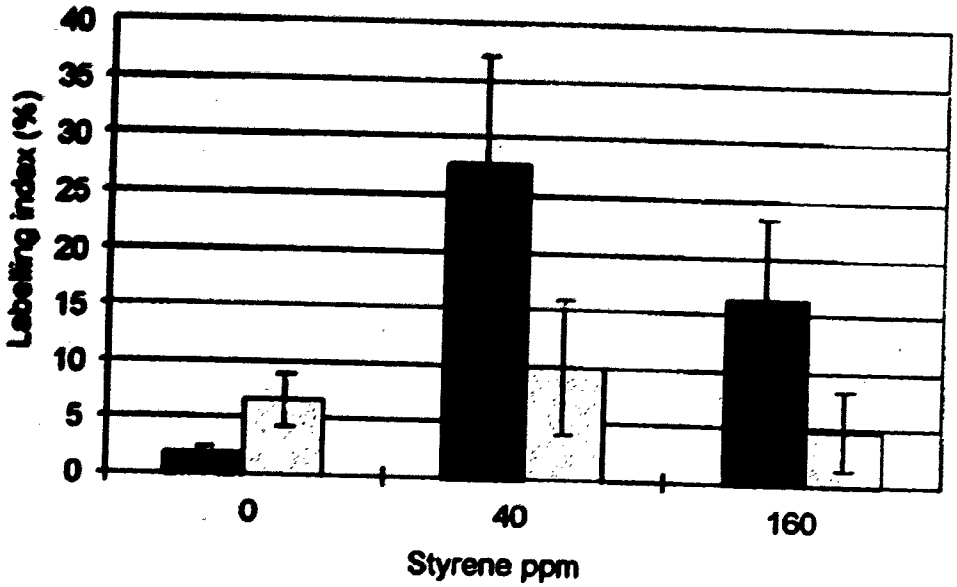


TABLE 1 - PULMONARY LABELLING INDICES IN MICE EXPOSED TO STYRENE DAILY FOR 4 DAYS

Time (h)	Control 0 ppm	Styrene 40 ppm	Styrene 160 ppm
Alveoli			
6	2.57 ± 1.15	2.86 ± 0.38	2.10 ± 0.36
24	3.26 ± 1.94	2.17 ± 0.54	1.89 ± 0.67
48	1.93 ± 0.63	2.14 ± 0.28	2.60 ± 0.83
72	2.85 ± 0.65	2.50 ± 0.64	2.30 ± 0.66
96	2.83 ± 0.80	2.69 ± 0.67	2.71 ± 1.01
Terminal bronchioles			
6	2.59 ± 1.49	3.55 ± 2.64	2.00 ± 0.64
24	2.29 ± 0.72	3.70 ± 1.85	1.55 ± 0.27
48	1.57 ± 1.57	4.94 ± 4.60	2.80 ± 0.57
72	2.84 ± 1.29	15.75 ± 9.66*	14.20 ± 9.81
96	2.16 ± 0.69	15.35 ± 2.16**	20.75 ± 15.80
Large bronchioles			
6	2.38 ± 0.99	3.05 ± 1.34	1.65 ± 0.60
24	2.45 ± 0.73	3.65 ± 1.36	1.60 ± 0.38
48	2.22 ± 1.09	9.75 ± 13.68	3.70 ±1.36
72	2.22 ± 0.73	20.00 ± 8.04**	23.80 ± 8.87**
96	1.97 ± 0.34	17.60 ± 5.31**	16.60 ± 9.69*

* Statistically significantly different from control values, Student's t-test, 2-sided $p < 0.05$.

** Statistically significantly different from control values, Student's t-test, 2-sided $p < 0.01$

TABLE 2 - MORPHOLOGICAL CHANGES IN MOUSE LUNG FOLLOWING EXPOSURE TO STYRENE, DAILY FOR 4 DAYS

Styrene ppm	Finding	Day 1	2	3	4	5
0	NAD	10	9	10	10	10
	Vacuolated cells - bronchiole	0	1	0	0	0
40	NAD	2	7	8	8	10
	Bronchiolar cell necrosis	5	1	0	0	0
	Vacuolated cells- bronchiole	1	2	0	0	0
	Loss of apical cytoplasm	1	0	2	1	0
	Desquamation of bronchiole	5	1	0	0	0
	Increased mitosis - bronchiole	0	0	0	1	0
	Disorganisation of bronchiole	0	0	0	2	0
160	NAD	7	9	7	8	5
	Vacuolated cells- bronchiole	2	1	0	0	1
	Desquamation of bronchiole	1	0	0	0	0
	Hypertrophy of Clara cells	0	0	3	0	0
	Increased mitosis	0	0	0	2	0
	Disorganisation of bronchiole	0	0	0	0	4
	Alveolitis	0	0	0	0	1

TABLE 3 - PULMONARY LABELLING INDICES IN MICE EXPOSED TO STYRENE AND TREATED WITH 5-PHENYL-1-PENTYNE

Styrene ppm	Treatment	Alveoli	Terminal bronchiole	Large bronchiole
0	Corn oil 5-P1-P	2.9±1.2 1.9±0.5	3.6±2.1 4.8±1.6	1.7±0.8 6.6±2.4
40	Corn oil 5-P1-P	1.8±0,5 1.8±0.9	8.9±5.7* 7.3±5.3	27.5±9.6*** 10.6±5.9
160	Corn oil 5-P1-P	1.4±0.6 2.0±0.6	8.9±3.4** 3.1±1.0	16.3±7.0** 4.8±3.5

* Statistically different from controls, students t-test, 2 sided, $p<0.05$

** Statistically different from controls, students t-test, 2 sided, $p<0.01$

APPENDIX 1 - SCHEDULE FOR THE CYTOCHROME P-450 INHIBITOR STUDY

Group	Dose Styrene ppm	Animal No.	Dose (oral)	Start date (oral dosing)	Implant date	Exposure date(s)	Sacrifice date
1	0	1-10	Corn oil	15.10.98	16.10.98	16,17,18	19.10.98
	0	11-20	5-Phenyl-1-pentyne	16.10.98	17.10.98	17,18,19	20.10.98
2	40	21-30	Corn oil	15.10.98	16.10.98	16,17,18	19.10.98
	40	31-40	5-Phenyl-1-pentyne	16.10.98	17.10.98	17,18,19	20.10.98
3	160	41-50	Corn oil	15.10.98	16.10.98	16,17,18	19.10.98
	160	51-60	5-Phenyl-1-pentyne	16.10.98	17.10.98	17,18,19	20.10.98

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REPORT NO: CTL/R/1410

THE EFFECTS OF STYRENE ON MOUSE LUNG FOLLOWING ORAL DOSING

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REPORT NO: CTL/R/1410

THE EFFECTS OF STYRENE ON MOUSE LUNG FOLLOWING ORAL DOSING

AUTHOR

Dr T Green

DATE OF ISSUE

June 21st 1999

STATEMENT OF DATA CONFIDENTIALITY CLAIM

THIS DOCUMENT CONTAINS INFORMATION CONFIDENTIAL AND TRADE SECRET TO CEFIC.

It should not be disclosed in any form to an outside party, nor should information contained herein be used by a registration authority to support registration of this product or any other product without the written permission of the Styrene Steering Committee, a CEFIC Sector Group.

I, the undersigned declare that this report constitutes a true record of the actions taken and the results obtained in the above study.

Dr T Green
(Study Director) ..... Date 21.06.99.

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

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Dr J R Foster/Mr A Soames	Pathologists

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1. SUMMARY

Male CD-1 mice orally dosed with 10, 100 and 200mg/kg styrene, daily, for five consecutive days exhibited a dose dependant increases in cell replication in the terminal bronchioles of the lung at the two higher dose levels. No increases in labelling indices were apparent in either the large bronchioles or the alveoli of the same animals. There was evidence of slight focal crowding of non-ciliated cells in the epithelium of the terminal bronchiole in 3/10 mice in the top dose group only. The 100 mg/kg dose was a no-effect level for morphological change and 10 mg/kg for both morphological damage and increased cell replication.

2. INTRODUCTION

Styrene is used in the production of polymers, copolymers and reinforced plastics. It is pneumotoxic in CD-1 mice when inhaled, with morphological changes and increases in cell division occurring after short-term exposure, and lung tumours after chronic exposure (Green, 1999; BIBRA, 1998). Whilst occupational exposure to vapours of styrene occurs mainly via inhalation, low levels of styrene may be ingested in food or drink contained in packaging made from styrene based polymers. The effects of ingested styrene on the mouse lung have not been previously assessed.

Following oral dosing of mice with styrene, very little styrene is exhaled via the lungs, even at dose levels approaching the LD_{50} (Csanady et al 1994). Indeed, the same authors also concluded that the amount of styrene exhaled following oral dosing could not be accurately predicted by modelling. Consequently, it is impossible to replicate, following oral dosing, the type of airway concentration seen after exposure to 40 or 160 ppm styrene by inhalation. In the absence of any clear correlation between airway exposure following oral and inhalation exposure a broad range of dose levels were chosen for the oral study. In this study, the effects of styrene on mouse lung morphology and cell replication have been assessed following oral doses of 10, 100 and 200mg/kg.

3. MATERIALS AND METHODS

3.1 Chemicals

Styrene (99%), stabilised with 10-15 ppm 4-tert-butylcatechol, was supplied by Sigma-Aldrich Co. Ltd, Poole, Dorset, UK. and assigned CTL reference number Y00115/003/009 and stored at 4°C. Corn oil was supplied by CPC UK, and assigned CTL reference number Y00790/007/008. 5-Bromo-2'-deoxyuridine (BrdU, 99%) was obtained from Sigma-Aldrich Co. Ltd, Poole, Dorset, UK.

3.2 Animals

Male CD-1 mice, 22-27g body weight, were supplied by Charles River, Margate, Kent. Prior to the study the animals were housed in stainless steel cages in rooms equipped with a 12 hour light-dark cycle. The environment of the animal room was controlled to provide conditions suitable for CD-1 mice with a temperature being maintained within a target range of 19-23 °C, a relative humidity within a target range of 40-70%, and between 25-30 air changes per hour. All conditions were monitored by the Honeywell site monitoring system and any excursions outside these ranges were noted by means of an external alarm and recorded.

Certified diet (CT1 diet, Special Diet Services, Witham, Essex, UK) and mains water were supplied *ad libitum* except during the exposure periods.

Animals were randomly assigned to treatment groups by a method based on individual bodyweights. Individual animals were identified by ear punching. All animals were observed to ensure they were normal before the start of the study and any showing adverse signs either before or during the study were killed.

3.3 Study design

Groups of 10 mice were given single oral doses of 10, 100 and 200mg/kg styrene in corn oil (10 ml/kg), daily, for 5 consecutive days. A control group of 10 mice were given single oral doses of corn oil (10ml/kg), also for 5 days. Three days prior to sacrifice each animal was fitted with an osmotic minipump (Alzet 7 day) containing 200ul 5-bromo-2'-deoxyuridine

(BrdU; 15mg/ml in 0.9% saline). The animals were killed with an overdose of halothane 24 hours after the last dose and exsanguinated. The lungs were immediately excised, infused and fixed via the trachea with 10% neutral buffered formalin and embedded in paraffin wax. Sections (5-7um) were cut and stained with haematoxylin and eosin. The quantitation of cell replication was undertaken as described by Foster et al. (1994). Areas for evaluation of labelling indices were randomly selected from each area of the lung.

4. RESULTS

Bodyweight gains and clinical signs in animals dosed with styrene were normal and comparable to those in the control group.

4.1 Lung pathology

There were no treatment-related findings visible macroscopically following dosing with any of the three doses of styrene. Microscopically there were no effects observed in mice administered 10 or 100mg/kg styrene (Table 1). In mice dosed with 200mg/kg styrene, 3/10 animals exhibited slight focal crowding of non-ciliated cells in the epithelium of the terminal bronchiole. There was no evidence of either cellular necrosis or damage.

4.2 Cell replication

Five hundred cells were counted for each compartment of the lung. The labelling indices in the large bronchioles, terminal bronchioles and alveoli of the lungs from the control (corn oil dosed) mice were 1.2%, 1.8% and 2.8% respectively (Table 2). There was no evidence of an increase in cell replication in either the large bronchioles or the alveolar region at any of the dose levels in this study. There was, however, a marked dose dependant increase in the S-phase frequency in the terminal bronchioles at the 100 and 200 mg/kg dose levels. At 200mg/kg, the labelling index was 5-fold that measured in corn oil treated mice. A weaker, although still significant response, was seen in mice dosed 100mg/kg, while 10mg/kg proved to be a no effect level (Table 2).

5. DISCUSSION

Styrene is known to be a pulmonary toxicant in CD-1 mice when administered via the inhalation route (SIRC, 1998). Few studies have dealt with exposure to styrene via the oral route in laboratory animals, since exposure via this route was considered to be negligible when compared to occupational exposure by inhalation. In the present study, mice dosed orally with styrene at dose levels up to 200mg/kg, daily for five consecutive days, exhibited increases in cell division in the terminal bronchioles of the lung. There was a clear dose response at the two higher dose levels and a clear no-effect at the lower. Interestingly, the only region of the lung affected when styrene is administered orally, was the terminal bronchiole, whereas by inhalation, styrene also affected the epithelium of the large bronchioles (Green 1999). This difference is probably due to differences in the way styrene reaches the pulmonary epithelium following inhalation exposure or orally dosing. Following inhalation exposure all regions of the airways will be exposed to the chemical. After an oral dose very little styrene is exhaled in the mouse (Csanady et al. 1994) and hence the lung tissues are exposed to styrene primarily through the systemic circulation. The most highly perfused regions of the lung are the terminal bronchioles (Netter, 1980), which is consistent with the observed response in this study.

During inhalation exposure the cell type which is morphologically affected by exposure to styrene, and the one which undergoes an increase in cell division, appears to be the non-ciliated Clara cell. The same target cell, and a virtually identical response, is also seen after oral dosing with styrene. It can be concluded therefore that styrene has the potential to be toxic to the lungs of CD-1 mice irrespective of whether they are dosed via the inhalation or oral route.

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TABLE 1 - MORPHOLOGICAL CHANGES IN MOUSE LUNG FOLLOWING ORAL DOSING OF STYRENE, DAILY FOR 5 DAYS

Terminal Kill Microscopic Findings	Group 1 0 m/kg	Group 2 10 mg/kg	Group 3 100 mg/kg	Group 4 200 mg/kg
Males on study	10	10	10	10
Animals completed	8*	10	10	10
Lung -				
no abnormalities detected	8	10	10	7
focal crowding in terminal bronchioles	0	0	0	3
- minimal	0	0	0	1
- slight	0	0	0	2

Fisher's exact test:- Group 1 compared with all other groups (* $p < 0.05$ ** $p < 0.01$, one sided)

* there were two intercurrent deaths in the control group

TABLE 2 - PULMONARY LABELLING INDICES IN MICE FOLLOWING ORAL DOSING OF STYRENE, DAILY FOR 5 DAYS

Dose mg/kg	Alveoli	Terminal bronchiole	Large Bronchiole
0	2.8 ± 2.1	1.8 ± 0.9	1.2 ± 0.6
10	1.3 ± 0.6	1.1 ± 0.5	0.9 ± 0.4
100	1.3 ± 0.6	3.8 ± 2.5 *	0.8 ± 0.4
200	1.5 ± 0.6	9.9 ± 2.7 **	2.9 ± 2.1

* Statistically significantly different from control values, Student's t-test. 2-sided, $p < 0.05$

** Statistically significantly different from control values, Student's t-test, 2-sided, $p < 0.01$

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CENTRAL TOXICOLOGY LABORATORY
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CHESHIRE UK

REPORT NO: CTL/R/1411

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

E 02

CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

REPORT NO: CTL/R/1411

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

STUDY DETAILS

Sponsor:	Styrene Information and Research Centre
CTL Study Numbers:	XR6516
	XM6515

AUTHOR

Dr J R Foster

DATE OF ISSUE

October 5th 1999

I, the undersigned declare that this report constitutes a true record of the results obtained in the above study.

Dr T Green
(Study Director) ..... Date 5-10-99

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The following contributed to this report in the capacities indicated:

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1. SUMMARY

The purpose of these investigations was to assess the distribution of ^{14}C-styrene in the nasal tissue and lungs of male CD rats and male CD1 mice, by histoautoradiography, at time points up to 24 hours following a 6 hour inhalation exposure in a closed re-circulating system. Groups of 10 rats and 20 mice were exposed to 160 ppm styrene for 6 hours and killed at 5 minutes, 1 hour, 4 hours and 24 hours post exposure. Following sacrifice, cryosections of lungs and nasal passages were prepared for autoradiography in order to visualise the distribution of ^{14}C styrene and/or its metabolites. The sections were processed to distinguish between total radioactivity, volatile radioactivity and that which was covalently bound. The autoradiograms were examined and photographed by light microscopy using dark and light field photomicrography and the results recorded in terms of the relative intensity and distribution of radiolabel. Whole body autoradiograms were also prepared using a single rat and a single mouse at each time point.

The results show that there are significant differences both in the distribution and the persistence of radiolabel between the mouse and the rat in the lung but little discernible difference between the two species in the way in which styrene is handled in the nasal passages. In the mouse lung the results show that the bronchiolar mucosa preferentially accumulates radio-labelled ^{14}C styrene and/or its metabolites and that it retains this label throughout the period of study up to 24 hours. Although the rat shows a similar initial localisation of radiolabel to the bronchiolar mucosa, the walls of the pulmonary blood vessels also show large amounts of radiolabel and the label is more quickly redistributed to the airways of the alveoli and bronchioles than it is in the mouse. Of significance is the finding that in neither species is the radiolabel accumulated within the alveolar walls at any of the time periods studied. The whole body autoradiograms demonstrated localisation and persistence of radioactivity in the nasal passages at each time point.

2. INTRODUCTION

The purpose of these investigations was to assess the distribution of ^{14}C-styrene in the nasal tissues and lungs of male CD rats and male CD1 mice, by histoautoradiography and whole body autoradiography, at time points up to 24 hours following a 6 hour inhalation exposure to styrene in a closed re-circulating system. These strains of animals were used because adequate background data is available on the use of these strains within Central Toxicology Laboratory and because long term studies on styrene toxicology have been performed in these strains of rodents (SIRC, 1998; Cruzan et al. 1998).

Three methods of preparing the tissue for autoradiography were used in this study in an effort to study the localisation of radiolabel within the tissues. The first method localises all radioactivity by avoiding translocation or extraction of radiolabel from the tissue. It does this by maintaining the tissue in a frozen state by picking up the cryosections of nasal tissue onto pre-coated emulsion slides and then subsequently freeze-drying the tissue prior to the autoradiographic exposure period. Tissue prepared in this way is subsequently referred to in the study as the 'total' data. The second preparation method was designed to differentiate the volatile radioactive components present in the tissue sections from the total radioactivity present. The sections were prepared as for the first method but the slides were placed under vacuum for 24 hours prior to beginning the autoradiographic exposure. Hence any volatile components of styrene or its metabolites would be extracted from the tissue prior to development of the autoradiogram. What remains within the tissue would be the non-volatile components of styrene or its metabolites. Hence sections prepared by this method will be subsequently referred to as the 'non-volatile' data. Finally an attempt was made to distinguish that component of the radioactivity that was labile in the tissue from that which was 'tightly' bound within the sections. This was attempted by permitting the frozen sections to be fixed in an aqueous solution which is known to extract all but the most tightly bound components. This is referred to in the text as the 'fixed' data.

3. MATERIALS AND METHODS

3.1 Test substance and dose vehicle specification

[ring-U-^{14}C] Styrene (code: CFQ11087), which was stored at -20°C, was obtained from Amersham Life Sciences Ltd, Buckinghamshire as a custom synthesis with a radiochemical purity of 98.1% by radio-gas chromatography. It had a specific activity of 19mCi/mmol and a radioactive concentration of 5 mCi/mmol in ethanol. Unlabeled styrene was obtained from Sigma/Aldrich, (Poole, Dorset, UK) with a purity of 99.9% and given CTL reference number Y00115/003/009.

3.2 Animals

40 male Sprague-Dawley CD rats weighing 180-200g and 80 male CD1 mice weighing 20-25g were obtained from Charles River UK Ltd, Margate, Kent, UK. The animals arrived at the CTL animal accommodation approximately 5 days prior to the study and were housed in the J2.22 inhalation facility 24 hours prior to the start of the study. Randomisation of animals was achieved by assigning them to the designated number of cages as they were picked up from the carrier box until all cages contained a single animal. Subsequent animals were then placed in the cages in rotation, as they were picked up from the carrier box, until the requisite number of animals were present in each of the cages. The animals were housed 4 per cage and individual animals were identified by tail marking.

The animals received Rat and Mouse Number 1 (RM1) pelleted diet from Special Diet Services, Witham, Essex, UK., and mains water *ad libitum*, except during exposure periods. The environment of the animal room was controlled with the temperature being maintained within a target range of 19-23 °C, a relative humidity within a target range of 40-70%, an artificial light cycle of 12 hours light and 12 hours darkness and between 25-30 air changes per hour. All conditions were monitored by the Honeywell site monitoring system and any excursions outside these ranges were noted by means of an external alarm and recorded.

3.3 Experimental Procedures

3.3.1 Dose preparation

Approximately 2mCi of [ring-U-^{14}C] styrene in ethanol was diluted with 350 µl of unlabeled styrene to give a specific activity of approximately 0.66 mCi/mmol. The ethanol was removed by adding water (10 volumes) and extracting the styrene into pentane which was dried and removed under reduced pressure. The styrene was stored at -20°C and its purity checked by radio-gas chromatography immediately before use. Each rat was exposed to a body burden of 1.25 mCi/kg and each mouse to a body burden of 5 mCi/kg.

3.3.2 Study design

Rats and mice were exposed to 160 ppm C-14 styrene for 6 hours. At the end of the exposure period the animals were removed from the chambers and placed in stock cages with free access to diet and water. Groups of 10 rats and 20 mice were terminated at 5 minutes, 1 hour, 4 hours and 24 hours post exposure.

The clinical condition of all animals was monitored during the exposure and post-exposure periods.

At each time point a single animal was killed for whole body autoradiography.

3.3.3 Exposure system

Animals were exposed whole body in glass chambers of approximately 20 litres using a closed re-circulating system. Air was circulated in the system at approximately 1 l/min; carbon dioxide was removed with Carbosorb AS and oxygen added at a rate sufficient to maintain a concentration of between 20 and 21%. The animals were placed in the chamber and ^{14}C styrene injected into the chamber to give an atmospheric concentration of 160 ppm. Equilibrium was achieved within 15 min and both styrene and oxygen levels in the chamber were monitored throughout the experiment by gas chromatography. A Shimadzu GC-14BT dual column gas chromatograph was used. Oxygen was measured using a molecular sieve 5A column (1/8 inch x 6ft) at 115°C and a thermal conductivity detector. Styrene was measured using a 10% Carbowax 20M column (1/8 inch x 6ft) at 115°C and a flame ionisation detector.

3.3.4 Investigation post mortem.

Animals were terminated at the end of the study by exsanguination using cardiac puncture under terminal anaesthesia with halothane.

Following exsanguination the thoracic cavity was opened and the lungs examined for any gross changes. The lungs of each animal were then removed, together with the trachea, up to the epiglottis and laid onto a wax sheet for subsequent trimming. The left lobe and the largest of the right lobes was removed, the top and bottom of each lobe sectioned away with a scalpel blade and the middle portion of each lobe was then plunged into hexane/solid CO_2, in order to freeze the lobes. The lobes were stored at -80 °C until required for cryo-sectioning. The remainder of the lungs was discarded. The decision was subsequently taken to cryo-section only the left lobe from each animal.

For examination of the nasal cavity, the carcass of each animal was transected to remove the lower limbs and the whole of the front portion, including the head and nasal passages, was frozen in hexane/solid CO_2. In order to examine the levels of the nasal passages separately for the distribution of ^{14}C-styrene the nasal passages were subsequently sawed using a Dremel Multi power saw model 395 (Norfolk, UK) to give blocks approximating to those of level 2 and level 4 of the nasal regions designated by Morgan (1991) and Young (1981). Level 2 corresponds to that region in the front of the nose where the predominant epithelial type present is respiratory with a small amount of olfactory mucosa. Level 4 is taken from the back of the nose where the predominant epithelial type is olfactory mucosa with small amounts of respiratory mucosa.

