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Chemical Category		MKH 6561	

OFFICE OF TOXIC SUBSTANCES
CODING FORM FOR GLOBAL INDEXING

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9.0 CONTINUATION SHEET

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Continuation of 2.1

As there were no reproductive or developmental effects observed in this study, the results from this study do not warrant reporting under TSCA 8(e). However, in a one-generation reproduction study the litter size at birth and the viability index were reduced at a dose of 20,000 ppm, and there was a shift towards a reduced number of males born in the 20,000 ppm dose group. As the one generation reproduction study has been reported to the EPA (8(e)HQ-99-14385, this study is being reported as a follow-up.

Abstract

MKH 6561 was examined for possible effects on reproduction in a two-generation study using Wistar rats, with one litter per generation. This involved the administration of MKH 6561 to groups of 30 male and 30 female rats at concentrations of 0 (control), 1000, 4000, or 16000 ppm in the diet.

Parental F0 animals were pre-treated over a period of about 10 weeks and allowed to mate over a period of up to three weeks. F1 offspring were nursed up to four weeks of age. Some of the F1 offspring were selected for further treatment and for production of a F2 generation, as done with the F0 rats. F2 offspring were weaned at four weeks of age. F1 pups not kept to become F1 parents and F2 pups were killed and necropsied when the pups were 28 days old.

Clinical signs, body weights, food intake, mating performance, fertility, duration of pregnancy, estrous cycling, and sperm parameters were examined in F0 and F1 rats. Litter size, number of stillbirths, ratio of males to females, pup weight at birth, pup viability, and body weight gain were evaluated in F1 and F2 offspring. Developmental milestones were examined in F1 weanlings. Necropsies were done on all rats. Selected organs were weighed (F0, F1 adults and F1, F2 weanlings) and histopathological evaluations were performed on some organs from F0 and F1 adult rats.

Food intake was increased for F0 males, and F0 and F1 females in the 16000 ppm dose group.
There was no treatment-related effect on mortality, appearance, health, and behavior of the F0 and weaned F1 animals.
There was no treatment-related effect on the body weight of F0 and F1 rats.

There was no treatment-related effect on the following reproduction parameters: mating performance, fertility, duration of pregnancy, number of pups born, percentage of male and female pups, pup weights, litter size at birth, pup weight development, stillbirths, and pup survival rates (viability and lactation indices).

There were no treatment-related clinical or gross pathological findings, including malformations, for pups and weanlings. There was no treatment-related effect on the maturation of the external sexual organs of F1 rats.

There was no treatment-related effect on the estrous cycling of F0 and F1 females.
There was no treatment-related effect on sperm analyses.

For females in the 4000 ppm (F0) and 16000 ppm (F0 and F1) dose groups, there was an increase in the number of animals with a dilated cecum.

Histopathological examination revealed an increase in focal vacuolation of the forestomach epithelium of F0 and F1 males and females in the 16000 ppm dose group, as well as for F1 males in the 4000 ppm dose group.

No treatment-related morphological changes were observed in the reproductive organs of F0 and F1 parental animals in the 16000 ppm dose group.

Thus, the overall no-observed-effect level (NOEL) for the parental animals was 1000 ppm of MKH 6561, and the reproductive NOEL was 16000 ppm of MKH 6561.

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STUDY TITLE

MKH 6561
(c.n.: Procarbazon-Sodium)
Two-Generation Study in Wistar Rats

DATA REQUIREMENT

US EPA OPPTS Guideline No. 870.3800

AUTHOR

Dr. R. Eiben

109096

STUDY COMPLETION DATE

May 26, 1999



PERFORMING LABORATORY

BAYER AG
DEPARTMENT OF TOXICOLOGY
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D-42096 Wuppertal
Germany

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LABORATORY PROJECT ID

Bayer AG Report No. 28792
Bayer AG Study No. T1061527

STATEMENT OF DATA CONFIDENTIALITY

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d)(1)(A), (B), or (C):

BAYER CORPORATION

Dr. J.H. Thyssen:

J.H. Thyssen
Vice President, Toxicology

Date:

June, 28th, 99

GLP COMPLIANCE STATEMENT

This study was conducted in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1997) and with the Principles of Good Laboratory Practice according to Annex 1 German Chemicals Act * and meets the FIFRA Good Laboratory Practice Standards (= 40 CFR Part 160) and the GLP standards of the Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF, 59 NohSan No. 3850), with the exception that recognized differences exist between GLP principles/standards of OECD and FIFRA (for instance authority granted by Agency inspectors and certain record retention requirements).

STUDY DIRECTOR

BAYER AG



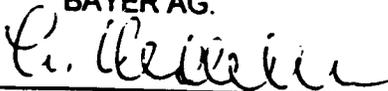
Dr. R. Eiben

March 22, 1999

Date

SPONSOR

BAYER AG.



Dr. L. Machemer

March 23, 1999

Date

SUBMITTER

BAYER CORPORATION


Dr. J.R. Thyssen
Vice President, Toxicology6-28-99

Date

* Bundesgesetzblatt, Part I of the 29th of July 1994)

FLAGGING STATEMENT

I have applied the criteria of 40 CFR 158.34 for flagging studies for potential adverse effects to the results of the attached study. This study neither meets nor exceeds any of the applicable criteria.

**SUBMITTER
BAYER CORPORATION**


Dr. J.H. Thyssen
Vice President, Toxicology

6-28-99
Date

**SPONSOR
BAYER AG**


Dr. L. Macheimer

March 23, 1999
Date

**STUDY DIRECTOR
BAYER AG**


Dr. R. Eiben

March 22, 1999
Date

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Quality Assurance Statement

Test Item : MKH 6561

Study No.: T1061527

Study-based inspections/audits were conducted by the Quality Assurance on the dates given below. Audit reports have been submitted in writing to the study director and, if necessary, also to the laboratory management, or other persons affected.

Date of audit		Date of report to study director and/or management
02/ 14/ 1997	(study plan)	02/ 14/ 1997
02/ 13/ 1997	(study conduct)	02/ 19/ 1997
04/ 15/ 1997	(study conduct)	04/ 15/ 1997
05/ 12/ 1997	(study conduct)	05/ 12/ 1997
05/ 22/ 1997	(study conduct)	05/ 22/ 1997
06/ 03/ 1997	(study conduct)	06/ 03/ 1997
06/ 20/ 1997	(study conduct)	06/ 23/ 1997
07/ 23/ 1997	(study conduct)	07/ 23/ 1997
08/ 06/ 1997	(study conduct)	08/ 06/ 1997
09/ 11/ 1997	(study conduct)	09/ 11/ 1997
10/ 09/ 1997	(study conduct)	10/ 09/ 1997
10/ 29/ 1997	(study conduct)	10/ 29/ 1997
11/ 05/ 1997	(study conduct)	11/ 05/ 1997
11/ 13/ 1997	(study conduct)	11/ 13/ 1997
11/ 28/ 1997	(study conduct)	11/ 28/ 1997
01/ 04/ 1999 - 01/ 26/ 1999	(first draft)	01/ 28/ 1999
05/ 19/ 1999	(final draft)	05/ 20/ 1999

The results of the study and the methods used have been correctly reported.

Quality Assurance Unit
PH-OP-QA-GLP, Bayer AG

Date: *May 20, 1999*

Responsible:

Lehn
Dr.H.Lehn

SIGNATURES

Study Director:

ER May 26, 1999
(Dr. R. Eiben) (Date)

Head of Carcinogenicity
and Genotoxicity

H. Enzmann May 26, 1999
(Dr. H. Enzmann) (Date)

1. SUMMARY

MKH 6561 was examined for possible effects on reproduction in a two-generation study in Wistar rats with one litter per generation. This involved administration of **MKH 6561** to groups of 30 male and 30 female rats at concentrations of 0 (control), 1000, 4000 or 16000 ppm in their diet (for actual doses in mg/kg body weight see Tables 5 and 19).

Parental F0 animals were pretreated over a period of about 10 weeks and allowed to mate over a period of up to three weeks. F1 offspring were nursed up to an age of four weeks. Some of them were selected for further treatment and for breeding a F2 generation as done with F0 rats. F2 offspring were weaned at an age of four weeks.

Clinical signs, body weights, food intake, mating performance, fertility, duration of pregnancy, estrus cycling and sperm parameters were examined in F0 and F1 rats. Litter size, number of stillbirths, ratio of males to females and pup weight at birth as well as viability, lactation, and body weight gain were studied in F1 and F2 offspring. Developmental milestones were examined in F1 weanlings. Necropsies were done in all rats. Selected organs were weighed (F0, F1 adults and F1, F2 weanlings) and histopathological evaluations were performed on some organs of F0 and F1 adult rats.

The food intake was not remarkably influenced up to 4000 ppm **MKH 6561** and increased at 16000 ppm.

The mortality of treated animals was not increased in any generation and there was no treatment effect on the appearance, health and behavior of the F0 and weaned F1 animals at levels of up to 16000 ppm.

Up to 16000 ppm there were no adverse effects on the body weight in F0 and F1 rats.

The reproduction parameters such as mating performance, fertility, duration of pregnancy, number of pups born, percentage of male and female pups, pup weights and litter size data at birth, pup weight development as well as stillbirths and pup survival rates (viability and lactation indices) showed no treatment-related effect at levels of up to 16000 ppm.

No test substance-related clinical or gross pathological findings including malformations were observed in pups or weanlings. No treatment effect on maturation of external sexual organs of F1 rats was apparent.

The estrus cycle staging in F0 and F1 females showed no abnormalities up to 16000 ppm.

Sperm analyses revealed no test substance-related changes.

At 4000 ppm (F0) and 16000 ppm (F0 and F1) more females with a dilated caecum were found than in the other groups.

Histopathological examination revealed an increase in focal vacuolation of the forestomach epithelium at 16000 ppm in F0 rats and F1 females as well as in F1 males of the 4000 and 16000 ppm group.

No morphological changes due to the treatment were detected in reproduction organs of parent F0 and F1 animals at 16000 ppm.

Thus, the dietary concentration of 1000 ppm MKH 6561 is established as the overall no observed adverse effect level (NOAEL=NOEL) for the parent animals under conditions as described. The reproduction was unaffected up to a concentration of 16000 ppm.

2. INTRODUCTION

MKH 6561 is a test substance presently under development as a herbicide.

In the following results of a two-generation study with **MKH 6561** on Wistar rats with one litter per generation are reported.

The conduct of this study complied with recommendations published by the U.S. EPA (Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Humans and Domestic Animals, Series 83-4: "Reproductive and Fertility Effects"; Revised Edition, November 1984) and the OECD (Guidelines for Testing of Chemicals, Section 4: Health Effects, No. 416: "Two-Generation Reproduction Toxicity Study", adopted May 26, 1983). Furthermore, this study was conducted according to the "Guidance on Toxicology Study Data for Application of Agricultural Chemical Registration", Society of Agricultural Chemicals, Japan 1985 (MAFF Requirements).

Moreover, additional investigations mentioned in newly issued draft guidelines such as sperm tests, estrus cycle determination, registration of developmental milestones and measurement of organ weights in weanlings were performed.

3. GENERAL INFORMATION

3.1 Test Facilities

The experimental phase and the evaluation of the study were conducted at the Institute of Toxicology, BAYER AG, Friedrich-Ebert-Straße 217-333, D-42096 Wuppertal, Germany. The histological technique was performed by Propath (UK) Ltd., Willow Court, Netherwood Road, HR 2 6Ju, Hereford, England. The histopathological examination was done at TPC Toxicologic Pathology Consultancy, Goethestr. 26, 24116 Kiel, Germany.

3.2 Duration of Study, Chronology

The dates of the experimental phase of the study and other important dates are summarized in Table 1.

Table 1 - Key Study Dates

Study Identification:	
Test Number:	T1061527
Pathology Number:	4997
Animals:	
Delivery of Animals:	February 5, 1997
Animal Age at Delivery:	4 - 5 weeks
Animal Age at Study Start:	5 - 6 weeks
Mean Initial Weights at Study Start:	
Males:	153 g (123 - 185 g)
Females:	102 g (87 - 120 g)
Study Initiation Date:	February 7, 1997
Total Duration of Study:	42 weeks
Study Start Date (First Day of Treatment):	February 12, 1997 (male)
(Experimental Starting Date)	February 13, 1997 (female)
Experimental Completion Date:	February 24, 1999
(Last Animal Necropsied)	
Study Completion Date	see signatures

The times (days or weeks) stated in the data listings are defined as follows:

a. Body weights, clinical findings and food intake:

F0 generation: Week 0 refers to results obtained before administration of test substance commenced on the first day of treatment. It may also be used to include occurrences during the first week of treatment. Week 1 refers to data recorded on day 8, etc., allowing for a margin of ± 3 days. Days of body weight or food consumption measured during pregnancy and lactation are given with respect to the first day of pregnancy or lactation. The food intake is designated according to the week/day on which the unconsumed food was weighed. In the calculations of mean food intake the actual number of days over which consumption took place was taken into account.

F1 generation: In principle the same time schedules are used for the F1 generation. However, week 0 means in this case the day on which all F1 weanlings were appointed to their group for further treatment.

b. Lists of surviving F0/F1 animals and dates of death:

These lists indicate the number of animals still alive on the last day of a given week (in respect to the first day of the treatment (F0) or the day, when the first F1 weanling was appointed to the group), and the day on which the animal died. Death days are given in the Pathology Report.

3.3 Archiving

The study plan, raw data, specimens and the final report are retained in the archives specified by Toxicology of Bayer AG. The storage of a retention sample of the test item and, if applicable, also of the reference item is in the responsibility of the sponsor.

3.4 Persons Involved, Responsibilities

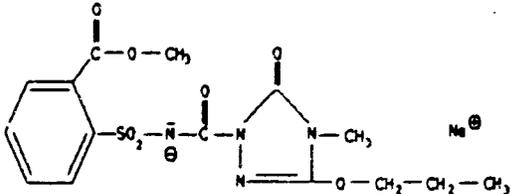
Study Director:	Dr. Eiben
Head of Carcinogenicity and Genotoxicity:	Dr. Bomhard (until October 31, 98) Dr. Enzmann (from November 1, 98)
Analyses of test substance:	Dr. Gau
Analyses of test substance in the diet:	Dr. Rüngeler
Procurement of laboratory animals:	Dr. Hoffmann
Animal housing:	Dr. Eiben
Gross pathology:	Dr. Rinke, Dr. Sander
Histopathology:	Dr. Vogel
Technical services:	Mr. Lömker, grad. engineer
Computer-assisted data recording and processing:	Dr. Klotz
Archiving:	Prof. Dr. Schlüter
Quality Assurance:	Dr. Lehn

4. MATERIALS, TEST SYSTEM, METHODS

4.1 Test Item and Administration

Data concerning the test item (=test substance) and administration are summarized in Table 2.

