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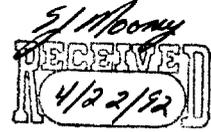


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Robert T. Drew, Ph.D.
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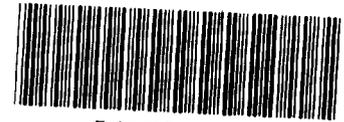
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Dear FYI Coordinator:

In accordance with API's policy of providing the federal government with copies of research designed to determine whether any chemical substance or mixture manufactured, processed or distributed by API member companies may cause risk of injury to health or the environment, we are enclosing a copy of the following draft report:

(Identification no.: FYI-not assigned) A 90-Day Feeding Study in the Rat with Six Different Mineral Oils, Three Different Mineral Waxes, and Coconut Oil, BIBRA Project No. 3.1010

We have not yet had an opportunity to conduct detailed review of study data or evaluate this report. Consequently, the conclusions must be viewed as tentative. This document does not contain confidential information. We will continue to keep you apprised of the progress of this research. If you have any questions about it, please communicate with me.

Sincerely,

Robert T. Drew, Ph.D.

CONTAINS NO CBI

A 90-day feeding study in the rat with six different mineral oils (N15(H), N70(H), N70(A), P15(H), N10(A) and P100(H), three different mineral waxes (a low melting point wax, a high melting point wax and a high sulphur wax) and coconut oil

BIBRA Project No: 3.101

DRAFT

VOLUME 1: Report Text and Tables

STUDY TITLE

A 90-day feeding study in the rat with six different white mineral oils
(N15 (H), N70 (H), N70 (A), P15 (H), N10 (A) and P100 (H)),
three different mineral waxes (a low melting point wax,
a high melting point wax and a high sulphur wax) and coconut oil

AUTHOR

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STUDY COMPLETED ON

31 March 1992

PERFORMING LABORATORY

BIBRA Toxicology International
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LABORATORY PROJECT IDENTITY

Project No. 3.1010

Report No. 1010/1/92

GOOD LABORATORY PRACTICE

Acceptance of Report

I hereby declare that, to the best of my knowledge and belief, the study described in this report was conducted in compliance with accepted international standards of Good Laboratory Practice.

Study Director:
N.R. Worrell, BSc, PhD.

Signature.....Date.....

This report is approved by BIBRA Management as an accurate reflection of the study conducted.

Senior Manager - Contracts:
P.G. Brantom, BSc, PhD, MIBiol, MCIM.

Signature.....Date.....

Director:
S.E. Jagers, BSc, PhD, MIBiol.

Signature.....Date.....

Quality Assurance Programme

The study critical phases were inspected according to the following programme. As far as can be reasonably established, the methods described and the results reported accurately reflect the raw data generated during the study.

Critical Phase

QAU Inspection
date

QAU Report
Acceptance date

Protocol deviations

Quality Assurance

Signature.....Date.....

CHRONOLOGY OF STUDY AND LOCATION OF RAW DATA

Delivery of first batch of animals : 18 February 1991

**First day of treatment
of male animals from first batch : 26 February 1991**

First day of necropsy : 28 May 1991

Archives

All raw data, any relevant specimens and a copy of the final report are retained in the BIBRA Archives under the appropriate reference unless specifically requested by the sponsor. Specimens will be retained as long as they afford evaluation.

SUMMARY

Groups of 20 male and 20 female Fischer 344 rats were fed diets containing one of six different white mineral oils (N15 (H), N70 (H), N70 (A), P15 (H), N10 (A), and P100 (H)) or one of three different mineral waxes (a low melting point wax, a high melting point wax and a high sulphur wax) at dose levels of 0.002%, 0.02%, 0.2% and 2.0% for 90 days. Groups of 60 males and 60 females were fed the control diet for the same period of time. A further group of 20 males and 20 females were fed diet containing 2% coconut oil as an oil of biological origin for comparison. These animals comprised the main study. Additional groups of 10 males and 10 females were fed diet containing test article at the 2% level or coconut oil at the 2% level for 90 days followed by a 28 day period on control diet. Groups of 30 male and 30 female animals were fed control diet for the same period of time. These animals comprised the reversal study.

An additional study was carried out simultaneously with groups of 5 male and 5 female animals receiving control diet, diet containing test article at the 2% level or coconut oil at the 2% level for 90 days (tissue level study) and further groups of 5 male and 5 female animals receiving control diet, diet containing test article at the 2% level or coconut oil at the 2% level for 90 days followed by a 28 day period on control diet (tissue level reversal study).

All animals were monitored for body weight, food intake and clinical condition and an ophthalmic examination was performed prior to treatment and prior to necropsy on the main and reversal study animals. Main study and reversal study animals were subject to a full necropsy with a selection of organs weighed and a comprehensive list of tissues retained. Blood samples were collected at necropsy and a full range of haematological parameters measured. Serum was separated from the blood samples and a range of clinical chemistry parameters measured. All tissues from the high dose and control groups were processed and examined by light microscopy. Liver, lymph nodes, spleen, kidney, small intestine and lung only from all the intermediate dose level animals were processed and examined by light microscopy.

A limited list of tissues were collected from the tissue level and tissue level reversal animals, weighed and the level of mineral hydrocarbon material present in the tissues determined. Serum vitamin E levels were measured in samples collected from these animals.

A wide spectrum of effects were seen during the course of the study. With some test articles (white oil P100 (H), high melting point wax and high sulphur wax) no treatment related effects were seen. For the other test articles the effects, when present, were the same regardless of the nature of the mineral hydrocarbon but the presence or absence of effects and their severity varied widely from test article to test article. Generally, effects were more severe in female than male animals.

The most significant effects were seen in liver and lymph node. The liver effects comprised increased organ weight, increases in serum liver enzyme levels, tissue accumulation of mineral hydrocarbon material and the appearance of granulomatous tissue. In general, the effects were confined to the higher dose levels. The effects in the lymph node were similar comprising increased organ weight, an accumulation of mineral hydrocarbon material and the appearance of histiocytosis. Where present, histiocytosis was often observed at the 0.02% dose level and occasionally at the 0.002% level.

With high doses of some test articles, kidney weight and spleen weight were both increased but with no associated histopathological findings.

Haematological changes were seen at the higher doses of some test articles and comprised a small decrease in red blood cell count in female animals only and an increase in white blood cell count in both sexes.

Most effects of treatment showed some signs of reversibility after the 28 day reversal period. The granulomatous lesions in the liver and lymph node did not, however, appear to be reversible.

No effects of treatment were seen with coconut oil.

The various test articles examined in the study varied widely in their toxicological potency. In general the most potent materials were the white oils N10 (A), P15 (H) and N15 (H) and the low melting point wax. The white oils N70

(A) and N70 (H) produced a spectrum of effects but were less potent. The high melting point wax and high sulphur wax and the white oil P100 (H) were without effect.

No-effect levels based on the presence of histiocytosis were 2.0% for white oil P100 (H), high melting point wax and high sulphur wax, 0.002% for white oils P15 (H), N70 (H), N70 (A) and N10 (A) and <0.002% for white oil N15 (H) and the low melting point wax.

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- Appendix 25. Individual body weights (g) of female rats from batch 4 on the reversal study fed either control diet, one of six white mineral oils at a level of 2.0%, one of three mineral waxes at a level of 2.0% or coconut oil at a level of 2.0% A370
- Appendix 26. Individual food intakes (g/rat/day) of male rats from batch 1 fed either control diet, one of six white mineral oils at levels of 0.002, 0.02, 0.2 or 2.0%, one of three mineral waxes at levels of 0.002, 0.02, 0.2 or 2.0% or coconut oil at a level of 2.0% A387
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- Appendix 29. Individual food intakes (g/rat/day) of male rats from batch 4 fed either control diet, one of six white mineral oils at levels of 0.002, 0.02, 0.2 or 2.0%, one of three mineral waxes at levels of 0.002, 0.02, 0.2 or 2.0% or coconut oil at a level of 2.0% A432
- Appendix 30. Individual food intakes (g/rat/day) of male rats from batch 4 on the reversal study fed either control diet, one of six white mineral oils at a level of 2.0%, one of three mineral waxes at a level of 2.0% or coconut oil at a level of 2.0% A447