3.3.4.1 Autoradiography.

Because of the technical difficulties inherent in the procedure not all of the rats or mice were examined at each time point post-exposure. The blocks from the remaining animals were stored at -80°C for possible future backup.

The frozen blocks of either lung or nasal passages were mounted in a Leitz cryostat 1720 and sectioned at 15 µm thickness. The section that was intended for evaluation of the distribution of the total radioactivity, henceforth referred to as the 'total' section, and the one intended for evaluation of the non-volatile radioactive component, henceforth referred to as

the 'non-volatile', were mounted directly onto glass slides, pre-coated with K5 nuclear photographic emulsion (Ilford , UK). This procedure was, of necessity, conducted under safe light conditions in a photographic darkroom. For the evaluation of non-volatile radioactivity, within the sections, the respective sections were placed into an evacuated bell jar and held overnight, in the dark, to remove any volatile radioactivity components present within the sections. For the evaluation of tightly bound radioactivity, henceforth referred to as 'fixed', the cryosections were mounted onto uncoated glass microscope slides, fixed for 5 minutes in 10% neutral buffered formalin solution, washed in distilled water and subsequently dipped into K5 nuclear photographic emulsion at 40°C. Following dipping the slides were rapidly cooled on an ice-cold steel plate. All slides, irrespective of procedures leading up to the next stage, were subsequently placed into light-proof black boxes, wrapped in light-tight polythene bags, and were placed into a cupboard at room temperature, for autoradiographic exposure, for periods of either 7 days or 4 weeks. Following the required exposure period, all slides were developed in undiluted D19 developer (Kodak, UK) for 6 minutes, washed in distilled water and fixed in Unifix photographic fixer (Kodak, UK) for 4 minutes. All slides were subsequently counter-stained in haematoxylin for 30 seconds before being dehydrated and mounted in xylene.

3.3.4.2 Light microscopic evaluation of autoradiography

All autoradiographs were viewed and evaluated for the distribution of radioactivity using a Leica DM RB photomicroscope using dark field illumination. The relative amounts of radiolabel in the sections was assessed qualitatively and given a grading from 1-4 corresponding to minimal (1), slight (2), moderate (3) and marked (4) with the specific distribution within each component tissue being evaluated. All gradings were done by a single person and slides were evaluated with the full knowledge of their group designation and time point. Representative photographs were taken of lung and nasal regions from both rats and mice at each of the time periods post exposure to ^{14}C styrene, and following each of the three autoradiographic preparations.

3.2.4.3 Whole body autoradiography

The carcasses were immediately frozen in hexane/dry ice, embedded in 2%(w/v) carboxymethyl cellulose and transferred to a cryostat (PMV, Stockholm, Sweden) at -20°C. Longitudinal sagittal sections were taken onto tape and freeze dried at -20°C for 48 hours. Autoradiographs were prepared using a Molecular Dynamics Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA) with exposure times typically of 7 days.

4. RESULTS

4.1 Exposures

The atmospheric concentrations of styrene in the chambers were 166±11.7 ppm for rats and 164±15.6 ppm for mice. Based on the uptake of styrene during the experiments, the radiochemical doses were 1.1 mCi/kg for rats and 3.76 mCi/kg for mice.

4.2 Histoautoradiography

Changes in levels of radioactivity (total, bound, volatile) with time are shown in Figures 1-6. Changes in radioactivity in each tissue compartment of the lungs and nasal passages are given in Tables 1and 2 respectively. Representative autoradiograms illustrating the results described in this section are shown in the photographic supplement.

4.2.1 Changes in the distribution of ^{14}C styrene/metabolites in the lung of the rat with time post exposure .

4.2.1.1 Distribution of Total Radioactivity (Fig. 1a).

At 5 minutes after cessation of exposure, radioactivity was specifically associated at very high levels with the bronchiolar mucosa (see photograph 1 in supplement) and the walls of pulmonary blood vessels.

At the 1 hour time point, the radiolabel remained at moderate levels within the bronchiolar mucosa and blood vessel walls although, in addition, label was now also present at moderate levels in the bronchiolar airway and the lumen of the blood vessels.

At the 4 hour time point, label was no longer associated with the bronchiolar mucosa but was now present at high levels in the alveolar airways and at moderate levels in the lumens of the blood vessels (see photograph 2 in supplement).

At the 24 hour time point, radiolabel was present at high levels in the airways of both the bronchioles and alveoli and specific tissue localisation was not seen.

From the 5 minute period on, the relative amount of radiolabel in the tissue decreased and there was a redistribution from the tissues into the airways at the later time points. At no time was radiolabel observed in the alveolar epithelium in these preparations.

4.2.1.2 Distribution of Non-volatile Radioactivity (Fig. 1b).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at very high levels within the bronchiolar mucosa, the bronchiolar airway, and within the walls of pulmonary blood vessels.

At the 1 hour time point, the radiolabel remained associated, at high levels, with the bronchiolar mucosa and associated airways, and at very high levels with the blood vessel walls although not with the vascular lumen as was seen for the total radioactivity at this time.

At the 4 hour time point, label was no longer associated with the bronchiolar mucosa but was now present at high levels in the airways of the bronchioles and alveoli, and at high levels in the walls of the pulmonary blood vessels.

At the 24 hour time point, radiolabel was present at high levels in the airways of both the bronchioles and alveoli and specific tissue localisation was not seen.

As with the results for the total radioactivity, the time course for the non-volatile radioactivity showed a redistribution from the tissues to the airways between the 5 minute and 24 hour time points. At no time was radiolabel localised to the alveolar mucosa in these preparations.

4.2.1.3 Distribution of Bound Radioactivity - from 'Fixed' sections (Fig 1c).

At 5 minutes after cessation of exposure, radioactivity was only found within the bronchiolar mucosa. From the 1 hour time point to the 24 hour time point, there was no specific

localisation of radiolabel to any specific site in the rat lung. At no time was any radiolabel localised to the alveolar wall or airways in these preparations of rat lung.

4.2.2 Changes in the distribution of ^{14}C styrene/metabolites in the lung of the mouse with time post exposure.

4.2.2.1 Distribution of Total Radioactivity (Fig 2a).

At 5 minutes after cessation of exposure, radioactivity was found specifically and at very high levels within the bronchiolar mucosa (see photograph 3 in supplement) and the bronchiolar airways and at more moderate levels in the blood vessel lumen of the lung.

At both the 1 and 4 hour time points, the radiolabel remained associated at very high levels with the bronchiolar mucosa with high levels also still present in the bronchiolar airways (see photograph 5 in supplement). No other tissues distribution was seen at these time points.

At the 24 hour time point, moderate levels of radiolabel were still associated with the bronchiolar mucosa and bronchiolar airway (see photograph 7 in supplement), and now also with the walls of the pulmonary blood vessels.

There was a gradual lessening of the amount of radiolabel present in the various tissues throughout the time course but label was consistently retained in the bronchiolar mucosa and airways while at no time point was there any specific association of radiolabel with the alveolar wall or alveolar airspace.

4.2.2.2 Distribution of Non-volatile Radioactivity (Fig 2b).

At all time points the distribution of the non-volatile component of the radiolabel showed the same pattern as that seen for the total radioactivity.

As with the distribution of total radioactivity, there was a gradual reduction in the amount of radiolabel present in the various tissues through the time course, but label was consistently retained in the bronchiolar mucosa and airways while at no time point was there any specific association of radiolabel with the alveolar wall or alveolar airspace.

4.2.2.3 Distribution of Bound Radioactivity - from 'Fixed' sections (Fig 2c).

The levels of radioactivity remaining, following brief fixation, were considerably lower than those seen in either of the two other procedures.

From the 5 minute time point to the 24 hour point, radiolabel remained present specifically in the bronchiolar mucosa with little found elsewhere in the lung (see photograph 4 in supplement). At the 1 hour time point onwards, label was observed in the lateral membranes of the bronchiolar cells, a distribution which was distinct from that seen at the 5 minute time point (see photograph 6 in supplement). This may represent an excretory pathway for the metabolic products of styrene as opposed to the radiolabel observed at the 5 minute time point which may represent the absorption route for the compound. No radioactivity was present in the alveolar walls or airways in these preparations.

4.2.3 A comparison of the distribution of ^{14}C styrene/metabolites in the lung between the rat and the mouse.

A summary of the changes observed in the distribution of ^{14}C styrene/metabolites between the tissues in the lungs of the rat and the mouse are shown in Tables 1a-e. For the bronchiolar mucosa (Table 1a) the rat and mouse initially showed similar levels of radiolabel but whereas in the rat the radiolabel had disappeared by the 4 hour time point, in the mouse, label was retained at all time points examined. It was also apparent that in the mouse a proportion of this radiolabel was bound to the tissue at all time points whereas in the rat only the 5 minute time point showed bound material.

For the bronchiolar airways (Table 1b), radiolabel was generally present in both the rat and the mouse at all the time points although no bound radiolabel was present in this region at any of the time points.

For the blood vessel lumen (Table 1c) radiolabel was only present at the early time points and there was no significant difference between the rat and the mouse.

For the blood vessel walls (Table 1d), the rat showed the presence of radiolabel at the earliest time point and this gradually disappeared with time. In contrast, in the mouse, no radiolabel was present at the early time points but at the 24 hour time point, radiolabel had now

appeared in this part of the lung. At no time was radiolabel bound in either the rat or the mouse.

For the alveolar airway (Table 1e), radiolabel appeared in the rat at the 4 and 24 hour time points but not before. None of this was in the 'bound' form. Radiolabel was not observed in this part of the lung in the mouse.

4.2.4 Changes in the distribution of ^{14}C styrene/metabolites in the nasal cavity of the rat with time post exposure - Level 2.

4.2.4.1 Distribution of Total Radioactivity(Fig. 3a).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at very high levels, with the surface of the olfactory and respiratory mucosa, within the olfactory mucosa itself and in Bowman's glands.

At the 1 hour time point, the radiolabel was specifically localised at high levels on the surface of the olfactory mucosa and in Bowman's glands, and at moderate levels on the surface of the respiratory mucosa.

At the 4 hour time point, moderate levels of radiolabel remained associated with the surface of the olfactory mucosa and with Bowman's glands with high levels on the surface of the respiratory mucosa and nasal airways. A low level of label had appeared in the respiratory submucosa at this time point.

At the 24 hour time point, low incidences of high levels of labelling were seen on the surface of the olfactory and respiratory mucosa and high levels were present in the sub-mucosal tissue of the respiratory area. Low levels were also present in the nasal airways.

From the 5 minute period, the relative amount of radiolabel in the tissue gradually decreased with some redistribution of radiolabel. At all time points the surfaces of both respiratory and olfactory tissue were labelled with essentially equal frequency and intensity and airway labelling was only seen at the later time points. The significance of the increasing amounts of label associated with the respiratory submucosa remains unknown.

4.2.4.2 Distribution of Non-volatile Radioactivity (Fig. 3b).

The distribution of the non-volatile component of the radiolabel essentially mirrored that of the total radiolabel at each time point.

From 5 minutes onward, the relative amount of radiolabel in the tissue gradually decreased but the olfactory mucosa and Bowman's glands were specifically targeted throughout the time course with the exception of the last time point.

4.2.4.3 Distribution of Bound Radioactivity - from 'Fixed' sections (Fig 3c).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at low levels, with the olfactory mucosa and its surface, with Bowman's glands, and with the surfaces of the respiratory mucosa.

Between 5 minutes and 1 hour, the amount of radiolabel in the tissue was relatively constant and remained specifically localised to the surface of the olfactory mucosa and to Bowman's glands, but was not seen in the olfactory mucosa itself.

At the 4 and 24 hour time periods label was not specifically associated with any tissue within the nasal cavity of level 2, but the majority of rats showed a diffuse labelling.

4.2.5 Changes in the distribution of ^{14}C styrene/metabolites in the nasal cavity of the rat with time post exposure - Level 4.

4.2.5.1 Distribution of Total Radioactivity(Fig. 4a).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at very high levels, with the olfactory mucosa, the surface of the olfactory and respiratory mucosa, and in Bowman's glands (see photograph 8 in supplement).

At the 1 hour time point, the radiolabel remained specifically localised at high levels with the surface of the olfactory mucosa and in Bowman's glands, at high levels with the surface of the respiratory mucosa and at moderate levels within the olfactory mucosa itself.

At the 4 hour time point, moderate levels of radiolabel continued to be present in Bowman's glands and within the olfactory mucosa, on the surface of the olfactory mucosa, or possibly in

the apical cytoplasm of the olfactory mucosa, with high levels being found on the surface of the respiratory mucosa and in the nasal airspace (see photograph 10 in supplement).

At the 24 hour time point, high levels of labelling were seen in the nasal airspace with moderate levels being present on the surface of the respiratory mucosa and submucosa. Low levels of labelling were seen on the surface of the olfactory mucosa but no label was present in Bowman's glands and the olfactory mucosa (see photograph 11 in supplement).

In summary, from the 5 minute period onwards, the relative amount of radiolabel in the tissue gradually decreased. The surface of the olfactory mucosa showed radiolabel throughout the time course but the mucosa itself was only labelled up to the 4 hour time. Radiolabel was associated with Bowman's glands at all but the longest time point examined. At all time points the surfaces of both respiratory and olfactory tissue were labelled with essentially equal frequency and intensity. The appearance of radiolabel in the nasal airspace at the later time points may reflect a possible excretory route of the metabolic products.

4.2.5.2 Distribution of Non-volatile Radioactivity (Fig 4b).

The distribution of the non-volatile component of the radiolabel essentially mirrored that of the total radiolabel at each time point.

From the 5 minute period on the relative amount of radiolabel in the tissue gradually decreased although more tissues were involved in the localisation of the label. The olfactory mucosa and Bowman's glands were specifically targeted consistently throughout the time course until the 24 hour time period when no labelling was observed.

4.2.5.3 Distribution of Bound Radioactivity - from 'Fixed' sections(Fig. 4c).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at low levels, within the olfactory mucosa, on the surface of the olfactory mucosa and Bowman's glands, and on the surfaces of the respiratory mucosa (see photograph 9 in supplement).

At the 1 hour, 4 hour and 24 hour time points the radiolabel was not specifically localised to any tissue within the nasal cavity at level 4 but the majority of rats showed a diffuse labelling.

From the 5 minute period, the relative amounts of radiolabel in the tissues were maintained although specificity for a particular tissue was only seen at the 5 minute time point and a diffuse distribution was the rule at the later time points.

4.2.6. Changes in the distribution of ^{14}C styrene/metabolites in the nasal cavity of the mouse with time post exposure - Level 2.

4.2.6.1 Distribution of Total Radioactivity(Fig 5a).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at very high levels, with the surface of the olfactory and respiratory mucosa and with Bowman's glands, at a high level with the respiratory submucosa, and at moderate levels with the olfactory mucosa itself and the nasal airways.

At the 1 hour time point a low level of diffusely distributed radiolabel was seen in the majority of rats examined. The radiolabel was specifically localised, at high levels, on the surface of the respiratory mucosa and submucosa but was not present elsewhere.

At the 4 hour time point, low to moderate levels of radiolabel were seen within the olfactory mucosa and on the surface of the olfactory mucosa respectively, although no label was seen elsewhere.

At the 24 hour time point, low incidences of low levels of labelling were seen in Bowman's glands with moderate levels being present in the nasal airways.

From the 5 minute period, the relative amount of radiolabel in the tissue gradually decreased with fewer tissues being targeted nor were tissues consistently radiolabelled from one time point to the next. For example, whereas Bowman's glands and the olfactory mucosa and surface were labelled at the 5 minute and 4 hour time periods, the specificity was lost at the 1 hour and 24 hour time points. The results showed considerable inter-animal variability.

4.2.6.2 Distribution of Non-volatile Radioactivity (Fig 5b).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at moderate to very high levels with the surface of the olfactory and respiratory mucosa, with the respiratory submucosa, within the olfactory mucosa itself and in Bowman's glands.

At the 1 hour time point, the radiolabel was remained specifically localised at high levels on the surface of the olfactory mucosa and in Bowman's glands but was not seen elsewhere.

At the 4 hour time point, moderate levels of radiolabel were seen on the surface of the olfactory mucosa and in Bowman's glands, with low levels being present within the olfactory mucosa.

At the 24 hour time point, moderate levels of labelling were seen in the nasal airways but no other tissue was labelled.

From the 5 minute period, the relative amount of radiolabel in the tissue gradually decreased with fewer tissues being specifically targeted. The surface of the olfactory mucosa and Bowman's glands was specifically targeted throughout the time course with the exception of the 24 hour time point. Diffuse labelling became the rule for the majority of animals after the 5 minute time point.

4.2.6.3 Distribution of Bound Radioactivity - from 'Fixed' sections (Fig 5c).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at low levels and high incidences, with the surface of the olfactory mucosa and respiratory mucosa, with the respiratory submucosa and with Bowman's glands.

At the 1 hour, 4 hour and 24 hour time points the radiolabel was not specifically localised to any tissue within the nasal cavity of level 2 but the majority of mice showed a diffuse labelling.

From the 5 minute period, the amount of radiolabel in the tissue was maintained relatively constant although specificity for a particular tissue was only seen at the 5 minute time point and a diffuse distribution was the rule at the later time points.

4.2.7 Changes in the distribution of ^{14}C styrene/metabolites in the nasal cavity of the mouse with time post exposure - Level 4.

4.2.7.1 Distribution of Total Radioactivity (Fig 6a).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at very high levels, with the surface of the olfactory and respiratory mucosa, with the respiratory submucosa, and with Bowman's glands (see photograph 12 in supplement). Moderate levels of radiolabel were present in the olfactory mucosa itself and the nasal airways.

At the 1 hour time point, the radiolabel was specifically localised, at high levels with the surface of the olfactory mucosa and respiratory mucosa, with the respiratory submucosa and with Bowman's glands (see photograph 14 in supplement). Moderate levels of labelling were seen within the olfactory mucosa itself.

At the 4 hour time point, a similar pattern of labelling to that seen at 1 hour was repeated for the olfactory mucosa, its surface and for Bowman's glands, but no labelling was now seen in the respiratory mucosa or submucosa and at this time. Very high levels of radiolabel were seen in the nasal airways (see photograph 15 in supplement).

At the 24 hour time point, the labelling was maintained in the olfactory mucosa and on its surface, with moderate levels in Bowman's glands. Moderate levels of label were seen in the nasal airways but no label was present in the respiratory mucosa or submucosa.

From the 5 minute period, the relative amount of radiolabel in the tissue gradually decreased with the incidences of specific tissue targeting becoming gradually fewer. The Bowman's glands and olfactory mucosa and surface were most frequently labelled throughout the time course up to the 24 hour time period.

4.2.7.2 Distribution of Non-volatile Radioactivity (Fig 6b).

The distribution of radiolabel in those sections prepared for the visualisation of the non-volatile components of the radiolabel gave a similar general pattern to that shown by the preparations designed to give the total radiolabel.

From the 5 minute period, the relative amount of radiolabel in the tissue gradually decreased with the incidences of specific tissue targeting becoming gradually fewer. The olfactory mucosa and surface, and Bowman's glands were labelled for the majority of the times

examined whereas the label associated with the respiratory portion were only seen up to the 1 hour time period.

4.2.7.3 · Distribution of Bound Radioactivity - from 'Fixed' sections (Fig 6c).

At 5 minutes after cessation of exposure, radioactivity was specifically associated, at low levels with the surface of the olfactory and respiratory mucosa, and Bowman's glands (see photograph 13 in supplement).

At the 1 hour, 4 hour and 24 hour time points the radiolabel was not specifically localised to any tissue within the nasal cavity of level 4 but the majority of mice showed a diffuse labelling.

From the 5 minute period on the amount of radiolabel in the tissue was maintained relatively constant although specific retention of label was not observed after this period.

4.2.8 A comparison of the distribution of ^{14}C styrene/metabolites in the nasal passages between the rat and the mouse.

The changes in the distribution of radiolabel between the different tissues in the rat and mouse nasal tissues are summarised in Tables 2a-f.

In the olfactory mucosa (Table 2a), the rat showed high levels of label at the early time points which was gradually lost with increasing time such that none was present at the 24 hour time period. The tissue in level 4 generally showed greater retention times than did that in level 2. Radiolabel was only present in the bound preparation at the five minute time point. In the mouse olfactory tissue, radiolabel was generally present at lower levels than in the rat but was retained for longer, particularly in the tissue in level 4. No radiolabel was present in the mouse olfactory mucosa in the bound preparation at any time point. Overall the mouse appeared to have less radiolabel but to retain it slightly longer than did the rat tissue.

Radiolabel was present on the surface of the olfactory mucosa in both the rat and the mouse at similar levels at the early time points which gradually got less with time (Table 2b). The mouse tissue showed variable amounts of radiolabel between level 2 and 4 with the latter retaining high levels while the former did not appear to show any label. Radiolabel was

present in both species in the bound preparation at the early time points but was generally lost after the 4 hour time point with no apparent species difference in response.

In the rat, Bowman's glands showed high levels of radiolabel in both levels 2 and 4 which gradually got less until at the 24 hour time point none was present (Table 2c). A low level of this label was present in the bound preparation. In the mouse, Bowman's glands in level 4 were generally more highly labelled than those in level 2 and they also retained the label for longer. In comparison to the rat, the mouse retained label at higher levels for longer periods. There was no difference in the proportion of bound label between the rat and the mouse.

In the rat high levels of radiolabel were present on the surface of the respiratory mucosa (Table 2d) with similar levels being present in level 2 and level 4. The label got gradually less with time although low levels were retained in the rat at 24 hours. Radiolabel was only present in the bound preparation at the 5 minute time interval. In the mouse label was only present on the surface of the respiratory mucosa at the 5 minute and 1 hour time point with similar amounts being present in both level 2 and level 4. No label was present in this compartment at 24 hours. Material was only present in the bound preparation at the earliest time point. The rat differed from the mouse by retaining the radiolabel for the period of the time course.

In the respiratory sub-mucosa of the rat (Table 2e), no label was present until 4 hours and this was retained, and in the case of the tissue in level 2 was increased, at 24 hours. None of this radiolabel was present in the bound preparation. In the mouse, radiolabel was present in the submucosa of the respiratory tissue in both level 2 and level 4 at the early time points but had disappeared at 4 and 24 hours. Radiolabel was present in the bound preparation at the 5 minute time period but was not seen at the later time points. There were distinct differences between the rat and the mouse for the distribution and tempora' sequence of appearance and disappearance of radiolabel in the respiratory submucosa with the mouse having the compound early on and losing it before the end of the time course, while the rat acquired the radiolabel late and retained it.

In the rat, radiolabel was not present in the airspace of the nasal cavity at the 5 minute and 1 hour time points but was present at high levels at 4 and 24 hours. No radiolabel was present in the bound preparation at any of the time points. In the mouse radiolabel was present in the

airways at both the early (5 minute) and later (4 hour and 24 hour) time points although no label was present at the 1 hour time point. There was no label in the bound preparation at any time point. There was a similar response for both rat and mouse in terms of the nasal airspace.

4.3 Whole body autoradiography

4.3.1 Rat.

The rat killed 5 minutes after exposure to ^{14}C-styrene (Photograph 16 in the photo-supplement) had high levels of radiolabel associated with the nasal passages, liver, kidney and intestines, with lower levels being present in the glandular stomach. The lungs had low overall levels although high local regions were present. No label was associated with the forestomach at this time.

At the 1 hour time point (Photograph 17 in the photo-supplement) the level of labelling was maintained in the nasal passages, while the liver, kidney and intestines also showed similar levels of radiolabel to those seen at the 5 minute time point. The lungs had low overall levels although high local regions were present. The glandular stomach had higher levels than those seen in the rat at 5 minutes. No label was associated with the forestomach at this time.