Table 2 - Test Item and Administration Data

Test Item:	MKH 6561
Chemical Name (CAS):	Benzoic acid, 2-[[[(4,5-dihydro-4-methyl-5-oxo-3-propoxy-1H-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-methyl ester, sodium salt
Structural Formula	
Common Name	Procarbazone-Sodium
CAS No.:	181274-15-7
Molecular Mass:	420.4 g/mol
Molecular Formula:	C ₁₅ H ₁₇ N ₄ O ₇ S.Na
Mixed Batch No.:	05649/0004 (reserve sample is stored)
Purity	97.6% (January 12, 1996)
Released for Toxicity Studies	until June 18, 1998
Appearance:	colorless crystalline powder
Storage:	refrigerator
Administration:	
Route and Frequency:	ad libitum in the diet up to necropsy
Excipient:	1% peanut oil (DAB 10)
Diet Preparation:	once weekly
Diet:	Altromin® 1321 meal
Storage of Formulation(s):	at room temperature
Formulation Stability:	at least 14 days

4.1.1 Dietary Preparation

MKH 6561 was blended (using a mixing granulator manufactured by Loedige, Paderborn) with Altromin[®] 1321 containing 1% peanut oil to minimize dust formation (including 0 ppm concentration). The amounts of test substance were calculated on the basis of an assumed 100% content of **MKH 6561**. The diet mixtures were prepared once weekly.

4.1.2 Analyses of Test Item in the Diet

Analytical investigations on homogeneity and stability (stability data were taken over from Study No. T0058151) of the test item in diet preparations were done prior to commencement of the study using samples from test mixtures (data see Analytical Report in Part 2). The test item content in the food given to the animals was checked at regular intervals throughout the study (start of study, randomly each 3 month period, end of study). This was done by analyzing samples of the diet mixes used. Per dose one sample of the food mixes was taken on the day the food was prepared, and another was taken after being kept under animal room conditions for the feeding period. All samples taken directly after diet preparation or at the end of the feeding period(s) were kept deep frozen (at temperatures of approx. -20°C) until examination.

Reserve samples from each mixture were stored at least for 8 weeks at about -20°C.

4.2 Dosages and Study Groups

The treated animals were dosed (not adjusted to body weight) with the test substance on a 7-day-a-week basis. The test substance was administered to parental (P = F0) animals prior to and during their mating, during the resultant pregnancy, and through the weaning of their F1 offspring. The substance was then administered to selected F1 offspring during their growth into adulthood, mating, and production of a F2 generation, until weaning of the F2 generation.

The dose schedule (nominal concentrations of **MKH 6561**) and the distribution of animals by study group are summarized in Table 3 on the next page.

Generally, treated and untreated rats were managed in the same manner.

Table 3 Dosing Scheme

Dosing Schedule and Group Allocation					
Group No.	Dose ppm	Sex	Number of Animals	Generation	Animal No.
1	0	m	30	F0	1 - 30
2	0	f	30	F0	31 - 60
3	1000	m	30	F0	61 - 90
4	1000	f	30	F0	91 - 120
5	4000	m	30	F0	121 - 150
6	4000	f	30	F0	151 - 180
7	16000	m	30	F0	181 - 210
8	16000	f	30	F0	211 - 240
9	0	m	30	F1	241 - 270
10	0	f	30	F1	271 - 300
11	1000	m	30	F1	301 - 330
12	1000	f	30	F1	331 - 360
13	4000	m	30	F1	361 - 390
14	4000	f	30	F1	391 - 420
15	16000	m	30	F1	421 - 450
16	16000	f	30	F1	451 - 480

Randomization

The F0 animals were randomly allocated to the individual study groups before treatment started. The rats were weighed individually, divided into two weight classes (light and heavy), and were kept further individually. Using a random list based on evenly distributed random numbers and especially generated for this study the animals were chosen individually from both collectives and assigned to the group specified by the random list. All animals of all test groups were of uniform weight and age.

The mean body weights at the start of the study can be found in Report Part 2.

The random list used to appoint F0 rats to groups or to select females for estrus cycle examinations were produced by using a program from the IBM Scientific Subroutine Package at the Institute of Biometrics, BAYER AG.

The random lists for F1 litter culling and for selections of F1 animals for further treatment were prepared on an HP 3000 computer system using a random-number generator.

4.3 Rationale for Dose Selection

Dose selection was based on the results of a one-generation pilot study in Wistar rats (EIBEN, R., Bayer Report No. 26743, 17. October 1997) performed with the dosage scheme 0, 1000, 5000 and 20000 ppm in the diet at the Institute of Toxicology, Bayer AG.

In this study a NOAEL level was established at 5000 ppm. At 20000 ppm reduced litter weights as a consequence of a smaller mean litter size at birth occurred. In addition, in this group a reduced viability and a shift towards a reduced number of males born per litter were noted. From these results the concentration of 20000 ppm was considered to be too high to be used in a two-generation study. Since from our experience a dietary concentration of 16000 ppm is high enough to correspond with the limit dose of 1000 mg/kg, 16000 ppm was established as high dose.

Therefore, the dietary concentrations of 0, 1000, 4000 and 16000 ppm were chosen for the present study.

4.4 Study Organization

The F0 animals were pretreated with the compound for about 10 weeks up to the cohabitation period. Within the last two weeks of this premating period investigations on estrus cycle were performed. During the following mating period the first male was co-housed with the first female F0 animal within the group and so on over night at a maximum of 12 times during the three-week mating period. Inseminated females were not further co-housed. Insemination was established by investigating vaginal smears prepared in the morning. After a gestation period of about 22 days litters were born and the dams were allowed to rear them. If necessary, four days after birth the F1 litters were reduced (= culled) to eight pups according to random lists. If possible, four male and four female pups remained per litter. Pups found in a moribund state at day 4 were excluded from lactation immediately after their body weight had been established. This was done to investigate possible malformations and to prevent cannibalism during further rearing period. The remaining F1 pups were raised to an age of four weeks and then necropsied. F0 females were killed and necropsied when 28 day old F1 animals had been weaned. F0 males were killed after mating period partly in the course of spermatological investigations.

Thirty male and 30 female F1 rats per group were selected for further treatment and to breed the F2 generation. This was done by randomly selecting one male and one female as far as possible from each litter. The weaned F1 offspring were treated further with the compound at least 13 weeks (including a two-week period for estrus cycle determinations). During the following mating period the third male was co-housed with the first female within the group and so on, and then co-housed as described in the case of the F0 animals. Sibling matings were thereby excluded since as far as possible already at weaning pups had been appointed within the group in a way that a F1 male was co-housed with a F1 female of the second litter next to one of the same group. The procedures during the mating, pregnancy and lactation period of F1 rats were the same as described for F0 rats. The F1 parent animals were killed as scheduled after their F2 litters had been weaned at day 28 p.p. as described for F0 rats.

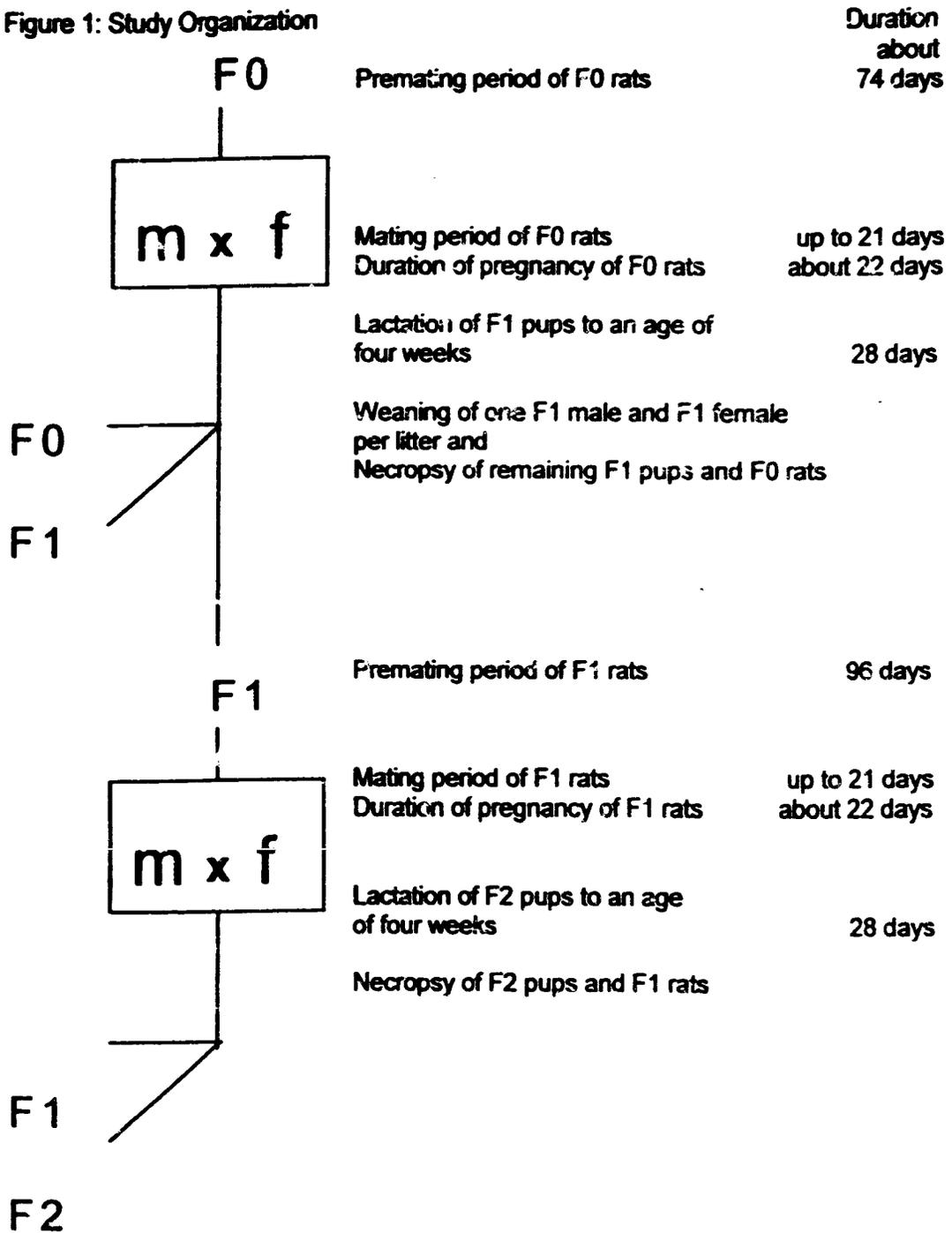
Additional mating procedures (= remating)

F0 and F1 females found sperm-positive after the first mating day but not pregnant were co-housed again over one week with the same male without checking insemination or measuring body weight and food intake during pregnancy. Litters born were nursed up to day 28 p.p. All data recorded from these litters were included to the litter data of their groups.

F0-female No. 44 (0 ppm) was not sperm positive with the male No. 14, which had shown signs of bad condition. However, when the male No. 15 was co-housed female No. 44 got pregnant. For calculation of fertility index, but not for the insemination index the pregnancy of female No. 14 was taken into account.

The following Figure 1 provides a schematic survey of the study organization.

Figure 1: Study Organization



4.5. Test System (Animals) and Housing Conditions

4.5.1. Test Animals

As in the case of the previous subchronic study with MKH 6561 (T0058151), this study was conducted with rats, a species recommended in guidelines for reproduction studies. In this study Wistar rats (strain ICO:WU (IOPS Cpb) of the breeder IFFA Credo/France, Belgium) were used. The rats had been sorted by the breeder in such a manner that no siblings were present in the animals used. Wistar rats have been used for reprotoxicological studies at Bayer AG for a number of years. Historical data of Wistar rats on the test parameters are available. Historical data of reproductive parameters in Wistar rats are given in the Report Part 2.

The state of health of the breed is monitored and the animals are routinely spot-checked for the main specific pathogens. The results of these tests are filed at Bayer AG. The animals were given no vaccinations or medical treatment.

After receipt, the animals were adapted to the animal room conditions, and carefully observed for signs of disease until treatment was initiated. Only healthy, symptom-free animals were used for the study. The females were nulliparous and non-gravid.

4.5.2 Housing Conditions

During the acclimatization period and study rats were housed singly under conventional conditions in Type IIa Makrolon[®] cages, which are larger Type II cages, (as described by SPIEGEL, A., and GÖNNERT, R., Z. Versuchstierkd. 1, 38 (1961) and MEISTER, G., Z. Versuchstierkd. 7, 144 (1965)). During the mating period females were kept (up to insemination) in Typ III cages and were co-housed overnight with their males.

As bedding material low-dust soft-wood shavings were used. When parturition was near cages of F1 females were provided with a special nesting material such as coarse wood shavings.

Both, bedding and nesting material were supplied by Ssniff GmbH, Soest, and tested for contaminants on a random basis (the results are held on file at BAYER AG).

The cages containing the experimental animals were separated by groups and placed on shelves in ascending order of animal number.

The animal room (No.: 254) was located in a special building area, separated from the rest by a barrier system. This area could be entered and supplied with materials only through lock systems and a clean corridor. For the disposal of used/soiled material and moribund/dead animals there was a separate transport route. Generally, used or unused cage material was moved around in closed containers in this building. To ensure optimal hygienic conditions during the study, people entering the barrier system had to change their clothes and to disinfect their hands and shoes.

Identification of Animals

The F0 and F1 animals were identified by cage cards stating the study number, test substance, animal number, sex and dose. The color of the cage cards varied according to the dosage group. Additionally, these animals were identified by ear tattoos.

Pups were identified by foot tattoos.

Cleaning, Disinfection and Pest Control

Cages, cage lids, food containers and drinking bottles were cleaned with hot water. The cage shelves were cleaned and disinfected with Tegel[®] 2000 at regular intervals.

Cages and food containers were replaced by clean ones weekly. Drinking bottles and caps were changed twice a month.