- Appendix 31. Individual food intakes (g/rat/day) of female rats from batch 1 fed either control diet, one of six white mineral oils at levels of 0.002, 0.02, 0.2 or 2.0%, one of three mineral waxes at levels of 0.002, 0.02, 0.2 or 2.0% or coconut oil at a level of 2.0% A455
- Appendix 32. Individual food intakes (g/rat/day) of female rats from batch 2 fed either control diet, one of six white mineral oils at levels of 0.002, 0.02, 0.2 or 2.0%, one of three mineral waxes at levels of 0.002, 0.02, 0.2 or 2.0% or coconut oil at a level of 2.0% A470
- Appendix 33. Individual food intakes (g/rat/day) of female rats from batch 3 fed either control diet, one of six white mineral oils at levels of 0.002, 0.02, 0.2 or 2.0%, one of three mineral waxes at levels of 0.002, 0.02, 0.2 or 2.0% or coconut oil at a level of 2.0% A485
- Appendix 34. Individual food intakes (g/rat/day) of female rats from batch 4 fed either control diet, one of six white mineral oils at levels of 0.002, 0.02, 0.2 or 2.0%, one of three mineral waxes at levels of 0.002, 0.02, 0.2 or 2.0% or coconut oil at a level of 2.0% A500
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- Appendix 36. Ophthalmological observations of male rats on the main and reversal studies fed either control diet, one of six white mineral oils at a level of 2.0%, one of three mineral waxes at a level of 2.0% or coconut oil at a level of 2.0% A523

- Appendix 37. Ophthalmological observations of female rats on the main and reversal studies fed either control diet, one of six white mineral oils at a level of 2.0%, one of three mineral waxes at a level of 2.0% or coconut oil at a level of 2.0% A531
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- Appendix 39. Individual organ weights (g) of male rats on the reversal study fed either control diet, one of six white mineral oils at a level of 2.0%, one of three mineral waxes at a level of 2.0% or coconut oil at a level of 2.0% A581
- Appendix 40. Individual organ weights (g) of female rats on the main study fed either control diet, one of six white mineral oils at levels of 0.002, 0.02, 0.2 or 2.0%, one of three mineral waxes at levels of 0.002, 0.02, 0.2 or 2.0% or coconut oil at a level of 2.0% A591
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- Appendix 60. Individual histological and necropsy findings in female rats on the main study fed either control diet, one of six white mineral oils at a level of 0.002, 0.02, 0.2 or 2.0%, one of three mineral waxes at a level of 0.002, 0.02, 0.2 or 2.0% or coconut oil at a level of 2.0% A1842

Appendix 61. Individual histological and necropsy findings in female rats on the reversal study fed either control diet, one of six white mineral oils at a level of 2.0%, one of three mineral waxes at a level of 2.0% or coconut oil at a level of 2.0%

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2. MATERIALS

2.1. Test articles

The test articles were supplied by the sponsor as clear colourless liquids (mineral oils) or as white solids (mineral waxes). The mineral waxes were powdered prior to use. The sponsors certificates of analysis are shown in Appendix 1.

Powdered wax was prepared by generating a fine spray of molten wax which, on cooling, formed a fine powder. The fine spray was generated by drawing molten wax through an atomiser with a stream of nitrogen. The atomiser was located in the base of a large glass chamber which contained the spray and resultant powder and prevented any contamination. The wax particles formed from the spray were allowed to settle and were removed from the chamber into clean containers for storage.

All waxes were generated using the same system with variation of temperature and flow rate to provide the finest possible particle size.

Coconut oil was purchased from Sigma Chemical Company Ltd., Poole, Dorset, England.

All test articles were stored at room temperature, in the dark and all containers were flushed with a stream of nitrogen prior to storage following each occasion of use.

2.2. Animals and maintenance

Male and female rats of the Fischer 344 strain aged approximately 4 weeks with a narrow weight and birth date range were obtained from a barrier-maintained colony (Harlan Sprague Dawley Inc, PO Box 29176, Indianapolis, IN 46229, USA). They were housed in polypropylene cages with stainless-steel grid tops and floors, suspended over paper for removal of excreta. Each cage housed five rats of the same sex and treatment group. The animals were kept in the study room for a minimum of 7 days (male) or 13 days (female) prior to the first treatment to allow them to acclimatize to the environmental conditions. Each cage carried a label identified with the following:

- BIBRA Project No. 3.1010
- Study Director (Nan Worrell)
- Project licence number
- identification numbers of animals contained
- sex of animals contained
- treatment of animals contained and the dates of starting treatment
- date of necropsy.

Labels were colour coded for groups and this code extended to the bulk containers of test article:diet mixture.

The cages were held on racks in rooms used solely for this study and controlled to the following conditions:

- temperature : 19-24°C
- relative humidity : 45-70%
- light : artificial, 12 hr light
(06.00-18.00 hr GMT);
12 hr dark
- air changes : minimum 15/hr with no recirculation using
high efficiency filters

The rats were fed a nutritionally adequate diet (Rat and Mouse No.1 maintenance diet, Special Diets Services Ltd, Witham, Essex) in stainless steel pots designed to limit spillage. Diets from batch Nos. 5587, 5786 and 6051 were used and copies of the manufacturer's analysis certificates are shown in Appendix 2. Domestic mains tap water was supplied to each cage in bottles fitted with a stainless steel drinking valve. Samples of this water are taken on a regular basis from the BIBRA facility by the supplying authority. The records of these analyses are retained on a central file.

It was not considered that any of the contaminants present in the food and water adversely affected the outcome of the study.

3. EXPERIMENTAL DESIGN

3.1. Assignment to treatment groups and animal identification

The study comprised a basic 90-day study at four dose levels for each test article and at the top dose level only for coconut oil with 20 animals per group and 60 control animals (the main study), a reversibility study at the top dose level only with ten animals per group and thirty control animals with a 90-day feeding period followed by a 28 day recovery period (reversal study), a tissue level study at the top dose level only with five animals per group taken for necropsy after 90 days (tissue level study) and a tissue level reversibility study at the top dose level only with five animals per group with a 90-day feeding period followed by a 28-day recovery period (tissue level reversal study).

The treatment groups and animal identification numbers are shown in the table overleaf. Each animal was given a unique identification number indicated by an ear-punch code.

3.2. Randomisation

On receipt of each batch of animals they were weighed and then allocated to five weight bands containing equal numbers of animals. Animals were assigned to cages by a random number technique such that each cage contained one animal from each weight band.

Treatment groups and animal identification

			Animal numbers					
			Main		Reversal		Tissue Level and Tissue Level Reversal*	
			M	F	M	F	M	F
Group	Treatment (% in diet)	Test article						
1	0	Control	1-20	801-820	1601-1610	1731-1740	1861-1870	1971-1980
2	0	Control	21-40	821-840	1611-1620	1741-1750		
3	0	Control	41-60	841-860	1621-1630	1751-1760		
4	0.002	Oil 1	61-80	861-880				
5	0.02	Oil 1	81-100	881-900				
6	0.2	Oil 1	101-120	901-920				
7	2.0	Oil 1	121-140	921-940	1631-1640	1761-1770	1871-1880	1981-1990
8	0.002	Oil 2	141-160	941-960				
9	0.02	Oil 2	161-180	961-980				
10	0.2	Oil 2	181-200	981-1000				
11	2.0	Oil 2	201-220	1001-1020	1641-1650	1771-1780	1881-1890	1991-2000
12	0.002	Oil 3	221-240	1021-1040				
13	0.02	Oil 3	241-260	1041-1060				
14	0.2	Oil 3	261-280	1061-1080				
15	2.0	Oil 3	281-300	1081-1100	1651-1660	1781-1790	1891-1900	2001-2010
16	0.002	Oil 4	301-320	1101-1120				
17	0.02	Oil 4	321-340	1121-1140				
18	0.2	Oil 4	341-360	1141-1160				
19	2.0	Oil 4	361-380	1161-1180	1661-1670	1791-1800	1901-1910	2011-2020
20	0.002	Oil 5	381-400	1181-1200				
21	0.02	Oil 5	401-420	1201-1220				
22	0.2	Oil 5	421-440	1221-1240				
23	2.0	Oil 5	441-460	1241-1260	1671-1680	1801-1810	1911-1920	2021-2030
24	0.002	Oil 6	461-480	1261-1280				
25	0.02	Oil 6	481-500	1281-1300				
26	0.2	Oil 6	501-520	1301-1320				
27	2.0	Oil 6	521-540	1321-1340	1681-1690	1811-1820	1921-1930	2031-2040
28	0.002	Wax A	541-560	1341-1360				
29	0.02	Wax A	561-580	1361-1380				
30	0.2	Wax A	581-600	1381-1400				
31	2.0	Wax A	601-620	1401-1420	1691-1700	1821-1830	1931-1940	2041-2050
32	0.002	Wax B	621-640	1421-1440				
33	0.02	Wax B	641-660	1441-1460				
34	0.2	Wax B	661-680	1461-1480				
35	2.0	Wax B	681-700	1481-1500	1701-1710	1831-1840	1941-1950	2051-2060
36	0.002	Wax C	701-720	1501-1520				
37	0.02	Wax C	721-740	1521-1540				
38	0.2	Wax C	741-760	1541-1560				
39	2.0	Wax C	761-780	1561-1580	1711-1720	1841-1850	1951-1960	2061-2070
40	2.0	Oil N	781-800	1581-1600	1721-1730	1851-1860	1961-1970	2071-2080

