

CONTAINS NO CBI



Allied-Signal Inc.
Engineered Materials Sector
P.O. Box 1139
Morristown, NJ 07962-1139

FYI-OTS-0692-0853 INITIAL
SEQUENCE (A)

June 8, 1992

Dr. Vanessa Vu
OTS/HERD
E.P.A. - Washington Information Center
E.P.A. Headquarters East
Tower Room 619A
Washington, D.C. 20460

RECEIVED
6/29/92
T081CSB

JUN 23 11:24:03

OTS DOCUMENT RECEIPT OFF.

Dear Dr. Vu:

Enclosed for your information are copies of the protocols for two studies currently planned for HCFC 123. The first is titled HCFC 123: Multigeneration Study in the Rat, the second, Neurotoxicity Study in Rats.

It is our intention to initiate the reproduction study in July and the Neurotoxicity study in August. The upper exposure level for the reproduction study were based on observations in a probe reproduction study which noted effects on plasma triglyceride levels (reduction), liver weights (increase), depressed body weight gain and a possible increased time maturation for the males of five days. (The latter finding may be related to body weight gain depression). At the higher level of exposure (5,000 ppm) similar but more pronounced effects were seen.

For the neurotoxicity study, we currently are considering exposure levels of 5,000, 1,000 and 300 ppm.

If you have comments, please call me at your convenience (201-455-3672). PAFT welcomes this opportunity to continue to work with U.S. EPA on this important program.

Sincerely,

George M. Rusch, Ph.D., D.A.B.T.
Chairman, PAFT Toxicology Program

GMR:rb
Enc.

cc: Dr. M.R. Harris
Dr. R. Rubenstein
PAFT I Tox. Comm.

c:6-8-92c.gmr



recycled paper



FYI - 853A

CONFIDENTIAL

Schedule No. ALS/5

DRAFT (JUNE 1992)
A STUDY OF THE EFFECT OF HCFC 123
ON REPRODUCTIVE FUNCTION OF
TWO GENERATIONS IN THE RAT

SPONSOR'S ADDRESS:

Dr. G.M. Rusch,
Chairman PAFT Toxicology Committee,
Department of Toxicology,
Allied Signal Inc.,
P.O. Box 1139R,
Morristown,
New Jersey,
07962,
U.S.A.

PROTOCOL

From:

Departments of Reproductive
Toxicology and Inhalation
Toxicology,
Huntingdon Research Centre Ltd.,
P.O. Box 2,
Huntingdon,
Cambridgeshire,
PE18 6ES,
ENGLAND.

Design ref: MG INHALATION

PR92101

PERSONNEL

Sponsor's Monitoring Scientist: Dr. G. Malinverno.

HRC Staff

Veterinary Director: D.P. Buist.

Head of Pathology: C. Gopinath.

Monitoring Toxicologist: D.F. Newton.

Inhalation Toxicology:

Head of Department: G.C. Clark.

Head of Industrial Chemicals
Unit: C.J. Hardy.

Study Supervisor
(Inhalation exposures): D. Coombs.

Reproductive Toxicology:

Head of Department: D.D. Cozens.

Study Director: E.W. Hughes.

Study Supervisor: D.P. Myers.

In the temporary absence of the
Study Director the responsibilities
will be taken over by: D.F. Newton.

Working document distributed to:

D.D. Cozens, D.F. Newton, C.J. Hardy, D. Coombs, E.W. Hughes, D.P. Myers,
N.T. Harman, I.J. Sharman, Animal Room, A.K. Palmer, R.J. Harling, C.A. Parker,
Archives, HRC-USA, W, Jorgeson (x2), L. Cooke, QAD.

Sponsor: Dr. G. Rusch.

Monitoring Scientist: Dr. G. Malinverno.

PURPOSE OF THE STUDY

The object of this study is to assess the effect of HCFC 123 on the growth and reproductive performance of two consecutive generations in the rat.

The recommended guidelines of the following agencies have been taken into consideration in the design of this study:

USA EPA TOSCA Guideline 798.4700 published in Federal Register Vol. 52 no. 97. May 30 1987 pg 19056.

Japan. Directive 62. Kikyoku No. 303. MITI. 31 March 1987.

TEST SPECIES

Rat - requested by Sponsor, a universally accepted species in multiple generation studies. In addition, Huntingdon Research Centre has background control data on reproductive performance, fertility and survival indices for rats of the proposed strain.

ROUTE OF ADMINISTRATION

By inhalation as this is a potential route of human exposure.

Concentrations (ppm)

0 Control
30 ppm HCFC 123
100 ppm HCFC 123
300 ppm HCFC 123
1000 ppm HCFC 123

Choice of Concentrations

The concentrations were chosen by the Sponsor based on the results of a dose range finding study performed in this laboratory and reported separately (ALS/4).

Absorption

It is not intended to determine the absorption of the test compound in this study.

TEST MATERIAL

Information, supplied by the Sponsor, regarding the test material is contained in the Test Substance Data Sheet (or Sponsor's Material Safety Data Sheet). The Sponsor is responsible for characterisation of the test material. The following information is given in summary.

Test material: HCFC 123.
(2,2-Dichloro-1,1,1-trifluoromethane).
CAS Reg. No. 306-83-2.

Supplier: ICI, England.

Action: Propellant/Blowing Agent.

Description of material: The test material will be supplied in cylinders.

Storage conditions: Cylinders will be stored in the open.

Stability of test material: Known by Sponsor to be stable for duration of study.

The following information refers to the original batch of material supplied for the start of the study. Further batches may be required during the course of the study. Full details of batch usage will be maintained but protocol amendments will not be issued.

Date of receipt at HRC: ICI 4 1 ton cylinders 8 May 1991.

Batch No.: Cylinder numbers recorded in raw data.

Purity: 99.86 - 99.95%.

Expiry date: Stable for duration of study.

EXPOSURE OF THE RATS TO THE TEST VAPOUR

1. Dosing regimen

During all the premating phases the animals will be exposed 6 hours/day, 7 days a week. Each animal will be held individually in stainless steel mesh cages during exposure and will be returned to its home cages overnight. For mating, designated pairs (1 male : 1 female) will be cohabited overnight for the 20 day mating period.

During the mating period and up to presumed day 20 of pregnancy the animals will be exposed 6 hours/day, 7 days a week, and will be held in individual cages during exposure.

At the end of the mating period, males will continue to be exposed 6 hours/day 7 days a week. Males will be returned to their home cages overnight.

From presumed Day 20 of pregnancy through to Day 4 post partum females will remain in their breeding cages and allowed to deliver their young and to establish lactation without exposure to vapour. From Day 5 post partum females will be exposed 6 hours/day 7 days a week and returned to their breeding cages overnight.

For those females that fail to deliver any young, exposure will be re-introduced 7 days after the presumed Day 20 of pregnancy (unless inspection of the available data suggests that parturition is imminent). If there is then further evidence of mating in animals that are re-exposed, exposure will continue until the second presumed Day 20 of pregnancy. Females that are considered to be non-pregnant will be exposed to vapour throughout. Full details of the exposure records will be maintained and will be presented in the final report.

2. Exposure systems, generation of test vapour and monitoring of chamber conditions are described in Addendum 1.

PARENT FO ANIMALS

One hundred and sixty four-week old (± 1 day) Specific Pathogen Free male and the same number of female rats (CrI: CD® (SD) BR VAF/Plus strain) within a 40 gram weight range will be ordered from Charles River Portage, Michigan, USA. Males and females will be obtained from separate litters and no more than 4 of each sex will be obtained from the same litter; litter mates will be identified.

An additional five males and five females will be ordered for health check purposes.

On arrival all animals will be examined for abnormalities and for signs of overt ill health. Those designated as health check animals will be killed within 24 hours after arrival at HRC and subjected to routine macroscopic examination. Any abnormalities seen will be processed immediately and examined microscopically. Lungs, liver, kidneys, spleen and heart will be preserved in fixative, but not processed further unless abnormalities are seen.

Animals will be weighed on arrival and after an acclimatisation period of 11 days they will be weighed again and assigned to five groups. Assignment will be by a stratified randomisation procedure which gives priority to distribution of litter mates across groups, than of animals of approximately equal weight across groups. Following allocation, the animals will be tattooed (earmarked) to give individual identification. A further acclimatisation period of 7 days will be allowed between allocation of animals to groups and commencement of treatment. Prior to the commencement of treatment, all animals will be inspected by a Veterinary Officer.