The floor of the animal room was disinfected once a week (Tegel[®] 2000). Walls were cleaned regularly in the same way. Continuous pest control was performed using a cockroach trap on pheromone basis. The traps were supplied by Killgerm GmbH, Neuss, placed in the animal room and replaced about every 6 weeks by new ones. A contact between animals and traps was excluded.

Environmental Conditions

The animal room had a standardized climate:

Room temperature:	23 ± 2°C
Air humidity:	55 ± 5%
Light/dark cycle:	12 hour rhythm from 6 a.m. to 6 p.m. CET (artificial illumination: approx. 140 LUX, for work in the room approx. 380 LUX). From 6 p.m. to 6 a.m. CET orientation light, approx. 3-5 LUX
Air exchange:	approx. 15-20 passages per hour

Occasional deviations from these standards occurred, e.g. during cleaning of the animal room. These did not have any apparent influence on the outcome of the study.

Diet

The diet consisted of a fixed-formula standard diet (Altromin®1321 meal, supplied by Altromin GmbH, Lage) and tap water during the acclimatization period and throughout the study. Food and water were available for the animals ad libitum.

The nutritional composition and contaminant content of the standard diet were routinely checked and analyzed on a random basis. The tap water complied with German drinking water standards¹. The results of the analyses of the diet and water are held on file. The data available provided no evidence of any effect on the study objective.

The food was provided in stainless steel dispensers. Water was supplied in polycarbonate bottles with a capacity of approx. 300 ml or 700 ml (as described by SPIEGEL, A. and GÖNNERT, R., *Z. Versuchstierkd.* 1, 38 (1961) and MEISTER, G., *Z. Versuchstierkd.* 7, 144 (1965)).

¹ German drinking water standards of December 5, 1990, Bundesgesetzblatt No. 65, 2612, issued on December 12, 1990.

4.6. General Investigations

4.6.1 Inspection of Animals

All experimental animals were inspected (cage side examination) twice daily (once daily on weekends and public holidays), and any findings such as mortality, morbidity, behavioral changes and signs of difficult or prolonged parturition observed were recorded. In F0 and F1 parental animals a detailed physical examination of the animals was made and recorded at the weekly change of cages as a rule. In this evaluation the general state of health, behavior, condition of the fur, and the body openings as well as excretory products were examined. During gestation periods females were clinically examined on day 0, 7, 14, 20, and during lactation on day 0, 4, 7, 14, 21, and 28. Findings and abnormalities were recorded on-line or off-line using a coding system and a free text.

For pup inspections see Chapter 4.7.3.

4.6.2 Determination of Body Weight and Food Intake of Parent Animals (F0/F1)

All F0 animals were weighed at the start of the study (first day of dosing). The male animals were weighed at weekly intervals up to week 17 (F0) or 23 (F1), and the female animals until week 10 (F0) or 13 (F1) (= end of pre-mating period). After insemination had been established, the female animals were weighed on postcoital days 0, 7, 14 and 20; and on days 0, 4, 7, 14, 21 and 28 after birth of their pups. F0 and F1 animals were weighed on the date of necropsy to permit calculations of the relative organ weights.

The food intake of F0 and F1 rats was measured as follows :

In F0 males measurements were done weekly during premating period up to week 10 and later in week 15 and 16.

In F1 males food intake were recorded during premating period (13 weeks) and thereafter in week 18 to 22.

In females determinations were performed weekly during the premating period. Furthermore, food intake was recorded on postcoital days 7, 14 and 20 as well as on days 4 and 7 after delivery of the litter.

The food intake was recorded by weighing the quantity of food provided and back-weighing the amount which remained unconsumed.

From these primary data the following were calculated:

- a) daily food intake per animal
- b) mean daily food intake per animal
- c) mean daily test substance intake per kg body weight
- d) mean daily food intake per kg body weight

Averaged for each premating period

- e) mean food intake per animal and day
- f) mean food intake per kg body weight and day
- g) cumulative food intake per animal
- h) cumulative food intake per kg body weight
- i) mean test substance intake per animal and day
- j) mean test substance intake per kg body weight and day
- k) cumulative test substance intake per animal
- l) cumulative test substance intake per kg body weight

The algorithm used for calculating intake of food and test substance is described in Report Part 2.

4.7. Reproduction Parameters

4.7.1 Estrus Cycle Staging

Vaginal smears (methodical details see Chapter 4.7.2.) were taken daily during the two last weeks of pre-mating period. 15 randomly selected F0 and F1 females per dose level were used. The vaginal smears were examined microscopically whether large serrated cells indicating estrus had occurred. These data were used to characterize the estrus cycle length and to determine if females were cycling properly.

4.7.2 Determination of Insemination Rate and Duration of Pregnancy

During the mating period the rats were co-housed overnight from 4 p.m. to 8 a.m. To determine the date of insemination, vaginal smears were taken from the females on the morning following each co-housing until evidence of copulation was observed. The date at which sperms were found by microscopical examination or a vaginal plug was detected was taken as gestation day 0 in calculating the gestation length. Females which exhibited marked weight gains although insemination had not been established were not further co-housed. No duration of pregnancy could be determined for these animals.

The vaginal smears were obtained using a flame-sterilized platinum loop, and were then plated out on slides. The smears were stained for about one minute in May-Grünwald solution, and then microscopically examined.

4.7.3 Recorded Data on Pups

The numbers of live and dead pups as well as the sex of the pups (including those of dead pups if possible) were determined shortly after birth (on postpartum day 0), day 4 (before and after reduction), 7, 14, 21, and 28. At these time points (but not after culling) individual body weights and clinical signs were recorded as well. Note was taken of any apparent malformations.

4.7.4 Calculation of Indices

The following indices were calculated for each dose group: insemination index, fertility index, gestation index, live birth index, viability index and lactation index. The following formulae were used:

$$\begin{aligned} \text{Insemination index (\%)} &= \frac{\text{No. of sperm positive females}}{\text{No. of females co-housed with a male}} \times 100 \\ \text{Fertility index (\%)} &= \frac{\text{No. of pregnant females}}{\text{No. of sperm positive females}^*} \times 100 \\ \text{Gestation index (\%)} &= \frac{\text{No. of females with live pups}}{\text{No. of pregnant females}} \times 100 \\ \text{Live birth index (\%)}^\S &= \frac{\text{No. of live pups at birth}}{\text{total No. of pups born}} \times 100 \\ \text{Viability index (\%)}^\S &= \frac{\text{No. of live pups on day 4 pre-culling}}{\text{No. of live pups born}} \times 100 \\ \text{Lactation index (\%)}^\S &= \frac{\text{No. of live pups after three/four weeks}}{\text{No. of live pups after four days (after culling)**}} \times 100 \end{aligned}$$

* including pregnant females that were not sperm positive.

** moribund pups died during the course of culling were not included.

§ Index calculation per groups is based on total pup number per group.

4.8 Necropsies

4.8.1 Necropsies of Parent Animals

Unscheduled Necropsies

Parent animals that died or were killed in moribund condition (under diethyl ether narcosis) during the study were necropsied and macroscopically examined.

Scheduled Necropsies

After the F1 or F2 pups had been weaned, the dams were anesthetized with carbon dioxide and killed by exsanguination and examined for gross pathology.

In F0 and F1 females implantation sites were counted and documented. In all cases this was done after the uterus had been stained with ammoniumsulfide. In very few cases, discrepancies between number of implantation sites and number of pups delivered per female may have occurred.

F0 and F1 males were killed as scheduled under carbon dioxide narcosis when they were not required for further treatment. They were necropsied and macroscopically examined in the same way. In some cases this was done during the course of spermatological investigations (s. Chapter 4.10).

Fixation

The following organs/organ specimen of the F0 and F1 animals were fixed in buffered 4 % formaldehyde solution: liver, stomach, caecum (only F1 animals), pituitary gland, vagina, uterus, ovaries with oviducts, mammary gland with skin, coagulation glands, seminal vesicles, prostate gland, tattooed ears and all organs/organ specimen exhibiting macroscopic changes (such as in intestine).

The testes and epididymides (if sperm analysis was done one organ only) were fixed in Davidson's solution.

4.8.2 Necropsies of Pups

Unscheduled Necropsies

Unless autolysis or cannibalism rendered examination impossible, pups that were found dead at birth, that died during the course of lactation as well as those killed (with carbon dioxide) in moribund condition were macroscopically inspected after opening the body cavities, with particular attention on the organs of reproduction.

A lung flotation in tap water was performed during the necropsy of pups found dead on the day of the first litter inspection. This was done to determine whether pups had breathed at birth or not.

Scheduled Necropsies

The pups selected for litter reduction were killed with carbon dioxide on postpartum day 4. Weanlings were killed under carbon dioxide anesthesia by head dislocation on postpartum day 28, if not used for further treatment. Both, pups selected for culling as well as weanlings were examined for macroscopical alterations.

Fixation

The brain, liver, stomach, spleen, thymus and testes/ovaries of the first F1/F2 male and F1/F2 female per litter (day 28 p.p.) were fixed in 4 % formaldehyde solution. In any case macroscopically changed organs/organ specimen of all pups were fixed in buffered 4 % formaldehyde solution.

4.9 Organ Weight Determinations

4.9.1 Organ Weights of F1 and F0 Rats

Organ weight determinations of the brain, liver, kidneys, adrenals, spleen, uterus, seminal vesicles, prostate, epididymides*, testes and ovaries were done during the scheduled necropsy.

4.9.2 Organ Weights of F1 and F2 Weanlings

The body weights and the weights of brain, liver, spleen, thymus, testes/ovaries were determined in the first male and female weanling per litter at scheduled necropsy.

* in 0 and 16000 ppm rats used for spermatology one epididymis only

4.10 Examination of Sperms

Spermatological investigations were performed in the first 20 living F0 and F1 males of the 0 and 16000 ppm group.

Determination of spermatozoa motility and viability:

After necropsy sperm motility and decrease in motility after 5 minutes were evaluated in a sperm sample collected from the right cauda epididymis in HAM's F10 tissue culture medium (37°C). A sample of about 20 µl of the culture medium was set on a 37°C warmed and siliconized slide and then covered with a warmed cover glass. Evaluation of motility was performed on 100 spermatozoa during minute 1 and minute 5 after preparation of the sample using semi-darkfield microscope. The microscope stage was warmed up at 37°C. Spermatozoa showing any kind of active movement were given a positive score. Furthermore, the difference between motility recorded during the first minute and that measured during the fifth minute was calculated in percentage.

Determination of spermatozoa morphology:

Spermatozoa morphology was evaluated in a formalin citrate fixed and Eosin G stained sperm sample collected from the right cauda epididymis. Morphological changes of the head, upper and middle tail were evaluated on 200 spermatozoa using the microscope as mentioned above.

Quantitative determination of spermatozoa in epididymis:

Determination of spermatozoa density was performed in a suspension (0.9% NaCl) of minced cauda epididymis tissue by counting of spermatozoa in a hemocytometer and calculation of spermatozoa density per mg epididymis. The samples used for counting had been heated (> 70° C) shortly.

Quantitative determination of homogenization resistant spermatid heads in the testis:

Determination of spermatid head density was performed in a suspension (0.9% NaCl plus 200 µl Triton x-100) of homogenized testis tissue by counting of spermatid heads in a hemocytometer and calculating of spermatid head density per mg testis.

4.11 Determination of Developmental Milestones (F1 Weanlings)

The age of preputial separation or vaginal opening was studied in all F1 weanlings selected for further treatment. This was done by investigation of the preputium and vagina up to occurrence of preputial separation or vaginal opening as far as had not happened on the day 28, 31, 34, 37 and 40 p.p.

4.12 Histopathological Examinations

The following organs of all F0- and F1- rats belonging to the control or high dose group were examined microscopically:

Liver, coagulation glands, epididymides, mammary gland area, ovaries in 2 steps each, oviducts, pituitary gland, prostate gland, seminal vesicles, skin, testes, uterus including cervix uteri and vagina.

The stomach was examined in all parent animals. Additionally, all gross lesions such as dilated caeci were evaluated microscopically.

Methods used are described in detail in the Pathology Report.

4.13 Statistics, Recording of Data and Presentation of Results

Statistics

The following methods were used to test for statistical significance:

- a. The Dunnett-Test in connection with a variance analysis for
 - Body weights of parent animals
 - Organ weights of parent animals
- b. The Kruskal-Wallis-Test with a Steel-Test for food consumption data
- c. The U-Test was used for the evaluation of the
 - Pup weights
 - Litter sizes
 - Litter weights

The mean pup weight of each individual litter was used as a basis for calculation of the pup weight means of the dose groups. The litter size calculation was based on the number of female animals with live pups.
- d. The T-Test was used for the evaluation of the organ weights of F1 of F2 weanlings
- e. The Fisher's exact probability test (two-tailed) at significance levels of $\alpha = 5\%$ and 1% was used for the evaluation of the
 - Insemination index¹⁾
 - Fertility index¹⁾
 - Gestation index¹⁾
 - Live birth index¹⁾
 - Viability index¹⁾
 - Lactation index¹⁾

Calculations to a, b and d were performed using an HP 3000 computer system. Calculations to c were performed using an HP Vectra personal computer (NPAR 1 way from SAS). Calculations to e were performed using an HP 3000 computer system.

- f. The CHI-Square test (corrected by Yates) performed on a HP Vectra personal computer (NPAR 1 way from SAS) was used for the evaluation of the postimplantation loss data, if dose-dependent means occurred.

¹⁾ (done if dose-related changes had occurred.)

Results of sperm analyses, observations concerning developmental milestones and number of implantation sites were not evaluated statistically.

Recording of Data

Body weights, food consumption data and clinical findings were recorded on-line up to pregnancy (females) or up to necropsy (males). During gestation and lactation these data were processed offline. Organ weights of F0 and F1 rats were recorded on-line or off-line. Inlife pup data with the exception of clinical findings were recorded off-line. All other data (belonging to adults or offspring) were documented without any computer assistance.

Details of processing of histopathological data are given in the Pathology Report.

Individual pup weights listed under "day 4 after culling" are taken from the same measurement as weights listed under "day 4 before culling".

Data not Presented

Body weights and/or food consumption data of females not found to be sperm positive or failed living pups and those of sperm-positive, but not pregnant females are not reported, since they are not useful for study evaluation.

Since food intake data recorded during pregnancy and lactation varies strongly from that measured during pre-mating period the calculation of cumulative and mean food/test substance consumption was limited to the pre-mating period.

In some very rare cases there could be gaps in individual data lists of Report 2, if an individual value was erroneously not recorded.