Oil 1 = White Oil N15 (H); Oil 2 = White Oil N70 (H); Oil 3 = White Oil N70 (A); Oil 4 = White Oil P15 (H);
 Oil 5 = White Oil N10 (A); Oil 6 = White Oil P100 (H); Wax A = Low Melting Point Wax; Wax B = High Melting
 Point Wax; Wax C = High Sulphur Wax; Oil N = Coconut Oil; M = Male; F = Female

* First five animals in each group of ten - tissue level study; last five animals in each group of ten - tissue level
 reversal study

3.3. Phasing of the study

Due to the large number of animals involved, the study was commenced over a period of eight weeks. The phasing was as follows:

Week 1 - groups 1-40 main study males with five lowest identification numbers

Week 2 - groups 1-40 main study females with five lowest identification numbers

Week 3 - groups 1-40 main study males with next five identification numbers and all males on tissue level and tissue level reversal study

Week 4 - groups 1-40 main study females with next five identification numbers and all females on tissue level and tissue level reversal study

Week 5 - groups 1-40 main study males with next five identification numbers

Week 6 - groups 1-40 main study females with next five identification numbers

Week 7 - groups 1-40 main study males with five highest identification numbers and all male animals on the reversal study

Week 8 - groups 1-40 main study females with five highest identification numbers and all female animals on the reversal study

All female animals were one week older than male animals at the start of treatment.

3.4. Administration of test article

The test articles were given as a constant concentration in the diet of each group.

3.5. Duration of treatment

Each test article was available continuously for at least 90 days. Animals designated for reversal studies reverted to control diet after 90 days of treatment and received control diet for either 29, 30, 31 or 32 days prior to necropsy.

4. METHODS

4.1. Method of analysis of mineral oils and waxes in rodent diet

Samples of diet (between 2 and 20 g, depending on dose level) were extracted on a multiple vortex mixer for 5 minutes and then in an ultrasonic bath at 40°C for 30 minutes with carbon tetrachloride (50 or 100 ml). An appropriate aliquot of the extract was mixed well with Florisil (6 g) and the mixture filtered to a known volume. The filtrate was mixed well with Florisil (1 g), filtered and the infra-red absorbance, in the C-H stretching region, of the second filtrate measured against a carbon tetrachloride background using a Fourier Transform infra-red spectrometer. The concentration of test article was determined by comparison with standard solutions. Samples of control diet were also extracted and the measured concentration of test article subtracted from the measured test article concentration in the test diets. Results were corrected for recovery using factors obtained by the analysis of separately prepared spiked diets.

To define whether the background level of hydrocarbon absorbance observed in the control diets during the feeding study was due to mineral oil contamination of the diet or to residual endogenous lipid material carried through the clean-up procedure further studies were conducted. Control diet, from a batch used for study diet preparation was spiked at 2% by weight with white oil N15 (H) and diluted with further diet to 0.02% and 0.002%. Samples of control, 0.002% and 0.02% diets were extracted with carbon tetrachloride, the extracts purified from endogenous lipid on Florisil and mineral hydrocarbon quantitated by Fourier Transform infrared spectrometry. Samples were then subject to a further treatment to remove any remaining endogenous lipid. This comprised either column chromatography on basic any remaining alumina with carbon tetrachloride extraction or alcoholic potassium hydroxide hydrolysis with Florisil treatment.

Basic alumina treatment permitted quantitative recovery of mineral oil but did not decrease the measured level of mineral hydrocarbon material in the control diet. Alkaline hydrolysis did reduce the apparent background level of absorbance in the control diet. However, the apparent level of

mineral hydrocarbon in the oil spiked diets was also reduced to a similar extent by this treatment.

4.2. Method of analysis of coconut oil in rodent diet

Samples of diet (2 g) were extracted on a multiple vortex mixer for 5 minutes and then in an ultrasonic bath at 40°C for 30 minutes with carbon tetrachloride (100 ml). A sample (10 ml) of this extract was filtered to a known volume and the infra-red absorbance, in the C-H stretching region, of the filtrate measured using a Fourier Transform infra-red spectrometer. The concentration of coconut oil in the diet was determined by comparison with standard solutions. Samples of control diet were also extracted and the measured concentration of test article subtracted from the measured test article concentration in the test diets. Results were corrected for recovery using factors obtained by the analysis of separately prepared spiked diets.

4.3. Mixing of mineral oils and waxes and coconut oil with rodent diet

The quantity of compound calculated to give a 10% mixture was weighed into a known quantity of diet and mixed gradually using an electric hand mixer. This was then blended with further diet using a paddle mixer to give a 2% mixture. The 0.2%, 0.02% and 0.002% diets were prepared by serial dilution. Each preparation was mixed in a mechanical blender for fifteen minutes. Each wax and the diet used to prepare the premixes were cooled overnight at approximately -20°C before use. The coconut oil was melted by heating to 40°C before mixing. The diet used to make the 10% coconut oil pre-mix was also warmed to 40°C prior to use to ensure even distribution of the test article whilst the coconut oil was still molten.

4.4. Efficiency of mixing mineral oils and waxes and coconut oil with rodent diet

Batches of diet were prepared to contain the test articles at the lowest and highest concentrations intended for use. Six samples taken from various parts of each mixture were analysed for the concentration of test article.

4.5. Stability of mixing mineral oils and waxes and coconut oil with rodent diet

For storage stability studies, samples of the diets prepared to contain test article at the lowest and highest concentrations intended for use were stored in closed metal containers at room temperature and the test article concentration measured after 3, 7 and 14, 15 or 16 days. Samples of the same diets were also placed in animal feed pots and exposed to the atmosphere in an animal room. They were analysed for test article after 1 and 4 days.

4.6. Preparation of study diets

Diet mixtures were prepared weekly. At approximately monthly intervals duplicate samples of study diets were taken for analysis. Diet mixtures were stored at room temperature in sealed metal containers prior to use.

4.7. Observations during the study

Daily records were kept of the humidity and maximum and minimum temperature of the animal room. Each animal was examined daily in its cage and any variation in its appearance, the appearance of its excreta or of its behaviour was noted. Once weekly a more detailed examination was carried out at the time of a weighing.

Body weight

The rats were weighed individually three or four days before treatment, on the first day of treatment and then twice weekly until killed.

Food intake

Food intake for each cage of rats was measured over the intervals between body weight measurements.

Ophthalmological examination

All high dose and control animals were examined during the week preceding treatment and during the last week of treatment. Both eyes were

examined by direct and indirect ophthalmoscopy. The examination included the lids, conjunctivae, cornea, anterior chamber, iris, lens, vitreous body and fundus.

4.8. Necropsy examination

Main study and reversal study animals

The main study examinations were phased over four days for each sex from each batch of animals. The reversal examinations were phased over three days for each sex. On each day the rats from the various groups were examined in random order. Prior to necropsy the rats were deprived of food overnight, with water available. On the day of necropsy each animal was weighed and then killed by exsanguination from the dorsal aorta under barbiturate anaesthesia. During the necropsy any abnormalities were noted.