GENERAL ANIMAL MANAGEMENT AND ACCOMMODATION

The study will be housed in barriered building Y11, room 009.

Animal room controls for temperature and relative humidity will be set at 20°C and 55% respectively, and lighting will be controlled to give 12 hours light (8 am to 8 pm) and 12 hours dark per 24 hours.

During the pre-mating period, males and females will be housed separately, four to a cage. The cages will be suspended on movable racks. Each cage will be constructed of stainless steel with a mesh front, back and floor and stainless steel sides, 53 cm long, 35 cm wide and 25 cm high. Plastic trays will be placed underneath and lined with paper to collect excreta. Cages of males will be interspersed amongst those holding females to promote development of regular oestrous cycles.

Each group of rats will be kept in a separate ventilated cabinet. These ventilated cabinets draw their air supply from the holding room. Exposure to the test vapour will take place in the same room as that in which the animal holding cabinets are situated. The positions of animals within the cabinets will be altered weekly during the exposure period according to a pre-designed pattern.

Suspended polypropylene cages (North Kent Plastics, RM-2 type), 38 cm long, 25 cm wide and 18 cm high, with solid floor and sides and fitted with a stainless steel wire top will be used during the mating period for both generations and for the first week for the F1A generation.

During the mating period, animals (the same mating pair throughout) will be housed on the basis of one male to one female in plastic breeding cages (North Kent Plastics, RM-2 type).

At the end of the mating period the males will be rehoused with their former cagemates in stainless steel cages and the females will be housed in individual breeding cages (North Kent Plastics, RM-2 type) for the birth and rearing of young. Suitable nesting material will be provided (see Addendum 2). All animals will be given free access to pelleted diet and tap water. Individual water bottles will be supplied to each cage of animals, except during the six-hour exposure period. (Quality Assurance Aspects for Food and Water are presented in Addenda 3 and 4). These analytical data will be lodged in HRC Archives.

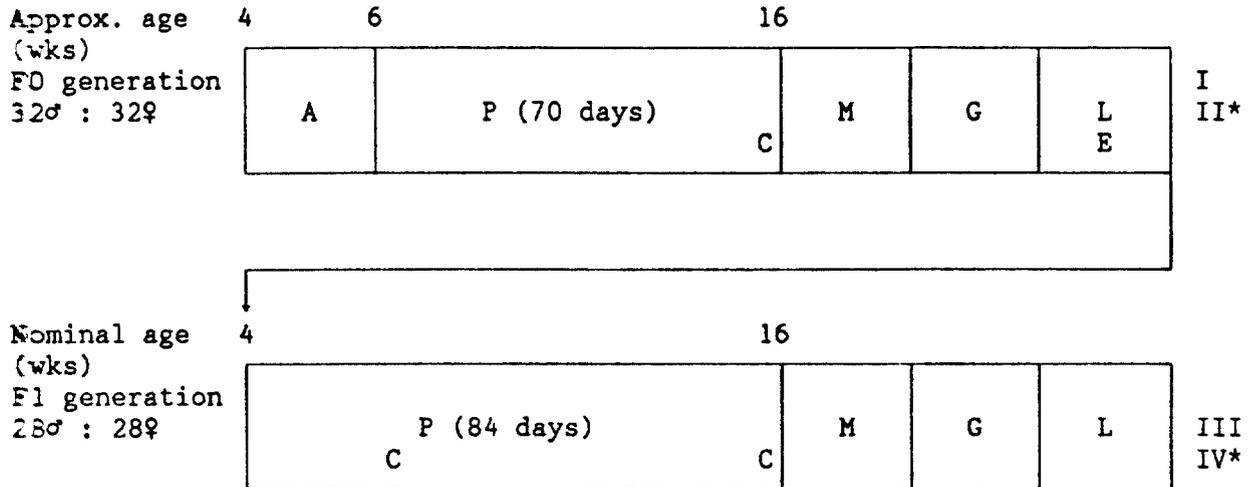
Throughout the study each cage will be identified by a label coloured according to group and recording study schedule number, animal number(s) and details of treatment.

EXPERIMENTAL DESIGN

The four experimental groups are constituted as follows:

Group/ colour code	HCFC 123 concentration of (ppm)	Number of rats per sex		Animal numbers			
				F0		F1	
		F0	F1	♂	♀	♂	♀
1: White	0	32	28	1- 32	161-192	321-348	461-488
2: Yellow	30	32	28	33- 64	193-224	349-376	489-516
3: Blue	100	32	28	65- 96	225-256	377-404	517-544
4: Green	300	32	28	97-128	257-288	405-432	545-572
5: Red	1000	32	28	129-160	289-320	433-460	573-600

Treatment of the F0 males and females by inhalation will begin when they are approximately 6 weeks of age, and will continue until all litters are weaned. Direct treatment of the F1A (F1B) males and females will be considered to commence when they are approximately 4 weeks of age (a typical age range being 23 to 32 days of age) and will continue until all F2 litters are weaned.

Diagrammatic Summary of Study SequenceFor each groupKey

- A = Acclimatisation period
 P = Premating treatment
 M = Mating
 G = Gestation
 L = Lactation
 C = Clinical chemistry
 E = Determination of fat content in milk

Notes

- I Select 28♂ + 28♀ for the F1A (F1B) generation. Macropathology, all excess pups. Organ weights + tissue preservation, 1♂ + 1♀ per litter.
 II Sacrifice FO males & females - macropathology, organ weights + tissue preservation all animals*.
 III Macropathology, all pups.
 Organ weights + tissue preservation 1♂ + 1♀ per litter.
 IV Sacrifice F1A males & females - macropathology, organ weights + tissue preservation, all animals.*
 * Histology; restricted, in the first instance, to tissues from control animals and at the highest exposure level concentration.

Initiation of 32 FO animals of each sex per group should allow 28 F1 animals of each sex to be obtained by selection of 1 male and 1 female from the appropriate number of litters with chronological outliers excluded. The use of 28 F1 animals of each sex should result, unless the pregnancy rate is adversely affected by treatment, in at least 20 pregnant F1 females per group.

SUMMARY OF STUDY SEQUENCE

I FO GENERATION

Rearing Animals of the FO generation will be 6 to 7 weeks of age at the commencement of exposure. They will be exposed to their test atmospheres for at least 70 days prior to mating, i.e. until they are approximately 16 weeks of age.

First mate During the mating period, males and females will be paired together on the basis of 1 male to 1 female. The same animals will be paired together throughout the 20 day mating period regardless of the outcome of the vaginal smear and will only be separated during the daily inhalation exposure regime.

At the end of the mating period, the females will be allowed to give birth.

The dams will be allowed to rear their young to Day 21 post partum.

Selection At Day 21 post partum 28 male and 28 female pups per group will be selected to form the basis of the F1A generation.

The pups will be selected from as many litters as possible.

Following selection of the F1A pups excess pups will be sacrificed and examined macroscopically. For one male and one female pup per litter, specified organs will be weighed and tissues preserved [against contingency of histopathological examination]. For each sex the animals selected will be the median weight weanling remaining after random selection of the F1 animal for rearing.

For Groups 1 and 5 only, a second weanling of each sex will also be chosen by random selection provided it is not the same weanling nominated by the median method.

[In the event of equivocal findings at the first mate it may be prudent to proceed to a second mate with retention of selected F1A pups.

In a study confined to a single mate and in which some partners fail to give birth it may be possible to remate with new partners].

[] Optional item - not covered in basic price

Sacrifice Shortly after the F1A pups have weaned, F0 males and females will be sacrificed and examined macroscopically. From all adults, specified organs will be weighed and a full range of tissues preserved. Histopathological examination will be confined to the reproductive tract.

II F1A GENERATION

Rearing The selected F1A animals will be exposed to their respective atmospheres from nominal week 4* for at least 84 days prior to mating, i.e. until they are approximately 16 weeks of age.

First mate During the mating period, males and females will be paired together on the basis of 1 male to 1 female. The same animals will be paired together throughout the 20 day mating period regardless of the outcome of the vaginal smear and will only be separated during the daily inhalation exposure regime.

The dams will be allowed to rear their young to Day 21 post partum.

On or shortly after Day 21 post partum pups will be sacrificed, and examined macroscopically. For one male and one female pup per litter specified organs will be weighed and tissues preserved [against contingency of histopathological examination].

[In the event of equivocal findings at the first mate it may be prudent to proceed to a second mate].