5. RESULTS

The results of the investigations are summarized below. Only group means are given in the tables of Report Part 1. Individual animal data and group means with statistical data can be found in Report Part 2. For the list of abbreviations used in the tables see Chapter 7.

5.1 Analyses of Test Substance in the Diet

All data of analytical investigations are given in detail in the analytical report to be found in Part 2 of this report.

Before the start of the study homogeneity and stability of MKH 6561 in the diet were examined using sample mixtures.

The results revealed that the test substance was homogeneously distributed in the diet amount used, and is stable in the concentration range used throughout the feeding period (1 week).

Five randomly taken samples of diet mixtures fed to the animals were analyzed for their content of MKH 6561 and stability over one week. All these food mixtures proved to be in the specified concentration range.

5.2 F0 Generation

5.2.1 Clinical Signs and Mortality

As can be seen from individual data and a list with cumulative clinical findings presented in Part 2 no test substance-related effects on the appearance, health or behavior were observed in male or female F0 animals at levels of up to 16000 ppm.

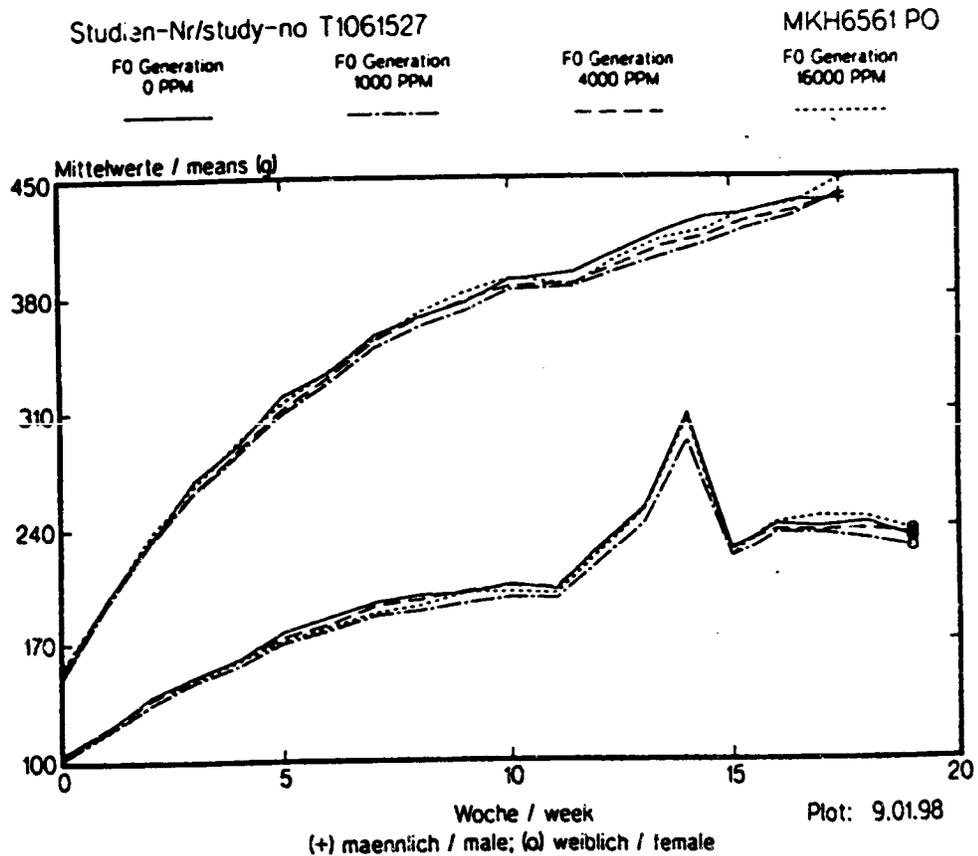
There is no evidence of treatment-related mortality in male and female F0 animals at levels of up to 16000 ppm since no F0 animal died unscheduled (see survival tables in Report Part 2 and death dates in the Pathology Report).

5.2.2 Body Weights of F0 Parent Animals

The group mean body weights of the male and female F0 rats are plotted against the time of the study in the following Figure 2.

As also shown in the tables with means and statistics in Report Part 2, the body weights of the male and female F0 animals receiving up to 16000 ppm did not differ significantly from those of the controls.

Figure 2 Body Weights of Male and Female F0 Parent Animals



5.2.3 Food Consumption and Test Substance Intake of F0 Parent Animals

Individual values measured weekly during the pre-mating period and those recorded in week 15 and 16 (males) or during gestation and lactation (females) are presented together with means and statistics in Report Part 2.

In the following the mean daily food intake per animal and per kg body weight, as well as the corresponding cumulative consumption figures for each study group averaged over the whole pre-mating period are listed.

Table 4

Mean Daily and Cumulative Intake of Food during the Pre-mating Period						
Dose ppm	Sex	Weeks	g/Animal		g/kg Body Weight	
			Total	per Day	Total	per Day
0	m	10	1503	21.5	4954	70.8
1000	m	10	1557	22.2	5239	74.8
4000	m	10	1554	22.2	5199	74.3
16000	m	10	1623	23.2	5384	76.9
0	f	10	1043	14.9	6211	88.7
1000	f	10	1058	15.1	6489	92.7
4000	f	10	1090	15.6	6536	93.4
16000	f	10	1151	16.4	7023	100.3

As can be seen from Table 4 and data given in the Report Part 2 the food intake per animal and related to body weight was similar to controls up to 4000 ppm.

Rats ingesting 16000 ppm consumed 8 - 10% (cumulative) more food per animal per day than the corresponding control rats with a maximum in week 3 (males 10%, females 18%, both $p < 0.01$).

Food intake per kg body weight was, therefore, increased as well in this group.

In the following the mean daily intake of test substance per animal and per kg body weight, as well as the corresponding cumulative consumption figures for each study group averaged over the whole (Table 5) pre-mating period are listed.

Table 5

Mean Daily and Cumulative Intake of Test Substance during the Premating Period						
Dose ppm	Sex	Weeks	mg/Animal		mg/kg Body Weight	
			Total	per Day	Total	per Day
1000	m	10	1557	22.2	5239	74.8
4000	m	10	6214	88.8	20794	297.1
16000	m	10	25964	370.9	86151	1230.7
1000	f	10	1058	15.1	6489	92.7
4000	f	10	4359	62.3	26145	373.5
16000	f	10	18423	263.2	112373	1605.3

As can be seen from Table 5 the intake of MKH 6561 in the treated groups up to 4000 ppm roughly corresponds to the theoretical dose intervals.

In both, males and females of the 16000 ppm group the intake of MKH 6561 exceeded the limit dose of 1000 mg/kg per body weight.

5.2.4 Gross Pathological Examinations in F0 Parent Animals

The individual gross pathological and histopathological findings, as well as the pertinent incidence tables are given in the Pathology Report (Report Part 3).

No significant gross pathological findings were made at necropsy in F0 males at levels of up to 16000 ppm and in F0 females at 1000 ppm. In 4000 ppm and 16000 ppm females (0-0-6-12) slightly to severely dilated caeca were noted.

Concerning uterine implantation sites counted during necropsy no conspicuous discrepancies between the number of implantation sites and that of delivered pups occurred if controls and dose groups are compared (see Table 6 and tables in Report Part 2). The postimplantation loss in treated rats was not increased (see Table 6).

Table 6

Evaluation of Implantation Sites in F0 Females			
Dose ppm	No. of Implantation Sites	No. of Pups at Birth	Postimplantation Loss (%)
0	333	320	3.9
1000	323	285	11.8
4000	331	306	7.6
16000	333	320	3.9

5.2.5 Organ Weights in F0 Parent Animals

The following Table 7 presents the absolute and relative mean weights of the brain, liver, adrenals, spleen, kidneys, epididymides, prostate, seminal vesicles, testes, uterus or ovaries of male and female F0 parent animals. Individual results and statistics are given in Report Part 2.

As can be seen from Table 7 none of the absolute or relative organ weights were changed in a toxicologically relevant manner up to 16000 ppm. The significantly lower means calculated for the epididymis weights of treated males do not reflect a treatment effect, since the very high control mean value results from four extremely high individual values and because a clear dose dependence is lacking. Other statistically significant differences between treatment groups and control are too small to bear any toxicological relevance.

Table 7

Absolute Organ Weights of F0-Rats											
Dose ppm	Sex	Body W. g	Brain mg	Adren. mg	Liver mg	Spleen mg	Kidneys mg	Testes mg	Epidid. mg	Prosta mg	Sem. V. mg
0	m	446	1982	46	15223	723	2718	3276	2050	661	2176
1000	m	439	1980	43	14805	733	2616	3393	1660++	770+	2156
4000	m	443	1913+	43	14770	705	2591	3311	1626++	810++	2157
16000	m	442	1901++	43	14286	699	2533+	3303	1547++	638	2111
								Ovaries mg	Uterus mg	-	-
0	f	233	1731	61	10371	510	1739	139	1472		
1000	f	228	1742	63	10624	519	1763	144	1433		
4000	f	235	1752	65	11310+	505	1804	141	1463		
16000	f	238	1722	65	10934	486	1814	134	1548		
Relative Organ Weights of F0-Rats (mg/100g body weight)											
Dose ppm	Sex	Body W. g	Brain	Adren.	Liver	Spleen	Kidneys	Testes	Epidid.	Prosta	Sem. V.
0	m	446	449	10	3390	163	614	731	450	147	483
1000	m	439	449	10	3369	167	596	777	379+	175++	491
4000	m	443	434	10	3330	160	586	751	369+	183++	485
16000	m	442	432	10	3228	158	573++	750	361+	145	479
								Ovaries	Uterus	-	-
0	f	233	744	26	4449	219	747	60	638		
1000	f	228	766	28	4671	228	775	64	628		
4000	f	235	747	27	4811+	215	767	60	620		
16000	f	238	726	27	4595	204+	763	56	653		

5.2.6 Spermatological Evaluations in F0 Males

Individual results of the spermatological evaluations such as sperm motility, after 1 and 5 minutes, sperm morphology, counts of sperms per mg epididymis and spermatids per mg testis are listed in tables to be found in Report Part 2. A summary of calculated means is given in the following Tables 8 and 9.

Table 8

Evaluation of Sperm Motility and Morphology (Mean Values of 19 (0 ppm) / 20 (16000 ppm) F0 Males)				
Dose ppm	Sperm Motility			Abnormal Sperms %
	1 st min %	5 th min %	Difference %	
0	82	76	7.4	0.74
16000	82	77	6.1	0.98

Table 9

Evaluation of Sperm and Spermatid Counts (Mean Values of 19 (0 ppm) / 20 (16000 ppm) F0 Males)		
Dose ppm	Mean Number of	
	Spermatids per mg Testis	Sperms per mg Epididymis
0	58095	810955
16000	55308	785384

In males receiving 16000 ppm there were no remarkable changes in motility and morphology of sperms. The mean frequency of sperm abnormalities in samples of the 16000 ppm group was comparable with that at 0 ppm.

With respect to the number of spermatids and sperms counted in the right epididymis or testis no compound-related effect is visible.

Therefore, males of the groups 1000 and 4000 ppm were not examined in this respect.

5.2.7 Determination of Estrus Cycle Length in F0 Females

Determinations of estrus staging were done two weeks before F0 rats were co-housed for mating.

Table 10

Dose ppm	Mean Length of Estrus Cycle days	Mean No. of Estri observed within 12 days	No. of Females Showing Irregular Cycle or a Pro- longed Estrus*
0	4.18	2.9	1
1000	3.95	2.9	1
4000	3.60	2.9	1
16000	3.99	2.6	2

* ≥ 5 days

As can be seen from Table 10 the average lengths of the estrus cycle of treated rats were roughly comparable with that of controls. The number of detected estri within 12 days and the frequency of females cycling irregularly were inconspicuous.

5.2.8 Histopathological Investigations in F0 Rats

The results and evaluation of the histopathological investigations are given in detail in the Pathology Report (Report Part 3). These investigations revealed an increased number of males (0-1-0-13) and females (0-0-0-3) showing a focal vacuolation of the forestomach epithelium at 16000 ppm.

There were no microscopical changes which could correlate with the caeca enlargement found in the female groups 4000 and 16000 ppm at necropsy.

In the remaining organs no treatment-related changes were evident.

5.2.9 Parameters of Reproduction in F0 Parent Animals

5.2.9.1 Insemination Index, Fertility Index, Gestation Index and Duration of Pregnancy

After a pre-mating period of about 10 weeks, pairs of one male and one female of the same group were co-housed for mating. The calculated indices of insemination, fertility and gestation, as well as the mean duration of pregnancy per group are listed in the following Table 11.

Table 11 Data Concerning Fertility and Gestation

Dose ppm		0	1000	4000	16000
Insemination index	%	100	100	100	100
Fertility index	%	96.7	100	96.7	100
Gestation index	%	100	100	100	100
Gestation length	Days	22.3	22.2	22.1	22.3
Mated females	n	30	30	30	30
Litters alive	n	29	30	29	30

The insemination, fertility and gestation indices as well as the mean duration of pregnancy did not differ to a toxicologically relevant extent from the pertinent control data at levels of up to 16000 ppm.

There were some F0 females (1-0-2-2 with ascending dose*) which had been found to be sperm-positive after the first day of co-housing but failed pregnancy. According to experience this would happen, if a male co-housed with a female for the first time inseminated the female outside the estrus. This was obviously the case, since these animals had pups when remated with the same male for one week following the three week co-housing period, except No. 163.

* 0 ppm: No. 36; 4000 ppm: No. 151, 163; 16000 ppm: No. 218, 229

Female number 44 (0 ppm) was remated with another male (No. 15) over one week, because the first male (No. 14) was in bad condition.

The mating performance of the F0 animals is given as cumulative percentages in Table 12. Only those mated females were included, in which the mating date could be determined by detection of sperms in the vaginal smear.

The mating performance was not affected by the treatment at levels of up to 16000 ppm.

Table 12

F0 Mating Performance				
Day of Mating Period	Cumulative Percentage of Sperm-Positive Females			
	Dose			
	0 ppm	1000 ppm	4000 ppm	16000 ppm
1	30.0	13.3	30.0	33.3
2	53.3	46.7	43.3	50.0
3	60.0	63.3	60.0	70.0
4	90.0	80.0	70.0	80.0
5				
6				
7	93.3	83.3	73.3	83.3
8				
9				
10			76.7	
11		93.3	83.3	
12		96.7		

5.2.9.2 F1 Litter Parameters at Birth

The following Table 13 lists the total numbers of pups born, those found dead, the live birth index, the percentages of male and female pups, and the litter size at birth.