The following organs were weighed from those animals scheduled for full necropsy:

- ▶ adrenal glands
- ▶ brain
- ▶ caecum (with and without contents)
- ▶ heart
- ▶ kidney
- ▶ liver
- ▶ ovaries
- ▶ spleen
- ▶ testes
- ▶ thymus

Samples of the following tissues were retained in 10% neutral buffered formalin:

- ▶ adrenal glands
- ▶ artery (aorta)
- ▶ bladder
- ▶ brain

- ▶ caecum
- ▶ colon
- ▶ cervix uteri
- ▶ diaphragm
- ▶ duodenum
- ▶ epididymis
- ▶ extra orbital lachrymal glands
- ▶ eye
- ▶ femur
- ▶ Harderian gland
- ▶ heart
- ▶ ileum (including Peyer's patches)
- ▶ jejunum
- ▶ kidneys
- ▶ liver (representative samples from each lobe)
- ▶ lungs (with main stem bronchi)
- ▶ lymph nodes (axillary, cervical and mesenteric)
- ▶ mammary gland (inguinal region)
- ▶ nasal bones
- ▶ nerve (sciatic taken together with surrounding muscle)
- ▶ oesophagus
- ▶ ovaries
- ▶ pancreas
- ▶ perirenal fat
- ▶ pinnae (retained for identification only)
- ▶ pituitary
- ▶ prostate
- ▶ rectum
- ▶ salivary gland
- ▶ seminal vesicles
- ▶ skeletal muscle
- ▶ skin (inguinal region)
- ▶ spinal cord

- ▶ spleen
- ▶ sternum
- ▶ stomach
- ▶ testes
- ▶ thymus
- ▶ thyroid/parathyroid glands (retained on trachea)
- ▶ tongue
- ▶ trachea
- ▶ uterine horns
- ▶ vagina
- ▶ vein (posterior vena cava)

In addition samples of the following tissues from high dose and control animals only were retained in formal calcium:

- ▶ liver
- ▶ spleen
- ▶ small intestine
- ▶ mesenteric lymph nodes

Tissue level study and tissue level reversal study animals

Animals designated to provide tissues for analysis were killed by exsanguination under barbiturate anaesthesia and samples of the following tissues taken for analysis:

- ▶ liver
- ▶ mesenteric lymph nodes
- ▶ kidney
- ▶ spleen
- ▶ perirenal fat

4.9. Haematology

Blood samples were collected from the aorta of all animals on the main and reversal studies at necropsy and examined for:

- ▶ total erythrocyte count
- ▶ total leucocyte count
- ▶ haemoglobin concentration
- ▶ mean cell volume
- ▶ haematocrit (by calculation)
- ▶ platelet count
- ▶ differential leucocyte count
- ▶ reticulocyte count
- ▶ prothrombin time

4.10. Clinical chemistry

Serum was separated from blood collected at necropsy from the main and reversal study animals and analysed for:

- ▶ glucose concentration
- ▶ urea concentration
- ▶ total protein concentration
- ▶ albumin concentration
- ▶ alkaline phosphatase activity
- ▶ alanine aminotransferase activity
- ▶ aspartate aminotransferase activity
- ▶ albumin/globulin ratio
- ▶ creatinine
- ▶ calcium
- ▶ phosphorus (as phosphate)
- ▶ chloride
- ▶ total bilirubin
- ▶ sodium
- ▶ potassium
- ▶ gamma glutamyl transferase

4.11. Tissue analysis for mineral hydrocarbon content

Samples of up to approximately 1 g of tissue were homogenised in 70% potassium hydroxide solution (1.5-5 ml) and homogenised using an

Ultraturrax single-blade homogeniser. The homogenate was sonicated for 10 minutes in an ultrasonic bath maintained at 60°C. Carbon tetrachloride (10 or 20 ml) was added to each sample and sonicated for 30 minutes at 60°C, mixing occasionally by hand. The layers were separated using centrifugation if necessary. Extraction columns were prepared by weighing 3-5 g deactivated Florisil into sintered glass filter tubes (one for each extract to be processed). An aliquot of the lower, organic phase was poured onto the extraction column, the eluate collected and the column washed with carbon tetrachloride to a known final volume. The infra-red absorbance, in the C-H stretching region, of the eluate was measured against a carbon tetrachloride background using a Fourier Transform infra-red spectrometer. The concentration of mineral hydrocarbon in the tissue was calculated by comparison with appropriate standards.

4.12. Vitamin E analysis

Plasma was separated from the blood collected at necropsy from the animals designated to provide tissues for analysis and analysed for vitamin E (α -tocopherol). Samples were saponified, extracted with hexane and analysed by HPLC using fluorimetric detection.

4.13. Histological examination of tissues

A microscopic examination was made of haematoxylin and eosin stained sections prepared from wax embedded samples of all the preserved tissues from the control group, the high dose group and from lung, liver, kidney, spleen, small intestine and mesenteric lymph nodes of all other groups. All lung sections were examined for evidence of infection.

4.14. Analysis of data

The continuous variable data from the control and test groups were tested for normality using the Kolmogorov-Smirnov (K.S.) test and homogeneity of variance using Bartlett's test. Statistical significance was determined to be at $p < 0.05$ in a K.S. test and at $p < 0.01$ in a Bartlett's test. If both tests

were non-significant, the control and test groups were compared using analysis of variance followed by the least significant difference (L.S.D.) test.

If either test produced a significant result, a suitable transformation was attempted. If the transformed data resulted in a non-significant Bartlett's test but a significant K.S. test, the Wilcoxon Mann-Whitney test was used. If the transformed data resulted in a non-significant K.S. test but a significant Bartlett's test, an appropriate t -test was used, based on whether a pooled variance was suitable or not.

If no suitable transformation could be made, one of the above tests was selected as the most appropriate based upon the nature and distribution of the data.

The results of the Mann-Whitney and t -tests were compared with the L.S.D. test. In most cases, the L.S.D. test was reported. However, if large differences between tests was evident, other test results were reported as appropriate unless the data was deemed to be highly variable and there was no evidence to justify the removal of outliers.

Incidence data from the histopathological examination was tested for differences between treated and control animals using Fisher's exact test. Mann-Whitney tests were performed on incidence data graded by severity.

In all test comparisons, a probability level of $p < 0.05$ in a two-sided test was taken to indicate statistical significance.

5. RESULTS

5.1. Homogeneity and stability studies

Preliminary investigations demonstrated that the method of test article analysis was insufficiently precise, particularly at lower dietary concentrations of test article, to support the protocol criteria for acceptability of the mixing procedure (mean concentration of 90-110% of expected, and a coefficient of variance of the six samples of less than 10%). All 2% diets did have a coefficient of variance of less than 10%, except for the low melting point wax where the value was 11.9% and all test diets at the 2% level had a mean analysed concentration between 80 and 120% of nominal. These results were considered adequate confirmation of homogeneity of the test diets and of a satisfactory method of mixing within the limitations of the analytical method. At the 0.002% level the analytical method was not capable of a precision better than $\pm 50\%$. The results all fell within these limits and suggest the dilution procedure was satisfactory. (Appendix 3). The stability studies were similarly constrained by the limitations of the analytical method. From Appendix 4 it is, however, apparent that there was no obvious loss of test article from the diet on storage in either open (4 days) or closed (14 days) containers. The method of analysis employed in the study measured total mineral hydrocarbon and would probably not have detected any change in the nature of the material.

5.2. Environmental conditions

The temperature and percentage relative humidity recorded in the animal rooms are shown in Appendices 5-8. Temperature and percentage relative humidity were slightly outside protocol limits on a number of occasions during the study. These minor deviations are not considered to have affected the outcome of the study.

5.3. Analysis of study diets

On five occasions during the study, test diets were analysed for test article concentration as a check of correct preparation. The results are shown in Appendix 9. In general, within the limitations of the analytical method

(2% diet \pm 0.4%, 0.2% diet \pm 0.04%, 0.02% diet \pm 0.004% and 0.002% diet \pm 0.001%) most study diets analysed were shown to contain the nominal concentration.

On occasions during the study samples of diet from batches which had been analysed prior to feeding were taken from the food pot at the end of the feeding period for analysis as a check on the stability of the test article/diet mixtures. These values are shown in Appendix 9. These results confirm the stability of the test article/diet mixtures on storage and during use.

Apparently high levels of mineral hydrocarbon in control diet were the subject of an additional study to elucidate the nature of the hydrocarbon and resolve whether the material was of mineral or biological origin. The results indicate that the background absorbance observed in the control diet throughout the study is due to the presence of mineral hydrocarbon and not residual lipid or an artefact of the sample preparation.

5.4. Observations of condition and behaviour

There were occasional abnormal observations of condition but none that were considered to be treatment related (Appendices 10 and 11).

5.5. Body weights

Body weights for the main study groups are shown in Tables 1 and 5, for the reversal study in Tables 2 and 6, for the tissue level study in Tables 3 and 7 and for the tissue level reversal study in Tables 4 and 8.

Body weights for some groups showed occasional periods when the values were statistically significantly different from control. These differences were not consistent between the sexes on the various studies and were not dose-related.

5.6. Food intakes

There were four cages of animals per group on the main study (twelve cages of controls) and the results for these groups are shown in Tables 9 and 13. There were two cages of animals per group on the reversal study

(six cages of controls) and the results for these groups are shown in Tables 10 and 14. There was one cage of animals per group on the tissue level and tissue level reversal studies thus statistical analysis on these groups could not be performed. The individual values for each of these cages are shown in Tables 11 and 15 (tissue level study) and 12 and 16 (tissue level reversal study).