[In a study confined to a single mate and in which some partners fail to give birth it may be possible to remate with new partners].

* Age at which direct treatment considered to commence

Sacrifice Shortly after the last F2A pups have weaned, F1A males and females will be sacrificed and examined macroscopically. From all adults, specified organs will be weighed and tissues preserved. Histopathological examination will be confined to the reproductive tract.

III HISTOPATHOLOGICAL EXAMINATION

1. The preservation of a large range of tissues (see Observations, Section 3) from adults and weanlings of all generations allows the appropriate degree of histopathological examination indicated by the results being obtained.

Any investigations would usually be restricted in the first instance to the control group and at the highest exposure concentration. In the event of any unusual or unexpected observations at the highest exposure level, examination would automatically proceed to the lower level.

The tissues to be submitted to histopathological examination are detailed in observations, Section 3, "Terminal Studies".

OBSERVATIONS

1. Adult animals

The following procedures will be performed on F0, F1A adults.

(a) Signs

All animals will be regularly handled and examined for obvious changes or signs of reaction to treatment.

(b) Mortalities

Any rat which shows marked signs of ill health or reaction to treatment will be isolated and/or killed to prevent cannibalism or autolytic degeneration.

All animals that die or are killed for reasons of animal welfare will be weighed and subjected to post mortem examination to establish, if possible, the cause of death.

(c) Food consumption

Food intake of rats will be recorded. However, at various phases, the data may be of limited value. Consumption will be measured in two day blocks for the first two weeks of treatment, thereafter weekly throughout the pre-mating and mating period. Measurement will continue for males on a weekly basis and for females will be re-introduced from Day 1 up to and including Day 14 post partum.

If there is any spillage of food within the cage, or onto trays under the cage, where possible this will be weighed together with the residue in the hopper. The raw data will be annotated if the spillage is considered excessive and the data will be excluded from calculation of means.

During the pre-mating phases the food conversion ratios will be calculated.

(d) Water consumption

Water consumption will be measured on a daily basis during the initial two and last two weeks of the pre-mating treatment period for each generation.

(e) Bodyweight change

All animals will be weighed at the start of each generation (i.e. approximately 6 to 7 weeks of age for F0 generation, nominal 4 weeks of age (a typical age range being 23 to 32 days of age) for the F1A generation). For the first two weeks of treatment each animal will be weighed on alternate days and, subsequently at weekly intervals.

During the mating periods all females will be weighed daily throughout and daily weighing will continue until parturition. The occurrence of a positive indication of mating (i.e. sperm or plug) will be considered as Day 0 of pregnancy. Weights will be reported for Days 0, 7, 14, 17 and 20 of pregnancy or those considered more appropriate. Weights of pregnant animals without a positive indication of mating will be reported for appropriate days taken retrospectively from birth.

Dams that litter will be weighed on Days 0, 4, 7, 14 and 21 post partum. Additional weigh days may be added for the F1A generation if effects are suspected in the F0 generation.

(f) Pregnancy rate

Pregnancy rate will be determined as the percentage of surviving paired females that become pregnant.

(g) Mating performance

Vaginal smears will be taken daily, prior to exposure, for the seven days before and during the 20-day mating period, or longer if indicated, to enable the number of animals that mate on specific days to be determined, this information will be used in conjunction with other data:

- (i) to determine whether or not pregnancy occurs and, if so, continues uninterrupted after mating.
- (ii) to detect marked anomalies of the oestrous cycle
- (iii) to determine the Day 20 of pregnancy at which time the females stop being exposed to test atmospheres
- (iv) to determine the median pre-coital time and duration of pregnancy for dams which litter.

(h) Duration of pregnancy

The duration of pregnancy for females which litter will be taken as the time between the day of successful mating and the day on which pups are first seen.

(i) Terminal sacrifice

From all adults, specified organs will be weighed and a full range of tissues preserved; for histopathological examination, see "Terminal studies". In addition, any tissue showing macroscopic abnormality will be similarly preserved.

The uteri of apparently non-pregnant females will be examined by the Salewski technique^a prior to storage in fixative. The reproductive tract of all apparently infertile males and females will be examined histologically.

2. Litter data

(a) Birth (Day 0) to Day 21 post partum

The following procedure applies to all litters.

As soon as possible after parturition, the young will be counted, individually identified within the litter by toe amputation, sexed, weighed and examined for external abnormalities. Keeping nest disturbance to a minimum all litters will be examined daily for dead and/or abnormal young. The pups will also be weighed on Days 2, 4, 7, 14, 18 and 21 post partum.

On Day 4 post partum the pups will be weighed and where possible the litter standardised to a total litter size of eight pups, four male and four female, if available by random selection for each sex. No pups will be culled from litters of eight or less regardless of sex ratio.

Culled young will be routinely subjected to an autopsy unless precluded by autolysis. Dead young will also be subjected to an autopsy. Pups with suspected abnormalities will be preserved either in Bouin's solution if it is considered that free-hand sectioning or histopathological examination would be of value or in alcohol for subsequent staining with alizarin if skeletal defects are suspected.

(b) Selection of pups for organ weight analysis and tissue preservation

One male and one female pup from each litter in the group will be selected by median bodyweight for organ weight analysis and preservation of tissues as detailed in Section 3, "Terminal studies" [against the contingency of histopathological examination]. In addition for Groups 1 and 5 only, one weanling of each sex will also be chosen by random selection provided it is not the same weanling nominated by the median method.

(c) Terminal sacrifice of excess pups

On or shortly after Day 21 all excess pups will be sacrificed and examined externally and internally for abnormalities. Sex of the pups will be confirmed by gonadal inspection. Any tissues showing macroscopic abnormality will be preserved in neutral buffered 10% formalin to permit histological examination if required.

(d) Selection of pups for next generation

The following procedure applies to F1A litters:

On Day 21 post partum one male and one female pup in each of 28 litters per group will be retained for further study. The 28 litters will be those weaned as close to the median weaning date as possible for the study or group but paying attention to retaining as wide a genetic pool as possible. Selection of pups will be made using computer generated random number tables. Where less than 28 litters in a group are weaned or are within a reasonable range of weaning dates, or where all pups in a litter are of one sex, the complement may be restored by taking a second pup from an appropriate litter. (see Assessment of Results). Records of lineage will be maintained.

Selected animals will be individually identified by a tail mark from selection at 21 days of age until week 4 when animals will be tattooed (earmarked).

(e) Post weaning development

The onset of vaginal opening will be monitored in all selected females from 28 days post partum.

The occurrence of cleavage of the balanopreputial skinfold will be monitored in all selected males from 35 days post partum.

3. Laboratory investigations

(a) Biochemical/hormonal assays

When F0 and F1 animals are 14 weeks of age, samples of blood will be obtained from 12 animals/sex/group and will be withdrawn under light ether anaesthesia, from the orbital sinus.

The blood samples will be collected into heparin anticoagulant.

Animals will be fasted overnight prior to the laboratory investigations.

The estimations to be performed are listed overleaf together with an abbreviated title (for use in appendices and tables), the methods and units of measurement applicable at the time.

	<u>Units</u>
Cholesterol - Total (Chol) (using a Hitachi 737 Clinical Chemistry Analyser)	mg/dl
Esterified Cholesterol (Est Chol) (by subtraction - Total Chol - Free Chol)	mg/dl
Free Cholesterol (Free Chol) (using a Hitachi 737 Clinical Chemistry Analyser)	mg/dl
High Density Lipoprotein Cholesterol (HDL Chol) (using a Hitachi 737 Clinical Chemistry Analyser)	mg/dl
Low Density Lipoprotein Cholesterol (LDL Chol) (using a Hitachi 737 Clinical Chemistry Analyser)	mg/dl
Very Low Density Lipoprotein and Chylomicron Cholesterol (by subtraction - Total Chol - {HDL + LDL Chol})	mg/dl
Follicle Stimulating Hormone (FSH) (radioimmunoassay using Amersham rat FSH kit)	ng/dl
Luteinising Hormone (LH) (radioimmunoassay using Amersham rat LH kit)	ng/ml
Oestradiol (Oestr) (radioimmunoassay using Diagnostic Products Corporation test kit)	pg/ml
Progesterone (Prog-st) (radioimmunoassay using Diagnostic Products Corporation test kit)	ng/ml
Testosterone (Testo-st) (radioimmunoassay using Farmes Diagnostic Products Corporation test kit)	ng/ml
(b) <u>Fat content of milk samples</u>	

During the second week of lactation (Day 11), samples of milk will be obtained from 12 litters/group (the method is still to be determined) and analysed for total fat content.