Table 13 Litter Parameters at Birth

Dose ppm	Number of Pups		Live Birth	Males	Females	Mean Litter
	Total	Dead	Index %	%	%	Size ¹⁾
0	320	1	99.7	49.4	50.6	10.96
1000	285	2	99.3	51.9	48.1	9.43++
4000	306	0	100	54.9	45.1	10.48
16000	320	0	100	50.3	49.7	10.66

¹⁾ viable pups only

None of the litter parameters shown in Table 13 were changed to a toxicologically relevant extent up to 16000 ppm.

5.2.9.3 Clinical Observations in F1 Pups

The individual pup findings are listed in the table "observation in F1 pups" given in the Report Part 2. This table also includes necropsy findings, if there are any, of all offspring and dates of pup necropsy.

No significant clinical findings were made in F1 pups during the four week lactation period at levels of up to 16000 ppm. Malformations were not observed.

5.2.9.4 Body Weights of F1 Pups

Table 14 lists the mean litter weights at birth and on day 28 p.p.. Up to 16000 ppm litter weights were not affected at both time points.

As shown in Table 15 the birth weights of treated pups were not affected. No effect on body weight gain was noted up to 4000 ppm. At 16000 ppm body weights were slightly lower ($p \leq 0.05$) in male pups on day 21.

Table 14

Dose ppm	Mean Litter Weight (g)	
	Day 0	Day 28
0	64.35	507.75
1000	56.87 +	492.05
4000	61.04	497.38
16000	61.52	496.95

Table 15

		Mean Pup Weight at Birth and during Lactation (g)					
Dose ppm	Sex	Day 0	Day 4 after culling	Day 7	Day 14	Day 21	Day 28
0	m	6.05	9.47	14.18	28.08	45.36	72.73
1000	m	6.31+	10.05	14.77	28.35	45.61	73.05
4000	m	6.01	9.79	14.32	27.33	44.29	70.97
16000	m	5.99	9.15	13.49	26.72	43.26+	70.51
0	f	5.73	9.25	13.87	27.96	44.82	68.03
1000	f	5.90	9.80	14.38	28.01	44.59	68.50
4000	f	5.73	9.46	13.89	26.47	42.94	66.23
16000	f	5.68	9.10	13.74	27.06	42.74	66.62

5.2.9.5 Viability and Lactation Indices of F1 Pups

The following Table 16 presents data concerning viability and lactation of F1 offspring.

Table 16 Viability and Lactation Indices

Dose ppm	Viability Index	Lactation Index
	% Day 4	% Day 28
0	94.4	92.9
1000	97.2	93.3
4000	96.7	93.4
16000	98.4	93.6

The viability and lactation indices of the treatment groups were comparable with those of the 0 ppm group.

5.2.9.6 Gross Pathological Changes in F1 Pups or Weanlings

Individual findings detected at scheduled or unscheduled necropsies are listed in the table "Observations in F1 Pups" given in Report Part 2. Incidences are given in the Report Part 2.

In F1 pups necropsied during the lactation period no macroscopical alteration due to the treatment was observed up to 16000 ppm.

Skeletal deviations were not determined in the F1 pups, which died before day four p.p., were killed in the process of litter reduction on postpartum day four, or were necropsied unscheduled during lactation at levels of up to 16000 ppm.

No treatment-related gross pathological findings were made in F1 weanlings at scheduled necropsy.

5.2.10 Organ Weights of F1 Weanlings

Individual organ weights determined during the scheduled necropsy of F1 weanlings are given in the Report Part 2. The following Table 17 summarizes the calculated means per group. As shown there no remarkable organ weight differences exist between the treatment groups and the control group.

Table 17

Absolute Organ Weights of F1 Weanlings							
Dose ppm	Sex	Body W. g	Brain mg	Liver mg	Spleen mg	Thymus mg	Testes mg
0	m	75	1485	3433	238	297	663
1000	m	76	1503	3659	284	294	666
4000	m	73	1502	3344	227	274	629
16000	m	71	1449+	3192	222	288	631
							Ovaries mg
0	f	71	1428	3180	223	284	47
1000	f	71	1443	3242	248	317	45
4000	f	67	1429	2999	208	269	41
16000	f	67	1407	2976	216	273	43
Relative Organ Weights of F1 Weanlings (mg/100g body weight)							
Dose ppm	Sex	Body W. g	Brain	Liver	Spleen	Thymus	Testes
0	m	75	2030	4578	319	398	884
1000	m	76	2011	4817+	348	388	874
4000	m	73	2102	4600	315	377	866
16000	m	71	2050	4474	311	377	882
							Ovaries
0	f	71	2045	4495	317	404	67
1000	f	71	2061	4583	352	450	63
4000	f	67	2162+	4514	314	405	61
16000	f	67	2108	4401	319	404	64

5.3 F1 Generation

5.3.1 Clinical Signs and Mortality

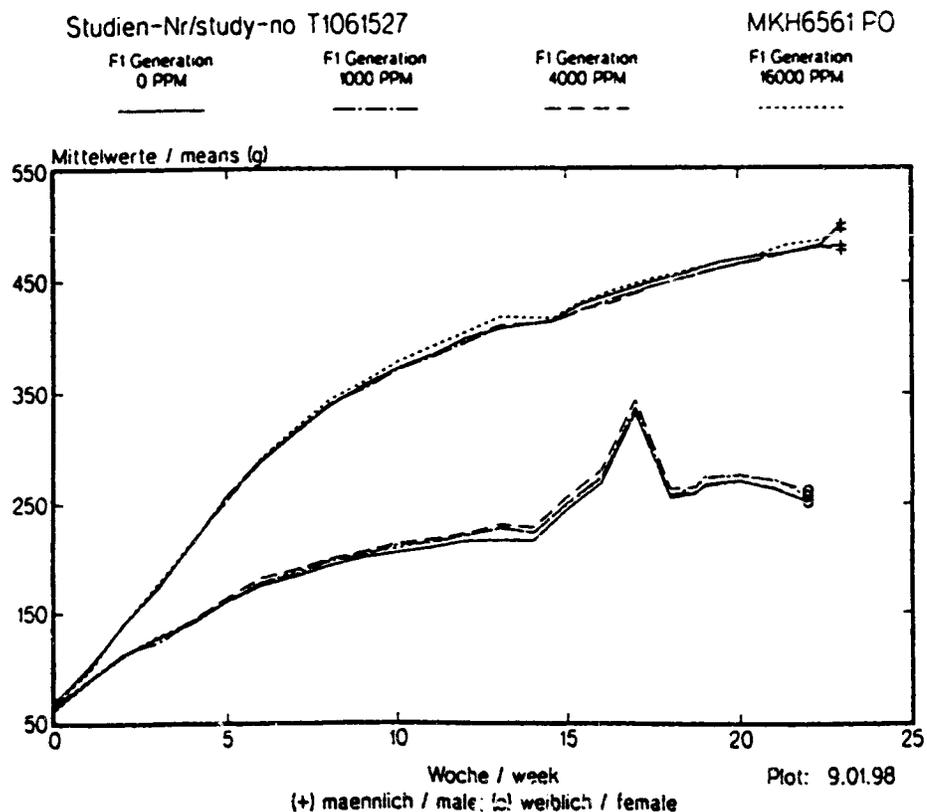
As can be seen from individual data and the list with cumulative clinical findings presented in Part 2, no test substance-related effects on the appearance or behavior were observed in male or female F1 animals at levels of up to 16000 ppm.

5.3.2 Body Weights of F1 Parent Animals

The group mean body weights of the weaned F1 rats are plotted against the time of the study in the following Figure 3.

As shown in Figure 3 and in tables with means and statistics presented in Report Part 2 treated males and females gained body weight comparable with that of the control group.

Figure 3 Body Weights of Male and Female F1 Parent Animals



5.3.3 Food Consumption and Test Substance Intake of F1 Parent Animals

The following Table 18 lists the mean food intake per animal or per kg body weight and day, and the corresponding cumulative consumption figures for each study group during the pre-mating period of F1 rats. Individual values and means per week including those recorded weekly beyond pre-mating period up to necropsy (males) or during the gestation and lactation (females) are presented in Report Part 2.

The food intake of treated F1 parent rats did not differ from the control data to a remarkable extent up to the concentration of 16000 ppm in males and 4000 ppm in females. F1 females receiving 16000 ppm ingested 17% more diet than controls, therefore, in this group the intake per body weight was enhanced as well.

Table 18

Mean Daily and Cumulative Intake of Food during the Pre-mating Period						
Dose ppm	Sex	Weeks	g/Animal		g/kg Body Weight	
			Total	per Day	Total	per Day
0	m	13	2048	22.3	7350	79.9
1000	m	13	2069	22.5	7326	79.6
4000	m	13	2080	22.6	7426	80.7
16000	m	13	2125	23.1	7555	82.1
0	f	13	1604	17.4	9472	103.0
1000	f	13	1659	18.0	9549	103.8
4000	f	13	1680	18.3	9510	103.4
16000	f	13	1872	20.4	10968	119.2

Table 19 lists the mean test substance intake per animal and day, per kg body weight and day, and the cumulative intakes for each study group during the pre mating period.

Table 19

Mean Daily and Cumulative Intake of Test Substance over the Premating Period						
Dose ppm	Sex	Weeks	mg/Animal		mg/kg Body Weight	
			Total	per Day	Total	per Day
1000	m	13	2069	22.5	7326	79.6
4000	m	13	8321	90.4	29706	322.9
16000	m	13	34000	369.6	120880	1313.9
1000	f	13	1659	18.0	9549	103.8
4000	f	13	6722	73.1	38039	413.5
16000	f	13	29955	325.6	175489	1907.5

Data of Table 19 show that the test substance intake in the treatment groups up to 4000 ppm roughly corresponds to the theoretical dose intervals. Female animals of the 16000 ppm group, which had shown a higher food intake ingested MKH 6561 more than the four fold than 4000 ppm females.

5.3.4 Gross Pathological Examinations in F1 Parent Animals

The individual gross pathological and histopathological findings, as well as the pertinent incidence tables are given in the Pathology Report (Report Part 3).

There were no treatment-related gross changes in males up to 16000 ppm. In females slightly to severely dilated caeca occurred in all treatment groups (0-3-2-17), however, a dose correlation is missing between group 1000 and 4000 ppm.

Up to the dietary concentration of 16000 ppm there were no compound related differences between the number of uterine implantation sites and that of delivered pups (see Table 20 and tables in Report Part 2). The means of the postimplantation loss in treated rats were comparable with that of controls (see Table 20). All females exhibiting implantation sites had born pups with the exception of No. 416.

Table 20

Evaluation of Implantation Sites in F1 Females.			
Dose ppm	No. of Implantation Sites	No. of Pups at Birth	Postimplantation Loss (%)
0	340	315	7.4
1000	361	334	7.5
4000	309	278	10.0
16000	321	275	14.3

5.3.5 Organ Weights in F1 Parent Animals

The following Table 21 presents the absolute and relative organ weights of male and female F1 parent animals as means. Individual results are included in Report Part 2.

Table 21

Absolute Organ Weights of F1 Rats											
Dose	Sex	Body W.	Brain	Adren.	Liver	Spleen	Kidneys	Testes	Epidid.	Prostate	Sem. V.
ppm		g	mg	mg	mg	mg	mg	mg	mg	mg	mg
0	m	487	2014	48	17568	755	3032	3569	1898	708	2292
1000	m	485	2042	47	17841	769	3093	3571	1704	940++	2286
4000	m	484	2007	45	16560	748	2955	3555	1774	918++	2157
16000	m	489	1984	50	16250+	727	3000	3454	1838	673	2337
								Ovaries	Uterus	-	-
								mg	mg		
0	f	251	1813	63	11478	572	1957	145	1230		
1000	f	253	1842	64	11827	575	1979	145	1178		
4000	f	261	1827	65	11253	568	1996	155	1351		
16000	f	258	1782	66	10939	532	1989	146	1202		
Relative Organ Weights of F1 Rats (mg/100g body weight)											
Dose	Sex	Body W.	Brain	Adren.	Liver	Spleen	Kidneys	Testes	Epidid.	Prostate	Sem. V.
ppm		g									
0	m	487	416	10	3609	155	625	737	400	147	474
1000	m	485	423	10	3668	159	638	740	353+	194++	473
4000	m	484	417	9	3414++	154	610	736	367	190++	447
16000	m	489	408	10	3322++	149	619	709	375	138	481
								Ovaries	Uterus	-	-
0	f	251	725	25	4561	227	781	58	489		
1000	f	253	731	25	4672	226	782	57	466		
4000	f	261	702	25	4315	218	765	59	521		
16000	f	258	693	26	4245	206++	772	57	466		

As shown in Table 21 there were no dose-correlated and/or significant differences among the absolute and relative organ weights up to the dose of 16000 ppm except lower ($p \leq 0.01$) means calculated for the relative liver weights (-5 and -8%) in males at 4000 ppm and above and decreased ($p \leq 0.01$) spleen weights (-9%) in 16000 ppm females. Since the differences to the control values were very small these deviations are considered to be of no toxicological relevance. Other means marked as significantly different not referred to in detail are of no toxicological relevance.

5.3.6 Determination of Developmental Milestones in F1 Weanlings

In all F1 weanlings selected for further treatment the age at which preputial separation and vaginal opening had occurred was recorded. The mean values are given in Table 22, individual dates are to be found in Report Part 2.

Table 22

Developmental Milestones in F1 Weanlings		
Dose ppm	Mean Age at Preputial Separation (days)	Mean Age at Vaginal Opening (days)
0	35.8	36.2
1000	36.1	37.3
4000	37.0	37.1
16000	37.0	37.0

As can be seen from Table 22 maturation of external sexual organs in F1 males and females was not remarkably influenced by the treatment with MKH 6561 up to 16000 ppm.

5.3.7 Spermatological Evaluations in F1 Males

Individual results of the spermatological evaluations such as sperm motility, (1st and 5th minute) and morphology as well as counts of sperms per mg epididymis and spermatids per mg testis are listed in tables in Report Part 2. A summary of calculated means is given in the following Tables 23 and 24.

No toxicological relevant effect could be seen on these sperm parameters at 16000 ppm. Therefore, males of the 1000 and 4000 ppm groups were not examined in this respect. All means are comparable with those of historical controls.