At the 4 hour time period high levels of radiolabel were still present in the nasal passages but liver levels were now low and kidney levels, although higher than in the liver, were decreased over that seen at the 1 hour period. The intestines had high levels of radiolabel while the lungs did not appear to have any radiolabel associated with them.

At 24 hours the nasal passages were one of the highest radiolabelled tissues, together with the forestomach, which had not been radiolabelled until this time. The liver, intestines and glandular stomach had low levels of labelling while no radiolabel was present in the kidneys or lungs.

4.3.2 Mouse.

At 5 minutes (Photographs 18 [head region only] & 19 in the photo-supplement), 1 hour (Photograph 20 in the photo-supplement) and 4 hours, a similar pattern of radiolabel

distribution was seen with high levels of labelling being present in the nasal passages, liver, kidney and intestines. Low levels were present in the glandular stomach and lungs while no label was present in the forestomach.

At 24 hours the nasal passages showed the highest levels of labelling in the mouse body, with some locally high levels being present in the lung and kidneys. No other tissues showed labelling at this time point.

5. DISCUSSION

A comparison of the results obtained from those sections which were prepared for the demonstration of total radioactivity to those prepared for evaluating only those non-volatile radioactive components has shown that there was little real difference between the distribution or content of radioactivity present in the sections of tissue prepared by the two different methods. This suggested that the majority of radiolabel present in the tissue was not parent styrene but a non-volatile product of styrene. A comparison of the distribution of radiolabel within the tissues following fixation versus that minus fixation has shown that the vast majority of radiolabel was extracted during the fixation process and that all that remains was very tightly bound product. The procedures involved in sectioning and fixing the sectioned cells are severe and may allow considerable loss of protein during subsequent fixation together with any bound radiolabel and hence the results may not accurately reflect protein-bound products. A better strategy may have been to fix the lung whole and then to section the fixed lung and conduct subsequent procedures for evaluating bound products.

The results have shown that for both the rat and the mouse considerable amounts of radio-labelled products were present in the lung and nasal passages up to 24 hours after cessation of a six hour exposure period to 160 ppm ^{14}C-styrene. The mouse lung has shown radiolabel specifically localised in the bronchiolar mucosa and airway and further showed that retention occurred at this site in the majority of animals up to and including the 24 hour observation period. In the rat a similar localisation was seen in the bronchiolar mucosa at 5 minutes and 1 hour but that this was lost at the later time points when distribution of radiolabel moved from the bronchiolar compartment to the vascular compartment where highly specific

localisation to the muscle wall of the blood vessels was seen up to 4 hours although this was lost at 24 hours. This distribution was also seen in the mouse but only at the 24 hour period.

Considerable amounts of radiolabel were also seen in both the bronchiolar and alveolar airways in the rat which increased with increasing time. Alveolar airway distribution of radiolabel was not seen in the mouse. The prolonged airway distribution of the radiolabel in the rat may reflect the excretion pathway for the chemical rather than any local retention of radiolabel. The specific retention of radio-label in the bronchiolar mucosa of the mouse, and the relatively quick loss of label from the same tissue in the rat, was better illustrated by reference to the data obtained from the fixed sections, where rats failed to show any specific bronchiolar-associated label after the 5 minute period.

Although transient localisation of radiolabel to the alveolar airways was observed, particularly in rats at the later time points, it was not seen in the mouse and perhaps more significantly, specific localisation of radiolabel to the alveolar wall in either species was never observed. Its appearance in the alveolar airspace may be a reflection of the excretory route of the products of styrene metabolism.

With reference to the nasal tissue, both the rat and the mouse showed similar profiles both for the initial distribution of ^{14}C-styrene and the relative changes in distribution that occurred with time. In both species the olfactory tissue and Bowman's glands showed high initial labelling with the surfaces of both the respiratory and olfactory areas showing specific radiolabel. Whether this surface localisation merely reflects the presence of radiolabel within the airways or whether it shows dissolution of the radiolabel amongst a surface mucous layer remains to be seen. The localisation and retention of radiolabel in the olfactory epithelium and in Bowman's glands (as shown by the 'total' and 'non-volatile' results) is consistent with the known target cytotoxicity which styrene shows for these tissues and with the distribution of the cytochrome P-450 enzymes known to metabolise styrene. The 'fixed' preparations showed, that at the early time periods radiolabel was bound tightly to the olfactory surfaces and Bowman's glands in both the rat and the mouse.

6. REFERENCES

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Table 1: Summary of the changes in the distribution of radiolabel with time in the lungs of the rat and the mouse.

1a. Changes in Bronchiolar mucosa.

RAT	Total	Non-volatile	Bound
5 min	4	4	1
1 hr	2	3	0
4 hr	0	0	0
24 hr	0	0	0
MOUSE	**Total**	**Non-volatile**	**Bound**
5 min	4	4	1
1 hr	4	3	1
4 hr	3	3	1
24 hr	2	2	1

1b. Changes in Bronchiolar airway

RAT	Total	Non-volatile	Bound
5 min	0	4	0
1 hr	2	3	0
4 hr	0	3	0
24 hr	3	3	0
MOUSE	**Total**	**Non-volatile**	**Bound**
5 min	4	4	0
1 hr	3	3	0
4 hr	3	3	0
24 hr	2	2	0

1c: Changes in Blood vessel lumen

RAT	Total	Non-volatile	Bound
5 min	0	0	0
1 hr	2	0	0
4 hr	0	0	0
24 hr	0	0	0
MOUSE	**Total**	**Non-volatile**	**Bound**
5 min	2	2	0
1 hr	0	0	0
4 hr	0	0	0
24 hr	0	0	0

1d: Changes in Blood vessel wall

RAT	Total	Non-volatile	Bound
5 min	4	4	0
1 hr	2	4	0
4 hr	2	3	0
24 hr	0	0	0
MOUSE	**Total**	**Non-volatile**	**Bound**
5 min	0	0	0
1 hr	0	0	0
4 hr	0	0	0
24 hr	2	2	0

1e. Changes in Alveolar airway

RAT	Total	Non-volatile	Bound
5 min	0	0	0
1 hr	0	0	0
4 hr	3	3	0
24 hr	3	3	0
MOUSE	Total	Non-volatile	Bound
5 min	0	0	0
1 hr	0	0	0
4 hr	0	0	0
24 hr	0	0	0

Table 2: Summary of changes in the distribution of radiolabel with time in levels 2 & 4 of the nasal passages of the rat and the mouse.

2a: Changes in Olfactory mucosa.

	Total		Non-volatile		Bound	
	Level 2	Level 4	Level 2	Level 4	Level 2	Level 4
RAT						
5 min	4	4	4	4	1	1
1 hr	0	2	2	0	0	0
4 hr	0	2	1	2	0	0
24 hr	0	0	0	0	0	0
MOUSE						
5 min	2	2	2	2	0	0
1 hr	0	2	0	2	0	0
4 hr	1	2	1	1	0	0
24 hr	0	2	0	0	0	0

2b: Changes in Olfactory mucosa - surface.

	Total		Non-volatile		Bound	
	Level 2	Level 4	Level 2	Level 4	Level 2	Level 4
RAT						
5 min	4	4	4	4	1	1
1 hr	3	3	2	4	1	0
4 hr	2	2	3	3	0	0
24 hr	1	1	1	2	0	0
MOUSE						
5 min	4	4	4	4	1	1
1 hr	0	3	3	3	0	0
4 hr	2	3	2	2	0	0
24 hr	0	3	0	2	0	0

2c: Changes in Bowman's glands.

	Total		Non-volatile		Bound	
	Level 2	Level 4	Level 2	Level 4	Level 2	Level 4
RAT						
5 min	4	4	4	4	1	1
1 hr	3	3	3	4	1	0
4 hr	2	2	2	2	0	0
24 hr	0	0	0	0	0	0
MOUSE						
5 min	4	4	2	4	1	1
1 hr	0	3	3	3	0	0
4 hr	0	3	2	2	0	0
24 hr	1	2	0	1	0	0

2d: Changes in Respiratory mucosa - surface.

	Total		Non-volatile		Bound	
	Level 2	Level 4	Level 2	Level 4	Level 2	Level 4
RAT						
5 min	4	4	4	4	1	1
1 hr	2	4	0	4	0	0
4 hr	3	3	3	3	0	0
24 hr	1	2	2	2	0	0
MOUSE						
5 min	4	4	3	4	1	0
1 hr	3	3	0	0	0	0
4 hr	0	0	0	3	0	0
24 hr	0	0	0	0	0	0

2e: Changes in Respiratory sub-mucosa.

	Total		Non-volatile		Bound	
	Level 2	Level 4	Level 2	Level 4	Level 2	Level 4
RAT						
5 min	0	0	0	0	0	0
1 hr	0	0	0	0	0	0
4 hr	1	0	2	2	0	0
24 hr	3	2	2	2	0	0
MOUSE						
5 min	3	4	3	4	1	1
1 hr	3	3	0	3	0	0
4 hr	0	0	0	0	0	0
24 hr	0	0	0	0	0	0

2f: Changes in Nasal airspace.

	Total		Non-volatile		Bound	
	Level 2	Level 4	Level 2	Level 4	Level 2	Level 4
RAT						
5 min	0	0	0	0	0	0
1 hr	0	0	0	0	0	0
4 hr	3	3	3	3	0	0
24 hr	1	3	2	2	0	0
MOUSE						
5 min	2	2	0	0	0	0
1 hr	0	0	0	0	0	0
4 hr	0	4	0	3	0	0
24 hr	2	2	2	2	0	0

Fig. 1: Summary of changes in the distribution of radiolabel with time in various tissues of the lung of the rat. a) Total; b) Non-volatile; c) bound.

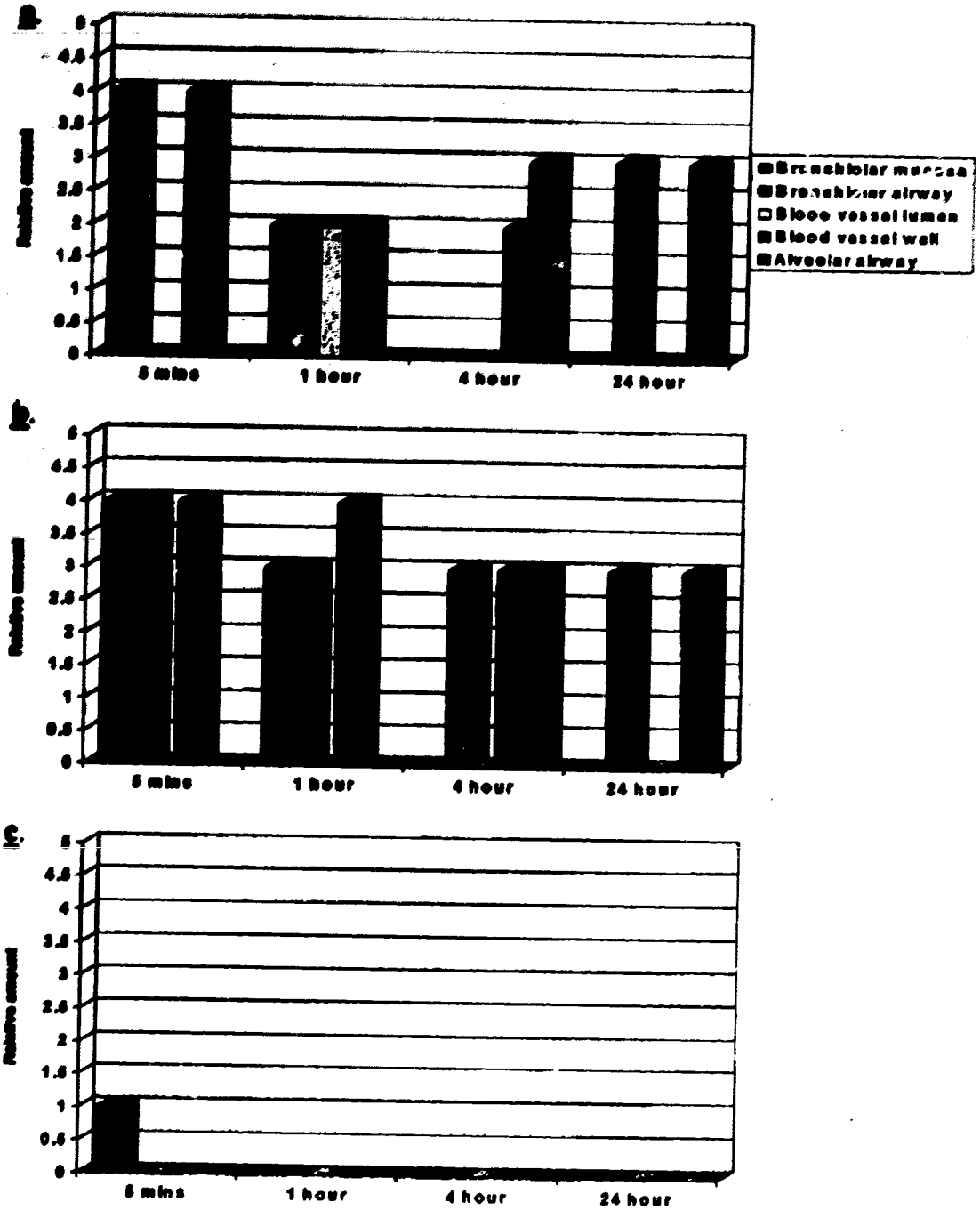


Fig. 2: Summary of changes in the distribution of radiolabel with time in various tissues of the lung of the mouse. a) Total; b) Non-volatile; c) Bound.

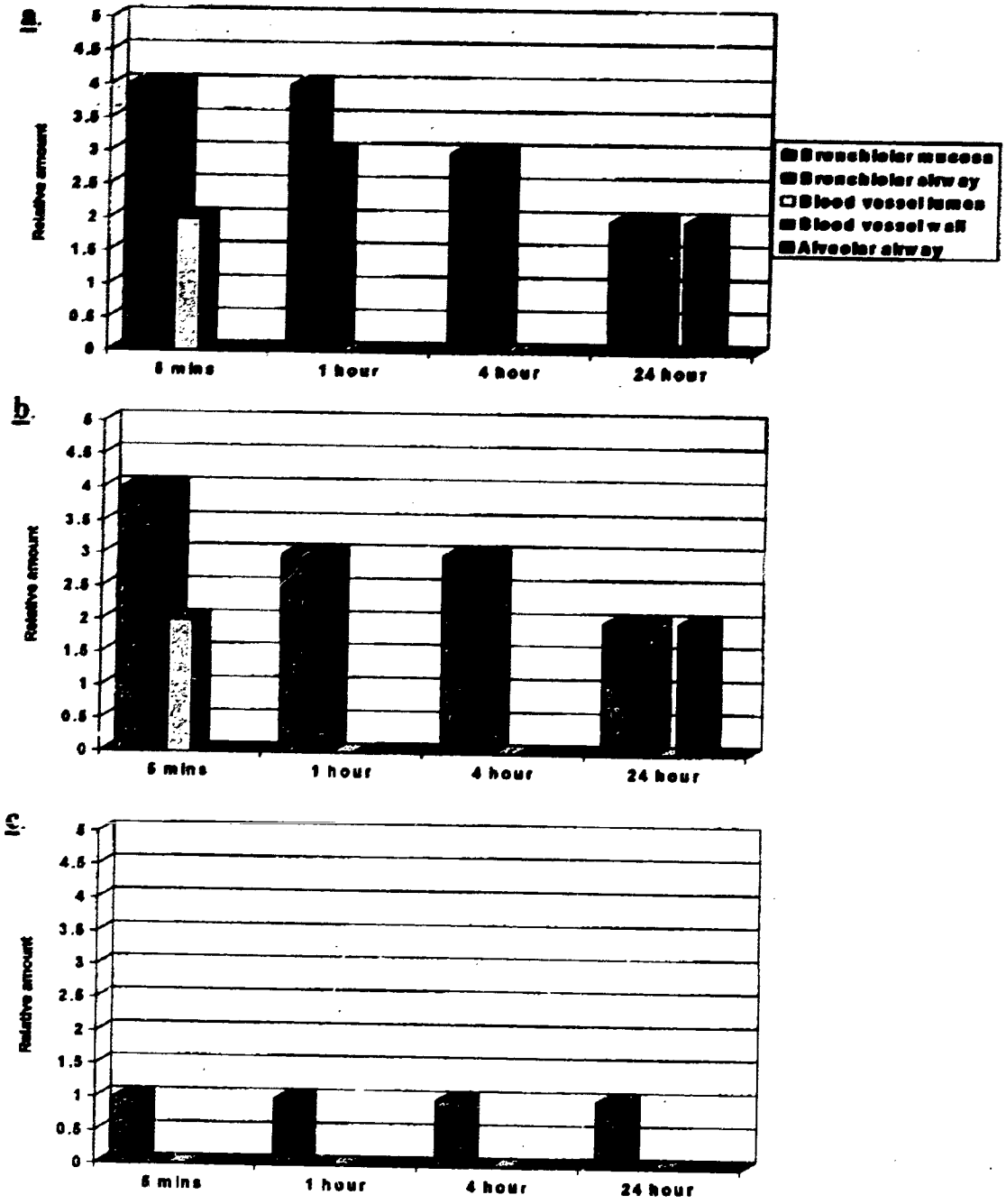


Fig. 3: Summary of changes in the distribution of radiolabel with time in various tissues in level 2 of the nasal passages of the rat. a) Total; b) Non volatile; c) Bound.

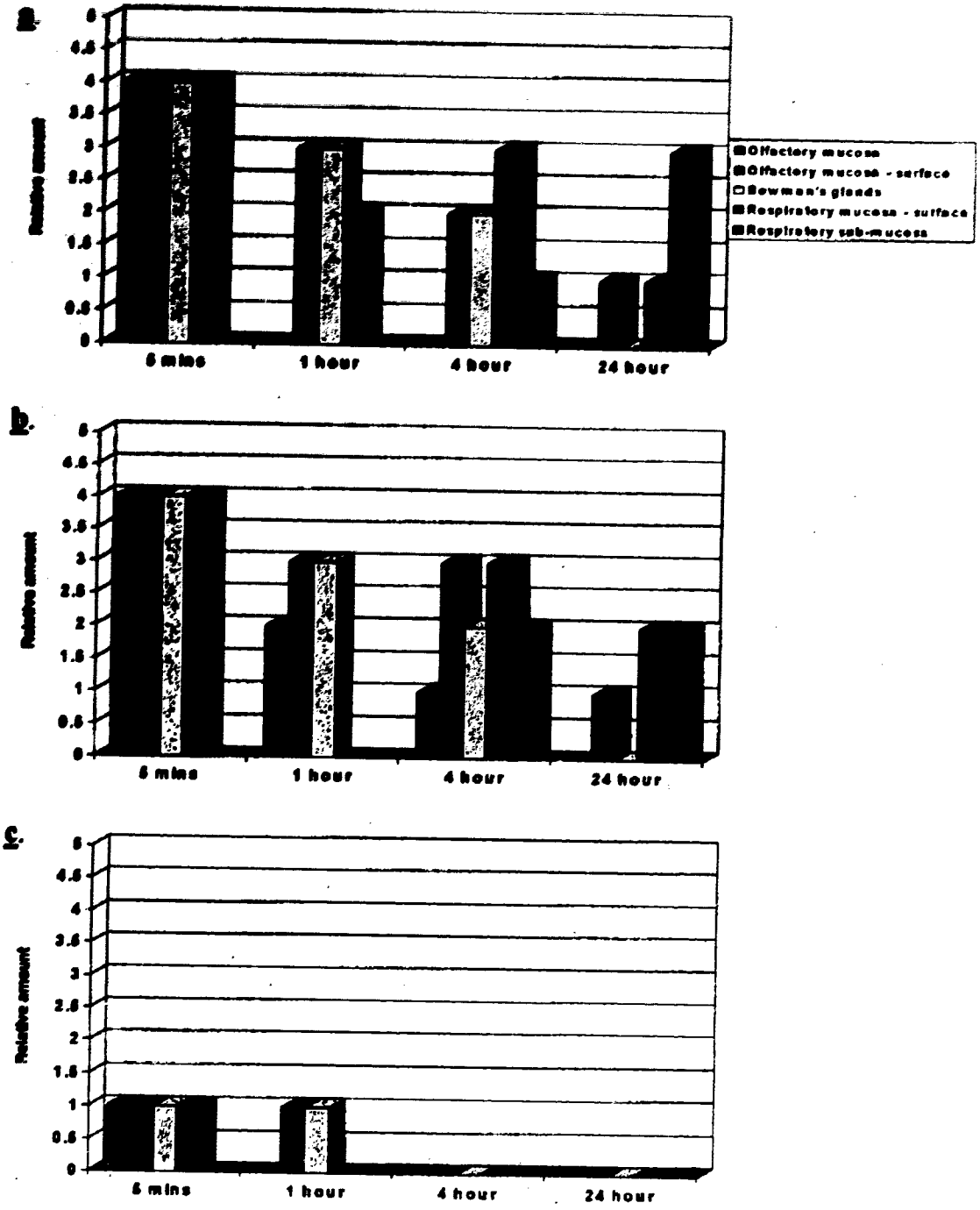


Fig. 4: Summary of changes in the distribution of radiolabel with time in various tissues in level 4 of the nasal passages of the rat. a) Total; b) Non-volatile; c) Bound.