4. Terminal studies

The following procedures apply to all F0, F1A adults and in selected weanlings.

The following organs will be weighed:

uterus (with cervix)
ovaries
prostate (with seminal vesicles coagulating gland) - adults only
pituitary - adults only
liver (adults only)

testes (weighed individually, identified as left or right and maximum length, breadth and width measurement recorded)
epididymides (weighed individually and identified as left or right)

Where appropriate, (paired) organs will be weighed individually.

Initially, histology will be restricted to the following tissues (see List of sample tissues) and will be performed as follows:

1. Reproductive tract associated tissues and target organs (indicated by asterisk (*))

all F0 and F1A adults of the control group and at the highest exposure concentration;

any apparently infertile male(s) or female(s) from the remaining concentrations.

The routine stain used will be haematoxylin and eosin.

For testes transverse paraffin embedded sections of all fixed testes will be made at 2 μ m and stained with PAS haematoxylin for examination of the seminiferous tubules. For examination of the interstitial cells, sections will be stained with haematoxylin and eosin.

Frozen sections of F0 liver (previously fixed in formalin) will be cut on a cryostat at 12 μ m and stained for glycogen using PAS.

List of sample tissues

The following tissues will be preserved in buffered 10% formalin (except eyes, preserved in Davidson's fluid and testes preserved in Bouin's fixative).

adrenals	oesophagus
aorta	*ovaries
bone (femur)	*pancreas
bone marrow (sternum)	*pituitary
brain	*prostate
cranial vault (for	rectum
lachrymal glands,	salivary gland
teeth, nasal turbinates,	*seminal vesicles (with coagulating
inner ear)	gland)
caecum	sciatic nerve
colon	skeletal muscle
duodenum	skin
eyes	spinal column (vertebral column)
heart	spleen
ileum	stomach
jejunum	*testes (both ts)
kidneys	*epididymides
*liver	thymus (if present)
lungs	thyroids (with parathyroids)
lymph nodes	tongue
(cervical/mesenteric)	trachea (with larynx and pharynx)
mammary gland	urinary bladder
macroscopically abnormal	*uterus (with cervix)
tissues	*vagina

* Microscopic examination restricted to identified tissues

Note: For weanling animals appropriate tissues will be removed and weighed prior to preservation and the carcass will be preserved with all other tissues in situ

ASSESSMENT OF RESULTSCaveat

The following should be considered as provisional or "first pass" methodology. Essentially it is general purpose and influenced by the prevalence of results that are "negative" in respect of demonstrating selective effects on reproduction. Where results are "equivocal" or "positive", alternative methods of data presentation and analysis may be more appropriate.

Individual litter values

In assessing litter parameters for each litter reared to weaning, pup loss at birth will be calculated as a percentage from the formula:

$$\frac{(\text{Total no. of young at birth} - \text{no. of live young})}{\text{Total no. of young at birth}} \times 100$$

Cumulative pup loss will be similarly calculated from the formula:

$$\frac{(\text{Total no. of young at birth} - \text{no. of live young at Day } x)}{\text{Total no. of young at birth}} \times 100$$

where x is the day of weighing the litter.

Litter weight and mean pup weight will be calculated from individual pup weight.

Sex ratios at birth will be calculated from the formula:

$$\frac{\text{Total no. of males}}{\text{Total no. of young}} \times 100$$

at weaning the formula will be:

$$\frac{\text{No. of males at weaning}}{\text{Total no. of young at weaning}} \times 100$$

The following reproductive indices will be calculated.

1. Copulation index

$$= (\text{number of rats with proven copulation} / \text{number of mated rats}) \times 100$$

2. Fertility index

$$= (\text{number of pregnant rats} / \text{number of rats with proven copulation}) \times 100$$

3. Gestation index

$$= (\text{number of females with live births} / \text{number of pregnant females}) \times 100$$

The following indices on the growth of pups will be calculated.

1. Viability index

$$= (\text{number of surviving pups at the day of examination} / \text{number of live borns or pups alive at weaning}) \times 100$$

2. Weaning index

$$= (\text{number of pups alive at weaning} / \text{number of live pups}) \times 100$$

Group values

Group mean values from individual litter values, may be presented where appropriate in two ways:

Mean A: includes all valid data from surviving animals that provide evidence of pregnancy including those subsequently losing the entire litter, i.e. total resorption or failure to wean any young.

Mean B: includes valid data from any animals with viable young at termination.

Mean B generally has more meaning when group size is low in which case the mean values would be unduly influenced by the presence of one or two animals totally losing their litters. Mean A may be a more accurate index when several litters are completely lost.

For litter and mean pup weights and abnormality values only Mean B values or the equivalent will be calculated.

Statistical analysis

Statistical analyses are routinely performed on litter data. If appropriate, other parameters may be analysed. Significance tests will normally be two-tailed. If distribution is markedly skewed one tailed criteria will apply.

Litter data

The basic sample unit is the litter and, due to the preponderance of non-normal distributions, non-parametric analyses have generally proved the most consistent and robust.

Mean values of litter size, post implantation loss, litter weight, mean pup weight and the incidence of anomalous offspring will generally be analysed by the Kruskal-Wallis test¹. Intergroup comparisons can be made either by the non-parametric equivalent of the 't' test together with the Jonckheere test for an ordered series of treatments¹ or by the non-parametric equivalent of Williams' test². Only the test(s) considered to be appropriate will be reported.

Where 75% of the values for a given variable consist of one value, a Fisher's exact test³ will be used and where appropriate, Mantel's test for trend in proportions⁴ may also be performed.

Other parameters

The following parameters may be analysed:

Pregnancy rate, maternal mortality, total litter loss and mean maternal bodyweight change.

Incidence values will usually be analysed using Chi-square test⁵ or Fisher's exact test³; and where appropriate Mantel's test for trend in proportions⁴ may also be performed.

Bodyweight data will usually be analysed using analysis of covariance (or variance)⁵ followed by Williams' test⁶.

Additional or alternative statistical methods will be used when considered appropriate. For example in a 2 generation study the true basic sample unit may be considered to be the F0 mating pair. The simple routine analysis may need to be adjusted or replaced in the event of uneven derivation of the F1A as may occur if animals are remated or if more than 1 F1A animal per sex is derived from a litter to restore the complement.

LOCATION OF STUDY RECORDS

All specimens, raw data and other documents generated at HRC during the course of this study will be lodged in the Huntingdon Research Centre Ltd., Archives, Huntingdon, England.

Any such material arising from investigations made by the Sponsor or other laboratory, the findings of which are included in the final report, will be retained either by the organisation conducting these investigations or by ERC.

All study related specimens and raw data lodged in the Huntingdon Research Centre Ltd., Archives will be kept for ten years after the issue of the final report and then discarded. The Sponsor will be notified of this date and given the option of receiving this material into their own archives.

A hard copy of the final bound report will be kept in the Huntingdon Research Centre, Archives.

GOOD LABORATORY PRACTICE

This study will be conducted in compliance with the principles of Good Laboratory Practice as set forth in:

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision Department of Health, 1989.

Organisation for Economic Co-operation and Development ISBN 92-64-12367-9, Paris 1982.

United States Environmental Protection Agency, (FIFRA) Title 40 Code of Federal Regulations Part 160, Federal Register, 29 November 1983, and subsequent amendment Federal Register 17 August 1989.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

STAFF SAFETY

The standard safety precautions operating within the Department will apply to this study.

Personnel entering the animal room will wear protective clothing.

AMENDMENT TO PROTOCOL

The HRC Study Director or Study Supervisor will contact the Sponsor's Monitoring Scientist by telephone or telex in the event of any unexpected findings or occurrence considered likely to be of significance.

It is not intended to make any amendment to this protocol without authorisation of the Sponsor, however, in the event of difficulty in contacting the Sponsor, and/or for reasons of animal welfare and/or for the protection of scientific integrity, the testing laboratory must retain the right to take independent action.

QUALITY ASSURANCE DEPARTMENT REVIEW

The Quality Assurance Department will conduct inspections of the various phases of the study and of certain repetitive operations, at the intervals required by the FDA Good Laboratory Practice Regulations. The dates on which the findings of these inspections are reported to the Study Director and to HRC Management will be specified in the final report.

The final report will be reviewed by HRC's Quality Assurance Department, comparing individual findings against raw data and comparing the statements and results presented in the report with individual data presented in the Appendices of the report.