Table 23

Evaluation of Sperm Motility and Morphology (Mean Values of 20 F1 Males)				
Dose ppm	Sperm Motility			Abnormal Sperms %
	1 st min %	5 th min	Difference %	
0	88	74	15.9	1.08
16000	88	75	14.8	0.73

Table 24

Evaluation of Spermatid and Sperm Counts (Mean Values of 20 F1 Males)		
Dose ppm	Mean Number of	
	Spermatids per mg Testis	Sperms per mg Epididymis
0	52567	719292
16000	51271	623200

5.3.8 Determination of Estrus Cycle Length in F1 Females

Determinations of estrus staging were done over 12 days two weeks before F1 rats were co-housed for mating. The results are presented as mean values in Table 25.

Table 25

Dose ppm	Mean Length of Estrus Cycle days	Mean No. of Estri Observed within 12 days	No. of Females Showing Irregular Cycle or a Prolonged Estrus
0	4.12	2.8	2
1000	4.16	2.6	4
4000	3.69	2.5	3
16000	4.01	2.8	1

As can be seen from Table 25 no significant changes in the mean length of estrus cycle and the number of detected estri were observed in treated F1 females.

5.3.9 Histopathological Investigations in F1 Adult Rats

Results and evaluation of the histopathological investigations are given in detail in the Pathology Report (Report Part 3). These investigations revealed that clearly more 4000 and 16000 ppm males exhibited a focal vacuolation of the forestomach epithelium (1-2-5-17) than in the other groups. In females this lesion was noted (0-0-0-4) in the high dose only.

No treatment-related pathological findings were noted among the other organs at 16000 ppm. There were no microscopic findings which correlate with the caecal enlargement recorded macroscopically in some 4000 and 16000 ppm females.

5.3.10 Parameters of Reproduction in F1 Parent Animals

5.3.10.1 Insemination Index, Fertility Index, Gestation Index and Duration of Pregnancy

After a pre-mating period of about 13 weeks, pairs of one male and one female F1 animal of the pertinent group were co-housed for mating. The calculated indices of insemination, fertility and gestation, as well as the mean duration of pregnancy are listed in the following Table 26.

Table 26 Data Concerning Fertility and Gestation

Dose ppm		0	1000	4000	16000
Insemination index	%	100	96.7	93.3	96.7
Fertility index	%	93.3	100	100	96.5
Gestation index	%	100	100	96.4	100
Gestation length	Days	22.0	22.1	22.3	22.3
Mated females	n	30	30	30	30
Litters alive	n	28	29	27	28

The insemination, fertility and gestation indices as well as the mean duration of pregnancy did not differ from the pertinent control figures to a toxicologically relevant extent at levels of up to 16000 ppm.

There were some F1 females (2-0-1-2 with ascending dose*) which had been found to be sperm-positive after the first day of co-housing but failed pregnancy. According to experience this would happen if a male co-housed with a female for the first time inseminated the female outside the estrus. This was obviously the case, since these females had pups when remated with the same male for one week that followed the three week co-housing period.

* 0 ppm: No. 274, 275; 4000 ppm: No. 394; 16000 ppm: No. 452, 467

The mating performance of the F1 animals is given as cumulative percentage in Table 27. Only mated females, in which the mating date could be determined by detection of sperms in the vaginal smear, were included.

The mating performance was not affected by the treatment at levels of up to 16000 ppm.

Table 27

F1 Mating Performance				
Day of Mating Period	Cumulative Percentage of Sperm-Positive Females			
	Dose			
	0 ppm	1000 ppm	4000 ppm	16000 ppm
1	26.7	26.7	20.0	16.7
2	36.7	40.0	30.0	43.3
3	63.3	60.0	40.0	60.0
4	80.0	70.0	63.3	73.3
5			66.7	
6				76.7
7	83.3	80.0	76.7	83.3
8		83.3		
9	86.7		80.0	86.7
10			83.3	
11				
12	93.3			90.0

5.3.10.2 F2 Litter Parameters at Birth

The following Table 28 lists the total numbers of pups born, those found dead at birth, the live birth index, the percentages of male and female pups, and the litter size at birth.

Table 28 Litter Parameters at Birth

Dose ppm	Number		Live Birth	Males	Females	Mean Litter
	Total	Dead	Index %	%	%	Size ¹⁾
0	315	5	98.4	49.5	50.5	11.07
1000	334	7	97.9	51.5	48.5	11.27
4000	278	4	98.6	52.9	47.1	10.53
16000	275	0	100	55.6	44.4	9.82

¹⁾ viable pups only

None of the litter parameters shown in Table 28 were changed significantly up to 16000 ppm.

5.3.10.3 Clinical Observations in F2 Pups

The individual pup findings are listed in the table "Observations in F2 Pups" given in the Report Part 2. This table also includes necropsy findings, if there are any, of all offspring and it informs about their day of death. No significant clinical findings were made in F2 pups during the four week lactation period at levels of up to 16000 ppm. Malformations were not observed.

5.3.10.4 Body Weight of F2 Pups

Table 29 lists the mean litter weights at birth and on day 28 p.p.. Up to 16000 ppm litter weights were not changed significantly.

As can be seen from Table 30 the individual birth weights of F2 pups were not affected up to 16000 ppm. Regarding the mean body weights of male and female F2 pups during lactation no adverse effect could be detected up to the dose of 16000 ppm. From day 14 p.p. onwards pups of treated F1 females exhibited higher body weights than untreated pups.

Table 29

Dose ppm	Mean Litter Weight (g)	
	Day 0	Day 28
0	67.91	581.16
1000	66.97	564.20
4000	65.03	539.00
16000	61.59	562.15

Table 30

Mean Pup Weight at Birth and during Lactation (g)							
Dose ppm	Sex	Day 0	Day 4 after culling	Day 7	Day 14	Day 21	Day 28
0	m	6.34	10.26	15.69	31.01	50.64	78.78
1000	m	6.11	9.91	15.74	32.78	54.14+	84.12++
4000	m	6.35	10.54	15.85	34.17++	55.64++	84.00++
16000	m	6.46	10.88	16.63	34.22++	53.48+	84.50++
0	f	5.99	9.98	15.36	31.19	50.07	75.13
1000	f	5.81	9.75	15.38	31.27	51.27	76.76
4000	f	5.97	9.78	14.76	31.86	51.48	78.91+
16000	f	6.15	10.60	16.32	33.61+	52.11	78.79

5.3.10.5 Viability and Lactation Indices of F2 Pups

The following Table 31 presents data concerning viability and lactation of F2 offspring.

Table 31 Viability and Lactation Indices

Dose ppm	Viability Index	Lactation Index
	% Day 4	% Day 28
0	99.3	95.0
1000	96.3	89.9
4000	97.4	83.7
16000	97.8	89.5

The viability (day 4 p.p.) of treated F2 pups was comparable with that of the controls. Up to the dose of 16000 ppm there was no dose-dependent reduction in the lactation indices.

5.3.10.6 Gross Pathological Changes in F2 Pups or Weanlings

Individual findings detected at necropsies are listed in the table "Observations in F2 Pups" given in Report Part 2. Incidences are given in the Report Part 2.

In F2 pups necropsied during the lactation period no macroscopical alterations due to the treatment were observed. No skeletal deviations were seen in the F2 pups that had died before postpartum day four, were killed in the process of culling, or were necropsied unscheduled during lactation at levels of up to 16000 ppm.

No treatment-related gross pathological findings were made in F2 weanlings at scheduled necropsy.

5.3.11 Organ Weights of F2 Weanlings

Individual organ weights determined during the scheduled necropsy of F2 weanlings are given in the Report Part 2. The following Table 32 summarizes the calculated means per group.

Table 32

Absolute Organ Weights of F2 Weanlings							
Dose	Sex	Body W.	Brain	Liver	Spleen	Thymus	Testes
ppm		g	mg	mg	mg	mg	mg
0	m	80	1485	3698	280	324	699
1000	m	86	1545+	3948	285	341	738
4000	m	94	1525	4177+	304	373 ++	787
16000	m	89	1521	3838	272	371 +	749
							Ovaries
							mg
0	f	77	1445	3481	254	335	41
1000	f	78	1477	3478	268	341	44
4000	f	82	1469	3585	264	356	48+
16000	f	81	1459	3538	260	364	44
Relative Organ Weights of F2 Weanlings							
(mg/100g body weight)							
Dose	Sex	Body W.	Brain	Liver	Spleen	Thymus	Testes
ppm		g					
0	m	80	1909	4653	358	409	878
1000	m	86	1813	4593	332	397	860
4000	m	94	1646++	4450	327	401	836
16000	m	89	1727++	4315++	308++	419	837
							Ovaries
0	f	77	1908	4553	333	440	54
1000	f	78	1905	4459	342	435	56
4000	f	82	1815	4388	323	437	59
16000	f	81	1823	4361	322	454	55

Regarding the mean weights of the brain, thymus, testes and ovaries no conspicuous differences could be detected between the controls and treatment groups. There were significantly reduced relative liver (-7%) and spleen (-14%) weights in male pups receiving 16000 ppm. These deviations to control values are too small to reflect a toxic effect of the compound.

6. DISCUSSION AND EVALUATION OF THE RESULTS

MKH 6561 was examined for possible effects on reproduction in a two-generation study in Wistar rats with one litter per generation. This involved administration of **MKH 6561** to groups of 30 male and 30 female rats at levels of 0, 1000, 4000 or 16000 ppm in their diet.

Parental F0 animals were pretreated over a period of about 10 weeks and allowed to mate over a period of up to three weeks. F1 offspring were nursed up to an age of four weeks. Some of them were selected for further treatment and for breeding a F2 generation. F2 offspring were weaned at an age of four weeks.

Clinical signs, body weights, food intake, mating performances, fertility, duration of pregnancy, estrus cycling and sperm parameters were examined in F0 and F1 rats. Reproduction parameters such as litter size, relation of males to females and pup weight at birth as well as viability, lactation, body weight gain were studied in F1 and F2 offspring. Developmental milestones were evaluated in F1 weanlings. Necropsies were done in all rats. Implantation sites in F0 and F1 females were recorded. Selected organs were weighed (F0 and F1 adults as well as F1 and F2 pups) and histopathological evaluations were performed on some organs of F0 and F1 parent rats.

There was no increase in the mortality of treated F0 or F1 parent rats.

The body weight development of F0 and F1 animals was comparable with that of the controls at levels of up to 16000 ppm.

The food intake was not changed in a remarkable manner up to 4000 ppm. At 16000 ppm there was a slight (F0 males: +8%) to moderate (F0 and F1 females: 10 - 17%) increase in food intake, which is, however not considered as an adverse effect. In any way rats of the 16000 ppm group ingested more than 1000 mg/kg per day (limit dose) **MKH 6561**.

The appearance and behavior of the F0 and F1 parent animals were not changed by treatment at levels of up to 16000 ppm.

No changes in estrus cycling due to the treatment could be detected.

Analyses of sperm parameters such as number per mg epididymis, morphology, motility and viability and determination of testicular spermatid head counts revealed no signs of a treatment effect at 16000 ppm.

The reproduction parameters insemination index, mating performance, fertility index, gestation index, duration of pregnancy, total number of pups born, number of dead pups at birth (live birth index), percentages of male and female pups, litter size at birth, and birth weights of pups as well as pup survival rate (viability and lactation indices) were not changed at levels of up to 16000 ppm.

The body weight gain of pups was not affected in the dose range investigated. The slightly reduced ($p \leq 0.05$) mean body weight of male F1 pups on day 21 does not reflect a treatment effect, since male F2 pups of all treatment groups exhibited higher body weights than untreated F2 pups from day p.p. 14 onwards.

With respect to the maturation of external sexual organs of F1 rats a treatment effect could not be seen either in male or in female F1 rats.

No test substance-related clinical or gross pathological findings were observed in F1 and F2 offspring up to 16000 ppm.

The skeletal development of the pups or weanlings was unaffected at levels of up to 16000 ppm.

The number of uterine implantation sites was not remarkably different from the number of pups born.

At necropsy no treatment-related gross pathological findings were made in parent F0 and F1 males at levels of up to 16000 ppm. In parent females treatment-related gross pathological findings were absent at 1000 ppm (F0) or at 1000 and 4000 ppm (F1). At higher concentrations more females exhibited a dilated caecum. As outlined in the Pathology Report caecal enlargement can be interpreted as either a toxic or an adaptive phenomenon. No histopathological correlate to this finding was detected. Due to the lack of a clear dose response in the low and mid doses of the F1 generation, a relationship to the treatment with MKH 6561 can only be assumed for the mid and high dose females of the F0 generation and the high dose females of the F1 generation. This effects probably reflect an adaptative reaction to changes in the physiological intestinal function. There might be a similar situation in the present study as described for a wide range of non- or low digestible compounds (Cook et al. 1992*), which induce caecal enlargements without microscopical changes in rats, as well. As discussed by Bär et al. 1995** caecal enlargement resulting from the ingestion of non- digestible compounds has no relevance for human safety and has not been evaluated as an adverse effect (Cook et al. 1992). Therefore, in this study an adverse effect on the intestine is not assumed.

Histopathological investigations revealed an increase in the incidence of focal vacuolation in the forestomach epithelium in 16000 ppm F0 males and F1 males receiving 4000 and 16000 ppm. Comparable lesions were noted in high dose F0 and F1 females. The occurrence of this alteration is considered to be related to the treatment with MKH 6561.

Histopathological investigations and organ weight measurements revealed no toxicological relevant changes in the remaining organs due to the treatment.

Thus, the dietary concentration of 1000 ppm MKH 6561 is established as the overall no observed adverse effect level (NOAEL=NOEL) for the parent animals under conditions as described. The reproduction was unaffected up to a concentration of 16000 ppm.