During the first month of the study the only consistent statistically significant effect on food intake in male animals was an increase in food intake by animals receiving white oil N70 (A) at 2% in the diet. Subsequently, a statistically significant increase in food intake in male animals was a common finding in the high dose test article groups and in the coconut oil treated group. In the second and third months of the study males receiving the highest dose of all test articles including coconut oil generally consumed more food than controls. The difference was frequently statistically significant.

There was evidence for a similar effect in female animals but the effect was less pronounced. Statistically significant increases in food intake occurred with fewer test articles but was evident for some part of the study in animals receiving 2% N70 (H), N70 (A), N15 (H), P15 (H) and the high melting point wax.

In general, statistically significant differences in food intakes between treated groups and controls disappeared during the reversal period.

5.7. Calculated intakes of test article

The intakes of each test article for the main, reversibility, tissue level and tissue level reversal studies were calculated as mg/kg bodyweight/day for each cage of rats over each food intake interval. Intakes were calculated on the basis of the nominal concentration of test article in the diet. The mean intakes per group are shown in tables 17-24. The average intake during the whole study for each test article for each separate study is shown overleaf. During the early part of the study animals received approximately 25% more than the mean figure and during the later weeks of the study approximately 25% less than the mean figure.

Mean calculated intakes of test articles over 90-day feeding period

MALE

TREATMENT	DOSE LEVEL (% IN DIET)						
	MAIN				REVERSAL	TISSUE LEVEL	TISSUE LEVEL REVERSAL
	0.002	0.02	0.2	2.0	2.0	2.0	2.0
White oil N15 (H)	1.677	17.96	175.7	1814	1787	1837	1792
White oil N70 (H)	1.696	17.80	181.4	1832	1882	1875	1910
White oil N70 (A)	1.652	17.61	180.7	1866	1874	1888	1854
White oil P15 (H)	1.664	17.70	176.8	1831	1854	1886	1898
White oil N10 (A)	1.723	17.77	175.0	1816	1856	1813	1761
White oil P100 (H)	1.679	17.88	176.4	1813	1809	1844	1803
Low melting point wax	1.702	17.78	180.0	1793	1855	1816	1816
High melting point wax	1.662	17.59	177.6	1837	1815	1816	1810
High sulphur wax	1.679	17.61	177.8	1821	1853	1871	1861
Coconut oil				1817	1777	1772	1780

FEMALE

TREATMENT	DOSE LEVEL (% IN DIET)						
	MAIN				REVERSAL	TISSUE LEVEL	TISSUE LEVEL REVERSAL
	0.002	0.02	0.2	2.0	2.0	2.0	2.0
White oil N15 (H)	2.037	19.35	187.7	1946	1950	1923	1951
White oil N70 (H)	2.019	19.01	193.6	1967	2011	1966	1939
White oil N70 (A)	1.990	18.61	188.0	2025	1959	2004	1962
White oil P15 (H)	1.967	18.93	187.7	1952	1925	1952	1935
White oil N10 (A)	2.056	18.59	182.9	1914	1926	1910	1883
White oil P100 (H)	1.960	18.98	186.3	1951	1956	1991	2004
Low melting point wax	1.998	18.81	191.3	1869	1901	1949	1836
High melting point wax	1.958	19.14	190.4	1952	1988	1970	1976
High sulphur wax	1.946	18.68	190.9	1934	1932	1922	2006
Coconut oil				1992	1915	1864	1943

Values shown are in mg/kg body weight/day
Data derived from Tables 17-24

5.8. Ophthalmological observations

Occasionally, abnormalities were found both prior to and following treatment. However, there were no findings that were indicative of a treatment related effect.

5.9. Organ weights

The weights of adrenals, kidneys, testes/ovaries, thymus, heart, brain, liver, spleen and full and empty caecum were measured for animals on the main and reversibility studies (Tables 25-32). For animals on the tissue level and tissue level reversal studies only liver, kidney, spleen, lymph node and perirenal fat were weighed (Tables 33-40). The organ weight data for each of the studies are considered below on an organ by organ basis.

Adrenals

There were occasional statistically significant findings but none that were considered to be treatment related.

Kidneys

There were a number of significant findings and these are summarised overleaf:

Animals receiving the highest dose of white oils N15 (H), P15 (H) and N10 (A) had higher kidney weights than controls. The differences were also present when these weights were expressed relative to body weight.

Following the reversal period, there were no statistically significant differences between kidney weights of control and treated females. A number of male reversal groups showed a statistically significant difference but these were, with the exception of the N10 (A) group, in the groups which had shown no effect in the main study.

The animals on the tissue level study, in general, showed similar kidney weight increases to animals on the main study. A tendency for these groups to show lower levels of significance reflects the much smaller group size.

MALE: KIDNEYS

TREATMENT	DOSE LEVEL (% IN DIET)													
	MAIN								REVERSAL		TISSUE LEVEL		TISSUE LEVEL REVERSAL	
	0.002		0.02		0.2		2.0		2.0		2.0		2.0	
	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL
White oil N15 (H)							**	***				**		
White oil N70 (H)		*												
White oil N70 (A)									***					
White oil P15 (H)							**	***			**	***		
White oil N10 (A)					*		**	***	**	*		**		
White oil P100 (H)									**	**				
Low melting point wax									*	***				
High melting point wax					*	*								
High sulphur wax									**					
Coconut oil														

FEMALE: KIDNEYS

TREATMENT	DOSE LEVEL (% IN DIET)													
	MAIN								REVERSAL		TISSUE LEVEL		TISSUE LEVEL REVERSAL	
	0.002		0.02		0.2		2.0		2.0		2.0		2.0	
	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL
White oil N15 (H)							**	***				*		
White oil N70 (H)							*	*						
White oil N70 (A)								*				*		
White oil P15 (H)							**	***						
White oil N10 (A)		*					**	***				*		
White oil P100 (H)														
Low melting point wax														
High melting point wax														
High sulphur wax					*									
Coconut oil														

ABS = Absolute organ weight; REL = Relative organ weight
Asterisks represent level of significance (* p < 0.05; ** p < 0.01; *** p < 0.001)
Data summarised from Tables 25-40

Testes

There were occasional small but statistically significant changes in testis weight. The only treatment that produced an effect in the high dose group was the low melting point wax where both absolute and relative organ weight were higher than controls. Relative testis weight (but not absolute) was also higher than controls in the reversal group of the same treatment.

Ovaries

There were occasional small but statistically significant changes in relative ovary weight. These findings are not considered to be treatment related.

Thymus

There were a number of small but statistically significant changes in thymus weight but none that were considered to be treatment related.

Heart

There were a number of small but statistically significant changes in heart weight but none that were considered treatment related.

Brain

There were occasional statistically significant findings but none that were considered to be treatment related.

Liver

There were a number of statistically significant findings and these are summarised overleaf:

MALE: LIVER

TREATMENT	DOSE LEVEL (% IN DIET)													
	MAIN								REVERSAL		TISSUE LEVEL		TISSUE LEVEL REVERSAL	
	0.002		0.02		0.2		2.0		2.0		2.0		2.0	
	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL
White oil N15 (H)				***			***	***				***		
White oil N70 (H)		*		*		**								
White oil N70 (A)									*		*			
White oil P15 (H)				*	**	***	***	***			*	***		
White oil N10 (A)					***	***	***	***			*	***		
White oil P100 (H)														
Low melting point wax				*	***	***	***	*	***	***	*			*
High melting point wax				*	*									
High sulphur wax				*	*				*					
Coconut oil														

FEMALE: LIVER

TREATMENT	DOSE LEVEL (% IN DIET)													
	MAIN								REVERSAL		TISSUE LEVEL		TISSUE LEVEL REVERSAL	
	0.002		0.02		0.2		2.0		2.0		2.0		2.0	
	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL
White oil N15 (H)				*	***	***	***	***	**	**	***	***		**
White oil N70 (H)							***	***	*	***	*			
White oil N70 (A)			*	**	*	**	***	***	**	*		***		
White oil P15 (H)				***	***	***	***	***	*	**	**	***		
White oil N10 (A)					**	***	***	***			***	***		*
White oil P100 (H)														
Low melting point wax					***	***	***	***	***	***	***	***	***	***
High melting point wax														
High sulphur wax		**												
Coconut oil														

ABS = Absolute organ weight; REL = Relative organ weight
Asterisks represent level of significance (* p < 0.05; ** p < 0.01; *** p < 0.001)
Data summarised from Tables 25-40

At the highest dose, four treatments resulted in higher liver weights in both sexes (white oils N15 (H), P15 (H), N10 (A) and the low melting point wax). In addition, higher weights were found in high dose females given N70 (H) and N70 (A). Particularly in females these differences were also present at lower dose levels of the same treatments, however, the lowest dose (0.002%) showed no evidence of this.