REFERENCES

- a Salewski, E. 1964 Farbemethode zum makroskopischen Nachweis von Implantationsstellen am Uterus der Ratte. Naunyn-Schmiedeberg's Arch. exp. Pathol. Pharmacol. 247 : 367.

STATISTICAL REFERENCES

1. Hollander, M & Wolfe, D.A. Non-parametric statistical methods 1973 Publ. J. Wiley & Sons, NY.
Kruskal-Wallis & Jonckheere tests: page 114-132.
Wilcoxon test : page 68-75.
2. Shirley, E. 1977 A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. Biometrics 33 : 386-389.

3. Fisher, R.A. 1950 Fisher's exact test for 2 x 2 contingency table : 'Statistical Methods for Research Workers' para. 21.01 Oliver & Boyd, Edinburgh.
4. Mantel, N. 1963 Chi-square tests with one degree of freedom: Extensions of the Mantel Haenszel procedure. J. Amer. Stat. Ass. 58 : 690-700.
5. Snedecor, G.W. & Cochran, W.G. 1967 'Statistical Methods' 6th ed. The Iowa State University Press.
6. Williams, D.A. 1971/2 Williams' test for comparing the effect of increasing doses of a substance with a zero dose. Biometrics 27 : 103-117. Biometrics 28 : 519-531.

ADDENDUM 1

Inhalation exposure method

Exposure chambers

The test substance will be administered by employing the whole-body exposure technique. The chambers are manufactured from stainless steel, and have stainless steel framed, glass panelled doors. The internal volume of each chamber is approximately 2.43 cubic metres. The air supply to the chambers is independent of the animal room supply. Both air supplies are filtered and conditioned. Operation of the chambers will ensure approximately 16 air changes an hour and uniform distribution of the test vapour throughout the body of each chamber. Chamber temperature and humidity will, ideally, remain within the limits $21 \pm 3^{\circ}\text{C}$ and $55 \pm 15\% \text{RH}$. Excursions outside these ranges will be documented as to extent and duration. The temperature and humidity within each chamber will be recorded at 30-minute intervals.

The volume flow of air will be monitored by a Venturi nozzle flow meter. Chamber air flows will be monitored and recorded automatically. Inlet and outlet air flows will be adjusted so that the internal pressure of the chambers is maintained 1 to 10 mm water below exposure room ambient.

Animal housing during exposure

The rats will be housed in stainless steel suspended cages during exposure. Each rat is housed individually. The position of the rats will follow a pre-arranged distribution designed to minimise possible spatial bias and to maximise male/female proximity without resulting in bodily contact. The position of the rats will change on a weekly basis.

Generation of the test atmosphere

The test liquid, under pressure in stainless steel reservoirs, will be fed through fine control needle valves to each of 4 coiled copper tubes, one for each level of exposure, immersed in a water bath maintained at 80°C . The vapour produced will be mixed with diluent air at 25 litres/minute and conveyed through stainless steel tubing to the inlet air duct of each appropriate chamber.

Chamber monitoring

Chamber environmental parameters including airflow, temperature, relative humidity, test substance usage, nominal and analysed vapour concentrations will be displayed and recorded at intervals throughout exposure. Control of chamber monitoring is effected by a computer program linked to the various monitoring devices, and containing the relevant calibration/calculation data necessary for the presentation of the recorded output.

ADDENDUM 2

Quality assurance aspects of nesting material

The nesting material used, designated "Goldchip" sawdust, is produced by SDS Ltd., P.O. Box 70, Witham, Essex, CM8 3AD. The sawdust is principally derived from UK grown Norway Spruce, Picea abies, the addition of small amounts of UK grown Scots Pine, Pinus sylvestris is permitted. Combination with hardwood species or imported wood is not permitted. No chemical preservative is applied to timber during processing or storage. The standards of production adopted by the manufacturers have been approved by the Quality Assurance Department.

As a precautionary measure a batch of sawdust is analysed for chemical contaminants every 3 months at a laboratory approved by HRC.

The maximum permitted levels of contaminants are:

Polychlorinated biphenyls	10 ppm
Pentachlorophenols	2.0 ppm
Dieldrin	0.1 ppm

The certificate of analysis is made immediately available to HRC.

ADDENDUM 3

Biosure Laboratory Animal Diet No. 1Composition and quality assurance aspects of diet

Biosure LAD is a fixed formula diet suitable for normal health, growth and reproduction of laboratory rats and mice. Each batch of diet is analysed for nutrients, possible contaminants and micro-organisms, likely to be present in the diet, and which, if in excess, may have an undesirable effect on the test system.

Prior to release of diet for use HRC Quality Assurance Department checks each certificate of analysis for conformity with the specification detailed below. Occasional slight deviations to this specification may be permitted.

<u>Nutrients</u>	<u>Target level</u>	<u>Tolerance %</u>	<u>Acceptable range</u>
Moisture	9.5	+10	10.5 % max
Crude fat	3.7	±15	3.1 - 4.3 %
Crude protein	21.5	±10	19.4 - 23.7 %
Crude fibre	2.0	±40	1.2 - 2.8 %
Ash	5.5	±15	4.7 - 6.3 %
Calcium	1.0	±20	0.8 - 1.2 %
Phosphorus	0.9	±20	0.7 - 1.1 %
Sodium	0.3	+100-50	0.15 - 0.60 %
Chloride	0.5	+100-50	0.25 - 1.0 %
Potassium	0.8	+100-50	0.4 - 1.6 %
Magnesium	0.15	±50	0.08 - 0.23 %
Iron	220	±50	110.0 - 330 mg/kg
Copper	15	±50	8.0 - 23 mg/kg
Manganese	70	±50	35.0 - 105 mg/kg
Zinc	60	±50	30.0 - 90 mg/kg
Vitamin A	12	+50-20	9.5 - 18 iu/g
Vitamin E	35	+150-20	28 - 88 mg/kg

ContaminantsMaximum concentration

Fluoride	40 mg/kg
Nitrate (as NaNO ₃)	200 mg/kg
Nitrite (as NaNO ₂)	10 mg/kg
Lead	2.5 mg/kg
Arsenic	1.5 mg/kg
Cadmium	0.5 mg/kg
Mercury	0.1 mg/kg
Selenium	0.6 mg/kg
Total Aflatoxins	5 mcg/kg
Total P.C.B.	50 mcg/kg
Total D.D.T.	150 mcg/kg
Dieldrin	50 mcg/kg
Lindane	150 mcg/kg
Heptachlor	50 mcg/kg
Malathion	5000 mcg/kg

ADDENDUM 3
(continued)

Microbiological contents

Maximum concentration

	LAD 1 (nuts)
Total viable organisms	10,000
Mesophilic spores	30,000
Salmonellae species	0
Presumptive E. coli	0
E. coli type 1	0
Fungal units	1,000
Antibiotic activity	0

Date ref: 01/91

ADDENDUM 4

Quality assurance aspects of water

Results of the routine physical and chemical examination of drinking water at source (Grafham Final Water - Huntingdon North supply zone) as conducted usually weekly by the supplier - Anglian Water Services Ltd. - were made available to HRC as quarterly summaries.

These summaries of source water included levels of:

Nitrites		Potassium	(K)
Nitrates		Phosphorus	(P)
Calcium	(Ca)	Chlorine	(Cl)
Magnesium	(Mg)	Silicon	(Si)
Sodium	(Na)	Iron	(Fe)

Additionally, levels of substances which are known to be present occasionally in local water and which, if present at levels in excess of recommended maxima (for humans) might have had undesirable effects on the test system, were determined in HRC tap water at approximately 6-monthly intervals.

Six-monthly analyses of HRC tap water included levels of:

Arsenic	(As)	Barium	(Ba)
Selenium	(Se)	Silver	(Ag)
		Antimony	(Sb)

organophosphorus, organochlorine and other pesticides, haloforms, chlorophenols, polychlorinated biphenyls and polycyclic aromatic hydrocarbons.