* Cook, W.M., Purchase, R., Ford, G.P., Cressy, D.M., Brantom, P.G. and Gangoli, S.D. (1992): *Fd. Chem. Toxic.* Vol 30, 7, 567-573

** Bär, A., Til, H.P. and Timonen, M. (1995): *Fd. Chem. Toxic.* Vol. 33, 11, 909-917

7. ABBREVIATIONS

Adren.	adrenal glands
aft.	after
Anim.No./anim.no.	animal number
App/adm	administration
bef.	before
body w./Body W./BW	body weight
Epidid./Epididym.	epididymides
f; w/f	female
m	male
M	mean
m.k.	moribund killed
n	number of litters examined
N	number of animals examined
n.d.	sex not detectable
p.c.	post coitus
PO.	oral in diet
p.p.	postpartum
Prosta.	prostate
red.	reduction (=culling)
S.D./s	standard deviation
Sem. Ve./Sem. V	seminal vesicles
study no	study number
TS 1 %	test result at significance level of $\alpha = 1\%$
TS 5 %	test result at significance level of $\alpha = 5\%$
-	negative
+	difference against control for $p \leq 0.05\%$ significant
++	difference against control for $p \leq 0.01\%$ significant
0	no test assessment

MKH 6561
(c.n. Procarbazone-Sodium)

Two-Generation Study in Wistar Rats

by

Dr. R. Eiben

T1061527

Part 2 of 3

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T1061527

MKH 6561

Bayer AG
PF-PM/PPA

06.01.98

Approval of Active Ingredient Sample

Active Ingredient Sample TOX 4015

Sample: MKH 6561

Development-No.: 0142835

Indication: Herbicide

Mixed Batch No.: 05649/0004

Origin of sample: PF-PIVE

Responsible Analyst: Dr. Gau

Laboratory: PB-A

Analytical Methods: HPLC, int. Std.

Approvals:

<u>TOX</u>	<u>Purity</u>	<u>Approved until</u>	<u>Date of Analysis</u>	<u>Comment</u>
4015-00	97.6 %	12.07.96	2.01.96	
4015-01	98.0 %	26.12.96	26.06.96	
4015-02	98.0 %	06.07.97	07.01.97	
4015-03	98.1 %	27.12.97	30.06.97	
4015-04	98.6 %	18.06.98	19.12.97	

M. Haug
(M. Haug, PF-PM/PPA)

A reserve sample will be retained.

**Analytical Method Validation
Homogeneity, Stability Data, and Dose Verification
in Animal Ration**

ANALYTICAL REPORT

Dr. W. Rüngeler

Study-No.:T1061527

Test Substance:MKH 6561

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1 SUMMARY (ANALYTICAL REPORT)

A liquid chromatographic method for the quantification of MKH 6561 in animal ration was developed. This work was conducted for tests on stability, homogeneity, and for verification of nominal concentration of this test compound in mixtures fed to animals. The method, its validation, and the analytical results on the actual study are presented in this report.

MKH 6561 was extracted from animal ration at room temperature with acetonitrile/milli-Q-water (9:1/v:v) as a solvent. It was analyzed on a reversed phase (C18) column and ultraviolet (UV) detection.

For this extraction method a recovery procedure was established for preparing the analytical test samples. For the extraction (ambient conditions) with the solvent the animal feed was blended with several amounts of the active ingredient. For data evaluation a mean recovery values for concentration ranges were determined.

The limit of quantification for MKH 6561 was approximately 2.13 µg/ml. Under the conditions of this method, the limit of reliable measurement of MKH 6561 was approximately 50 ppm for rodent ration.

The analytical data verify that the test material was homogeneously distributed and chemically stable within the concentration range of 100 ppm to 20000 ppm. Under current sample preparation and handling conditions stability in the diet was assured for a period of at least 14 days.

The test material content in the diet mixtures fed to the animals, prepared during the study, agreed with the target concentrations within defined limits.

2 INTRODUCTION (ANALYTICAL REPORT)

A liquid chromatographic method for quantifying MKH 6561 in animal ration was developed. This work was conducted for tests on stability, homogeneity, and for verification of nominal concentration of this test compound in the mixtures fed to the animals. The method, its validation, and the analytical results on the actual study are presented in this report.

MKH 6561 was blended with Altromin® 1321, which was mixed with 1% peanut oil (DAB 10). For analytical investigations, representative samples, produced under the study director's responsibility, were taken at different points of time from the diet fed to animals. These samples were extracted and diluted with acetonitrile/milli-Q-water (9:1/v:v). After optionally filtration subsequently quantified by high-performance liquid chromatography (HPLC) with UV-detection (DAD; wavelength: 230 nm). Standard solutions of approved MKH 6561 were used as a basis for evaluation.

For the analytical quantification of the test compound a concentration range of 2.13 to 204.9 µg/ml was covered. The calibration curve, produced from standard solutions, was prepared anew for each analytical sequence. The linearity of the calibration curve, however, must be given. Essentially, all sample concentrations were always within the calibration range documented for each sample sequence.

3 GENERAL INFORMATION

The experimental standard of this part of the study was conducted in compliance with the OECD Principles of Good Laboratory Practice (as revised in 1997) and with the Principles of Good Laboratory Practice (GLP) according to Annex 1 German Chemicals Act (Bundesgesetzblatt Part I of the 29th of July 1994).

Investigations necessary for drafting the analytical method and performing analyses were conducted from February to November 1997 at the Department of Industrial Toxicology, Institute of Toxicology of Bayer AG, D-42096 Wuppertal-F.berfeld, Friedrich-Ebert-Strasse 217-333.

The study documentation (raw data and final analytical report) are retained in the archives specified by Toxicology of Bayer AG. The storage of a retention sample of the reference item is in the responsibility of the sponsor.

4 MATERIALS AND METHODS

4.1 TEST SUBSTANCE

Test material: **MKH 6561**

Batch No.: 05649/0004
 Purity: 98.1%
 Origin of sample: Bayer AG, PF-P/VE
 Stability approved until: Dec. 27, 1997
 Test material storage: room temperature
 Stability of analytical samples: was ensured throughout the test period

Toxicology feed mixtures: in Altromin® 1321; mixed with 1% peanut oil DAB 10

4.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

4.2.1 INSTRUMENTS

Ambient Extraction: empty PTFE-column ..
 High performance liquid chromatograph HP1090 equipped with ..
 - Autosampler ..
 - DAD (Diode array Detector) ..
 - Integration: HP 3365 DOS-WorkStation/ChemServer ..
 supplied by Hewlett-Packard Inc.
 Standard laboratory equipment and glassware ..
 (Gas tight) Syringes (25 µl; 100 µl; 250 µl, 10 ml; Hamilton) ..

4.2.2 METHOD

Column: Hypersil ODS 5 µm; L = 125 mm; ID = 4 mm; Grom ..
 Oven temperature: 40°C or OFF ..
 Flow rate: 1.00 ml/min ..
 Mobile phase: A: buffer solution ..
 B: acetonitrile ..
 gradient program: time 0 --> %B=20 (start conditions) ..
 time 1 --> %B=20 ..
 time 10 --> %B=70 ..
 time 12 --> %B=90 ..
 Injection volume: 20.0 µl (Autosampler) ..
 Detector: wavelength: 230 nm ..
 band width (BW): 8 nm ..
 reference: 450 nm / 80 nm BW ..

4.2.3 SOLVENTS AND CHEMICALS

Acetonitrile Lichrosolv; Merck No. 30	**
o-Phosphoric acid (85%); H ₃ PO ₄ ; Merck	**
Sand; Riedel de Haen No. 18649	**
Sodium sulfate Na ₂ SO ₄ ; Merck	**
Deionized water (Milli-Q-water), available from Millipore unit, Fa. Millipore**	**
Buffer composition: Milli-Q-water adjusted to pH=3 with H ₃ PO ₄	**
Solvent for extraction and dilution: acetonitrile/ Milli-Q-water (9:1/v:v)	**
	** or equivalent

4.2.4 SAMPLE PREPARATION - AMBIENT EXTRACTION

The test material - animal feed mixtures were prepared under the study director's responsibility. For sample preparation 10 g of test material were mixed with 20 g of a mixture of sodium sulfate/sea sand (1:1/w:w) and filled in an empty PTFE-column. 100 ml of acetonitrile/Milli-Q-water (9:1/v:v) were used for extraction, the extracts were filled in a volumetric flask, diluted and brought up to volume with solvent. These solutions were injected onto the HPLC after appropriate dilution.

4.2.5 CALIBRATION OF THE ANALYTICAL METHOD

To set-up the calibration series, test material solutions in the solvent were prepared with appropriate concentrations. The stability of these solutions was checked at room temperature over a period of 9 days. No decrease in concentration was observed [1]. The method-specific parameters were adjusted on the HPLC instrument. 20.0 µl of each calibration concentration was injected for preparation of the calibration curve. Measurement wavelength: 230 nm (UV spectrum see Fig. A1)

Fig. A2 and A3 show a typical chromatogram of these external calibration solutions and additionally a food extract chromatogram. A statistically evaluated calibration curve was shown in Figure A4. This curve was plotted by the integrator and was based upon the injected concentrations. The calibration line was plotted anew for each analysis sequence, and deviations from this calibration range were therefore possible. All sample concentrations were always within the calibration range documented for each sample sequence. The quantitative evaluation was performed by determination and comparing the peak area of **MKH 6561** of the analytical solution with the peak areas of the external standard solutions.

Retention time:

MKH 6561 approx. 8.3 min;

concentration range: 2.13 to 204.9 µg/ml

2.13 µg/ml was the limit of quantification of the analytical measurement using this method.

Figure A1 - UV-spectrum of **MKH 6561**

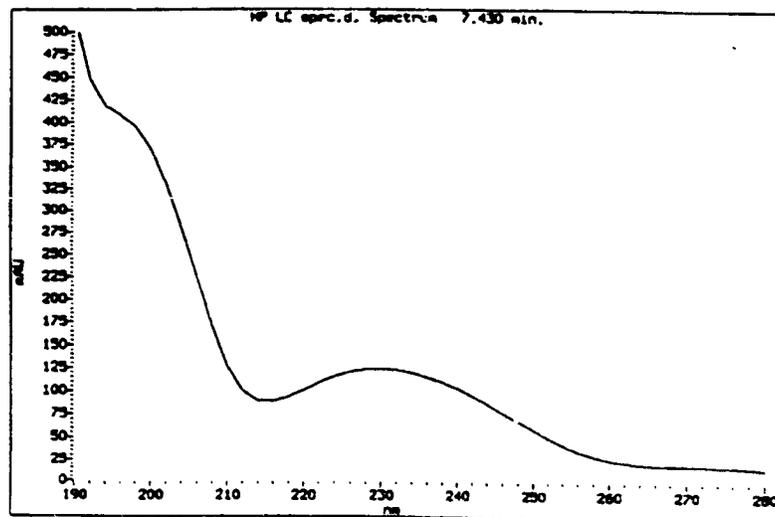


Figure A2 - Typical LC-chromatogram of the test substance as calibration standard
test material concentration: 18.48 $\mu\text{g/ml}$

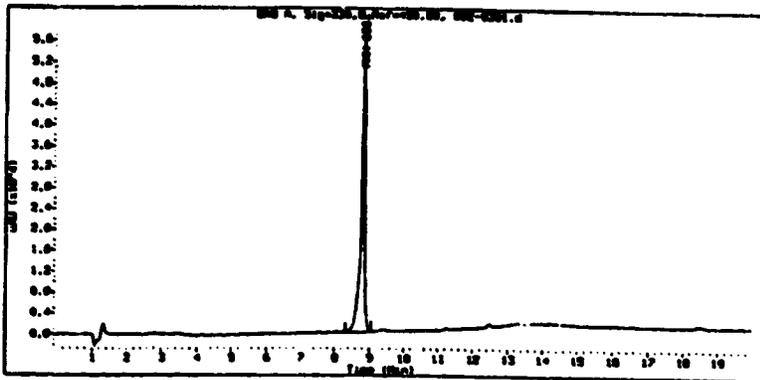


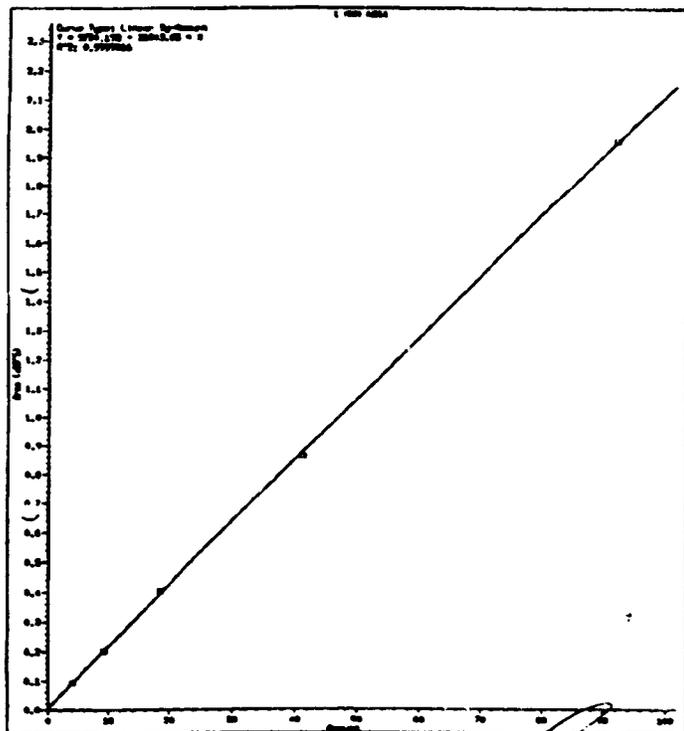
Figure A3 - Typical LC-chromatograms of rodent ration samples
a) test material concentration: 0 ppm (untreated control sample)



b) test material concentration: 1000 ppm



Figure A4 - Calibration curve of the analytical method (Date: April 14, 1997)



The calibration function was linear in the range shown. The linear regression value was $r^2 = 0.9999016$

4.2.6 PRECISION

The precision of this analytical method was assessed by 10 separate injections for two relevant concentrations of the calibration standards. The concentration values obtained with a statistical evaluation (coefficient of variation, = %RSD) were presented in Table A1 [3]. The precision of this method was found to satisfy the analytical requirements.

Table A1 - Precision of the analytical method

20.490 [$\mu\text{g/ml}$]	204.900 [$\mu\text{g/ml}$]
20.071	204.245
20.225	200.602
20.057	200.540
20.159	200.519
20.191	199.165
19.837	200.241
20.123	196.963
20.216	199.163
19.330	199.352
20.060	200.854
MEAN = 20.027	MEAN = 200.164
%RSD = 1.3%	%RSD = 0.9%

4.2.7 RECOVERY

The recoveries from rodent ration were evaluated in fortification tests. Known amounts of test compound were added to untreated control feed - Altromin® 1321 - prior to extraction.