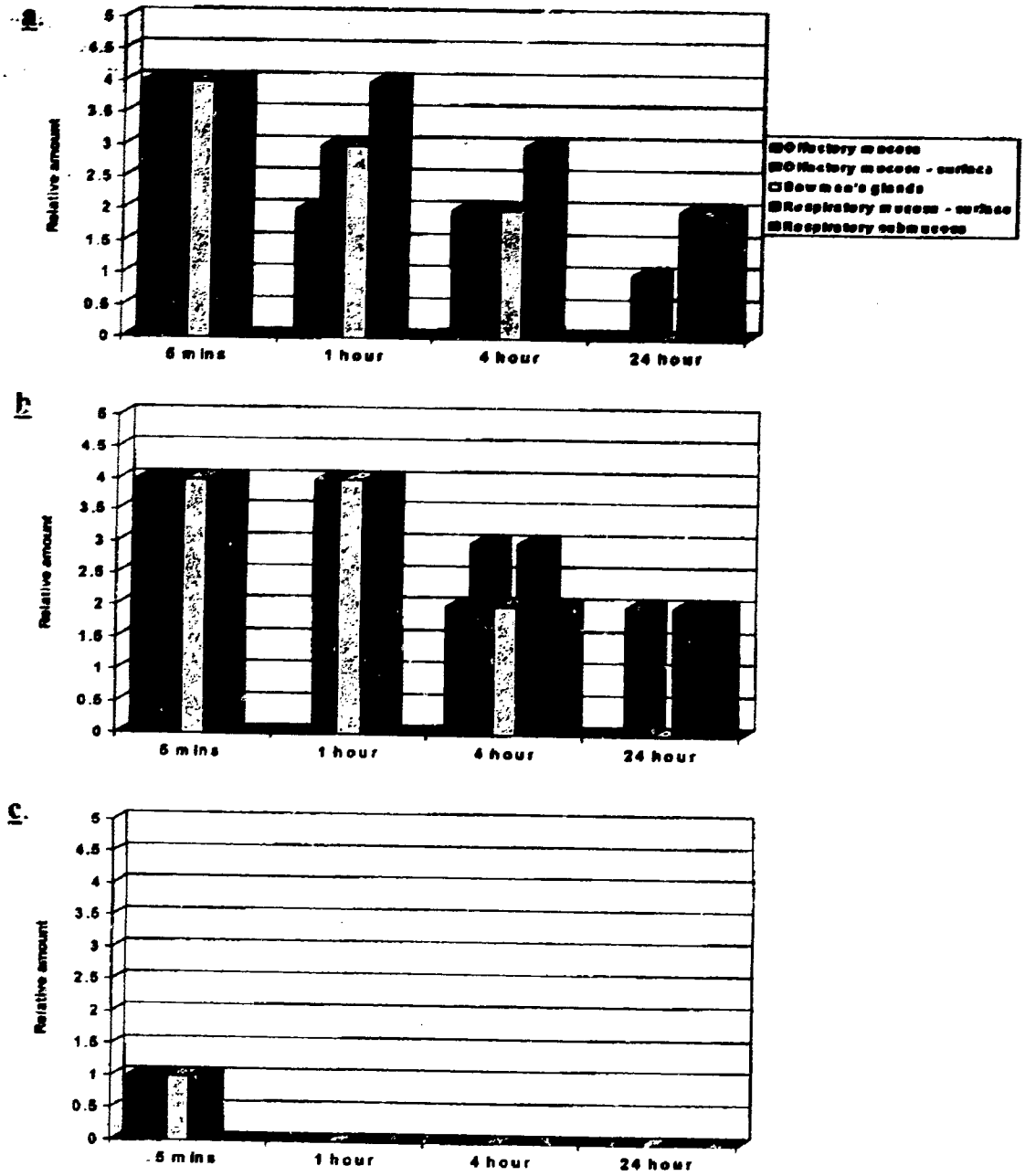


Fig. 5: Summary of changes in the distribution of radiolabel with time in various tissues in level 2 of the nasal passages of the mouse. a) Total; b) Non-volatile; c) Bound

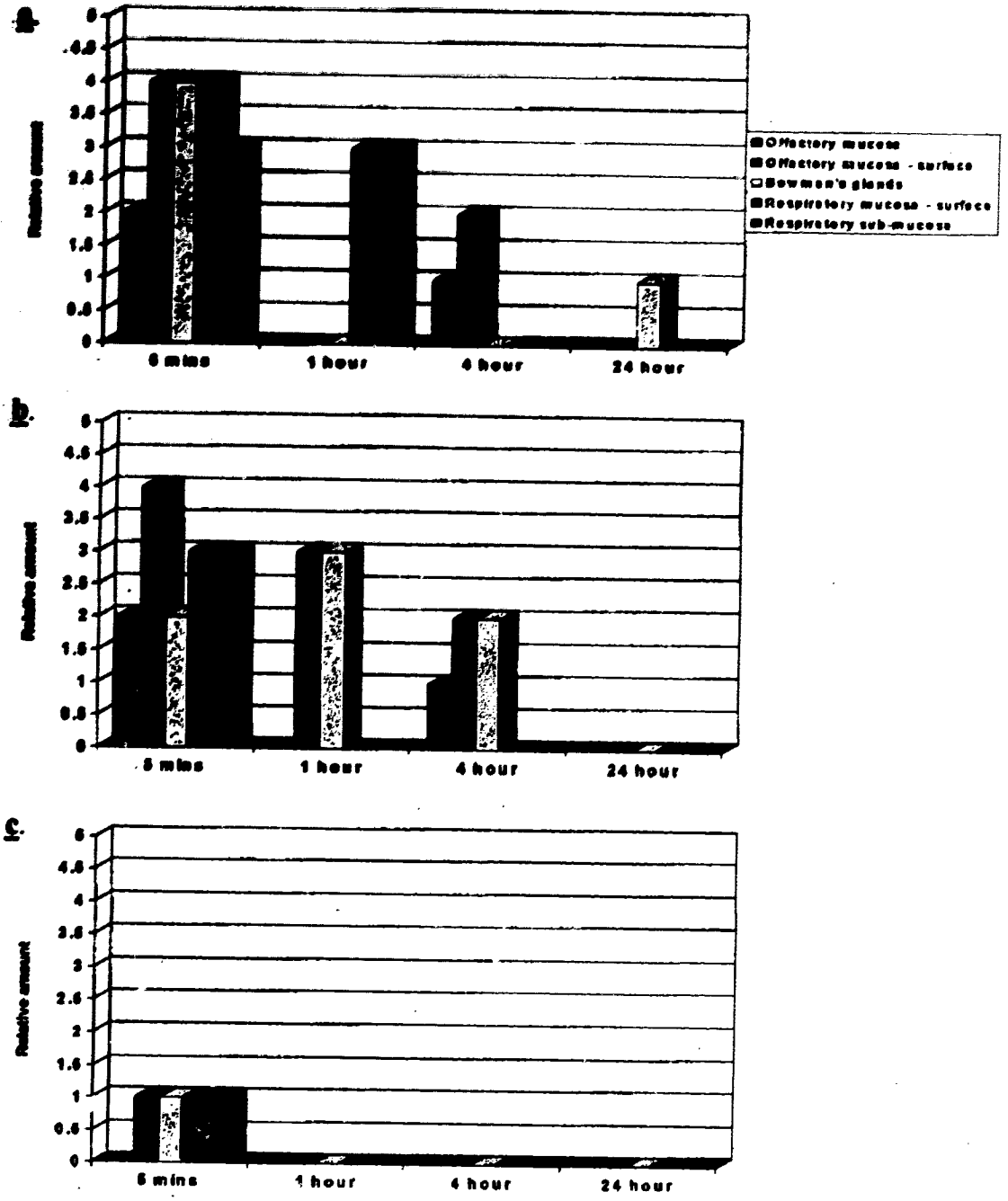
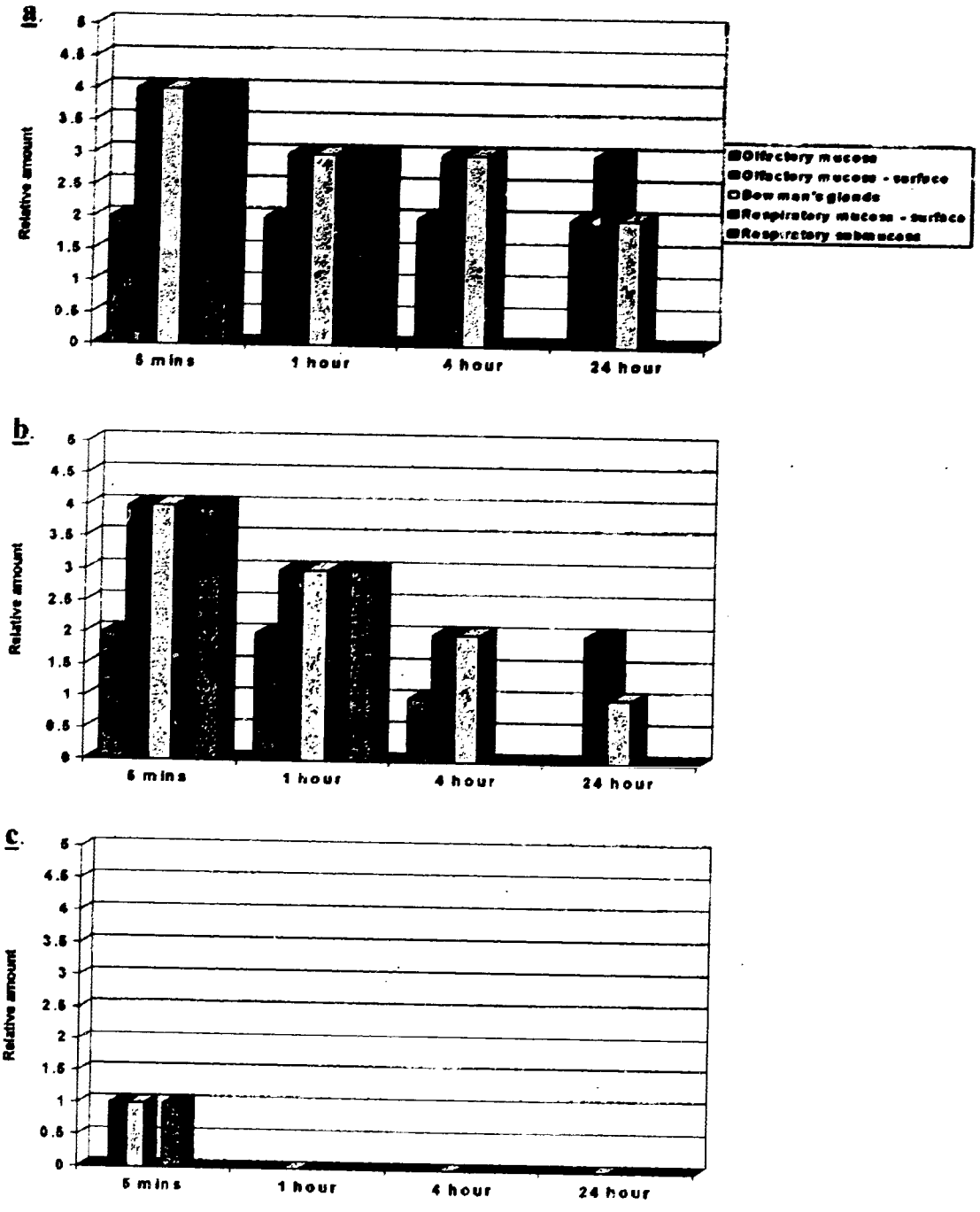


Fig. 6: Summary of changes in the distribution of radiolabel with time in various tissues in level 4 of the nasal passages of the mouse. a) Total; b) Non-volatile; c) Bound.



Appendix 1. Histoautoradiography - individual animal data

XR6516: INDIVIDUAL ANIMAL DATA - RAT
5 minute sacrifice

ANIMAL No	LUNG	NASAL
1		
Total	High amounts preferentially present in blood vessel wall and in bronchiolar epithelium	Poor section - no specific label (L2). Label present on mucosal surface of respiratory and olfactory mucosa with lesser amounts in Bowman's glands (L4).
Fixed	Low amounts preferentially present in bronchioles	No specific label (L2). Small amounts of label present on mucosal surface, in olfactory mucosa and in Bowmans glands (L4).
Volatile	High amounts preferentially present in blood vessel wall and in bronchiolar epithelium	Label present on mucosal surface of respiratory and olfactory mucosa with lesser amounts in Bowmans glands (L2&L4).
2		
Total	High amounts preferentially present in blood vessel wall and in bronchiolar epithelium	Poor ARG (L2) Label present on mucosal surface and in olfactory mucosa with lesser amounts in Bowmans glands (L4).
Fixed	Low amounts preferentially present in bronchioles	Small amounts of label present on mucosal surface, in olfactory mucosa and in Bowmans glands (L4&L2).
Volatile	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium and bronchiole lumen.	Poor ARG (L2)
3		
Total	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium and bronchiole lumen.	Large amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2&L4)
Fixed	Small amounts of label associated with bronchiolar epithelium.	Small amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2&L4)
Volatile	Very high amounts preferentially present in blood vessel wall and in	Large amounts of label present on mucosal surface of respiratory and

	bronchiolar epithelium and bronchiole lumen.	olfactory, in olfactory mucosa and in Bowmans glands (L2&L4).
4		
Total	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium and bronchiole lumen.	Large amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L4). No specific label (L2).
Fixed	No specific label.	Small amounts of label present on mucosal surface of olfactory mucosa and in Bowmans glands (L4&L2)
Volatile	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium and bronchiole lumen.	Large amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2&L4).
5		
Total	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium.	Large amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2&L4)
Fixed	Small amounts preferentially present in blood vessel wall and in bronchiolar epithelium	Small amounts of label present on mucosal surface of olfactory mucosa and in Bowmans glands (L2&L4).
Volatile	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium	Large amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2&L4)
6		
Total	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium	Moderate amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L4&L2)
Fixed	Small amounts preferentially present in bronchiolar wall	No specific label (L4). Label present in airways and on surface of olfactory mucosa and in Bowman's glands (L2).
Volatile	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium	Poor section (L4). Moderate amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2)
7		
Total	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium.	Moderate amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa

		and in Bowmans glands (L2&L4)
Fixed	Small amounts preferentially present in bronchiolar wall	Small amounts of label present on mucosal surface of olfactory mucosa and in Bowmans glands (L2 &L4)
Volatile	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium.	Moderate amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2&L4)
8		
Total	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium.	Moderate amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2&L4)
Fixed	Small amounts preferentially present in bronchiolar wall	No specific label - melt (L2)? Some focally high label on surface of respiratory and olfactory mucosa (L4).
Volatile	Very high amounts preferentially present in blood vessel wall and in bronchiolar epithelium.	Moderate amounts of label present on mucosal surface of respiratory and olfactory, in olfactory mucosa and in Bowmans glands (L2&L4)
9		
Total	High levels in blood vessel walls and lumen - little in bronchioles.	Label present on mucosal surface and in Bowmans glands (L2). Label present on mucosal surface and in olfactory mucosa with lesser amounts in Bowmans glands (L4).
Fixed	Small amounts preferentially present in bronchiolar wall	Label present on mucosal surface and in Bowmans glands (L4). No label (L2)
Volatile	Higher levels in bronchioles with lower levels in blood vessel walls and lumen.	Specific label over respiratory and olfactory surface mucosa and in Bowman's glands (L2). Melted autoradiogram (L4).
10		
Total	High levels in smooth muscle of blood vessel wall	Specific label over respiratory and olfactory surface mucosa and in Bowman's glands (L2). No specific label (L4).
Fixed	No specific label.	No specific label (L2). No specific label (L4).
Volatile	High levels in smooth muscle of blood vessel wall	Very high label over respiratory and olfactory surface mucosa and in Bowman's glands (L2). No specific label (L4).

ANIMAL No	LUNG	NASAL
11		
Total	No label	No specific label (L2&L4)
Fixed	No label	No specific label (L2&L4)
Volatile	No label	No specific label (L2&L4)
12		
Total	No label	
Fixed	Low amounts preferentially associated with bronchiolar epithelium in terminal airways in lateral membranes.	
Volatile	No label	
13		
Total	No specific label	Low amounts preferentially localised on surface of respiratory mucosa (olfactory not present) (L2). High amounts preferentially localised on surface of respiratory an olfactory mucosa (L4).
Fixed	No specific label	No specific label (L2&L4)
Volatile	No specific label	No specific label (L2). High amounts preferentially localised on surface of respiratory an olfactory mucosa and Bowman's glands (L4).
14		
Total	Low amounts preferentially present in arterial muscle and blood vessel lumen and in bronchiole airways and bronchiolar epithelium	No specific label - melt? (L2&L4).
Fixed	Low amounts preferentially associated with bronchiolar epithelium in terminal airways in lateral membranes.	No specific label (L2&L4)
Volatile	No specific label	High amounts preferentially localised on surface of olfactory mucosa and Bowman's glands (L2&L4).
15		
Total	Low amounts preferentially present in arterial muscle and blood vessel lumen	
Fixed	No specific label	

Volatile	No specific label	
16		
Total	Low amounts preferentially present in blood vessel lumen and in bronchiole airways and bronchiolar epithelium	Low amounts preferentially localised on surface of respiratory and olfactory mucosa and Bowman's glands (L2). Poor section - L4)
Fixed	No specific label	No specific label (L2&L4)
Volatile	Low amounts preferentially present in bronchiole airways and bronchiolar epithelium	No specific label (L2). Poor section (L4)
17		
Total	Diffuse label - melt?	
Fixed	No specific label	
Volatile	No specific label	
18		
Total	Low amounts preferentially present in blood vessel wall and in bronchiole airways and bronchiolar epithelium	No specific label (L2&L4)
Fixed	No specific label	No specific label (L2&L4)
Volatile	No label present	No specific label (L2&L4)
19		
Total	Low amounts preferentially present in blood vessel wall.	Label present on olfactory mucosal surface and Bowman's glands but little in olfactory epithelium itself. None in respiratory mucosa (L2). Label present on olfactory mucosal surface and Bowman's glands with less in olfactory epithelium itself. None in respiratory mucosa (L4)
Fixed	No specific label	Label present on olfactory mucosal surface and Bowman's glands but little in olfactory epithelium itself. None in respiratory mucosa (L2&L4).
Volatile	High amounts preferentially present in blood vessel wall and in bronchiole airways and bronchiolar epithelium	Label present on olfactory mucosal surface and Bowman's glands with less in olfactory epithelium itself. None in respiratory mucosa (L2).
20		
Total	Moderate amounts preferentially present in blood vessel wall and lumen and in bronchiolar epithelium.	
Fixed	No specific label	
Volatile	No label present	

4 Hour Sacrifice

ANIMAL No	LUNG	NASAL
21		
Total	Low levels preferentially present in submucosa of bronchioles	Label present on olfactory mucosal surface and Bowman's glands but little in olfactory epithelium itself. None in respiratory mucosa (L2&L4)
Fixed	Few grains - no specificity.	Few grains no specific (L2&L4)
Volatile	High levels preferentially present in bronchiole and alveolar airways and bronchiolar submucosa and in blood vessels	Label present on olfactory mucosal surface and Bowman's glands but little in olfactory epithelium itself. None in respiratory mucosa (L2). Label present on olfactory mucosal surface and Bowman's glands with lots in olfactory epithelium. Little in respiratory mucosa (L4)
22		
Total	Low levels preferentially present in smooth muscle of blood vessels	Poor section/ARG. (L2&L4)
Fixed	Few grains - no specificity.	
Volatile	High levels preferentially present in alveolar airways and lumen of blood vessels	Poor section/ARG. (L2&L4)
23		
Total	Low levels preferentially present in smooth muscle of blood vessels	
Fixed		
Volatile	No specific label.	
24		
Total	Few grains no specificity	No specific label (L2). Label present on olfactory mucosal surface and Bowman's glands with lots in olfactory epithelium. Little in respiratory mucosa (L4)
Fixed	No specificity	
Volatile	Few grains no specificity	No specific label (L2). Label present on olfactory mucosal surface and Bowman's glands with lots in olfactory epithelium. Little in respiratory mucosa (L4)
25		
Total	High levels preferentially present in muscle wall of blood vessels	
Fixed	Little label - no specificity	

Volatile	High levels preferentially present in muscle wall of blood vessels	
26		
Total	Little specificity - melt?	Low label present on olfactory mucosal surface and Bowman's glands but little in olfactory epithelium itself. None in respiratory mucosa (L2). High label present on olfactory mucosal surface but little in olfactory epithelium and Bowman's glands. None in respiratory mucosa (L4).
Fixed	No specific label present.	No specific label present (L2&L4).
Volatile	Little specificity - melt?	Label present on olfactory mucosal surface and Bowman's glands but little in olfactory epithelium itself. None in respiratory mucosa (L2). Very high label present on olfactory mucosal surface and basement layer of mucosa, less in Bowman's glands and little in olfactory epithelium. None in respiratory mucosa (L4).
27		
Total	Low levels preferentially present in smooth muscle of blood vessels and bronchioles	
Fixed	Few grains - little specificity	
Volatile	Little specificity - melt?	
28		
Total	Some preference for blood vessel lumen and smooth muscle.	Very high label present on olfactory and respiratory mucosal surface, less in Bowman's glands and olfactory and respiratory epithelium itself. Squamous epithelium of mouth has high levels (L2&L4).
Fixed	Random distribution of label.	Little label - non-specific
Volatile	Preference for air spaces in alveoli and bronchioles and blood spaces - bronchiolar epithelium is only place where no label.	Very high label present in airspace and on olfactory and respiratory mucosal surface, and in olfactory and respiratory mucosa. Squamous epithelium of mouth has high levels (L2&L4).
29		
Total	Fairly random - Some preference for blood vessel lumen and smooth	

	muscle	
Fixed	Preference for lateral membranes of bronchiolar epithelium	
Volatile	Random distribution of label	
30		
Total	High levels in smooth muscle of blood vessels and air spaces in both alveoli and bronchioles - melt?	Very high label present in airspace and on olfactory and respiratory mucosal surface, low levels in submucosa but little in mucosa itself. (L2&L4).
Fixed	Very low levels - no specific.	Little label - non-specific (L2). ARG poor (L4).
Volatile	High levels in smooth muscle of blood vessels and bronchioles, also air spaces in both alveoli and bronchioles - melt?	Very high label present in airspace and on olfactory and respiratory mucosal surface, lower levels in submucosa and little in mucosa itself. (L2&L4).

24 Hour Sacrifice

31		
Total	Labelling preferentially present in smooth muscle of blood vessels - random elsewhere.	Label present in airspace and sub-mucosa - not in mucosa itself (L4). No specific label - melt (L2)?
Fixed	Random distribution	Random distribution (L4&L2)
Volatile	Labelling preferentially present in smooth muscle of blood vessels - random elsewhere but obviously absent from bronchiolar epithelium.	Label present in airspace and sub-mucosa - not in mucosa itself (L2&L4).
32		
Total	Random distribution	Low levels in airspace (L2)
Fixed	Random distribution	Low levels - random (L4&L2).
Volatile	Label in airspace and interstitium but definitely not in bronchiolar epithelium.	High levels in airspace and submucosa but definitely not in mucosa (L2). No specific label - melt (L4)?
33		
Total	High levels in airspace of bronchioles and alveoli - none in tissue itself or in blood vessels.	Poor ARG (L4&L2).
Fixed	Low levels - random label.	
Volatile	High levels in airspaces but definitely missing from bronchiolar epithelium itself.	Random label (L4). Low levels on mucosal surface (L2).
34		
Total	Low levels - random distribution.	
Fixed	Random distribution of label.	
Volatile	Poor ARG.	
35		
Total	High levels in airspaces but definitely missing from bronchiolar epithelium itself.	Very high label present in airspace and on olfactory and respiratory mucosal surface and in submucosa but little in mucosa itself. (L2&L4).
Fixed	Random distribution of label.	Random distribution of label. (L4&L2)
Volatile	High levels in airspaces but definitely missing from bronchiolar epithelium itself.	High levels on tissue but no specific distribution (L2). High levels on mucosal surface - elsewhere label is random (L4).
36		
Total	High levels in airspaces but definitely missing from bronchiolar epithelium itself.	
Fixed		

Volatile	High levels in airspaces but definitely missing from bronchiolar epithelium itself.	
37		
Total		
Fixed	Random distribution of label	
Volatile	Random distribution of label.	
38		
Total	Random label - melt?	
Fixed		
Volatile		
39		
Total	Label specifically over bronchiolar epithelium in lumen and mucosa.	Label present in squamous epithelium of mouth - low levels on surface of mucosa - no specific label elsewhere (L2).
Fixed	No specific distribution	Low levels - no specific (L2)
Volatile	No specific distribution	Label present in squamous epithelium of mouth - low levels on surface of mucosa - no specific label elsewhere (L2).

XM6515: INDIVIDUAL ANIMAL DATA - MICE

5 minute sacrifice

ANIMAL No	LUNG	NASAL
1		
Total	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Very high amounts on surface of respiratory and olfactory mucosa and in Bowman's glands and sub-mucosa (L4). No specific label (L2).
Fixed	Low levels associated with bronchiolar mucosa.	Low levels present on surface of olfactory mucosa and in Bowman's gland (L4). No specific label (L2).
Volatile	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Very high amounts on surface of respiratory and olfactory mucosa and in Bowman's gland and sub-mucosa (L4). No specific label (L2).
2		
Total	High levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland and sub-mucosa (L4). Random label (L2).
Fixed	Low levels associated with bronchiolar mucosa.	Low levels of label on surface of olfactory mucosa and in Bowman's gland and sub-mucosa (L4&L2).
Volatile	High levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessels.	Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland and sub-mucosa (L4). Random label (L2).
3		
Total	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland and sub-mucosa (L4). No specific label (L2).
Fixed	Low levels associated with bronchiolar mucosa.	Low levels of label on surface of olfactory mucosa and in Bowman's gland (L4). Low levels of label on surface of olfactory and respiratory mucosa and in

		Bowman's gland (L4).
Volatile	High levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland and sub-mucosa (L4). No specific label (L2).
4		
Total	Very high levels in blood vessel lumen with high levels in bronchioles and bronchiolar mucosa.	Missing
Fixed	Low levels associated specifically with bronchiolar mucosa.	Missing
Volatile	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Missing
5		
Total	High levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland (L2). Very high levels of label on surface of olfactory mucosa and in Bowman's gland (L4).
Fixed	Low levels associated with bronchiolar mucosa.	No specific label (L2). Low levels of label on surface of olfactory mucosa and in Bowman's gland (L2&L4).
Volatile	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	High levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland (L2&L4).
6		
Total	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Very high levels on surface of mucosa throughout (L2). Poor ARG (L4).
Fixed	Low levels associated with bronchiolar mucosa.	Low levels associated with surface of mucosa (L2). Low levels associated with surface of olfactory mucosa and Bowman's glands (L4).
Volatile	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Very high levels on surface of mucosa throughout (L2). Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland (L4).