Date ref: 09/90



Protocol reference: INH0512

HUNTINGDON RESEARCH CENTRE LIMITED
DEPARTMENTS OF INHALATION TOXICOLOGY AND
REPRODUCTIVE TOXICOLOGY
HUNTINGDON, ENGLAND

FINAL PROTOCOL
(24 pages)

HCFC 123

NEUROTOXICITY STUDY IN RATS

HRC Schedule Number:

Sponsor:

Dr G M Rusch
Chairman, PAFT Toxicology Committee
Department of Toxicology
Allied Signals Inc.
PO Box 1139R
Morristown
New Jersey 07962
USA

Date:

5 June 1992

Circulation list: Client (2), G Malinverno (Domicare), QAD, Main Files,
D W Coombs, C J Hardy, G C Clark, I J Sharman, T J Kenny, W A Gibson,
Slide Processing, L Cooke, C Gopinath, D J Lewis, E W Hughes



INTRODUCTION

The purpose of this study, to be performed at the Huntingdon Research Centre Limited, Huntingdon, England (HRC), is to assess the possible neurotoxic effects of HCFC 123 in rats, when administered by inhalation, for 13 weeks. The study includes rats which will be kept for a 4-week withdrawal period. The rats will be exposed on 5 days of each week.

The test substance is to be administered by inhalation, a possible route for exposure in man. The rat is the species of choice due to regulatory requirements and the strain is selected on account of the availability of background data, relating to neuropathology and behavioural parameters, at our laboratories.

The procedures to be used during the course of this study are documented in the relevant HRC Standard Operating Procedures Manuals.



1. Personnel

1.1 Huntingdon Research Centre Limited

1.1.1	Study Director:	C.J. Hardy.
1.1.2	Study Supervisor:	D.W. Coombs
1.1.3	Veterinary Director:	D.P. Buist
1.1.4	Principal Pathologist:	C. Gopinath
1.1.5	Scientist responsible for behavioural observations:	E.W. Hughes

NOTE In the temporary absence of the Study Director, the responsibilities of this role will be assumed by G. C. Clark, Head, Department of Inhalation Toxicology.

1.2 Sponsor

1.2.1	Study Monitor for PAFT:	Dr. G. Malinverno, Occupational Health Department, Domicare-Ferruzz/Montedison Group, Via Principe Eugenio 5, 201555 Milano, ITALY.
-------	-------------------------	--

2. Personnel safety

HCFC 123 is a low boiling point liquid of low toxicity. All precautions will be taken to minimize the risk of exposure of personnel involved in the study.



3. Test system

- 3.1 Animals: Crl:CD®(SD) BR rats, a Caesarean-derived strain of Sprague-Dawley origin.
- 3.2 Supplier: Charles River, Portage, Michigan, USA.
- 3.3 Number: 90 (45 males and 45 females).
- 3.4 Age: Approximately 6 weeks old on delivery.
- 3.5 Weight: According to age. Animals will be ordered within a weight range of ± 10 g for each sex.

The rat is selected for use in this study for the following reasons:

- to satisfy regulatory guidelines for testing in a rodent species;
- methods for assessing neurotoxic endpoints are well-defined in this species;
- the rat can be used in sufficient numbers to permit statistical evaluation of the study results.

The strain is selected for reason of the availability of comprehensive data, relating to behavioural and neuropathological parameters, at this laboratory.

- 3.6 Health inspection: The animals delivered for the study will be inspected by a senior animal technician prior to their allocation to the experimental groups. Any unsuitable animals will be discarded at this time. However, if 10% or more of the animals show signs of ill-health rather than physical damage the Veterinary officer will be informed. If, in the opinion of the Veterinary Officer, the animals are considered to be not suitable for use in the study, the entire batch of animals will be discarded and a new batch ordered.
- 3.7 Group allocation: Each animal will be assigned a temporary number, weighed and, when applicable, the required number of animals (including those intended for the reserve group) selected by, for each sex, discarding those animals furthest from the mean body weight. The remaining animals will then be randomly assigned to cages, stratified by body weight, in such a way that the cage means will be approximately equal. The appropriate numbers of cages will then be allocated to the experimental groups.
- 3.8 Identification: Each rat will be individually identified by a number tattooed on the ear pinnae.



- 3.9 Acclimatisation: The period of acclimatisation will be 2 weeks approximately.
- 3.10 Reserve animals: Rats allocated to the reserve group will be used to replace any rat considered unsuitable for experimentation, (e.g. low body weight gain and/or inappetence, unusual behaviour pattern) or which dies during the acclimatisation period. Remaining reserve animals will then be killed.

4. Accommodation and husbandry

- 4.1 Location of study: Building Y14.
- 4.2 Cage type and size: Stainless steel mesh on front, back and floor with stainless steel sheet sides: 35 cm wide, 53 cm long and 25 cm high. The cages are suspended on racks. Plastic trays, lined with absorbent paper, will be placed below the cages to collect animal waste.
- 4.3 Number of animals per cage: Five of the same sex.
- 4.4 Cage identification: Each cage will bear a coloured label identifying the group (see Appendix 1) and the numbers of the animals contained within it.
- 4.5 Cleaning of cages: The paper in the trays below the cages will be changed daily. The cages will be changed for clean cages at approximately 2-week intervals.
- 4.6 Room temperature and relative humidity: Will ideally be maintained within the limits $21 \pm 3^{\circ}\text{C}$ and $55 \pm 15\%$. Excursions outside these ranges will be documented as to time and duration of the event.
- Temperature and relative humidity will be monitored and recorded continuously using a Kent Clearspan recorder.
- 4.7 Lighting: Will be on a controlled 12-hour cycle; on 0800 - 2000 hours, off 2000 - 0800 hours.
- 4.8 Dry Diet: SDS Rat and Mouse No. 1 modified diet. An excess amount of diet will be available to the animals at all times except during inhalation exposures and as specified elsewhere in this protocol.
- 4.9 Water: Tap water, from individual bottles will be available to the animals at all times except during inhalation exposures and as listed elsewhere in this protocol. The bottles will be rinsed and refilled daily.



- 4.10 Analysis of food: The diet is a closed formula diet suitable for normal health, growth and reproduction of laboratory rats and mice. The standards of production adopted by the manufacturers have been approved by the HRC Quality Assurance Department.

Analyses will be made of all batches of diet for most nutrients and for specific substances and micro-organisms likely to be present in feed ingredients or the finished diet and which, if in excess of specified amounts, might have an undesirable effect on the test system. Batches of diet will conform with the standards agreed by the manufacturers and the HRC Quality Assurance Department

- 4.11 Analysis of water: The water supplied to HRC, by Anglian Water, is potable water for human consumption. Anglian Water takes its guidelines on water quality from the EEC directive relating to water for human consumption, viz: Council Directive 80/778/EEC.

Results of routine physical and chemical examination of drinking water at source as conducted, usually weekly by the supplier, are made available to HRC as quarterly summaries.

These results include levels of:

Nitrites	Potassium	Chloride
Nitrates	Silicon	Iron
Calcium	Arsenic	Selenium
Magnesium	Barium	Silver
Sodium	Antimony	Phosphorus

as well as concentrations of pesticides, related products, polycyclic aromatic hydrocarbons, haloforms, chlorophenols and polychlorinated biphenyls.

**5. Test substance****5.1 Test substance:**

- (a) Common name: HCFC 123
- (b) Chemical name: 2,2 Dichloro-1,1,1-Trifluoromethane
- (c) Presentation: Liquid
- (d) Received from: ICI Chemicals and Polymers Limited
The Heath
Runcorn
Cheshire
- (e) On: 8 May 1991
- (f) Drum numbers: 16005
5803
10593
16723
- (g) Purity: 99.86 - 99.95%
- (h) Expiry date: Not stated.

5.2 Storage: Ambient, outdoor temperature and humidity.

5.3 Disposal: Unused test substance remaining at the end of the study will be returned to the Sponsor. Costs incurred in return of the test substance will be the responsibility of the Sponsor and will be advised separately. Any such costs are not included in the study cost.

6. Route of administration

The inhalation route is selected since this is a possible route of accidental exposure in man.



7. Administration

- 7.1 Exposure levels: The exposure levels have been selected by the Sponsor and are 300, 1000 and 5000 ppm.
- 7.2 Groups and exposure levels: The allocation of animals to the experimental groups, group numbers and exposure levels are shown in Appendix 1.
- 7.3 Exposure: The test substance will be administered by inhalation as described below.
 - 7.3.1 Exposure duration: A single six hour exposure on 5 days (Monday to Friday) of each week for 13 weeks.
 - 7.3.2 Exposure system. Rats will be exposed in whole-body exposure chambers constructed from stainless steel and glass. The internal volume of the chamber is approximately 750 litres.
 - 7.3.3 Atmosphere generation. Details of the atmosphere generation system are shown in Appendix 2.