Animals in the reversal groups generally showed an overall decrease in the magnitude of the effect but liver weight was still increased at the end of the reversal period in male animals receiving low melting point wax and in female animals receiving white oils N15 (H), N70 (H), N70 (A) and P15 (H) and low melting point wax.

The animals on the tissue level and tissue level reversal studies, in general, showed similar liver weight increases to animals on the main and reversal studies. A tendency for these groups to show lower levels of significance reflects the much smaller group size.

Spleen

There were a number of statistically significant findings and these are summarised overleaf.

Relative spleen weights were higher than control in both sexes given 2% white oils N15 (H), N70 (H), N70 (A), P15 (H), N10 (A) and in groups receiving low melting point wax at the 2% and 0.2% levels with the differences being much more marked in the female groups.

At the end of the reversal period the only group where a difference between treated males and controls was apparent was in the group receiving low melting point wax. Relative spleen weight was still statistically significantly increased in the reversal female groups that showed an effect in the main study with the exception of the females receiving white oil N70 (A) where there was no statistically significant difference from controls.

MALE: SPLEEN

TREATMENT	DOSE LEVEL (% IN DIET)													
	MAIN								REVERSAL		TISSUE LEVEL		TISSUE LEVEL REVERSAL	
	0.002		0.02		0.2		2.0		2.0		2.0		2.0	
	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL
White oil N15 (H)							*	*						
White oil N70 (H)							*	*						
White oil N70 (A)								*						
White oil P15 (H)							*	*						
White oil N10 (A)								*						
White oil P100 (H)														
Low melting point wax					***	***	***	***	***	***	**	**	***	***
High melting point wax														
High sulphur wax														
Coconut oil														

FEMALE: SPLEEN

TREATMENT	DOSE LEVEL (% IN DIET)													
	MAIN								REVERSAL		TISSUE LEVEL		TISSUE LEVEL REVERSAL	
	0.002		0.02		0.2		2.0		2.0		2.0		2.0	
	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL	ABS	REL
White oil N15 (H)							***	***	*	*		**		
White oil N70 (H)							**	**	*	***				
White oil N70 (A)							***	***						
White oil P15 (H)					*	**	***	***	***	***	**	*		
White oil N10 (A)							***	***		**	**	*	**	
White oil P100 (H)														
Low melting point wax					***	***	***	***	***	***	***	***	***	***
High melting point wax														
High sulphur wax														
Coconut oil														

ABS = Absolute organ weight; REL = Relative organ weight
 Asterisks represent level of significance (* p < 0.05; ** p < 0.01; *** p < 0.001)
 Data summarised from Tables 25-40

Caecum Full

A few very small but statistically significant increases in organ weight (absolute and relative) were observed in some of the high dose main study groups. Both absolute and relative organ weights were statistically significantly increased in males treated with 2% white oil P 15(H) both at 90 days and following the reversal period.

Caecum Empty

There were occasional statistically significant findings but none that were considered to be treatment related.

Lymph Node

Since lymph nodes were only weighed in the tissue level and tissue level reversal groups the group size is small and there is no information on treatments other than the highest dose. However, there were substantial and statistically significant increases in both absolute and relative organ weight in male and female animals receiving white oils N15 (H), P15 (H) and N10 (A) or low melting point wax at 2% in the diet.

The effect was still apparent in the male reversal animals treated with white oils P15 (H) and N10 (A) or the low melting point wax and in the reversal females treated with white oils N15 (H), P15 (H) and N10 (A) or low melting point wax and additionally white oils N70 (A) and N70 (H) (not significant at the end of the 90-day treatment period). There was a small but statistically significant increase in relative organ weight only, in reversal females receiving high melting point wax.

Perirenal Fat

There were occasional statistically significant findings but none that were considered to be treatment related.

5.10. Haematology

The haematology results are presented in Tables 41-44. A number of haematological parameters were affected by treatment. These findings for the main study are summarised in the Tables overleaf. Occasional statistically significant findings that are not considered to be treatment related are not included in these summary tables.

At the highest dose of many of the test articles females had a small but statistically significant decrease in red blood cell count with an concomitant reduction in haemoglobin concentration and haematocrit. Similar changes were also seen in the 0.2% group for white oils P15 (H) and N10 (A). In the 2% low melting point wax group the effect was severe enough to be accompanied by an increased reticulocyte count. Similar differences in red cells were not seen in the male groups.

An increase in white blood cell count was also present in many of the high dose female groups. This was generally a consequence of increased neutrophil and monocyte counts, often associated with increased eosinophils and basophils. Similar changes were also seen at the 0.2% level for the females given low melting point wax. Occasionally similar changes were seen in male groups but the effects were less severe.

The reticulocyte count was still elevated in the female low melting point wax reversal group along with the white blood cell count. White blood cell count was also elevated in the white oils P15 (H), N15 (H) and P100 (H) reversal female groups.

Summary of haematological findings considered to be treatment related : males

Treatment	Dose (% in the diet)	RBC	Retics	Hb	HCT	MCH	WBC	N	L	M	E	B	Platelets
White oil N15 (H)	2.0%						†°	†°°°		†°°			†°
White oil N70 (H)	2.0%												
White oil N70 (A)	2.0%			†°									
White oil P15 (H)	2.0%						†°	†°°°		†°°			
White oil N10 (A)	2.0%							†°°°		†°		†°	
White oil P100 (H)													
Low melting point wax	0.2%			†°		†°°°		†°°					†°°
	2.0%			†°°		†°°°		†°°					†°°°
High melting point wax	2.0%			†°°									
High sulphur wax	2.0%			†°°									
Coconut oil	2.0%			†°									

° = p < 0.05; °° = p < 0.01; °°° = p < 0.001; † = decreased compared with control; †° = increased compared with control.

Summary of haematological findings considered to be treatment related : females

Treatment	Dose (% in the diet)	RBC	Retics	Hb	HCT	MCV	MCH	WBC	N	L	M	E	B	Platelets
White oil N15 (H)	2.0%	↓***		↓***	↓*		↑*	↑**	↑***		↑**			
White oil N70 (H)	2.0%	↓**		↓**										
White oil N70 (A)	2.0%	↓*				↑*								
White oil P15 (H)	0.2%	↓*		↓*										
	2.0%	↓***		↓***	↓*			↑**	↑***		↑***	↑***	↑**	
White oil N10 (A)	0.2%	↓*		↓**	↓**									
	2.0%	↓***		↓***				↑**	↑***		↑***	↑**		
White oil P100 (H)														
Low melting point wax	0.02%										↑*			
	0.2%							↑***	↑***	↑*	↑**		↑***	↑***
	2.0%	↓***		↓***	↓***		↓***	↑***	↑***	↑***	↑***	↑*	↑***	↑***
High melting point wax														
High sulphur wax	0.2%													↓*
	2.0%							↑***		↓**	↑*			↓**
Coconut oil	2.0%	↓*		↓***										

* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ↓ = decreased compared with control; ↑ = increased compared with control.

5.11. Clinical Chemistry

The clinical chemistry results are presented in Tables 45-48. A number of parameters were affected by treatment. These findings for the main study are summarised in the tables overleaf. Other occasional statistically significant findings that are not considered to be treatment related are not included in these summary tables.

A number of parameters showed changes that occurred mainly in the highest dose groups of some test articles (e.g. lower alkaline phosphatase, increased γ -glutamyl transferase, alanine aminotransferase and aspartate aminotransferase). A lower albumin : globulin ratio seen in some groups generally reflected decreased total protein and albumin levels, thus only the ratio is included in the summary tables. Glucose levels were often higher than control in all but the highest dose group of many of the test articles. Males showed a much more limited range of differences.

After one month without treatment both sexes given low melting point wax had statistically significantly increased levels of alanine aminotransferase, aspartate aminotransferase and γ -glutamyl transferase. γ -Glutamyl transferase was also higher than control in the female N10 (A) group at this time.

Alkaline phosphatase was significantly raised in the female low melting point wax reversal group and significantly reduced in the male reversal groups receiving white oils N15 (H), P15 (H), N10 (A) and P100 (H) or low melting point wax. Glucose was statistically significantly increased in the reversal female white oils N15 (H) and N10 (A) reversal groups and also in the female reversal group receiving low melting point wax. The albumin:globulin ratio was significantly reduced in all the reversal female white oil groups apart from the P100 (H) group and in the female reversal low melting point wax group.