7		
Total	High levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	No specific label (L2). Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland (L4).
Fixed	Low levels associated with bronchiolar mucosa.	No specific label (L2). Low levels associated with surface of olfactory mucosa and Bowman's glands (L4).
Volatile	High levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	No specific label (L2). Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland (L4).
8		
Total	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Poor section - No specific label (L2). Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland (L4).
Fixed	Low levels associated with bronchiolar mucosa	Poor section - No specific label (L2). Low levels associated with surface of olfactory mucosa and Bowman's glands (L4).
Volatile	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Poor section - No specific label (L2). Very high levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland (L4).
9		
Total	Over-exposed ARG (4wk). No specific label.	Poor section - no specific label (L2). Very high levels associated with tissue - melt? (L4).
Fixed	Low levels associated with bronchiolar mucosa.	Low levels associated with surface of olfactory mucosa and Bowman's glands (L4&L2).
Volatile	High levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	High levels associated with surface of mucosa (L2). High levels associated with olfactory surface and Bowman's glands (L4).
10		
Total	High levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	High levels of label associated with surface of respiratory mucosa (L2). Poor section - no specific label (L2&L4).
Fixed	Low levels associated with bronchiolar mucosa.	Poor section - no specific label (L2). Low label associated with surface of olfactory mucosa and

		Bowman's glands (L4).
Volatile	Very high levels in bronchioles and bronchiolar mucosa with moderate amount associated with blood vessel lumen.	Poor section - no specific label (L2). High levels of label on surface of respiratory and olfactory mucosa and in Bowman's gland (L4).
11		
Total	Very high levels present in bronchiole airways and associated with bronchiolar mucosa.	Very high label associated with tissue (L2). Poor section (L4).
Fixed	None remaining	None remaining (L2&L4)
Volatile	Very high levels present in bronchiole airways and associated with bronchiolar mucosa.	Very high levels associated with respiratory mucosal surface and sub-mucosa (L2). Poor section (L4).
12		
Total	Very high levels present in bronchiole airways and associated with bronchiolar mucosa.	High label associated with submucosa in respiratory portion (not respiratory mucosa), moderate amounts in airways (morphology poor L2&L4).
Fixed	None remaining	None remaining (L2&L4)
Volatile	Very high levels present in bronchiole airways and associated with bronchiolar mucosa and moderate levels associated with blood vessel lumen.	High label associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands. Low amounts present in respiratory mucosa and sub-mucosa (morphology reasonable on second section L2&L4)
13		
Total	Very high levels present in bronchiole airways and associated with bronchiolar mucosa.	High levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands. Low levels associated with respiratory with no preferential localisation (L2 &L4).
Fixed	None remaining	None remaining (L2&L4)
Volatile	Section poor	High levels with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands. Low levels associated with

		respiratory with no preferential localisation. (L2)
14		
Total	Very high levels present in bronchiole airways and associated with bronchiolar mucosa and moderate levels in blood vessel lumen.	Very high levels associated with Bowman's glands, high levels in olfactory mucosa with low levels in lumen (L2). Very high levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands Low levels associated with respiratory with no preferential localisation (L4).
Fixed	None remaining	None remaining (L2&L4)
Volatile	Very high levels present in bronchiole airways and associated with bronchiolar mucosa - Very high levels present in airway lumen of alveoli.	High levels associated with surface in respiratory mucosa and very high levels in submucosa (L2). High levels associated with surface in olfactory mucosa and very high amounts in submucosa and Bowman's glands (L4).
16		
Total	More evenly spread through tissue - melting?	Very high amounts associated with surface mucous in olfactory and moderate amounts with tissue itself with preference for Bowman's glands (L2).
Fixed	Little remaining - no specificity	Little remaining - no specificity (L2&L4)
Volatile	More evenly spread through tissue - melting?	Very poor section (L2). Very high levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for Bowman's glands (L4).
17		
Total	Very high levels present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessel lumens.	Very high levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands (L4). Very high levels diffusely associated with soft tissue, not cartilage (L2).
Fixed	Moderate levels present in	Low amounts in surface of

	bronchiole airways and associated with bronchiolar mucosa.	olfactory mucosa and Bowman's glands (L4). Little remaining - no specificity (L2)
Volatile	Very high levels present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessel lumens.	Very high levels associated with surface mucous in olfactory and moderate amounts with tissue itself with preference for mucosa and Bowman's glands (L4 & L2).
18		
Total	Very high levels present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessel lumens.	Very high levels associated with surface mucous in olfactory and moderate amounts with tissue itself with preference for mucosa and Bowman's glands (L4). Very high levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands (L2).
Fixed	Low levels associated with bronchiolar mucosa	Little remaining - no specificity (L2&L4)
Volatile	Very high levels present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessel lumens.	Very high levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands (L2). Section poor (L4).
19		
Total	High levels present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessel lumens.	Very high levels associated with tissue in a diffuse way (L2). Very high levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands (L4).
Fixed	Little remaining - no specificity	Little remaining - no specificity
Volatile	High levels present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessel lumens.	Very high amounts associated with tissue in a diffuse way (L2). High levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands (L4).
20		

Total	Very high levels present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessel lumens.	Very high levels associated with tissue in a diffuse way (L2). Very high levels associated with surface mucous in olfactory and in moderate amounts with tissue itself with preference for mucosa and Bowman's glands (L4).
Fixed	Little remaining - no specificity	Little remaining - no specificity
Volatile	High levels present in bronchiole airways and associated with bronchiolar mucosa and high in associated blood vessel lumens.	Poor section (L2). Very high levels associated with surface mucous in olfactory and in lesser amounts with tissue itself with preference for mucosa and Bowman's glands (L4).

1 Hour Specific

ANIMAL No	LUNG	NASAL
21		
Total	Very high levels preferentially present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessel lumens.	Poor section - no specific label (L2&L4).
Fixed	Low levels in bronchiolar mucosa	No specific label (L4&L2)
Volatile	High levels preferentially present in bronchiole airways and associated with bronchiolar mucosa and moderate in associated blood vessels.	Low label - preferentially present on surface of olfactory and in Bowman's glands (L2) No specific label (L4).
24		
Total	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	High label preferentially on surface of respiratory epithelium and sub-mucosa (no olfactory present) (L2). High label preferentially on surface of respiratory and olfactory epithelium and Bowman's glands and sub-mucosa (L4).
Fixed	No specific label.	No specific label (L4&L2)
Volatile	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	No specific label (L2). High label preferentially on surface of respiratory and olfactory epithelium and Bowman's glands and sub-mucosa (L4).
26		
Total	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa	Poor section (L2).High label preferentially on surface of olfactory epithelium and Bowman's glands (L4).
Fixed	Some present but little specificity	No specific label (L4&L2)
Volatile	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	No specific label (L4&L2)
27		
Total	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	High label preferentially on surface of olfactory epithelium and Bowman's glands (L4).
Fixed	Low levels in bronchiolar mucosa.	
Volatile	High levels preferentially present in bronchiole airways and	High label preferentially on surface of olfactory epithelium and

	associated with bronchiolar mucosa.	Bowman's glands (L4).
28		
Total	High levels preferentially present in bronchiole airways and bronchiolar mucosa.	No specific label (L&L4).
Fixed	Low levels in bronchiolar mucosa.	No specific label (L4&L2)
Volatile	High levels preferentially present in bronchiole airways and bronchiolar mucosa.	High label preferentially on surface of olfactory epithelium and Bowman's glands (L2&L4).
29		
Total	Random grains - melt?	No specific label (L2&L4).
Fixed	Few grains - no specificity	No specific label (L4&L2)
Volatile	High levels preferentially present in bronchiole airways and bronchiolar mucosa.	High label preferentially on surface of olfactory epithelium and Bowman's glands (L4). No specific label (L2).
30		
Total	High levels preferentially present in bronchiole airways and bronchiolar mucosa.	No specific label (L2). Moderate label present on olfactory mucosal surface, in olfactory epithelium and Bowman's glands (L4).
Fixed	Few grains - no specificity	No specific label (L4&L2)
Volatile	High levels preferentially present in bronchiole airways and bronchiolar mucosa.	High label present on olfactory mucosal surface, in olfactory epithelium and Bowman's glands (L2). No specific label (L4).
36		
Total	Very high levels preferentially present in bronchiole airways and bronchiolar mucosa.	No specific label (L2&L4)
Fixed	No specific grains.	No specific grains (L2&L4).
Volatile	High levels preferentially present in bronchiole airways and bronchiolar mucosa and high levels in alveolar airways.	No specific label (L2). Moderate levels of label present on olfactory mucosal surface, in olfactory epithelium and Bowman's glands (L4).
38		
Total	Random grains - melt?	Moderate levels - no specific (L2&L4).
Fixed	No specific label.	No specific grains (L2&L4).
Volatile	Random grains - melt?	Moderate levels - no specific (L2&L4)
40		
Total	High levels preferentially present in bronchiole airways and bronchiolar mucosa.	High levels in Bowman's glands (L4). No specific label (L2).

Fixed	Low levels preferentially present in bronchiole airways and bronchiolar mucosa	No specific grains (L2&L4)
Volatile	High levels preferentially present in bronchiole airways and bronchiolar mucosa.	Moderate amount of label present on olfactory mucosal surface, in olfactory epithelium and Bowman's glands (L4&L2).

4 Hour Sacrifice

ANIMAL No	LUNG	NASAL
44		
Total	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	Moderate label present on olfactory mucosal surface and Bowman's glands but little in olfactory epithelium itself. None in respiratory mucosa (L2). L4 poor section
Fixed	Few grains - no specificity.	Poor section (L2). Moderate label present on olfactory mucosal surface and Bowman's glands but low levels in olfactory epithelium itself (L4).
Volatile	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	Moderate label present on olfactory mucosal surface and Bowman's glands but low levels in olfactory epithelium itself. None in respiratory mucosa (L2 & L4).
51		
Total	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	Poor section - no specific label (L2&L4).
Fixed	Few grains - no specificity	Poor section - no specific label (L2). No specific label (L4).
Volatile	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	Poor section - no specific label (L2). High levels of label present on olfactory mucosal surface and Bowman's glands but low levels in olfactory epithelium itself. None in respiratory mucosa (L4).
52		
Total	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	Poor section - no specific label (L2&L4).
Fixed		No specific label (L2). Poor section - no specific label (L4).
Volatile	Random - melt?	Poor section - no specific label (L2&L4).
53		
Total	High levels preferentially present in bronchiole airways and associated bronchiolar mucosa.	High levels present on olfactory mucosal surface and Bowman's glands but low levels in olfactory epithelium itself. None in respiratory mucosa (L2). Poor

		section (L4).
Fixed	Low levels preferentially present in bronchiole airways and associated with bronchiolar mucosa.	No specific label (L2&L4).
Volatile	Very high levels of label - Preferentially present in bronchiole airways and associated with bronchiolar mucosa.	No specific label (L2&L4).
54		
Total	Very high levels of labelling preferentially present in bronchiolar mucosa.	Moderate levels present on olfactory mucosal surface and Bowman's glands but low levels in olfactory epithelium itself (L2&L4).
Fixed		No specific label (L2&L4).
Volatile	Very high levels of labelling preferentially present in bronchiolar mucosa.	Random label - melt? (L4). No specific label (L2).
56		
Total	High levels preferentially present in bronchiolar mucosa.	No specific label (L2&L4).
Fixed	Random distribution of grains	No specific label (L2&L4).
Volatile	High levels of labelling preferentially present in bronchiolar mucosa.	No specific label (L2&L4).
57		
Total	High levels preferentially present in bronchiolar mucosa.	No specific label (L2&L4).
Fixed	Random distribution of grains	No specific label (L2&L4).
Volatile	High levels preferentially present in bronchiolar mucosa.	No specific label (L2). Moderate label preferentially on surface of olfactory and in Bowman's glands (L4).
58		
Total	High levels preferentially present in bronchiolar mucosa and lumen.	High label preferentially on surface of olfactory and in Bowman's glands with moderate amounts in the olfactory mucosa (L4). No specific label (L2).
Fixed	Low levels preferentially present in bronchiolar mucosa.	No specific label (L2&L4).
Volatile	High levels preferentially present in bronchiolar mucosa and lumen.	Random label - melt (L4&L2)?
59		
Total	High levels preferentially present in bronchiolar mucosa and lumen.	Moderate amounts on surface of olfactory mucosa and in

		Bowman's glands (L2). Very high amounts of label present in airways and on surface of olfactory mucosa and in Bowman's glands.
Fixed	Random distribution of grains	No specific label (L2&L4).
Volatile	High levels preferentially present in bronchiolar mucosa and lumen.	Poor ARG - no specific label (L2). High amounts of label present in airways and on surface of olfactory mucosa and in Bowman's glands.
60		
Total	High levels preferentially present in bronchiolar mucosa and lumen.	No specific label (L2). Moderate amounts on surface of olfactory mucosa, in mucosa itself and in Bowman's glands
Fixed	Random distribution of grains	No specific label (L2&L4).
Volatile	High levels preferentially present in bronchiolar mucosa and lumen but more diffuse distribution - some melting?	Moderate amounts on surface of olfactory mucosa and in Bowman's glands (L2&L4).

[redacted]

61		
Total	Moderate levels preferentially present in blood vessel wal' with low amounts in bronchiolar mucosa and lumen	None present (L2). None present (L4). Fixed?
Fixed	Random distribution of grains	
Volatile	Missing section	None present (L2). Poor section - overdeveloped ARG (L4).
63		
Total	missing section	Poor section - no specific label (L2&L4)
Fixed	Low levels preferentially present in mucosa	Poor section - no specific label (L2&L4).
Volatile	Moderate levels preferentially present in bronchiolar mucosa	No specific label present (L2). Moderate label preferentially over surface of olfactory epithelium and in airway with much less in Bowman's glands (L4).
64		
Total	Moderate levels preferentially present in basement membrane of bronchiolar mucosa and in wall of blood vessels	None present (L2). None present (L4). Fixed?
Fixed		No specific label (L2&L4).
Volatile	Moderate levels preferentially present in basement membrane and apical cytoplasm of mucosa but absent from mucosa and moderate in wall of blood vessels	None present (L2). None present (L4). - fixed?
68		
Total	Moderate levels present in bronchiolar lumen and basement area but absent from epithelium - also moderate levels present in blood vessel walls - artefact of melting?	No specific label (L2&L4)
Fixed	Random distribution.	
Volatile	Moderate levels present in bronchiolar lumen and basement area but low in epithelium - also moderate levels present in blood vessel walls.	No specific label (L2&L4)
69		
Total	Moderate levels present in bronchiolar lumen, basement area	No specific label (L2). High label preferentially localised on surface

	and epithelium - also moderate levels present in blood vessel walls.	of olfactory mucosa with moderate levels in Bowman's and mucosa itself (L4).
Fixed	Low levels associated with mucosa - lateral areas of cells	No specific label (L2&L4)
Volatile	Melting artefact.	No specific label (L2&L4).
70		
Total		Poor section - No specific label (L2&L4)
Fixed	Low levels associated with mucosa - lateral areas of cells	Poor section - No specific label (L2&L4)
Volatile		Poor section - No specific label (L2&L4)
71		
Total	High levels associated with blood vessel walls and lumen and moderate amounts with bronchiolar wall.	Melted section - non specific labelling (L2 & L4). High levels associated with blood vessels and squamous epithelium of skin and hair follicles. Moderate levels in airway overlying respiratory and olfactory tissue (L4).
Fixed	Little label left - non specific.	Little label left - non specific (L2&L4).
Volatile	High levels associated with larger alveolar areas - melting artefact?	Moderate levels associated with blood vessels and squamous epithelium of skin. Moderate levels in airway overlying respiratory and olfactory tissue (L2).
72		
Total	No label present. - fixed?	No label present. - fixed (L2&L4)?
Fixed	No label present.	No label present (L2&L4).
Volatile	No label present. - fixed?	No label present. - fixed (L2&L4)?
73		
Total	No label present.	Moderate levels associated with squamous epithelium of skin. Moderate levels in airway (L2). No label (L4).
Fixed	No label present.	No label present (L2&L4).
Volatile	No label present.	poor section (L2). No label (L4)
74		
Total	No label present.	No label present (L2&L4).
Fixed	No label present.	No label present. (L2&L4)
Volatile	No label present.	No label present. (L2&L4)

B 11

CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

REPORT NO: CTL/ R/1411

PHOTOGRAPHIC SUPPLEMENT TO STYRENE: NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

B 12

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STUDY DETAILS

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Pathologist: Dr J R Foster

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I, the undersigned declare that this supplement constitutes a true record of the results obtained in the above study.

Dr T Green
(Study Director) ..... Date 5.10.99

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

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NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

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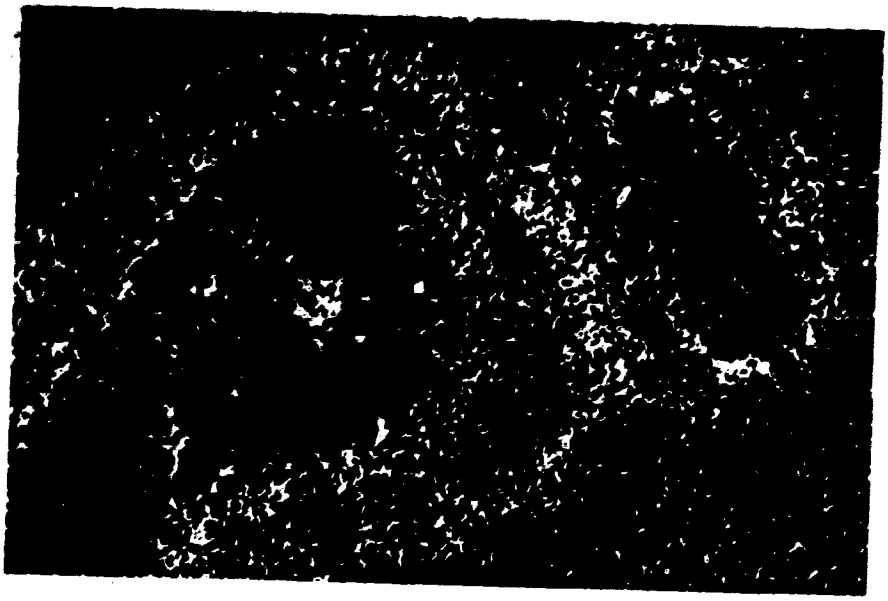
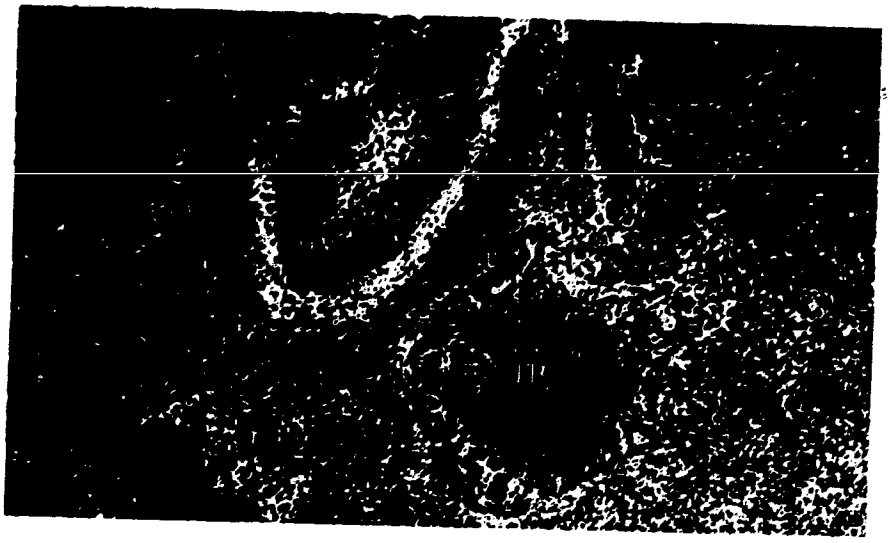
NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE

PHOTOGRAPH 20 Whole body autoradiographs from mice at 1 hour following inhalation exposure to ^{14}C Styrene. The arrow shows the labelled nasal passages. 16

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

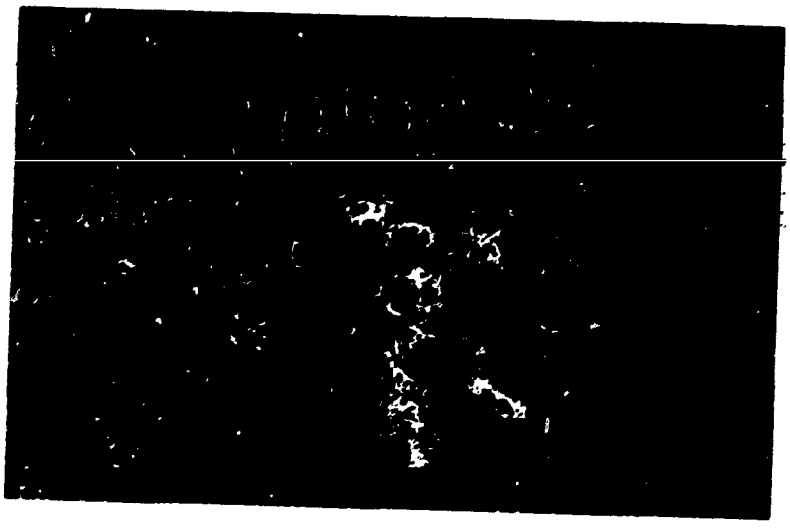


PHOTOGRAPH 1 **Rat lung ^{14}C Styrene distribution by autoradiography - 5 minutes post exposure. Total ^{14}C-Styrene. Labelled structures are terminal bronchioles.**



PHOTOGRAPH 2 **Rat lung, ^{14}C-Styrene distribution by autoradiography - dark field illumination. Total ^{14}C Styrene. 4 hours post exposure. Label is randomly distributed in the lung. TB; terminal bronchiole, BV; blood vessel.**

PHOTOGRAPH 5 **Mouse lung, ^{14}C-Styrene distribution by autoradiography - 1 hours post-exposure. Total ^{14}C Styrene. Dark field illumination. TB; terminal bronchiole, BV; blood vessel.**



PHOTOGRAPH 6 **Mouse lung, ^{14}C-Styrene distribution by autoradiography - 1 hours post-exposure. Bound ^{14}C Styrene. Label is in intercellular spaces.**

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

PHOTOGRAPH 7 **Mouse lung, ^{14}C-Styrene distribution by autoradiography - 24 hours post-exposure. Total ^{14}C styrene. Label is in bronchioles.**

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE

PHOTOGRAPH 8 **Rat nasal, ^{14}C-Styrene distribution by autoradiography - 5 minutes post-exposure. Total ^{14}C Styrene. Area shows olfactory epithelium**

PHOTOGRAPH 9 **Rat nasal, ^{14}C-Styrene distribution by autoradiography - 5 minute post-exposure. Bound ^{14}C Styrene. Label is in lumen and Bowman's glands.**

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE

PHOTOGRAPH 10 **Rat nasal, ^{14}C-Styrene distribution by autoradiography - 4 hours post-exposure. Total ^{14}C styrene. Label is on surface of epithelium and in Bowman's glands, with lesser amounts in mucosa itself.**

PHOTOGRAPH 11 **Rat nasal, ^{14}C-Styrene distribution by autoradiography - 24 hours post-exposure. Total ^{14}C styrene. Label is as above.**