8. Chamber conditions

- 8.1 Generation: Samples will be taken from each chamber at approximately hourly intervals using gas-tight syringes.
- 8.2 Method of analysis: Samples will be analysed immediately following collection by gas chromatography. The samples will be injected onto the GC-column via a gas loop. Details of the GC conditions will be provided in an amendment to this protocol.
- 8.3 Airflow: The total airflow through the chamber will be 150 litres per minute. This will be 40 litres per minute from the vapour generator and 110 litres per minute of diluent air. Airflow will be monitored by tapered tube flow meters and recorded at 30 minute intervals.
- 8.4 Chamber temperature and humidity: Chamber temperature and relative humidity will be monitored continuously using wet/dry bulb hygrometers and recorded at 30 minute intervals.



9. Clinical observations

Dated and signed records of all activities relating to the day by day running and maintenance of the study, as well as to the group observations and examinations as listed in this protocol, will be recorded in the study Day Book.

Dated and signed records of appearance, change and disappearance of clinical signs will be maintained on clinical history sheets for individual animals.

9.1 Daily checks: Throughout the study, all cages will be checked in the morning and again in the afternoon for dead or moribund animals.

9.2 Mortality and morbidity: Any animal found dead or dying during the acclimatisation period will be discarded and replaced by an animal from the reserve group. Any animal found dead or dying at any other time will be examined macroscopically and a full spectrum of tissues preserved.

Where possible, dead and moribund animals will be subjected to macroscopic examination on the day of death. Otherwise, the carcass will be placed inside a polythene bag and kept in a refrigerator (4°C) until examination on the following day.

9.3 Clinical signs: Signs of ill-health, together with any behavioural change or response to treatment, will be recorded. Individual animal records will be maintained on the basis of:

- any observation, considered to be of possible importance, made at any time during the study;
- a careful external examination made at weekly intervals commencing one-week prior to the start of dosing when special attention will be given to the detection of audible respiratory sounds.

During exposures, signs will be recorded as a group response where all visible rats appear to be reacting similarly to the test substance.

9.4 Body weight: The weight of each rat will be recorded weekly beginning one week before the start of dosing. During the dosing period, the body weights will be recorded before dosing on the day.

9.5 Food consumption: Food consumption by each cage of rats will be determined weekly commencing one week before exposures commence.



10. Behavioural observations

A functional observation battery as described in Appendix 3 will be carried out on the following occasions.

- prior to the start of exposures (all rats in the test groups)
- after 4 weeks exposure (all rats)
- after 8 weeks exposure (all rats)
- after 13 weeks exposure (all rats)
- after 4 weeks withdrawal (5 males and 5 females from each group)

Animals will not be exposed on the days on which the examinations are undertaken.

11. Terminal studies

Following behavioural examination, performed after 13 weeks of exposure, five male and five female rats in each group will be sacrificed and fixed by whole-body perfusion. The remaining 5 male and 5 female rats in each group will be killed and perfused at the end of a 4-week withdrawal phase.

11.1 Whole-body perfusion

Each rat will be anaesthetised by intraperitoneal injection of pentobarbitone sodium (Expiral® 200 mg/ml) at a dose of 2 ml/kg.

As soon as the pupillary reflex is abolished, thoracotomy will be performed and infusion will take place. Initially, heparinised saline will be infused followed by buffered 4% paraformaldehyde at a rate of approximately 25 ml/minute. The fixative flows under gravity from a 1 litre reservoir from a height of approximately 18 inches through a 16 gauge needle. The fixative will be introduced into the left ventricle and leave the right atrium of the heart.

Following perfusion/fixation, the brain of each rat will be removed and will be weighed. The carcasses will be stored in fixative prior to teasing out of nervous tissue in preparation for sectioning. The following tissues will be dissected out prior to embedding and sectioning:

- spinal cord
- sciatic and tibial nerves
- dorsal and ventral root fibres



11.2 Microscopic examination

Processing of nervous tissue will initially be confined to rats killed after the final exposure, during Week 14. Extension to rats killed at the end of the 4-week withdrawal phase will be carried out, at additional cost, if a treatment-related change is noted in rats killed during Week 14.

Sections for examination will be prepared as follows:

Brain	6 levels	H&E
Nerves - sciatic	2 levels	TB
sural	1 level	TB
tibial	1 level	TB
Spinal cord	2 levels (cervical and lumbar)	H&E
Dorsal root fibres	2 levels (One from C ₃ -C ₆ , One from L ₁ -L ₄)	H&E
Ventral root fibres	2 levels (One from C ₃ -C ₆ , One from L ₁ -L ₄)	H&E
Dorsal root ganglion	2 levels	H&E
Gasserian ganglion	1 level	H&E

H&E indicates paraffin wax sections stained with haematoxylin and eosin.

TB indicates plastic sections stained with toluidene blue



12. Reports

- 12.1 Interim reports: The Sponsor will be informed as soon as possible of any unexpected toxicity and/or response to administration of the test substance.
- 12.2 Final report: Five bound copies and one unbound copy of the report will be provided. The data presented in the report will include but not be limited to the following:

Strain, age, weight and source of animals used;
Description of the method of allocation of animals to groups;
Environmental conditions;
Description of system and procedures for inhalation exposure;
Results of the chamber concentration analyses;
Clinical signs;
Individual animal and group mean body weights;
Food consumption data by cage and group;
Individual histopathological findings;
Description of all statistical procedures used.

13. Archives

All raw data collected and a sample from each batch of test substance supplied during the study will be stored in our purpose-built archive facility. Study files will be microfilmed, with one copy stored in a separate location from the archive. Raw data will be stored for as long as the quality permits evaluation. Wet tissues will be stored no longer than 10 years from the date of submission of the study report. The Sponsor will be approached prior to disposal of any raw data.



14. Good Laboratory Practice and Quality Assurance

This study will be conducted in compliance with the principles of Good Laboratory Practice as set forth in:

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

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982.

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

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health and Social Security 1986 and subsequent revision. Department of Health, 1989.

The HRC Quality Assurance Department will review the protocol for GLP completeness, maintain a record of the study on its master schedule, inspect the study procedures and audit the final report for accuracy of presentation and collation of the raw data.

15. Amendments

Amendments to this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorises a protocol change verbally, such change will be honoured by HRC. However, it then becomes the responsibility of the Sponsor to follow such verbal authorisation with a written verification. All protocol modifications will be issued and signed by the Study Director and Sponsor.



APPENDIX 1

Experimental groups, animal numbers and exposure concentrations and schedule

<u>Group</u>	<u>Rat numbers</u>		<u>Concentration of the test substance in air</u>
	<u>Male</u>	<u>Female</u>	<u>ppm</u>
1 (Control)	1-10	41-50	0
2 (Low dose)	11-20	51-60	300
3 (Inter. dose)	21-30	61-70	1000
4 (High dose)	31-40	71-80	5000
Reserve	81-85	86-90	

*To be advised

Rats will be exposed for 6 hours a day for 5 days a week for 13 weeks.

The following colour codes will be used to identify cage labels and documentation associated with each group:

<u>Group</u>	<u>Colour code</u>
1	White
2	Yellow
3	Green
4	Red



INH0512

APPENDIX 2

EQUIPMENT AND PROCEDURES EMPLOYED IN THE GENERATION
HCFC 123 VAPOUR



INH0512

APPENDIX 2

(continued)

The vapour generation system comprises reservoirs of liquid HCFC 123, liquid delivery lines containing particulate filters, metering valves and copper coils immersed in a water bath maintained at a temperature of $60 \pm 2^\circ\text{C}$. Liquid is forced from the reservoir (under pressure with nitrogen) through the metering valves into the copper coils where evaporation takes place. The vapour is mixed with diluent air at 40 litres/minute and carried to the air entry points on the inhalation chambers. The liquid reservoir, liquid and vapour transport lines and metering valves are all constructed from stainless steel.

The upper curved surface of each liquid reservoir¹ is fitted with a central, screw thread, "O"-ring seal filler cap, a 3-way valve to allow pressurisation with nitrogen gas and pressure release following exposures and a safety "pop-off" valve set to operate at 5 p.s.i.g. above operating pressure. Each reservoir is mounted on an electronic load cell² and the weight of the contents is displayed continuously. The reservoirs are tared empty on the load cells prior to use.