Summary of clinical chemistry findings considered to be treatment related

Males

Treatment	ALKP U/l	ALAT U/l	ASAT U/l	Gamma-GT U/l	Alb/Glob mmol/l
0.2% White oil N15 (H)	↓*				
2.0% White oil N15 (H)	↓***				
0.2% White oil P15 (H)	↓***				
2.0% White oil P15 (H)	↓***				
0.2% White oil N10 (A)	↓*				
2.0% White oil N10 (A)	↓***				
0.2% Low melting point wax		↑***	↑***	↑*	
2.0% Low melting point wax	↓***	↑***	↑***	↑***	↓***

Females

Treatment	ALKP U/l	ALAT U/l	ASAT U/l	Gamma-GT U/l	Glucose mmol/l	Bilirubin umol/l	Alb/Glob ratio
0.002% White oil N15 (H)					↑*		
0.02% White oil N15 (H)					↑***		
0.2% White oil N15 (H)	↓***				↑**		
2.0% White oil N15 (H)	↓***		↑***	↑***			↓**
0.002% White oil N70 (H)					↑**		
0.02% White oil N70 (H)					↑*		
0.2% White oil N70 (H)					↑***		
0.02% White oil N70 (A)		↑*	↑***				
0.2% White oil N70 (A)		↑*					
0.002% White oil P15 (H)					↑*		
0.02% White oil P15 (H)					↑**		
0.2% White oil P15 (H)							↓*
2.0% White oil P15 (H)	↓***			↑***			↓***
0.2% White oil N10 (A)	↓***				↑*		
2.0% White oil N10 (A)	↓***		↑*	↑***			↓**
0.002% White oil P100 (H)					↑**		
0.02% White oil P100 (H)			↑*	↑***			
2.0% White oil P100 (H)			↑*				
0.002% Low melting point wax					↑*		
0.02% Low melting point wax					↑*		
0.2% Low melting point wax		↑***	↑***	↑**	↑**		↓***
2.0% Low melting point wax		↑***	↑***	↑***		↑**	↓***
0.002% High melting point wax					↑***		
0.2% High melting point wax					↑**		
2.0% High melting point wax					↑*		
0.002% High sulphur wax					↑*		
0.02% High sulphur wax					9↑*		
0.2% High sulphur wax					↑**		
2.0% High sulphur wax	↑*						

* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ↓ = decreased compared with control; ↑ = increased compared with control

5.12. Tissue Analysis

Tissue levels of mineral hydrocarbon were measured using a carbon tetrachloride extraction and infra-red detection technique. The exact nature of the material measured and its similarity to the test article cannot therefore be precisely defined. The material is, however, almost certainly mineral hydrocarbon material of some kind. The results for male and female animals are shown in Table 50.

Kidney

The only animals showing appreciable levels of mineral hydrocarbon were the females receiving white oil N70 (H). Elevated levels of mineral hydrocarbon material were also present in the kidneys of the reversal animals receiving this treatment.

Liver

Mineral hydrocarbon material was detected in male groups treated with white oil N15 (H) and with low melting point wax. There was also an indication of mineral hydrocarbon material present in the livers of male animals receiving white oils N70 (H) and N70 (A). Substantially more mineral hydrocarbon material was present in the livers taken from the female animals. Mineral hydrocarbon material was detectable in the female low melting point wax group and in all female white oil groups except for white oil P100 (H). There was evidence in both sexes for a lowering of levels after the one month reversal period but there was still a substantial amount of mineral hydrocarbon material present in all groups where mineral hydrocarbon material was detectable at the end of the 90-day period.

Spleen

A marked deterioration of the spleen tissue occurred on storage. Dessication of the tissue occurred to some extent in almost all of the samples and the

degree of dessication varied considerably. Thus the results shown in Appendix 56 and 57 indicate a wide variation in the measured levels of mineral hydrocarbon in the tissue. No attempt has been made to express these results in tabulated form. From inspection of the individual data there is very little evidence of appreciable accumulation of mineral hydrocarbon material in the spleen of either sex.

Lymph Node

Very substantial amounts of mineral hydrocarbon material were detectable in the animals receiving the low melting point wax and in those receiving all white oils with the exception of P100 (H). There was very little indication of any reduction in mineral hydrocarbon levels following the reversal period.

Perirenal Fat

There was evidence of a small accumulation of mineral hydrocarbon material in perirenal fat particularly in the white oils N15 (H), P15 (H) and N10 (A) groups and in the low melting point wax group, in both sexes. There was some evidence for a reduction in mineral hydrocarbon levels following the reversal period but elevated levels of mineral hydrocarbon were still present in many of the groups.

5.13. Serum Vitamin E analysis

Serum concentrations of vitamin E were measured in the animals on the tissue level and tissue level reversal studies. The treated groups received test article at the 2% level only. The results are shown in Table 49. Serum concentrations of vitamin E were statistically significantly reduced in all groups treated with white mineral oils. Serum vitamin E was statistically significantly increased in males and females receiving low melting point wax and in female animals receiving coconut oil. Males receiving high melting point wax showed a statistically significantly lower serum vitamin E level

than controls.

The only group to show an effect of treatment after the four week reversal period was the group treated with white oil N 10(A) where serum vitamin E levels still remained statistically significantly reduced.

5.14. Histopathological examination of tissues

Details of the histopathological findings are presented in Tables 51-54. Histopathological lesions were present in a number of tissues. Many of these were of a nature or severity expected in animals of this age and strain and most are not considered further. All findings that were statistically significant or considered to be treatment related are described below on a tissue by tissue basis.

Liver

A statistically significantly increased incidence of microgranuloma and granuloma was seen as shown below:

	Dose level at which granulomatous lesions present (% in diet)	
	Males	Females
White oil N15 (H)	-	2.0
White oil N70 (H)	-	2.0
White oil N70 (A)	-	2.0
White oil P15 (H)	-	2.0*
White oil N10 (A)	-	2.0
White oil P100 (H)	-	-
Low melting point wax	0.2, 2.0	0.2, 2.0
High melting point wax	-	-
High sulphur wax	-	-
Coconut oil	-	-

Results summarised from Tables 51 and 53.

* Statistically significant only when incidences of microgranuloma few, microgranuloma many and granuloma few are combined.

Lesions were classified as microgranuloma if the average diameter was less than 25% of the average hepatic lobule or as granuloma if larger. For each animal bearing granulomatous lesions an estimate was recorded of the number of lesions present, and scored as either few or many. The granuloma were not homogeneously distributed between lobes or even within one lobe, frequently being concentrated in a limited area, with the caudal lobe often the most severely affected. The most severe lesions were distributed around portal areas with a number of granulomatous foci surrounded by a sheath of inflammatory cells. In contrast, the smallest lesions were small collections of macrophages (3 to 5) occasionally with a few lymphocytes at the periphery. In Tables 51-54 the incidence of microgranuloma and granuloma have been compared with the control incidence for each category of lesion.

The only male groups showing a statistically significantly increased incidence of microgranuloma or granuloma were those receiving low melting point wax at the top two dose levels. The severity of the lesion increased with dose and at the highest dose centrilobular vacuolation was also present. A few microgranuloma were also found in one rat receiving white oil N70 (A).

Granulomatous lesions were found in the livers of females given white oils N15 (H), N70 (H), N70 (A), P15 (H) and N10 (A) and in the low melting point wax group. A granulomatous lesion was seen in a single animal given high melting point wax. The most severely affected females were in the group given low melting point wax. This was reflected both in the number of lesions/rat and in the size of lesions.

A number of other lesions were seen in the liver of treated animals at incidences which were occasionally statistically significantly higher than in the control group. Since the lesions were generally those which occurred frequently in controls these occasional differences are considered unlikely to be an effect of treatment.

One month after cessation of treatment granulomatous lesions were present in the livers of animals from all the groups in which lesions were seen at the end of the 90 days treatment. The severity of the lesions seen at the end of the reversal period was similar to that observed immediately after the end of treatment.

Lymph node

The lymph node lesions comprised focal collections of macrophages, often in the cortical region. The macrophages were lightly vacuolated, giving a slightly foamy appearance to their cytoplasm. Some macrophages had a yellowish-brown pigmentation of varied intensity. The focal collections of macrophages were classified as histiocytosis and were scored "minimal", "mild", "moderate" or "marked" on the basis of size and abundance.

The foci of histiocytosis were not homogeneously distributed but were often restricted to one node or even to part of one node, with some areas severely affected and others relatively free of any effect. Since these lesions were unevenly distributed their observation is subject to chance variation related to selection of tissue and sectioning. Indeed, the lymph nodes from high dose and control animals were divided into two samples at necropsy and there were processed separately for conventional histopathology and for special fat stain. This reduced the amount of tissue available for histopathological examination in these groups and may have led to under-reporting of the incidence of lesions.