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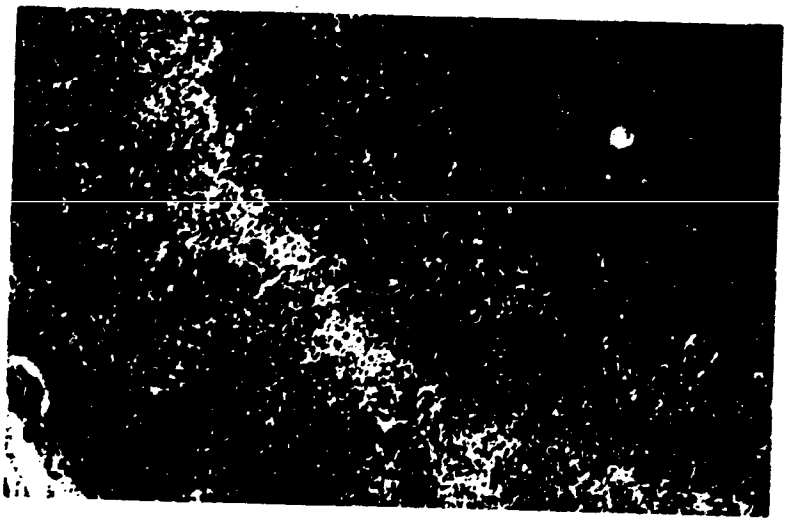
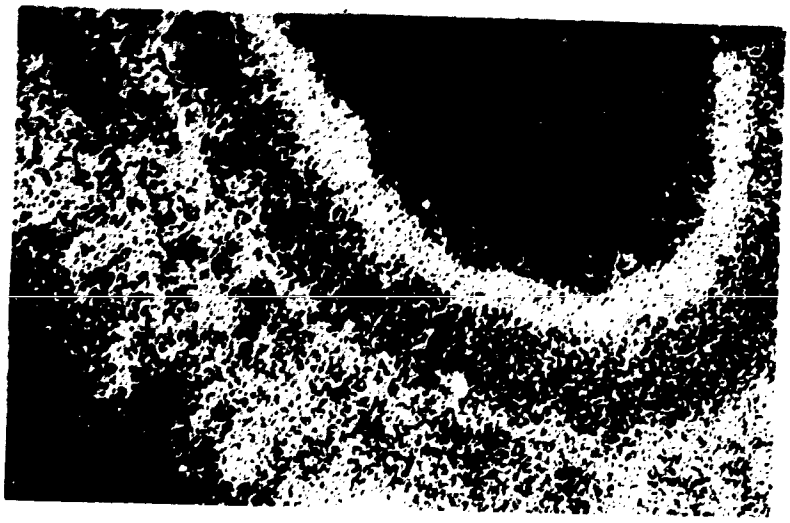
PHOTOGRAPH 12 **Mouse nasal, ^{14}C-Styrene distribution by autoradiography - 5 minutes post-exposure. Total ^{14}C styrene. Label is on surface and in Bowman's glands with lesser amounts in mucosa itself.**



PHOTOGRAPH 13 **Mouse nasal, ^{14}C-Styrene distribution by autoradiography - 5 minutes post-exposure. Bound ^{14}C Styrene only. Label is as in photograph 12.**

PHOTOGRAPH 14 **Mouse nasal, ^{14}C-Styrene distribution by autoradiography - 1 hours post-exposure. Total ^{14}C styrene.**



PHOTOGRAPH 15 **Mouse nasal, ^{14}C-Styrene distribution by autoradiography - 4 hours post-exposure. Total ^{14}C styrene. Label is on surface of mucosa and in Bowman's glands with lesser amounts in mucosa itself.**

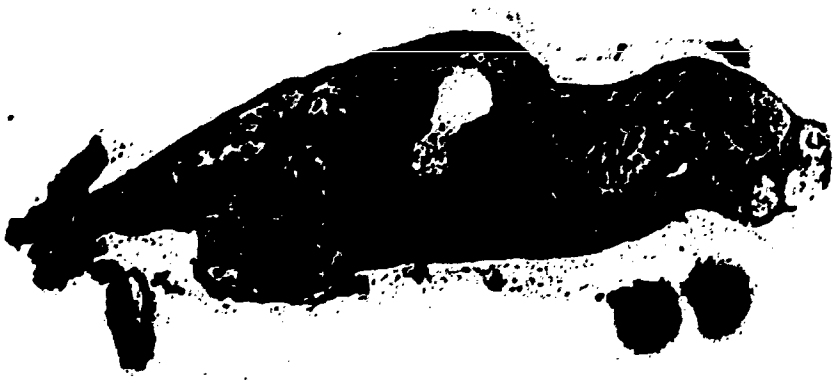
NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

PHOTOGRAPH 16 **Whole body autoradiographs from rats at 5 minutes following inhalation exposure to ^{14}C Styrene. The arrow shows the labelled nasal passages.**

PHOTOGRAPH 17 **Whole body autoradiographs from rats at 1 hour following inhalation exposure to ^{14}C Styrene. The arrow shows the labelled nasal passages.**

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

PHOTOGRAPH 18 **Whole body autoradiographs from mice at 5 minutes following inhalation exposure to ^{14}C Styrene. The arrow shows the labelled nasal passages.**



PHOTOGRAPH 19 **Whole body autoradiographs from mice at 5 minutes following inhalation exposure to ^{14}C Styrene.**

NASAL AND LUNG HISTOAUTORADIOGRAPHY IN MALE CD RATS AND MALE CD1 MICE FOLLOWING A 6 HOUR INHALATION EXPOSURE TO [^{14}C] STYRENE.

PHOTOGRAPH 20 **Whole body autoradiographs from mice at 1 hour following inhalation exposure to ^{14}C Styrene. The arrow shows the labelled nasal passages.**

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CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

REPORT NO: CTL/R/1412

THE METABOLISM OF STYRENE BY RAT, MOUSE AND HUMAN NASAL CYTOCHROME P-450's.

D 02

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THE METABOLISM OF STYRENE BY RAT, MOUSE AND HUMAN NASAL CYTOCHROME P-450's.

AUTHOR

Dr T. Green

DATE OF ISSUE

Oct 20th 1999

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It should not be disclosed in any form to an outside party, nor should information contained herein be used by a registration authority to support registration of this product or any other product without the written permission of the Styrene Information and Research Centre.

I, the undersigned declare that this report constitutes a true record of the actions taken and the results obtained in the above study.

Dr T Green
(Study Director)

Date 20/10/99

STUDY CONTRIBUTORS

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1. SUMMARY

The metabolism of styrene to styrene oxide has been compared in vitro in nasal tissues from mice, rats and humans. The cytochromes P-450 responsible for this metabolism have been identified and their distribution studied in nasal tissues from the three species. Covalent binding of styrene metabolites to nasal tissues has been determined at intervals up to 24 hours following a 6 hour exposure to radiolabelled styrene.

Styrene was rapidly metabolised to styrene oxide, predominantly the R enantiomer, in rat and mouse olfactory microsomal fractions at rates which were higher than those in hepatic microsomes. The rates in respiratory fractions were approximately half those in olfactory and were comparable with the hepatic rates. Styrene metabolism could not be detected in human nasal fractions. The difference in metabolic rate between rodents and humans, based on the highest rate in rodents (rat olfactory) and the limit of detection of the assay, was 186.

The distribution of the two major isoforms of cytochrome P-450, CYP2E1 and CYP2F2 that were found to be responsible for the metabolism of styrene in nasal tissue were studied using antibodies. Both enzymes were localised in the apical cytoplasm of the olfactory region and whilst CYP2E1 tended to be more localised to the olfactory region of the mouse, CYP2F2 showed a wider distribution with high levels also in the rat olfactory region and significant concentrations in the respiratory regions of both species.

When rats and mice were exposed to radiolabelled styrene in vivo, the levels of covalently bound material were significantly higher in mouse, than rat, nasal tissues.

It was concluded that styrene is metabolised to styrene oxide at high rates in rodent nasal tissues due to high concentrations of two cytochrome P-450 isoforms, CY2E1 and CYP2F2. A lack of detectable styrene metabolism in human nasal tissue suggests that the nasal toxicity seen in the rodent is unlikely to occur in humans.

2. INTRODUCTION

The primary metabolic pathway for styrene is the oxidation by cytochromes P-450 to two enantiomeric forms of styrene oxide (Bond, 1989). This metabolic transformation has been measured in a number of tissues (Carlson 1997), but not in nasal tissues, a site of toxicity in rodents exposed to this chemical (Cruzan et al. 1997). Rodent nasal tissues are known to have high concentrations of metabolising enzymes, including cytochromes P-450, particularly in the olfactory epithelium, which may explain the sensitivity of this tissue to styrene. In humans, the levels of these enzymes appear to be greatly reduced or even absent, suggesting that human nasal tissue will be much less sensitive than rodents to chemicals such as styrene (Reed, 1993). However, there is a paucity of good metabolic data in humans due to the difficulty in obtaining fresh human nasal tissue, and hence the metabolism of styrene in this tissue has not been studied previously. In this report, the metabolism of styrene has been compared in rat, mouse and human nasal tissues in vitro. In addition, nasal tissues from rats and mice were also analysed for volatile, water soluble, and covalently bound metabolites, following in vivo exposure to 160 ppm radiolabelled styrene by inhalation.

Having established metabolic rates in nasal tissues, a number of chemical inhibitors of cytochromes P-450 have been used in an attempt to identify the major isoforms involved in the metabolism of styrene in these tissues. The choice of inhibitors was based on the reported involvement of certain isoforms (CYP's 2E1 and 2F2) in styrene metabolism (Guengerich et al 1991; Carlson, 1997; Green, 1999) or on the basis that certain isoforms are heavily localised in rodent nasal tissues (isoforms of the CYP2A family). The inhibitors used were chlorzoxazone (CYP2E1), 5-phenyl-1-pentyne (CYP2F2) and coumarin and 8-methoxypsoralen (CY2A's).

Based on the results of these and previous studies (Green, 1999), the distribution of CYP's 2E1 and 2F2 have been determined in sections of nasal tissues using immunohistochemistry.

3. MATERIALS AND METHODS

3.1 Chemicals

[ring-U-^{14}C] Styrene (code: CFQ11087) was obtained from Amersham Life Sciences Ltd, Buckinghamshire as a custom synthesis with a radiochemical purity of 98.1% by radio-gas chromatography. It had a specific activity of 19mCi/mmol and was supplied as a solution in ethanol (5 mCi/mmol). Styrene (99%), chlorzoxazone (CZO), 8-methoxypsoralen (8-MP), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and diaminobenzidine tetrahydrochloride (Sigma DAB Peroxidase substrate) were all obtained from Sigma Chemical Company, Dorset, UK. R and S styrene oxide (R-SO, S-SO, both 98%), coumarin and 1,2-epoxy-3,3,3-trichloropropene oxide (TCPO) were obtained from Aldrich, Dorset, UK. 5 Phenyl-1- pentyne (5P-1-P) was obtained from Lancaster synthesis, Morecambe, UK. Radiolabelled and non-radiolabelled styrene, and styrene oxides, were stored at -20°C, the remaining chemicals at 4°C. All other chemicals used were of the highest grade available.

An polyclonal antibody against cytochrome P-4502E1, which was raised in sheep against purified rat cytochrome P4502E1, was purchased from Cambridge Biosciences, Cambridge, UK. An antibody against cytochrome P-4502F2 was a gift from Dr A Buckpitt, School of Veterinary Medicine, University of California, Davis, CA, USA. This antibody was raised in rabbits against a purified preparation of cytochrome P4502F2.

3.2 Animals

Fifty male Sprague-Dawley CD rats weighing 180-200g and 110 male CD1 mice weighing 20-25g were obtained from Charles River UK Ltd, Margate, Kent, UK 7 days before the start of the in vivo study and housed in standard stock cages. The animals were moved to the inhalation facility 24 hours before the start of exposure. Animals used to provide tissues for in vitro experiments were killed 24 hours after they arrived at the laboratory. The clinical condition of all animals was monitored prior to the start of the experiments and any animal showing adverse clinical signs was removed.

The animals received Rat and Mouse Number 1 (RM1) pelleted diet from Special Diet Services, Witham, Essex, UK., and mains water *ad libitum*. The environment of the animal

room was controlled with the temperature being maintained within a target range of 19-23 °C, relative humidity within a target range of 40-70%, an artificial light cycle of 12 hours light and 12 hours darkness, and between 25-30 air changes per hour. All conditions were monitored by the Honeywell site monitoring system and any excursions outside these ranges were noted by means of an external alarm and recorded.

3.3 Preparation of tissue fractions

Human nasal explants were obtained from the Royal National Throat, Nose and Ear Hospital, London, UK in compliance with local ethical guidelines. The tissues, which were received at the laboratory between 7th June and 5th July, 1999 and were described by the surgeon as morphologically normal, had been removed during surgery and immediately frozen in liquid nitrogen before being stored at -70°C until use. The donors (4 female, 3 male, 2 unknown) had no recent history of either drug or radio-therapy.

Rats and mice were sacrificed by asphyxiation with a rising concentration of carbon dioxide and respiratory and olfactory nasal tissues removed separately. The tissues, pooled by tissue type from either 40 rats or 100 mice, were thoroughly washed in ice cold 10 mM potassium phosphate buffer, pH 7.4 containing 1.15% w/v potassium chloride and a 20 % w/v homogenate prepared in the same buffer using a Potter Elvehjem homogenizer. The homogenates were centrifuged at 9000g for 20 minutes and the supernatant transferred to fresh tubes. A portion of the supernatant (S9) was stored at -70°C and the remainder centrifuged at 100,000g for 1 hour. The supernatant (cytosol) was removed and discarded. The microsomal pellet was resuspended in the same volume of buffer, centrifuged at 100,000g for an hour and the pellet resuspended in the phosphate/KCl buffer (1:1 w/v original tissue wet weight to buffer). The microsomes were stored at -70°C until required. Liver was also removed from the animals and microsomes prepared in the same way. Protein concentrations were determined by the method described by Lowry et al. (1951).

Only one of the human nasal tissue samples was available in sufficient quantity for the preparation of a microsomal fraction. This was prepared in a manner identical to that used for the rat tissues. For the remaining samples, an S9 fraction was prepared as above and stored at -70°C. A quantity of rat S9 fraction was retained from the above preparation for

comparison with these human S9 fractions. S9 fractions were dialysed to remove glutathione prior to the analysis.

Human CYP2E1 was expressed and purified from E. coli. Two plasmid *Escherichia coli* JM109 strains were provided by the Dundee LINK scheme, Ninewells Hospital, Dundee. CYP2E1 with OmpA(+2) leader sequence together with ampicillin resistance was expressed on one plasmid and *pelB*-human P450 reductase (pJR4) together with chloramphenicol resistance on the second. *E.coli* were stored at -80°C in LB broth containing 15% v/v glycerol. Cells were grown in Terrific broth containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol. Expression was induced by addition of 0 5 mM δ-ALA and 1 mM IPTG. Cells were harvested by centrifugation and spheroplasts produced by incubation with 0.25 mg/ml lysozyme and further centrifugation. Membranes containing P450 were produced by the sonication of the spheroplasts in the presence of 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 mM PMSF. Sonicated suspensions were centrifuged in spheroplast resuspension buffer at 180,000g for 1 h 10 min at 4°C. The supernatant was discarded, and the pellet resuspended in 1 ml ice cold TSE using a Dounce tissue grinder. Total P450 was determined by CO difference spectra and protein by the method of Bradford (1976). Membranes were stored at -80°C.

3.4 Styrene metabolism to styrene oxide

The metabolism of styrene to styrene oxide was determined using methodology based on that described by Carlson (1997). Tissue fractions containing the following protein concentrations; rat, olfactory, 0.25 mg; respiratory, 0.1 mg; mouse, olfactory, 0.055 mg; respiratory, 0.046 mg; rat and mouse liver, 0.25 mg, human, nasal S9, 0.25 mg; microsomes, 0.20 mg, were incubated in sealed glass tubes at 37°C with 5mM magnesium chloride, 1mM styrene and various concentrations of TCPO (0.025 - 0.1 mM added in DMSO) in 100mM potassium phosphate buffer pH 7.4 (total volume of 1ml). The flasks were preincubated at 37°C for 5 minutes before the reaction was started by the addition of NADPH (2mM). The reaction was stopped after 12 min by the addition of 2ml ice cold n-hexane.

In a second experiment the tissue fraction was replaced with 40pmol (0.1mg) of purified CYP2E1 protein.

3.6 Styrene metabolism in the presence of cytochrome P-450 inhibitors

The experiments described above measuring the formation of styrene oxide in various rat and mouse nasal tissue fractions and with purified human CYP2E1 were repeated in the presence of a number of cytochrome P-450 inhibitors. Chlorzoxazone (0.1-1.0 mM), 8-methoxypsoralen (0.005 -0.3 mM), coumarin (0.005-1.0 mM) and 5-phenyl-1-pentyne (0.005-1.0 mM) were added in DMSO (5 ul) at the start of the experiment.

3.8 Measurement of R and S-styrene oxides

Styrene and styrene oxide were extracted from the incubation into hexane (2 ml) by rotary mixing. The mixture was centrifuged and the hexane fraction analysed for R and S styrene oxide using a Chirex 3007 (Phenomenex, U.K.) guard (4.6 x 30mm) and analytical column (4.6 x 250mm) on a Shimadzu LC-10 system, with UV monitoring at 219nm. The column was eluted with 0.02% propan-2-ol in n-hexane. The retention times of R and S-styrene oxides were 23.0 and 24.5 min respectively. Standards were prepared from R and S-styrene oxide in n-hexane.

3.7 In vivo metabolism and covalent binding

3.7.1 Dose preparation

Approximately 2mCi of [ring-U-^{14}C] styrene in ethanol was diluted with 350 µl of unlabelled styrene to give a specific activity of approximately 0.66 mCi/mmol. The ethanol was removed by adding water (10 volumes) and extracting the styrene into pentane which was dried and removed under reduced pressure.

3.7.2 Study design

Rats (n=8) and mice (n=16) were exposed to 160 ppm C-14 styrene for 6 hours. At the end of the exposure period the animals were removed from the chambers and placed in stock cages with free access to diet and water. Groups of 2 rats and 4 mice were terminated at 5 minutes, 1 hour, 4 hours and 24 hours post exposure.

The clinical condition of all animals was monitored during the exposure and post-exposure periods for adverse clinical signs which may have exceeded permissible severity bands of the licence under which these experiments were carried out. Clinical signs were normal for all animals before and during the study.

3.7.3 Exposure system

Animals were exposed whole body in glass chambers of approximately 20 litres using a closed recirculating system. Air was circulated in the system at approximately 1 litre/min; carbon dioxide was removed with Carbosorb AS and oxygen added at a rate sufficient to maintain a concentration of between 20 and 21%. The animals were placed in the chamber and C-14 styrene injected into the chamber to give an atmospheric concentration of 160 ppm. Equilibrium was achieved within 15 min and both styrene and oxygen levels in the chamber were monitored throughout the experiment by gas chromatography. A Shimadzu GC-14BT dual column gas chromatograph was used. Oxygen was measured using a molecular sieve 5A column (1/8 inch x 6ft) at 115°C and a thermal conductivity detector. Styrene was measured using a 10% Carbowax 20M column (1/8 inch x 6ft) at 115°C and a flame ionisation detector.

3.7.4 Tissue analysis

Olfactory and respiratory nasal tissues were dissected from the heads and combined by species and tissue type at each time point. The tissues were homogenised in 1 ml of 10 mM potassium phosphate buffer, pH 7.4. Aliquots (50 µl) of each nasal homogenate were assayed for radioactivity by dissolving in Soluene followed by scintillation counting. Further aliquots (0.25 ml) were extracted with 2 volumes of hexane and the volatile radioactivity (assumed to be unchanged styrene) determined. Aqueous fractions were also analysed by high performance liquid chromatography (hplc) using a Waters Spherisorb 5 micron ODS-1 column and a UV detector monitoring at 210nm. The column was eluted at 1.1 ml/min with a solution containing 37.5mM phosphoric acid (neutralised with Tris base to pH 7.0), 25mM sodium sulphate and 0.5% methanol as described by Hernandez et al. (1983). The radioactive content of individual peaks was determined by radioactive flow detection on a Canberra Packard Flo-One detector with 1ml of column eluent being mixed with 3ml of Ultima-Flo M scintillant (Canberra Packard).

Covalently bound radioactivity was assayed in 0.25 ml of nasal homogenates. Protein was precipitated with an equal volume of trichloroacetic acid (10%) and centrifuged at 1,000g for 10 min. The precipitate was washed with 4 x 4 ml hexane, 3 x 4 ml methanol, dried, dissolved in 1 ml Soluene 350 and the radioactivity measured by scintillation counting.

3.8 The distribution of cytochromes P450's 2E1 and 2F2

Tissues were obtained from control rats and mice terminated by exsanguination by cardiac puncture under terminal anaesthesia with halothane according to standard procedures.

For examination of the nasal cavity, the skin was resected from the head and the brain and lower jaw were removed prior to the nasal passages being flushed in a retrograde direction by inserting a blunt needle into the pharyngeal duct and instilling approximately 10 ml of 10% neutral buffered formalin through the nasal passages for the rats and approximately 5 ml for the mice. The nasal passages were then immersed in the same fixative for a further 24 hours before being processed further. After fixation the heads were immersed in 14% disodium EDTA and decalcified for a minimum of 10 days with the solution being changed every two days. When the heads were ready they were sliced into four levels according to the convention of Young (1981) before being dehydrated and embedded in wax.

The human samples of nasal tissue were received frozen. A slice approximately 4 mm thick was removed from the tissue and immersed into 10 % phosphate buffered formalin in phosphate buffer and fixed for 24 hours before being processed into paraffin wax as for the animal samples.

The wax blocks were sectioned at 7 μm thick, the sections flattened on a water bath at 40 °C and then picked up onto glass slides and dried at room temperature. Sections of tissue were dewaxed and rehydrated into distilled water. They were then placed into 1% hydrogen peroxide in 95% methanol for 30 minutes to block endogenous peroxidase activity. Following rehydration, the sections were placed into 1% bovine serum albumin in phosphate-buffered saline (PBS/BSA), then into a 1/30 dilution of normal swine serum in PBS/BSA to suppress any background staining. The sections were then covered with a 1/200 dilution of the antibody to either cytochrome P450 2E1 or P450 2F2 for 16 hours at 4°C. At the end of the incubation period the slides were washed in PBS/BSA before being covered with a

solution of a swine anti-rabbit IgG (CYP2F2) or swine anti-sheep IgG (CYP2E1), both at a 1/100 dilution. Finally a solution of a rabbit (CYP2F2) or sheep (CYP2E1) peroxidase anti-peroxidase preparation was added, again diluted to 1/100 in PBS/BSA. The sites of primary antibody binding were revealed by developing the peroxidase enzyme with a buffered solution of diaminobenzidine tetrahydrochloride. All slides were subsequently counter-stained in haematoxylin for 30 seconds before being dehydrated and mounted in xylene.

All preparations were viewed and evaluated by a single person who had knowledge of the treatment groups. The relative amounts of immunoreactive protein in the sections was assessed qualitatively using a Leica DM RB photomicroscope and given a grading from 0-4 ranging from absence to very high staining intensity, with the specific distribution within each component tissue of the nasal passages being evaluated. Representative photographs were taken of nasal regions from rats, mice and the human samples.

4. RESULTS

4.1 Styrene metabolism to styrene oxide

The rates of metabolism of styrene to the R and S enantiomers of styrene oxide in microsomal fractions prepared from rat, mouse and human nasal and liver tissues are shown in Table 1 together with that measured using purified cytochrome P-4502E1. The rates were comparable in rat and mouse olfactory microsomes as were those in respiratory microsomes. The metabolic rates in respiratory fractions were approximately half of those seen in olfactory fractions. Nine human nasal samples were analysed, 8 as S9 fractions and one as a microsomal fraction. Styrene metabolism could not be detected in any of the samples. The limit of detection of the assay was 0.04 nmol/min/mg protein.

In both rats and mice the rates of styrene metabolism were higher in nasal tissues than those seen in the liver (Table 1). In nasal tissues from both species, and in mouse liver, the ratio of the R and S enantiomers was approximately 3:1. In contrast, the ratio in rat liver was 0.72 and that found for purified CYP2E1 even lower at 0.48, suggesting that this isoform of

cytochrome P-450 predominantly formed the S enantiomer and that the R enantiomer was formed by a different isoform.