¹ Newson Gale Ltd., 51, Norsey Road, Billericay, Essex CM11 1BG, England

² Huntleigh Industrial Controls Ltd., Load Cell Division, Portman Moor Ind. Estate, East Moors, Cardiff, South Glamorgan, CF22 2HB



APPENDIX 2

(continued)

Liquid is transported from the base of each reservoir to a metering valve³. Particulate filters (stainless steel, 7 μm pore size³) are incorporated into the liquid lines between the manifold and each valve to protect the valves. The liquid passes by the valves is led to the generator through tubing approximately 3 mm in diameter.

The copper coils are standard tubing of 1 cm diameter. The distal end of the liquid feed lines enters one end of the coiled tubing. Air, at a metered flow of approximately 40 l/min, measured using in-line rotameters, passes through a second copper coil prior to mixing with the vapour⁴. The vapour mixture produced leaves the generator and passes through stainless steel tubing to the exposure chamber.

Toggle valves in the liquid delivery lines allow any one or all 3 lines to be shut down quickly. All connections in the liquid delivery system are either welded or made with compression fittings.

To initiate vapour generation, the liquid reservoirs are filled with HCFC 123 by forcing the liquid, under pressure with nitrogen, from one of the supply drums; the amount of liquid in each reservoir is indicated by the load cell display. Sufficient liquid to produce vapour during the 6-hour exposure is transferred. The weight of each reservoir is recorded.

The temperature of the water bath is checked and the air supply to the generator is turned on and adjusted to the desired flow rate.

Each reservoir is pressurised with nitrogen gas and the toggle valves opened. At this stage, liquid flows from the reservoir through the control valves to the generator where the vapour was produced. Any adjustments to the liquid flows during the study, are made on the basis of the concentrations of HCFC 123 measured within the inhalation chambers⁵.

³ Nupro Co., Willoughby, Ohio 44094, U.S.A.

⁴ Compressed air source, particulate and carbon filtration, dew point approximately 2°C

⁵ Initial control valve settings will be established during trials which precede the start of animal exposures. Following the introduction of animals into the inhalation chambers, some adjustments to the control valve settings may be necessary. Thereafter, minimal adjustments only are usually required.



INH0512

APPENDIX 2

(continued)

Following daily exposures, the toggle valves are closed and the weight of each reservoir again recorded. The system is then depressurised.

The copper coils are cleared of residual liquid by continuing to pass air through them for approximately 20 min. The air supplies are then closed down. Any liquid left in the reservoir was is not drained but is simply topped up.



APPENDIX 3

Functional observational battery

This battery comprises 3 sets of observations. The first set of observations are performed while the animal is in its home cage. The second set of observations are performed in a test arena and the third set comprises handling/specific testing of the animal. The entire evaluation procedure requires 6 - 8 minutes per rat.

Home cage observations

Posture

- F = flattened, limbs may be spread out
- L = lying on side, limbs in air
- A = asleep, lying on side or curled up
- C = crouched over
- S = sitting or standing normally
- R = rearing
- J = jumping

Note: only A, S or R are normal

Convulsions - If present classify as:

Clonic

1. "chewing", clonus of the jaws
2. normal quivers of limbs, ears, head or skin
3. mild tremors or jerkings (stronger than seen normally)
4. severe whole-body tremors

Tonic

1. tonic, constant contraction and extension of hind limb muscles
2. opisthotonus - head, body and limbs rigidly arched backwards
3. emprosthotonus - head, body and limbs extended forward
4. popcorn - rat repeatedly pops in air
5. asphyxial - bout of severe clonic-tonic convulsions resulting in difficult respiration, post-ictal depression or death

Vocalisations (spontaneous) - present or absent

Palpebral closure - present or absent



APPENDIX 3

(continued)

If present rate as:

- 1 eyelids slightly drooping
2. eyelids drooping approximately half-way
3. eyelids completely shut

Now remove rat from cage and record:

Ease of removing rat from cage

1. very easy (totally limp)
2. easy (little/no resistance to being picked up)
3. moderately difficult (rat rears, often following investigator's hand)
4. rat freezes
5. difficult (runs around cage, or is hard to grab)
6. very difficult (tail and throat rattles)

Now while holding rat, note such things as bite marks, soiled fur appearance, missing toenails, emaciation (shallow stomach, prominent spinal vertebrae).

Now rate ease of handling rat in hand:

1. very easy (rat is totally limp)
2. easy (alert, limbs may be pulled up against body)
3. moderately easy
4. rat freezes (rigid in hand, with or without vocalisations)
5. difficult (squirming or twisting)
6. very difficult (excessive squirming/twisting, attempting to bite)

Salivation - present or absent

If present rate as:

1. slight
2. moderate
3. severe



APPENDIX 3

(continued)

Lacrimation - present or absent

If present rate as:

1. slight
2. moderate
3. severe

Palpebral closure (same as before)

Exophthalmus - present or absent

Pilo-erection - present or absent

Vocalisation on handling - present or absent

Now place rat on a flat surface covered with clean paper and observe for 3 minutes. During this time, the following observations are made:

Convulsions (same as before).

Level of activity in arena

1. none - animal does not move from where originally placed
2. some movement - some locomotor activity but mainly still
3. moving around arena with bouts of freezing/inactivity
4. moving around whole arena
5. hyperactive

Arousal - alertness of animal

1. very low (coma)
2. low (slight head and body movements)
3. somewhat low (relaxed, active vibrissae, reduced head/body movements)
4. normal (alert, calm/slight freezing)
5. somewhat high (slight excitement, tense, sudden movements (darting) or freezing)
6. very high (tense appearance, excited, sudden bursts of movement)

Rearing, count number of rears (defined as each time the rat raises up and both front legs come completely off the surface. This can be free standing or with support).



APPENDIX 3

(continued)

Gait

- A = excessive sway, rocks or lurches
- H = hind limbs splayed or dragging
- HU = hunched body
- F = front limbs dragging unable to support weight
- D = body drags/flattened against surface
- FO = feet markedly pointing out from body
- T = walks on tiptoe

Gait if abnormal - ranking of gait abnormalities

1. slightly abnormal
2. moderately abnormal
3. totally abnormal

Mobility score - ability of rat to move despite gait abnormalities

- N. normal
- 1. slightly impaired
- 2. Moderately impaired
- 3. totally impaired

At the end of 3 minutes, count the number of faecal boluses, note if diarrhoea is present. Count the number of pools of urine, note if pools overlap (polyuria).

Record any abnormal behaviours:

Record excessive or repetitive behaviour such as circling, stereotypic grooming, pacing, repetitive sniffing or head weaving. Record any unusual behaviour such as self-mutilations, straub tail, retropulsions, writhing, flopping.

Now proceed with the following:

Approach response - approach rat head-on with blunt object, such as pencil, holding approximately 3 cm from face.

1. no reaction
2. slowly approaches and sniffs
3. pulls away
4. freezes, actual muscle contractions
5. jumps/turns away
6. attacks



APPENDIX 3

(continued)

Touch response - touch rump gently with blunt object

1. no reaction
2. slowly turns to side
3. walks away
4. freezes
5. freezing with ear twitch
6. turns to opposite side
7. violent reaction

Startle response

1. no reaction
2. normal flinch reaction
3. noticeable jump
4. violent reaction

Righting reflex - rat is placed supine, on a flat surface. Score the response as:

1. normal, rat immediately turns over
2. rat slowly turns over
3. rat does not move for full 20 seconds

Tail pinch response - metal tweezers are used to squeeze the tail approximately 5 cm from tip

1. no reaction
2. turns round
3. walks away
4. freezes
5. jumps forward
6. bizarre response

If 2, rate time of response

1. slow/sluggish
2. quick response
3. immediately/violent turning



INH0512

APPENDIX 3

(continued)

Pupil response - present or absent

Grip strength - a strain gauge is used with a forelimb grip ring and a hind limb grip bar for rats to grab. Forelimb and hind limb strength is measured, with two readings taken for each response. An average is calculated for each response. Reference Meyer, O.A., Tilson, H.A., Byrd, W.C., and Ridey, M.T. Neurobehav. Toxicol. 1: 233-236 (1979).

Landing foot splay - the pads of the hind feet are painted with a "finger paint". Rat is dropped from a prone position 30 cm from paper, and ink spots where it lands are noted. Repeat. Measure distance between middle of ink blots, and average. Reference: Edwards, P.M., Parker, U.H., Toxicology and Applied Pharmacology 40: 589-591 (1977).

Body temperature - rectal temperature is taken, allowing 45 seconds before recording the reading.

Now weigh the animal.

If other observations made, enter these as free text on the bottom of the observation sheet.