Histiocytosis was occasionally found in the lymph nodes of control rats, but this was generally restricted to isolated foci and was always classified as "minimal".

Histiocytosis was increased compared with controls in males given white oils N10 (A), N70 (A) and N70 (H) at dose levels down to 0.2%, P15 (H) and low melting point wax at dose levels down to 0.02%, and in those given white oil N15 (H) at dose levels down to 0.002%. Histiocytosis was seen in females given white oils N15 (H), N70 (A), N70 (H), P15 (H),

and N10 (A) at dose levels down to 0.02% and in those given low melting point wax at all dose levels. In each case the severity increased with dose and the effect was much more severe in the females compared with the males. High sulphur wax, high melting point wax and white oil P100 (H) were indistinguishable from controls at any dose level in either sex.

A statistically significantly increased incidence of pigmented macrophages was a frequent finding in many of the treatment groups. Statistical significance was almost invariably confined to the intermediate dose groups and there was no apparent increase in the severity of the finding compared with controls. Thus, it is likely that this observation is a consequence of under-reporting in the high dose and control groups and is not a treatment related effect.

Histiocytosis was found in the lymph nodes one month after cessation of treatment in all groups in which lesions were seen at the end of 90 days. The general pattern of effect and severity was similar on both occasions apart from the effect in males given white oil N70 (H) which was noticeably more marked at the end of the reversal period.

Ileum and Jejunum

A statistically significantly increased incidence of macrophage accumulation in Peyer's patches was observed in both male and female animals receiving low melting point wax at the top two dose levels. A significant increase in macrophage infiltration of the lamina propria was observed in the high dose female low melting point wax group while significantly increased vacuolation of the lamina propria was seen in the female high dose white oil N10 (A) group.

Heart

An increased incidence of an inflammatory reaction around the base of the mitral valve in the heart and some periarteritis of the coronary vessels were seen in high dose animals given low melting point wax.

Kidney

There were a number of histopathological findings in the kidney but none that were considered to be related to treatment.

Lung

There were a number of histopathological findings in the lung but none that were considered to be related to treatment.

6. DISCUSSION

A wide spectrum of toxicological effects were observed during the course of the study. In general, when seen, the effects were similar regardless of the nature of the mineral hydrocarbon but the presence or absence of effects and their severity varied widely from test article to test article. Indeed whilst some test articles produced no effect at all, severe lesions of lymph node and liver were observed with others. For almost all findings the effects were more severe in female animals than in males.

Effects on the liver comprised an increase in organ weight and the formation of granuloma. Although the granuloma were characterized as microgranuloma or granuloma depending on size the essential histological features of the two were similar being formed by macrophages with a variable lymphocytic infiltrate (MacSween, 1979). In general the larger the granuloma the greater the lymphocytic component. The raised levels of aspartate aminotransferase, alanine aminotransferase and γ -glutamyl transferase and reduction in the activity of alkaline phosphatase are indicative of relatively mild hepatic damage (Benjamin and McKelvie, 1978) and are consistent with the degree of histological change. An increase in organ weight was apparent at dose levels lower than those at which granulomatous lesions were identified. Although this liver enlargement was not apparently accompanied by any evidence of other histological changes it is possible that such changes are present but are indistinguishable from the background lesions present in animals of this age and strain.

Lymph node effects comprised focal histiocytosis, increased organ weight and the presence of measurable amounts of mineral hydrocarbon material. Focal histiocytosis is a common non-specific reaction in lymph nodes resulting from the accumulation of non-degradable, often particulate, (Henry and Farrer-Brown, 1981) material within macrophages (Gopinath, Prentice and Lewis, 1987). It is probable therefore that the increased weight of the lymph nodes found in this study (only recorded in animals used for tissue analysis) is caused by the presence of histiocytosis resulting

from the accumulation of mineral hydrocarbon material. Histiocytosis of the type seen has been previously described following treatment with lipids and with phospholipids and vacuoles present within the macrophages have been shown to contain lipid material when stained with oil red O (Gopinath, Prentice and Lewis, 1987).

Kidney weight was increased following treatment with a number of the test materials. However the increase in the weight relative to body weight was never more than 108% of the control value and was not associated with any histopathological findings. As detectable amounts of mineral hydrocarbon material were only found in female animals given white oil N70 (H) this increase in kidney weight cannot be accounted for by accumulation of mineral hydrocarbon material. Since there were no changes in any of the clinical chemistry parameters associated with kidney function these findings are considered to have little toxicological significance.

The reason for the increase in splenic weight in a number of the treated groups is unclear as there were no concomitant histopathological changes and no appreciable accumulation of mineral hydrocarbon material in the tissue. There is, therefore, no indication of a primary cause for the organ weight differences. However, it is possible that the differences seen are secondary to other changes induced by treatment and must therefore be considered as treatment related.

All haematological effects observed appear to be related to the histopathological findings in the lymph node and liver and are typical of a relatively mild inflammatory reaction (Howarth, 1991). Raised white cell counts may represent a systemic effect as a result of the changes found in the lymph node and liver. Reduced red blood cell count may have been due to either red cell destruction or reduced haemopoiesis. However, the reticulocytosis seen in female rats given the low melting point wax suggest an unimpaired haemopoietic capacity. The slight reduction in red cell number may therefore be due to an increased red blood cell loss. This may

have resulted from an increased non-specific activity of the reticuloendothelial system. This finding is, however, considered to be of limited toxicological significance since the reduction in the red blood cell count was never more than 4%.

Serum glucose levels were often raised in a number of female treatment groups, in particular in the low dose and intermediate dose groups. The effect was never more than 116% of the control value and such a value is well within the range given for fasting glucose in a number of rat strains (Ringler and Dabich, 1979). Such an increase is therefore considered to be of little clinical significance, and of limited relevance to the toxicological assessment.

There appeared to be a very small effect of treatment on full caecum weight but not on empty caecum weight indicating an effect on caecum contents. This finding is considered to be of limited toxicological significance.

As an additional part of the study serum vitamin E levels were measured in animals receiving test article at the top dose level only. Serum vitamin E levels were reduced in all white oil treated groups including those receiving white oil P100 (H). Since levels were increased in the low melting point wax treated group there can be no simple direct relationship between serum vitamin E levels and the major toxic effects seen during the study. The depletion of serum vitamin E in the oil treated animals may be a consequence of the preferential solubility of the vitamin in the white oils with a consequent reduction in its bioavailability.

Almost without exception, the toxicological effects seen during the study were much more severe in females than in males. Since the test articles were fed at a constant concentration in the diet to the two sexes the female animals did indeed receive approximately 10% more test article than the male animals. It seems unlikely that an increase in intake of this magnitude is solely responsible for the difference in effects seen. Levels of mineral hydrocarbon material in the liver and lymph node were

considerably greater in female animals than in males and this probably accounts for the increased severity of the toxic effects in these organs in females. Why there should be a greater accumulation of mineral hydrocarbon material in these tissues in females compared with males is less clear. It has previously been suggested that since oestrogens are known to enhance the phagocytic activity of macrophages a more effective uptake of white oils from the lymphatics by these cells in females could explain the findings (Shell Report, SBER.87.010; unpublished data).

While there was evidence for a reduction in severity of some of the effects (e.g. organ weight increases, haematological findings) four weeks after cessation of treatment, there was no evidence of a reduction either in extent or severity of the histological changes seen in the lymph node and liver.

In general, the results of this study are in good agreement with those previously reported with white mineral oils in the same strain of rat (Shell Report, SBER.87.010; unpublished data).

The various test articles examined in the study varied in their toxicological potency. In general the most potent materials were the white oils N10 (A), P15 (H) and N15 (H) and the low melting point wax. The white oils N70 (A) and N70 (H) also produced a spectrum of effects but, in general, were less potent. The two microcrystalline waxes and the P100 (H) white oil appeared to be without effect. These results would suggest that as far as the white oils are concerned viscosity (which is in turn related to molecular weight) plays a major role in determining the toxicity of these materials. In the case of the waxes molecular weight may also play an

important role in determining toxicological potency but there is insufficient data to be sure that this is so.

On the basis of the histiocytosis seen in the lymph node no effect levels were as follows:

	<u>No effect level</u> <u>(% in the diet)</u>
White oil N15 (H)	<0.002
White oil N70 (H)	0.002
White oil N70 (A)	0.002
White oil P15 (H)	0.002
White oil N10 (A)	0.002
White oil P100 (H)	2.0
Low melting point wax	<0.002
High melting point wax	2.0
High sulphur wax	2.0

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