4.2 Cytochrome P-450 inhibitors

All of the cytochrome P-450 inhibitors reduced the rate of metabolism of styrene in rat and mouse nasal microsomal fractions to some extent, although by far the most efficient was 5-phenyl-1-pentyne, an inhibitor of CYP2F2 (Fig 1). Using purified CYP2E1 it was clear that these chemical inhibitors are not totally specific for the individual isoforms. All of them were effective inhibitors of CYP2E1 to some extent, particularly at the higher concentrations (Table 2). Some of the inhibitors also increased the R to S ratio of the styrene oxide enantiomers (>5:1) by selectively inhibiting the formation of the S-enantiomer. Chlozoxazone, coumarin and 8-methoxypsoralen were all active in this respect whilst 5-phenyl-1-pentyne showed no stereo-selectivity in its inhibition (Table 3). These observations are consistent with the experiment using pure CYP2E1 which showed this isoform to be primarily responsible for the formation of the S-enantiomer.

4.3 The distribution of cytochromes P450's in the nasal passages of the mouse, rat and human.

4.3.1 Cytochrome P-4502E1

In the mouse, high levels of expression of this protein were observed in the apical cytoplasm of the olfactory tissue with low to moderate levels being locally expressed in some basal cells and in Bowman's glands (Fig 2). The ciliated cells of the respiratory epithelium generally failed to express P4502E1 but some of the non-ciliated cells in the respiratory epithelium and some of the sub-mucosal ducts showed moderate levels of expression. The protein was not expressed in the transitional epithelium, the sub-mucosal glands, the squamous epithelium or in the blood vessels or nerve bundles within the tissue (Table 4).

In the rat low levels of expression of P4502E1 were observed in the apical cytoplasm of the olfactory tissue although no protein was immuno-localised in the basal cells or in Bowman's glands. Low levels of expression was also observed in the sub-mucosal ducts lying beneath the respiratory epithelium. The protein was not expressed in the transitional epithelium, the

respiratory epithelium, the sub-mucosal glands, the squamous epithelium or in the blood vessels or nerve bundles within the tissue (Fig 3, Table 4).

The samples of human nasal tissue were restricted in terms of the number of tissues which it contained. Hence olfactory tissue proper was not present in the samples although areas of respiratory epithelium, transitional epithelium, sub-mucosal ducts and glands, and blood vessels were present depending on the sections that were stained. P4502E1 was expressed at low levels in the respiratory epithelium of one sample, but not in the other, and at low levels in a small number of cells in the sub-mucosal glands but not in the majority of glands. The transitional epithelium and the majority of the sub-mucosal glands and the associated ducts did not express this protein (Fig 4, Table 4).

4.3.2 Cytochrome P4502F2

In the mouse, very high levels of expression of P4502F2 were observed in the apical cytoplasm of the olfactory tissue and in Bowman's glands with moderate levels being locally expressed in some basal cells. The ciliated cells of the respiratory epithelium and the associated sub-mucosal ducts also expressed high to very high levels of P4502F2. The protein was not expressed in the transitional epithelium, the squamous epithelium or in the blood vessels or nerve bundles within the tissue (Fig 2, Table 4).

In the rat, high levels of expression of P4502F2 were observed in the apical cytoplasm of the olfactory tissue although no protein was immuno-localised in the basal cells or in Bowman's glands (Fig 3). High levels of expression were also observed in the respiratory epithelium and in the sub-mucosal ducts lying beneath the respiratory epithelium, and low levels of P4502F2 were also seen in the transitional epithelium. The protein was not expressed in the walls of the blood vessels, in the squamous epithelium or in the nerve bundles within the tissue (Table 4).

In the human samples P4502F2 was expressed at low levels in the transitional epithelium and sub-mucosal glands. The majority of the sub-mucosal glands and the associated ducts did not express this protein (Fig 4, Table 4).

4.4 In vivo metabolism and covalent binding

The atmosphere concentrations during exposure were 166±15.2 ppm and 152±10.2 ppm for rats and mice respectively.

4.4.1 Nasal tissues

The amount of olfactory and respiratory tissue available, particularly from mice, precluded separation into heavy, cytosolic and microsomal fractions. Consequently, the analysis at each time point was done on a whole tissue homogenate. The analysis separated radioactivity as volatile (extractable into hexane), aqueous metabolites and covalently bound material. The data (Fig 5) are presented as ug styrene equivalents per gram of tissue.

The bulk of the radioactivity in both respiratory and olfactory tissue from rats and mice was present as water soluble metabolites which were rapidly cleared from the tissues. The concentrations of these metabolites were similar in both mouse tissues and in rat olfactory tissue (Fig 5). Volatile radioactivity, assumed to be mainly unchanged styrene, was low and generally cleared by 24 hours. Covalently bound radioactivity on the other hand remained at a constant level over the duration of the study. The levels of bound radioactivity in mouse respiratory and olfactory tissues were higher (3-5 fold) than in their equivalent rat tissues (Fig 5). The levels of radioactivity in these tissues were insufficient for the identification of metabolites by hplc.

5. DISCUSSION

Exposure to styrene has been shown to cause toxicity to the nasal epithelium of rodents after 3 consecutive daily exposures and after daily exposure for 90 days (Foster, 1999, Cruzan et al. 1997). In the study by Foster et al. (1999) the toxicity in mice was prevented by treatment with 5-phenyl-1-pentyne an inhibitor of the cytochrome P-450 mediated metabolism of styrene to styrene oxide. Comparisons of the relative rates of metabolism of styrene to styrene oxide in different species can, therefore, be used to estimate the sensitivity of those species to styrene induced nasal toxicity. Identifying the specific cytochrome P-450 isoforms responsible for the activation of styrene provided an opportunity to study, in a semi-

quantitative manner, the distribution of those enzymes in nasal tissues from species of interest, in this case mice, rats and humans.

The rates of metabolism of styrene to styrene oxide in rat and mouse olfactory tissues were similar, and remarkable, in that they were higher than those seen in the liver, whereas the rates in respiratory tissue were approximately half those in the olfactory region and comparable to those in the liver. This pattern is consistent with the expression of the cytochrome P-450's, particularly CYP2F2, which showed similar levels of expression in both rat and mouse olfactory epithelium. Although the high metabolic activity explains the sensitivity of the rat and mouse nasal epithelium to styrene induced toxicity, the greater sensitivity of the mouse (Cruzan et al. 1997) is not reflected by these metabolic rates. The most likely explanation for this apparent discrepancy is the differences in air flow patterns and flow rates between the two species resulting in a higher dose in the mouse than the rat.

Chemical inhibitors of cytochromes P-450 are rarely, if ever, totally specific for a single isoform. For example, 5-phenyl-1-pentyne was an excellent inhibitor of CYP2F2, yet it also had some inhibitor effect with CYP2E1. However, the results of these studies were consistent with reports in the literature (Guengerich et al. 1991; Carlson, 1997) that CYP's 2E1 and 2F2 are the two major isoforms responsible for styrene metabolism. This was confirmed by the in vivo study of Foster (1999) in which the nasal toxicity of styrene was prevented by treatment of mice with 5-phenyl-1-pentyne.

Antibodies to CYP's 2E1 and 2F2, showed that both enzymes were heavily expressed in rat and mouse nasal tissues with the highest protein levels being found in the olfactory regions of both species. Generally, the expression of CYP2F2 was greater than that of CYP2E1.

Of the two enantiomeric forms of styrene oxide produced by the metabolism of styrene, the R enantiomer is reported to be the more toxic (Bond 1989). Interestingly, the ratio of the R and S enantiomers of styrene oxide formed in all nasal tissues was in the order of 3:1. A similar ratio was seen in mouse liver, but that in rat liver was markedly different, 0.72:1, suggesting that the complement of P-450's metabolising styrene varies between species and between organs. When purified CYP2E1 was used to catalyse the metabolism of styrene, the R:S isomer ratio was 0.48. This would suggest that CYP2E1 mainly forms the S enantiomer and

CYP2F2 the R enantiomer, the more toxic form. Thus, the high nasal CYP2F2 activity, and the fact that this isoform predominantly forms the toxic R isomer of styrene oxide, provides a satisfactory explanation for the sensitivity of rodent nasal tissues to styrene.

The in vivo metabolism studies were generally supportive of the results seen in vitro and with the enzyme distribution studies. Although insufficient quantities of metabolites were produced by the very small amounts of tissue available to enable metabolite structures to be identified, it was clear that more active metabolites were formed in the mouse in vivo than in the rat. The levels of covalent binding in the nasal tissues of mice were significantly higher than those in the rat which is consistent with the higher sensitivity of the mouse to styrene.

Access to fresh, metabolically viable human nasal tissue is difficult, and the opportunity to use fresh tissue collected during surgery is extremely rare. The amount of tissue, which is usually predominantly respiratory in character, also varies considerably between individuals and in all cases was insufficient for full enzyme kinetics. When the amount of nasal tissue available precluded the preparation of microsomal fractions, it was considered desirable to assay individual samples as S9 fractions rather than to combined then to give sufficient material for the preparation of microsomes. Styrene metabolism could not be detected in these fractions even though very low levels of both CYP2E1 and CYP2F2 were detected with antibodies in tissues from some individuals. It was concluded therefore, that any metabolism of styrene, catalysed by these very low levels of enzyme, was below the limit of detection of the assay for styrene oxide. Based on the limit of detection, the differences between rodents and humans were considerable, at least 80-fold compared with rodent (rat) respiratory tissue and 160-fold compared with rodent (mouse) olfactory tissue.

In conclusion, styrene is metabolised to styrene oxide at high rates in rodent nasal tissues. The high rates are due to high concentrations of two cytochrome P-450 isoforms, CY2E1 and CYP2F2. Of the two isoforms the higher activity of CYP2F2 is believed to result in the observed preferential formation of the toxic R isomer. Assays of the metabolism of styrene in human nasal tissue suggests that the nasal toxicity seen in the rodent is unlikely to occur in humans.

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7. TABLES

TABLE 1. THE METABOLISM OF STYRENE TO STYRENE OXIDE BY RAT, MOUSE AND HUMAN NASAL AND LIVER MICROSOMAL FRACTIONS

Species	Tissue Fraction[a]	R (nmol/min/mg)	S (nmol/min/mg)	R : S ratio
Rat	Olfactory	7.456	2.512	2.97
Rat	Respiratory	3.250	1.216	2.68
Rat	Resp S9	0.320	0.120	2.68
Mouse	Olfactory	6.587	2.240	2.97
Mouse	Respiratory	3.640	1.245	2.92
Human	S9 (n=8)	ND	ND	-
Human	Microsomes (n=1)	ND	ND	-
Rat	liver	1.119	1.563	0.72
Mouse	liver	3.045	1.129	2.70
CYP2E1[b]		1.295 ± 0.115	2.848 ± 0.321	0.48

a. Fractions are microsomal unless otherwise stated.

b. Human CYP2E1 expressed and purified from E. coli.

N.D. not detected. Limit of detection 0.04 nmol/min/mg

TABLE 2. THE EFFECT OF CHEMICAL INHIBITORS ON THE METABOLISM OF STYRENE TO STYRENE OXIDE BY PURIFIED CYP2E1.

Chemical	R-SO nmol/min/mg	S-SO nmol/min/mg	R-SO % control	S-SO % control	R:S ratio
CYP2E1	1.295 ± 0.115	2.848 ± 0.321	100	100	0.458
0.05mM CZO	0.997 ± 0.067	2.232 ± 0.204	77.8	79.0	0.450
0.5mM CZO	0.864 ± 0.044	1.977 ± 0.181	67.5	69.9	0.443
0.05mM Coumarin	1.328 ± 0.136	2.984 ± 0.400	102.1	103.8	0.451
0.5mM Coumarin	0.980 ± 0.111	2.166 ± 0.260	76.2	76.7	0.454
0.005mM 5P-1-P	1.185 ± 0.196	2.712 ± 0.524	89.8	92.7	0.445
0.05mM 5P-1-P	0.844 ± 0.104	1.887 ± 0.246	63.3	63.8	0.448
0.005mM 8MP	1.238 ± 0.199	2.663 ± 0.445	93.8	91.7	0.467
0.05mM 8MP	0.889 ± 0.071	2.041 ± 0.242	68.7	71.4	0.442

TABLE 3. STYRENE METABOLISM TO STYRENE OXIDE: THE EFFECT OF CYTOCHROME P-450 INHIBITORS ON THE R:S STYRENE OXIDE ENANTIOMER RATIO.

Chemical	Rat olfactory microsomes	Rat respiratory microsomes	Mouse olfactory microsomes	Mouse respiratory microsomes
control	2.972	2.676	2.967	2.924
0.1mM CZO	3.598	not assayed	not assayed	not assayed
0.25mM CZO	3.870	not assayed	3.539	not assayed
0.5mM CZO	4.271	3.424	4.589	4.192
1.0mM CZO	5.006	3.948	5.429	4.975
0.005mM Coumarin	3.076	not assayed	not assayed	not assayed
0.05mM Coumarin	3.313	not assayed	not assayed	not assayed
0.5mM Coumarin	4.557	2.661	not assayed	not assayed
1.0mM Coumarin	4.064	2.968	not assayed	not assayed
0.005mM 5P-1-P	3.126	2.878	3.123	3.274
0.05mM 5P-1-P	2.984	3.034	2.283	2.898
1.0Mm 5P-1-P	3.021	not assayed	1.746	not assayed
0.005mM 8MP	3.181	2.997	3.163	3.304
0.05mM 8MP	4.182	3.511	5.667	4.208
0.3mM 8MP	4.560	not assayed	8.696	not assayed

TABLE 4: SUMMARY OF THE DISTRIBUTION AND RELATIVE INTENSITIES OF CYTOCHROMES P-450 2E1 AND 2F2 IN VARIOUS PARTS OF THE NASAL PASSAGES IN THE MOUSE, RAT AND HUMAN.

Cytochromes P-450 2E1 and 2F2 were localization and quantified with antibodies as described in the methods section.

	P450 2E1			P450 2F2		
	Mouse	Rat	Human	Mouse	Rat	Human
Nasal passages:						
Olfactory apical cytoplasm	3	1	N	4	3	N
Olfactory - basal cells	1	0	N	2	0	N
Bowman's glands	2	0	N	4	0	N
Respiratory epithelium - ciliated	0	0	1	3	3	N
Respiratory epithelium - non-ciliated	2	0	N	0	3	N
Sub-mucosal ducts	2	1	0	4	3	1
Sub-mucosal glands	0	0	1	0	0	N
Transitional epithelium	0	0	0	0	1	1
Blood vessel walls	0	0	N	0	0	1
Squamous epithelium	0	0	N	0	0	N
Nerves	0	0	N	0	0	N

Notes: N = tissue not identified; 0= P450 not detected; 1 = low levels; 2=moderate levels; 3=high levels, 4= very high levels.

FIGURE 1. THE EFFECT OF CYTOCHROME P-450 INHIBITORS ON THE METABOLISM OF STYRENE TO STYRENE OXIDE IN RAT (A) AND MOUSE (B) OLFACTORY MICROSOMES.

CZO, chlorzoxazone; 5P-1-P, 5 phenyl-1-pentyne; 8MP, 8-methoxypsoralen.

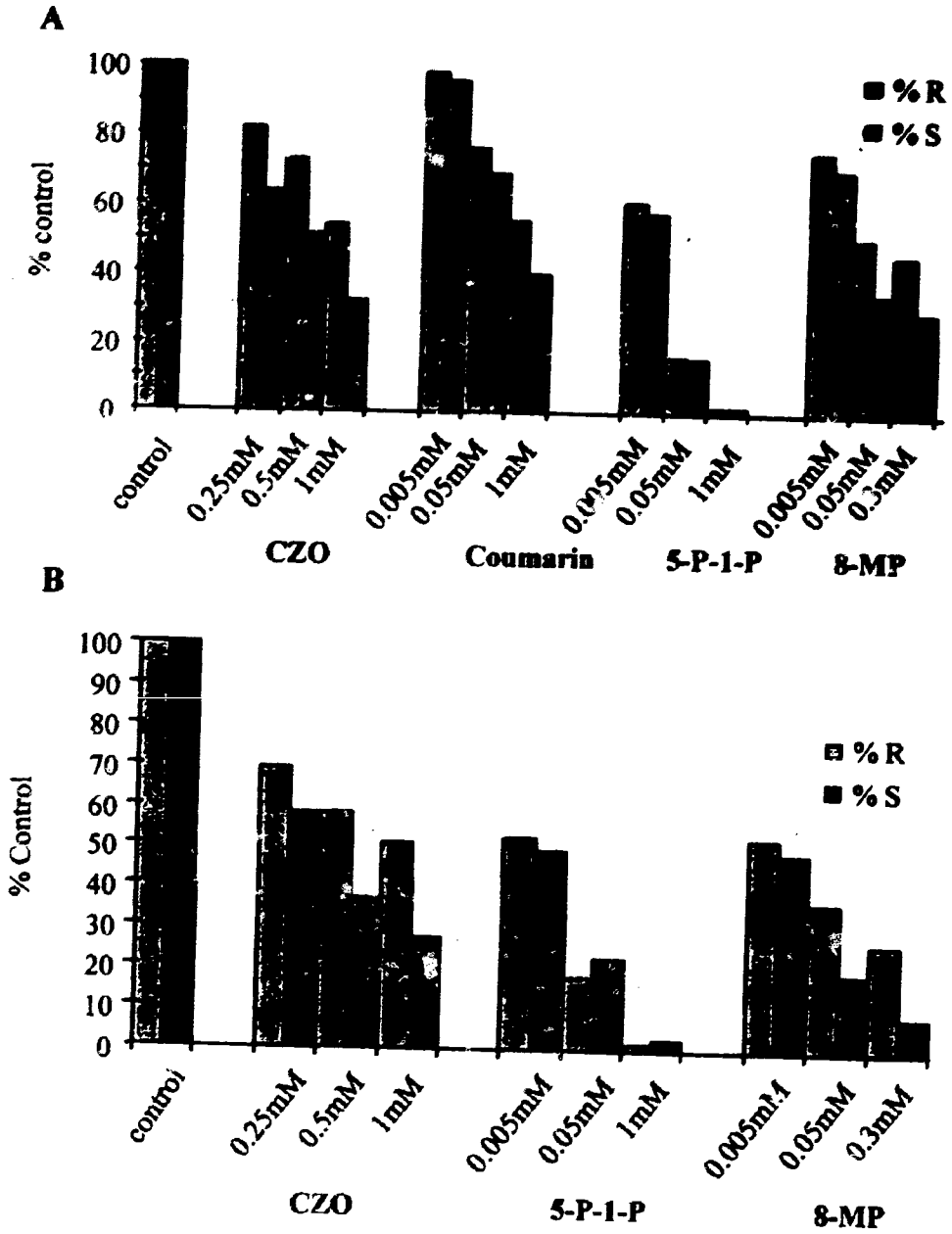
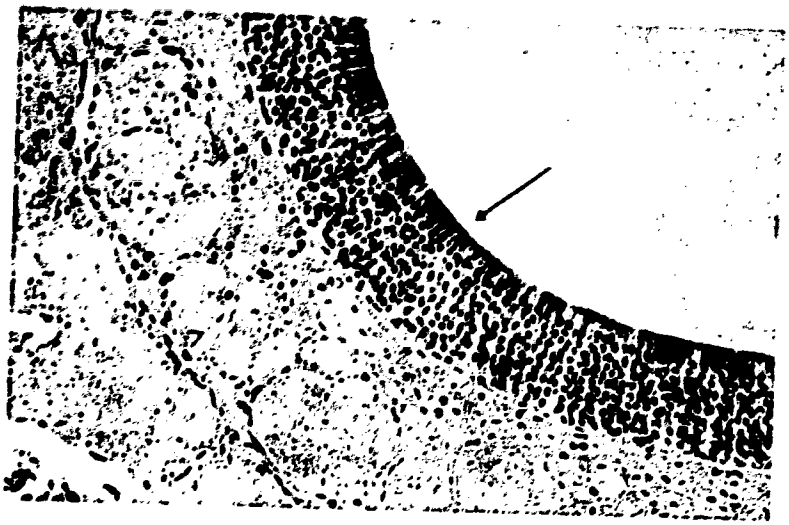
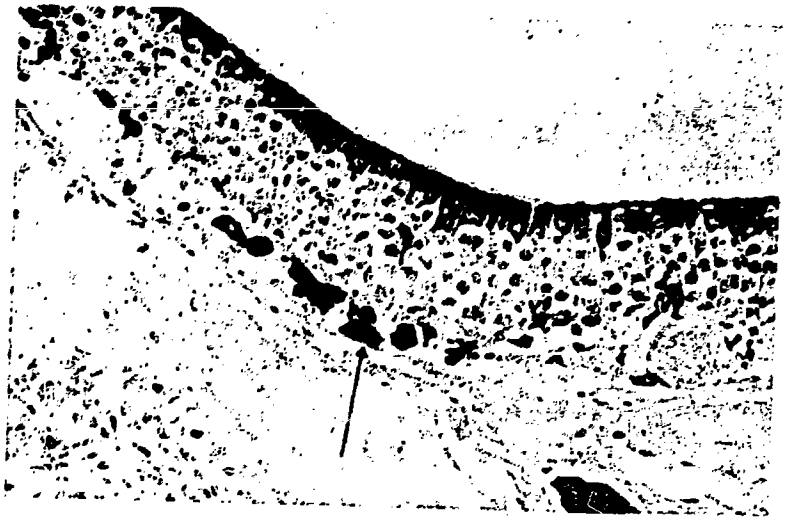


FIGURE 2. THE DISTRIBUTION OF CYTOCHROME P-450's 2E1 AND 2F2 IN MOUSE NASAL TISSUES.



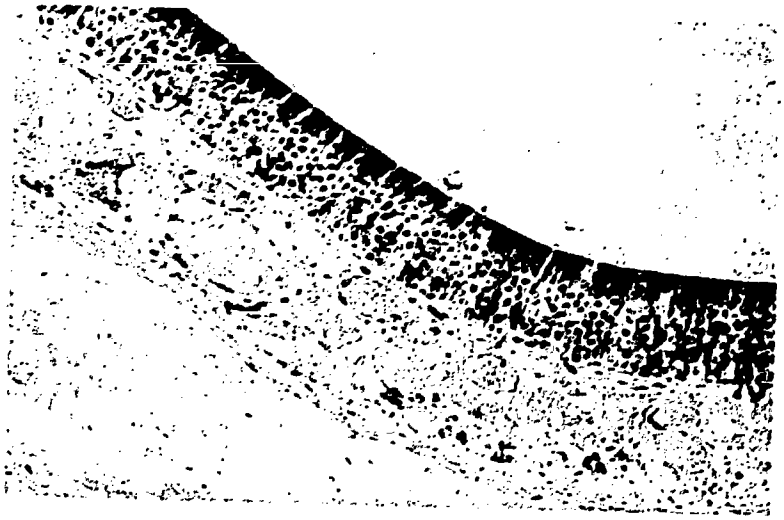
Mouse Olfactory Tissue P450 2E1; arrow shows apical staining for P450 2E1.



Mouse Olfactory Tissue P450 2F2; arrow shows Bowman's gland staining.

FIGURE 3. THE DISTRIBUTION OF CYTOCHROME P-450's 2E1 AND 2F2 IN RAT NASAL TISSUES .

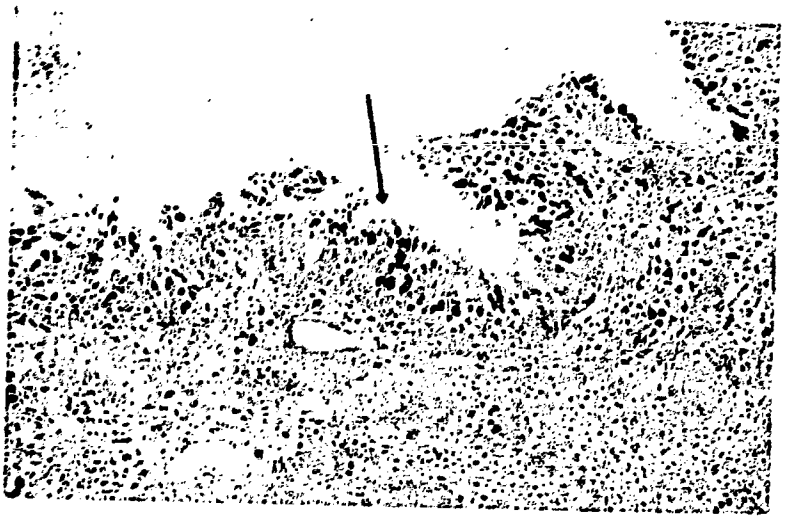
Rat Olfactory Tissue P450 2E1; arrow shows Bowman's gland staining.



Rat Olfactory Tissue P450 2F2; there is no Bowman's gland staining.

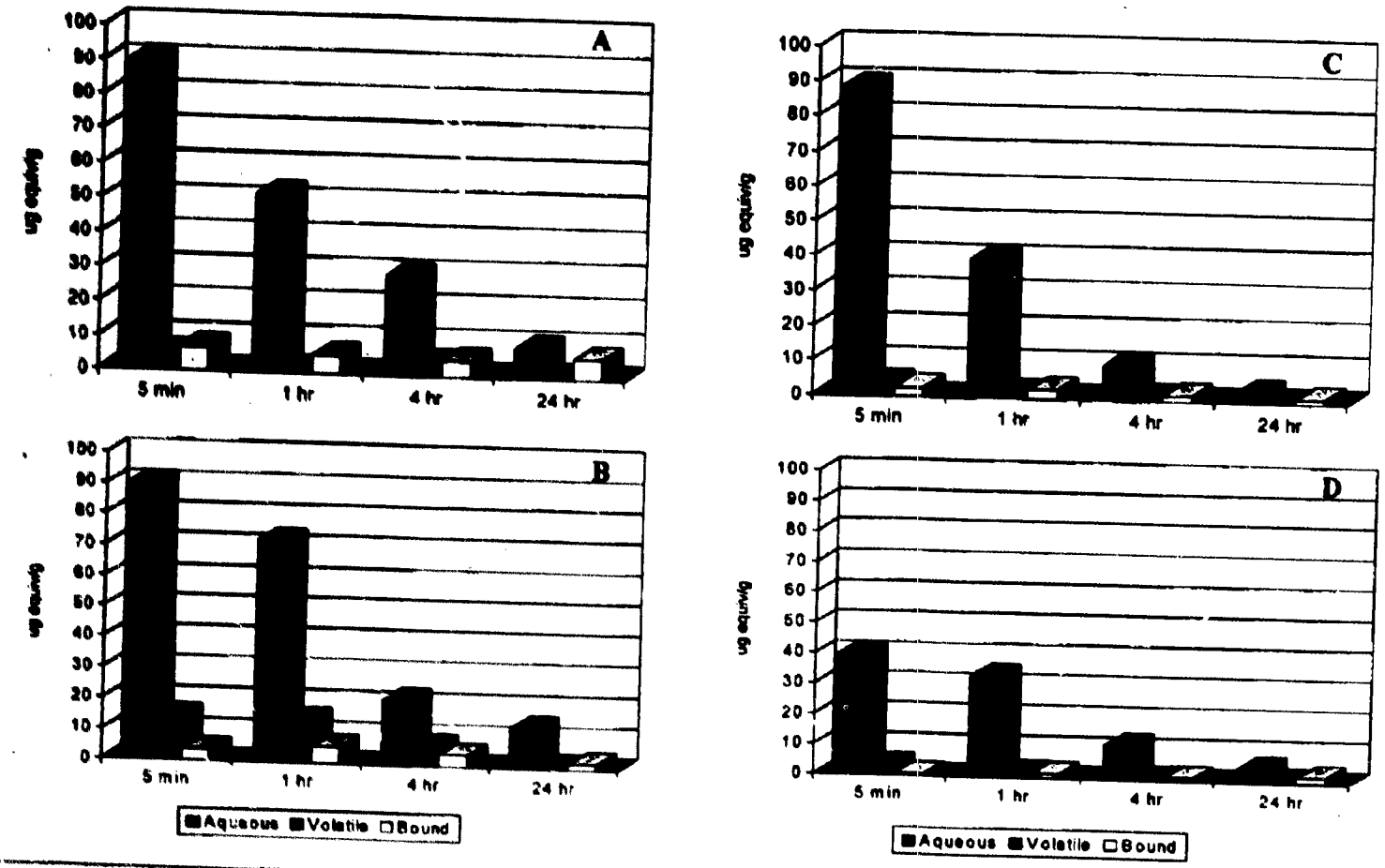
FIGURE 4. THE DISTRIBUTION OF CYTOCHROME P-450's 2E1 AND 2F2 IN HUMAN NASAL TISSUES.

Human nasal P450 2E1. The epithelium is pseudo-stratified and high background staining is seen.



Human nasal P450 2F2; arrow shows staining for P450 2F2.

FIGURE 6. THE DISTRIBUTION OF RADIOACTIVITY IN RAT AND MOUSE NASAL TISSUES AFTER EXPOSURE TO C-14 STYRENE FOR 6 HOURS. A mouse olfactory; B mouse respiratory; C rat olfactory; D rat respiratory



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THE ROLE OF CYTOCHROME P450 IN STYRENE-INDUCED NASAL LESIONS IN THE MOUSE.

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THE ROLE OF CYTOCHROME P450 IN STYRENE-INDUCED NASAL LESIONS IN THE MOUSE.

CTL Study Number: MM0200

AUTHOR

J R Foster

DATE OF ISSUE

August 9th 1999

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THE ROLE OF CYTOCHROME P450 IN STYRENE-INDUCED NASAL LESIONS IN THE MOUSE.

I, the undersigned, declare that this report constitutes a true record of the actions taken and the results obtained in the above study.

Dr T Green
(Study Director) ..... Date Aug 3rd 1999

STUDY CONTRIBUTORS

The following contributed to this report in the capacities indicated:

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Fig. 1: Control olfactory epithelial mucosa from level 4 of mouse nasal tissue. Haematoxylin and eosin, x 400 14

Fig. 2: Olfactory epithelial mucosa from level 4 of mouse nasal tissue following exposure to 160 ppm Styrene for 3 days. Haematoxylin and eosin, x 400 14

Fig. 3: Olfactory epithelial mucosa from level 4 of mouse nasal tissue following pre-dosing with 100 mg/kg 5P1P followed by exposure to 160 ppm Styrene for 3 days. Haematoxylin and eosin, x 400 15

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1 SUMMARY

Male CD-1 mice (10/group) were exposed to 0, 40 and 160 ppm styrene, 6 hours/day for three days and were killed 17 hours after the last exposure. Separate groups were pre-dosed with 100 mg/kg of the specific inhibitor of cytochrome P450 2F2, 5-phenyl-1-pentyne, before being exposed to 0, 40 and 160 ppm styrene. Following the designated exposure period all animals were killed and the nasal passages were removed and processed and examined histopathologically.

Administration of 160 ppm styrene to male CD1 mice caused a localised atrophy of the olfactory epithelium in the dorsal regions of levels 3 and 4 of the nasal passages. There was no effect on the other tissues of the nasal passages. Exposure of mice to three exposures of 40 ppm styrene did not cause any treatment-related effect on the olfactory mucosa or any other tissue in the nasal passages. Pre-administration of mice with 5-phenyl-1-pentyne and subsequent exposure to 160 ppm styrene prevented the styrene-induced nasal lesions and no other morphological effects were seen in other parts of the nasal passages with this combination of treatments. The administration of 5-phenyl-1-pentyne alone did not induce any effects in the nasal passages of mice.

The data suggest that the specific lesion in the olfactory tissue of mice following exposure to 160 ppm styrene is induced by a metabolite of styrene produced via the cytochrome P450 2F2 enzyme system, and that inhibition of this enzyme is able to prevent all the cytotoxicity seen when naïve mice are exposed to styrene.

2 INTRODUCTION

It has been previously shown that when male and female CD-1 mice were exposed, by inhalation, to 0, 50, 100, 150 or 200 ppm styrene for 6 hours/day for 13 weeks significant toxicity was seen in the nasal passages at the end of the study (Cruzan et al 1997). This toxicity included atrophy and respiratory metaplasia of the olfactory mucosa, atrophy of the olfactory nerves and hypertrophy and hyperplasia of Bowman's glands. These effects showed an exposure-dependent relationship and were present at the lowest exposure level. CD (Sprague-Dawley) rats exposed to 1000 and 1500 ppm styrene under the same protocol showed less severe changes to the olfactory epithelium and a clear NOEL for nasal changes at 500 ppm.

The mode of action of styrene in inducing nasal toxicity is unknown. The major route of styrene metabolism is by cytochrome P-450 enzymes to styrene oxide, a weak electrophile, which is potentially reactive with biological macromolecules (Bond, 1989), and nasal tissue, particularly the olfactory tissue, is known to have high levels of a number of these enzyme systems.

The purpose of the study was to examine the morphological events occurring within the nasal tissue after a three day exposure duration and to examine the influence of a specific inhibitor of cytochrome P450 2F2, the enzyme thought to be involved in the metabolism of styrene, on the expression of the nasal damage. Mice were pre-treated with the arylalkyne, 5-phenyl-1-pentyne, in order to block the activity of cytochrome P450 2F2 prior to being exposed, by inhalation, to styrene.

This study formed part of a larger study in which the effects of styrene on the mouse lung were evaluated. The results from that part of the study have been reported elsewhere (Green, 1999a). Details describing the selection, dose level and time course of inhibition of pulmonary cytochrome P4502F2 by 5-phenyl-1-pentyne are contained in that report.

3. MATERIALS AND METHODS

3.1 Chemicals

Styrene (99%), stabilised with 10-15 ppm 4-tert-butylcatechol, was supplied by Sigma-Aldrich Co. Ltd, Poole, Dorset, UK. and assigned CTL reference numbers Y00115/003/004-008 and Y00115/003/010-011 and stored at 4°C. 5-Phenyl-1-pentyne was obtained from Lancaster Chemical Co. UK.

3.2 Animals

Male CD-1 mice, 22-27g body weight, were supplied by Charles River, Margate, Kent. Prior to the study the animals were housed in stainless steel cages in rooms equipped with a 12 hour light-dark cycle. The environment of the animal room was controlled to provide conditions suitable for CD-1 mice with a temperature being maintained within a target range of 19-23 °C, a relative humidity within a target range of 40-70%, and between 25-30 air changes per hour. All conditions were monitored by the Honeywell site monitoring system.

Certified diet (CT1 diet, Special Diet Services, Witham, Essex, UK) and mains water were supplied *ad libitum* except during the exposure periods.

Animals were randomly assigned to treatment groups of 10 animals/group, by a method based on individual bodyweights. Individual animals were identified by ear punching. All animals were observed to ensure they were normal before the start of the study and any showing adverse signs either before or during the study were killed.

3.3 Study design

The nasal tissues analysed in this study were taken from a study conducted to investigate the effects of cytochrome P-450 inhibition on the development of pulmonary lesions in mice exposed to styrene. That study (MM0200) has been reported elsewhere (Green, 1999a). The experimental conditions with respect to exposure, cytochrome P-450 inhibition and the removal and processing of the nasal tissues were as follows:

Chambers of approximately 3.4m^3 capacity were used for the exposures (Doe and Tinston, 1981). Conditioned air was supplied to the chambers at a flow rate sufficient to give at least

12 changes per hour. Temperature, relative humidity and air flow were measured in the chambers every 30 minutes. Atmospheres were generated by allowing styrene to evaporate at 55°C into a stream of air at a controlled rate. The concentration of styrene in the chamber was measured by gas chromatography at hourly intervals throughout the experiment. A Hewlett Packard HP5890 gas chromatograph fitted with a CPSIL5CB column (25m x 530µm operated at 120°C) and flame ionisation detector was used.

Groups of mice (20 per dose group) were exposed to 0, 40 and 160 ppm styrene, 6 hours/day, for 3 days. Twenty four hours before the start of the experiment half of the mice in each group were given a single oral dose of 200 mg/kg 5-phenyl-1-pentyne in corn oil (10 ml/kg) and the remainder were given corn oil alone (10 ml/kg). These doses was repeated immediately after exposure on each of the 3 days. The mice were killed 18 hours after the third exposure.

Note: The mice used in these experiments were fitted with BrdU minipumps for quantitation of pulmonary cell replication rates (see Green 1999a).

Body weights and clinical signs were recorded daily, immediately before exposure. The animals were killed with an overdose of halothane, exsanguinated and decapitated. The lower jaw, skin and brain were then removed from the skull before the nasal passages were perfused, by instilling 10% neutral buffered formalin retrogradely, via the nasopharyngeal duct, through the nasal passages. The nasal passages were decalcified for 7 days in 20% formic acid before being trimmed into four levels according to Young (1981). The four levels were then dehydrated and embedded in paraffin wax. Sections (5-7µm) were cut and stained with haematoxylin and eosin.

4 RESULTS

The mean atmospheric concentrations of styrene over the 4 day study were 39.9±1.3 and 161±3.7 ppm. Temperature and humidity within the exposure chambers were within

acceptable limits (19-23°C; 40-70% respectively). Bodyweight gains and clinical signs in animals exposed to styrene were normal and comparable to those in the control group.

4.1 Nasal pathology

4.1.1 Macroscopical Findings (Tables 1a & 1b).

There were no treatment-related findings present macroscopically following exposure of mice to styrene.

4.1.2 Microscopical Findings (Table 2).

Administration of 5-phenyl-1-pentyne alone to mice exposed to 0 ppm styrene did not induce any effects in the nasal passages. In mice killed 17 hours after three exposures to 160 ppm styrene there were no changes in the tissues present in levels 1 and 2 of the nasal passages although in levels 3 and 4 the olfactory tissue showed a range of degenerative changes which were most commonly focal in nature. The most obvious effect of exposure was the presence of a mixed cellular and serous fluid exudate in the airways of the nasal passages in the olfactory epithelium of the dorsal meatus. The olfactory mucosa nearest to these exudates was often atrophied with a distinct loss of the normal cellular organisation present in this tissue. The Bowman's glands were also focally decreased in number in these areas. The nasal passages of those mice given 5-phenyl-1-pentyne preceding exposure to 160 ppm showed a normal histology.

Mice given 40 ppm styrene were largely unaffected by exposure although one animal showed a minimal atrophy of the olfactory mucosa in the level 4 section. The nasal passages of mice given a combination of 5-phenyl-1-pentyne followed by a three day exposure period to 40 ppm styrene were normal in appearance.

5 DISCUSSION

Exposure of mice to styrene at 160 ppm induced pathology of the nasal passages with a specificity for the olfactory epithelium in the region of the dorsal meatus. Although olfactory

epithelium was present throughout the dorsal and ventral parts of levels 3 and 4, with some also being present in level 2, only specific regions of olfactory tissue were targeted by exposure to the high level of styrene. The olfactory mucosa was more sensitive to the effects of styrene exposure than were the Bowman's glands although these too were affected where mucosal effects were more severe. The cellular exudate, present in the airways of the dorsal meatus, was considered, by virtue of the evidence of olfactory mucosal atrophy, to represent the desquamated remnants of damaged olfactory mucosa. No obvious loss of nerve bundles was observed in the sub-mucosal tissue in the regions of the olfactory damage.

The regional and tissue specific effects of styrene, reported in this study, are similar to those reported for CD1 mice and for the CD and F344 rat (Roycroft et al 1992; Cruzan et al 1997), although no NOEL was observed for nasal effects in the latter study at exposure levels down to 50 ppm when mice were exposed for 13 weeks, whereas over the three day exposure period used in the present protocol a NOEL of 40 ppm was achieved.

There are two enzymes thought to be responsible for the oxidative metabolism of styrene to styrene oxide, cytochromes P-450 CYP2E1 and CYP2F2, (Guengerich et al. 1991; ; Carlson 1997; Green et al. 1997). Pre-dosing of mice with the inhibitor of cytochrome P450 2F2, 5-phenyl-1-pentyne, was effective in preventing the nasal lesions induced by styrene. This is good supporting evidence that a metabolite of styrene is responsible for the nasal toxicity and further that it is a product of cytochrome P450 2F2 dependent metabolism that induces the nasal damage. Cytochrome P450 2F2 has been localised to the olfactory epithelium, using specific antibodies raised against the purified protein (Green, 1999b). The results provide additional support for the conclusion that it is this isozyme that is responsible for the activation and cytotoxicity induced in the nasal passages by styrene. The higher comparative levels of cytochrome P450 2F2 in the mouse versus the rat nasal passages would also support the former being more sensitive to the nasal toxicity of styrene that is the rat (Cruzan et al 1997). However the fact that the enzyme shows a diffuse localisation amongst most, if not all, of the olfactory epithelium, while the styrene-induced nasal lesions show a more restricted distribution suggests that the mere presence of an activating enzyme system does not, on its own, mean that cytotoxicity will result from exposure. The most probable

explanation for the localized nature of the nasal toxicity observed under the present short, and other 13 week, exposure regimes lies in a combination of the presence of the necessary enzyme systems for styrene activation, together with the presentation of a sufficient concentration of styrene to the target tissue by virtue of the airflow pattern of inspired air into the mouse nasal passages.

In conclusion, this study has demonstrated an early lesion in the mouse nasal passages which is localised not only to one tissue, the olfactory tissue, but also to a selective part of that tissue within the nasal turbinates. Furthermore the cytotoxicity was completely abrogated by pre-dosing the mice with 5-phenyl-1-pentyne, a specific inhibitor of cytochrome P450 2F2, this providing strong support that the cytotoxicity in the mouse is mediated via a metabolite of styrene generated by the enzymic action of P450 2F2. This conclusion is supported by the co-localisation of cytochrome P450 2F2 to the olfactory mucosal cells and Bowman's glands although the regional specificity of the lesions suggests that localised airflow probably also plays a critical role in delivering styrene at the appropriate concentration to the target cell population. The relative metabolic capacities of mouse and human nasal tissue for styrene (Green, 1999b), would suggest that the responses seen in the mouse nose are unlikely to occur in the human nasal passages where both the distribution of olfactory tissue, and the relative enzymic activity of the tissue is considerably less than that of the mouse.

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APPENDIX 1. PATHOLOGY TABLES

Table 1a: Comparison of Macroscopic Findings in mice exposed to styrene - intercurrent deaths.

ZENECA - CENTRAL TOXICOLOGY LABORATORY SECTION: SHORT TERM INHALATION TOXICITY

P A T H O L O G Y - INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS INCIDENCE

STUDY NO: MM0200 TITLE: STYRENE: 3 DAY INHALATION/INTERVENTION STUDY CD1 MICE

REMOVAL REASON: INTERCURRENT	SEX: MALES	GROUP 1 0 ppm	GROUP 2 40 ppm
	MALES ON STUDY	10	10
	ANIMALS COMPLETED	0	3[1]
	TISSUE NOT EXAMINED	0	3

Notes: (i); No tissue examined microscopically from these animals.

Table 1b: Comparison of Macroscopic Findings in mice exposed to styrene - terminal sacrifice.

ZENECA - CENTRAL TOXICOLOGY LABORATORY SECTION: SHORT TERM INHALATION TOXICITY

P A T H O L O G Y - INTERGROUP COMPARISON OF MACROSCOPIC FINDINGS INCIDENCE

STUDY NO: MN0200 TITLE: STYRENE: 3 DAY INHALATION/INTERVENTION STUDY CD1 MICE

REMOVAL REASON: TERMINAL	SEX: MALES	GROUP 1 0 ppm	GROUP 1 0 ppm +5P1P	GROUP 2[a] 40 ppm	GROUP 2 40 ppm +5P1P	GROUP 3 160 ppm	GROUP 3[a] 160 ppm +5P1P
	MALES ON STUDY	10	10	10	10	10	9
	ANIMALS COMPLETED	10	10	7	10	10	9
NASAL CAVITY							
SUBMITTED		10	10	7	10	10	9
NO.WITHOUT FINDINGS		10	10	7	10	10	9

a. Three mice from Group 2 and one mouse from Group 3 died during minipump surgery

Table 2: Comparison of Microscopic Findings in mice exposed to styrene.

DIVISION - GENERAL TOXICOLOGY LABORATORY SECTION: SHORT TERM INHALATION TOXICITY

P A T H O L O G Y - INTERGROUP COMPARISON OF MICROSCOPIC FINDINGS INCIDENCE

STUDY NO: MM0200 TITLE: STYRENE: 3 DAY INHALATION/INTERVENTION STUDY CD1 MICE

FISHER'S TEST:- GROUP 1 COMPARED WITH OTHER GROUPS (* P < 0.05 ** P < 0.01, ONE SIDED)

NON-NEOPLASTIC MORPHOLOGIES REMOVAL REASON: TERMINAL	SEX: MALES	GROUP 1 0 PPM	GROUP 1 0 PPM +5P1P	GROUP 2 40 PPM	GROUP 2[a] 40 PPM +5P1P	GROUP 3 160 PPM		GROUP 3[a] 160 PPM +5P1P
	MALES ON STUDY	10	10	10	10	10		10
	ANIMALS COMPLETED	10	10	10	7	10		9
NASAL CAVITY								
EXAMINED		10	10	10	7	10		9
NO ABNORMALITIES DETECTED		9	9	8	7	0		9
Cell exudate - dorsal meatus (TOTAL)		0	0	1	0	10	**	0
slight		0	0	1	0	1		0
moderate		0	0	0	0	3		0
marked		0	0	0	0	6	**	0
Loss of Bowman's glands (TOTAL)		0	0	0	0	9	**	0
minimal		0	0	0	0	2		0
moderate		0	0	0	0	7	**	0
Focal atrophy of olfactory mucosa (TOTAL)		1	1	1	0	10	**	0
minimal		1	1	1	0	1		0
slight		0	0	0	0	1		0
moderate		0	0	0	0	7	**	0
marked		0	0	0	0	1		0
Single cell necrosis - olfactory epithelium (TOTAL)		1	1	2	0	0		0
minimal		0	0	1	0	0		0
slight		1	1	1	0	0		0

a. Three mice from Group 2 and one mouse from Group 3 died during minipump surgery

THE ROLE OF CYTOCHROME P450 IN STYRENE-INDUCED NASAL LESIONS IN THE MOUSE.

Fig. 1: Control olfactory epithelial mucosa from the tip of an endoturbinate at level 4 of mouse nasal tissue. Haematoxylin and eosin stained section. X 400.

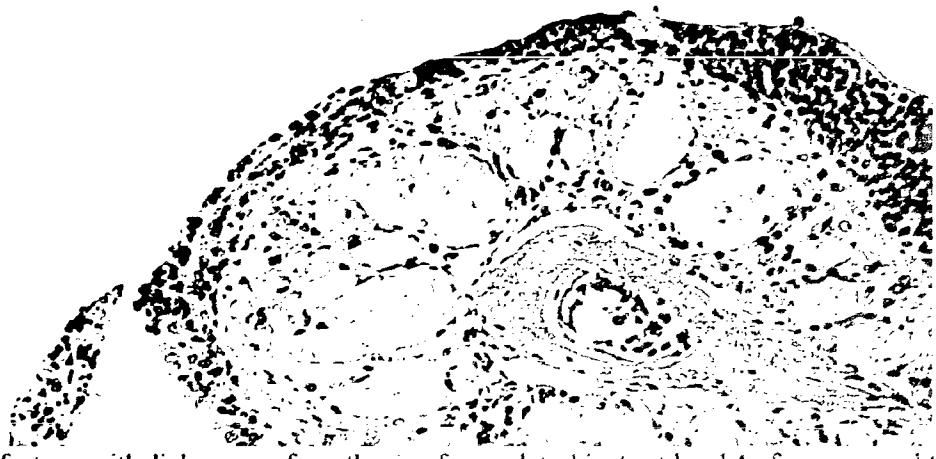

Fig. 2 Olfactory epithelial mucosa from the tip of an endoturbinate at level 4 of mouse nasal tissue following exposure to 160 ppm Styrene for 3 days. The mucosa is thin due to loss of cells and an exudate composed of dead cells is present in the airway adjacent to the mucosa. Haematoxylin and eosin. X 400

Fig. 3 Olfactory epithelial mucosa from the tip of an endoturbinate at level 4 of mouse nasal tissue following predosing with 100 mg/kg 5P1P followed by exposure to 160 ppm Styrene for 3 days. The mucosa has the same appearance as that in the control mouse nasal passages. Haematoxylin and eosin. X 400

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