Result (ambient extraction):

The analytical data verify that the test material recovery was assured within the concentration range from 100 to 20000 ppm, mean value: 94.7%

Table A2 - Recovery Test performed with three sample preparations

active ingredient added [ppm]	extraction procedure	Date of preparation	actual-concentration [%] from target/%RSD
100 ppm	ambient / T1061527	Jan. 31, 1997	96.1% ; 4.4%
250 ppm	ambient / T0058151	Jan. 22, 1995	97.2% ; 3.9%
20000 ppm	ambient / T0058151	Jan. 22, 1995	90.7% ; 2.5%

5 ANALYTICAL RESULTS

5.1 HOMOGENEITY AND STABILITY DATA

The analytical data verify that the test material in the animal ration was homogeneously distributed within the concentration range of 100 ppm to 20000 ppm (different diet quantities). Under current sample preparation and handling conditions comparable to those in the actual study the chemical stability was assured for a period of at least 14 days.

The homogeneity and stability data were presented by D.I.Riegner, PF-E/MR in the study no. T0058151 [1]. For stability testing the samples were stored under conditions comparable to those in the actual study and then frozen until analytical measurement.

From an analytical perspective there are no reservations concerning the acceptance of the homogeneity and stability data from T0058151 for the actual study, provided that the test mixture preparations follow the documented procedure.

5.2 ADDITIONAL HOMOGENEITY DATA (INCREASING OF DIET QUANTITY)

For homogeneity testing samples were taken from different locations within the sample containers. For calculations integral values from each sample were based on the external standard calibration curve of the active ingredient. The stability of these analytical solutions were ensured throughout the test period. For homogeneity assessment the percentage of active ingredient in the original test material was not included for calculations.

Table A3. presents the analytical results from three samples each collected from a high and low target concentration of diet rations. Each sample was prepared and injected twice.

**Table A3 Homogeneity tests; (diet Lot No.:0931; quantity: 20 kg)
date of preparation: Jan. 30, 1997**

Sample No.	target concentration		Sample No.
	100 ppm	20000 ppm	
1.1 - 1 st inj.	104	19697	1.1 - 1 st inj.
1.1 - 2 nd inj.	99	19658	1.1 - 2 nd inj.
1.2 - 1 st inj.	103	18535	1.2 - 1 st inj.
1.2 - 2 nd inj.	99	18578	1.2 - 2 nd inj.
2.1 - 1 st inj.	110	22203	2.1 - 1 st inj.
2.1 - 2 nd inj.	105	22143	2.1 - 2 nd inj.
2.2 - 1 st inj.	108	20103	2.2 - 1 st inj.
2.2 - 2 nd inj.	107	20090	2.2 - 2 nd inj.
3.1 - 1 st inj.	105	21731	3.1 - 1 st inj.
3.1 - 2 nd inj.	107	21696	3.1 - 2 nd inj.
3.2 - 1 st inj.	100	22008	3.2 - 1 st inj.
3.2 - 2 nd inj.	100	22733	3.2 - 2 nd inj.
Mean:	104 ppm	20765 ppm	
%RSD:	3.3%	7.1%	

5.3 CONTENT CHECK FOR DOSE VERIFICATION

The analytical data (LC) verify that the test material content in the diet mixtures agreed with the target concentrations within defined limits (Tab. A4).

Additionally a stability test of these samples was performed. The samples were stored for 7 days under conditions comparable to those in the actual study, and then quantified. The stability confirmed analytically.

For calculations integrator values from each sample were based on the external standard calibration curve of the active ingredient. For assessment of content checks the percentage of active ingredient in the original test material was not included for calculations.

Table A4 presents analytical results from sequential evaluations of the animal ration concentrations. These values were means of two individual samples for each concentration.

The calculation [%] of target concentration was based on the analytical result on day 0! In analyzed control samples amounts of active ingredient were not detected.

Table A4 - Content (in [%] of target concentration and actual weight units [ppm])
 date of preparation {I} / freezing {II} /
 date of measurement {III}

sample	1000 ppm	4000 ppm	16000 ppm
I+II: Feb. 10, 1997 III: Feb. 17, 1997 (stab. test-start)	108% (1076 ppm)	104% (4172 ppm)	104% (16558 ppm)
I: Feb. 10, 1997 III: Feb. 17, 1997 (stab. 7 days)	99% (1061 ppm) based on day 0	99% (4136 ppm) based on day 0	100% (16545 ppm) based on day 0
I+II: April 7, 1997 III: April 14, 1997 (stab. test-start)	104% (1035 ppm)	104% (4153 ppm)	104% (16573 ppm)
I: April 7, 1997 III: April 14, 1997 (stab. 7 days)	99% (1029 ppm) based on day 0	100% (4158 ppm) based on day 0	100% (16623 ppm) based on day 0
I+II: July 21, 1997 III: July 28, 1997 (stab. test-start)	100% (1000 ppm)	106% (4226 ppm)	103% (16417 ppm)
I: July 21, 1997 III: July 28, 1997 (stab. 7 days)	99% (995 ppm) based on day 0	98% (4156 ppm) based on day 0	99% (16259 ppm) based on day 0
I+II: Sep. 8, 1997 III: Sep. 15, 1997 (stab. test-start)	90% (905 ppm)	92% (3686 ppm)	98% (15672 ppm)
I: Sep. 8, 1997 III: Sep. 15, 1997 (stab. 7 days)	90% (815 ppm) based on day 0	101% (3733 ppm) based on day 0	101% (15838 ppm) based on day 0
I+II: Nov. 17, 1997 III: Nov. 24, 1997 (stab. test-start)	106% (1064 ppm)	106% (4242 ppm)	101% (16220 ppm)
I: Nov. 17, 1997 III: Nov. 24, 1997 (stab. 7 days)	98% (1039 ppm) based on day 0	98% (4161 ppm) based on day 0	99% (16013 ppm) based on day 0

6 LITERATURE

- [1] D.I.Riegner, PF-E/MR, report no. MR-0556/94; Nov. 9, 1994
Summary of Analytical Data on MKH 6561 (study no. T0058151)
- [2] D.I.Riegner, PF-E/MR, report no. RA-0359/94; Sep. 22, 1994
Summary of Analytical Data on MKH 6561 (study no. T2055651)
- [3] Dr. W. Rüngeler, PH-PDT/Tox. Analytic
Analytical data archived in study no. T7060911

END OF ANALYTICAL REPORT

NUTRIENT COMPOSITION OF DIET FOR RAT / MOUSE (Altromin 1321/1324)Ingredients *

Crude protein	19.0
Crude fat	4.0
Crude fiber	6.0
Ash	7.0
Moisture	13.5
Nitrogen-free extract	50.5

Metabolizable energy:

Kcal / kg	2850.0
KJ / kg	11900.0

Minerals *

Calcium	0.9
Phosphorus	0.7
Magnesium	0.2
Sodium	0.2
Potassium	1.0

Vitamins ***Standard-Diet

Vitamin A	15000.0 IU
Vitamin D3	600.0 IU
Vitamin E	75.0 mg
Vitamin K3	3.0 mg
Vitamin B1	18.0 mg
Vitamin B2	12.0 mg
Vitamin B6	9.0 mg
Vitamin B12	24.0 mcg
Nicotinic acid	36.0 mg
Pantothenic acid	21.0 mg
Folic acid	2.0 mg
Biotin	60.0 mcg
Choline	600.0 mg
Vitamin C	36.0 mg

Amino acids *

Lysine	0.90
Methionine	0.30
Cystine	0.30
Phenylalanine	0.80
Tyrosine	0.60
Arginine	1.10
Histidine	0.40
Tryptophane	0.20
Threonine	0.60
Isoleucine	0.80
Leucine	1.30
Valine	0.90

Trace elements **

Manganese	75.0
Iron	180.0
Copper	13.0
Zinc	70.0
Iodine	0.9
Fluorine	15.0

- * Average % content in the diet
 ** Average mg content in 1 kg diet
 *** Additive/1 kg diet

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- * Average % content in the diet
 ** Average mg content in 1 kg diet
 *** Additive/1 kg diet

CONTAMINANTS IN DIET FOR RAT/MOUSE (Altromin 1321/1324)

Contaminant	Detection Limit	Maximum Content
<u>Mycotoxins</u>		
Aflatoxin		
B1	2 ppb	10 ppb
B2	2 ppb	5 ppb
G1	2 ppb	5 ppb
G2	2 ppb	5 ppb
<u>Organo-Cl-Compounds</u>		
Tecnazene	0.001 mg/kg	not fixed
HCB (Hexachlorbenzene)	0.001 mg/kg	0.01 mg/kg
alpha-HCH	0.001 mg/kg	0.02 mg/kg
beta-HCH	0.001 mg/kg	0.02 mg/kg
gamma-HCH (Lindane)	0.001 mg/kg	0.10 mg/kg
delta-HCH	0.001 mg/kg	0.02 mg/kg
Quintozene	0.001 mg/kg	0.01 mg/kg
Heptachlor	0.001 mg/kg	0.01 mg/kg
Heptachlorepoxyde	0.003 mg/kg	as Heptachlor)
alpha-Chlordane	0.005 mg/kg	0.02 mg/kg
gamma-Chlordane	0.005 mg/kg	0.02 mg/kg
alpha-Endosulphane	0.005 mg/kg	0.10 mg/kg
beta-Endosulphane	0.005 mg/kg	0.10 mg/kg
Aldrin	0.003 mg/kg	as)
Dieldrin	0.003 mg/kg	Dieldrin)
Endrin	0.003 mg/kg	0.01 mg/kg
o,p-DDE	0.002 mg/kg)
p,p-DDE	0.002 mg/kg)
o,p-DDD	0.002 mg/kg	as)
o,p-DDT	0.002 mg/kg	DDT)
p,p-DDD	0.002 mg/kg)
p,p-DDT	0.002 mg/kg)
Methoxychlor	0.01 mg/kg	not fixed

CONTAMINANTS IN DIET FOR RAT/MOUSE (Altromin 1321/324)

Contaminant	Detection Limit	Maximum Content
<u>Organo-P-Compounds</u>		
Chlorthion	0.01 mg/kg	0.5 mg/kg
Disulfoton	0.005 mg/kg	0.5 mg/kg
Malathion	0.01 mg/kg	1.0 mg/kg
Parathion (-methyl)	0.005 mg/kg	0.5 mg/kg
Parathion (-ethyl)	0.01 mg/kg	0.5 mg/kg
Sulfotep	0.002 mg/kg	0.5 mg/kg
Fenthion	0.005 mg/kg	1.0 mg/kg
Dimethoate	0.005 mg/kg	1.0 mg/kg
Trichlorphon	0.01 mg/kg	1.0 mg/kg
Fenitrothion	0.01 mg/kg	1.0 mg/kg
Bromophos (-methyl)	0.01 mg/kg	1.0 mg/kg
Bromophos (-ethyl)	0.01 mg/kg	1.0 mg/kg
Chlorfenvinphos	0.01 mg/kg	0.5 mg/kg
Pirimiphos (-methyl)	0.01 mg/kg	1.0 mg/kg
Methidathion	0.01 mg/kg	1.0 mg/kg
Ethion	0.01 mg/kg	0.5 mg/kg
<u>Heavy Metals</u>		
Lead	0.1 mg/kg	1.5 mg/kg
Cadmium	0.01 mg/kg	0.4 mg/kg
Mercury	0.01 mg/kg	0.1 mg/kg
Arsenic	0.2 mg/kg	1.0 mg/kg
Selenium	0.1 mg/kg	0.6 mg/kg
Copper	1.0 mg/kg	not fixed
<u>PCB's</u>	0.01 mg/kg	0.05 mg/kg

Tolerance ranges of analysis:

Detection Limit	Tolerance
5 - 100 ppb	+/- 50 % relative
100 - 200 ppb	+/- 50 ppb absolute
above 200 ppb	+/- 25 % relative

SPECIFICATION OF TAP WATER

(according to "Trinkwasser-Verordnung" 12-05-90, BGBL No. 66 edited 12-12-90, page 2612-2629)

Limits of Chemical Substances in Tap-Water

Substance	Limit	corresponding	calculated as
	mg/l	mmol/m ³	
Arsenic	0.04 *	0.5	As
Lead	0.04	0.2	Pb
Cadmium	0.005	0.04	Cd
Chromium	0.05	1	Cr
Cyanide	0.05	2	CN (-)
Fluoride	1.5	79	F (-)
Nickel	0.05	0.9	Ni
Nitrate	50	806	NO ₃ (-)
Nitrite	0.1	2.2	NO ₂ (-)
Mercury	0.001	0.005	Hg
PAH **	0.0002	0.02	C
Organic Chloride Compounds ***			
- 1,1,1-Trichloroethane	0.025		
Trichloroethene			
Tetrachloroethene			
Dichloromethane			
- Tetrachloromethane	0.003	0.02	CCl ₄
Pesticides and similar compounds			
- per compound	0.0001		
- compounds in total	0.0005		

* from January 1, 1996: 0.01 mg/l

** PAH = Polycyclic Aromatic Hydrocarbons

*** from January 1, 1992: Compounds in total 0.01 mg/l
Tetrachloromethane 0.003 mg/l

Parameters and limits for the evaluation of the quality of drinking water
(appendix 4 of the "Trinkwasserverordnung")

I. SENSORY PARAMETERS

	Factor	Limit
1	Coloration	0.5 m ⁻¹
2	Turbidity	1.5 turbidity units / formazine
3	Odour threshold	2 at 12 °C 3 at 25 °C

II. PHYSICO-CHEMICAL PARAMETERS

	Parameters	Limit	calculated as
4	Temperature	25 °C	
5	pH	not less than 6.5 not more than 9.5	
6	Conductivity	2000 μ S cm ⁻¹ at 25 °C	
7	Oxidizability	5 mg/l	O ₂

III. LIMITS FOR CHEMICAL SUBSTANCES

	Parameters	Limit mg/l	calculated as	corresponding to approx. mmol/m ³
8	Aluminium	0.2	Al	7.5
9	Ammonium	0.5	NH ₄ ⁺	30
10	Iron	0.2	Fe	3.5
11	Potassium	12	K	300
12	Magnesium	50	Mg	2050
13	Manganese	0.05	Mn	0.9
14	Sodium	150	Na	6500
15	Silver	0.01	Ag	0.1
16	Sulphate	240	SO ₄ ²⁻	2500
17	Surfactants			
	a) anionic	0.2	a) Methyleneblue active substances	
	b) non-ionic	0.2	b) Bismuth active substances	

IV. MICROBIOLOGICAL PARAMETERS

Parameters	Volume of sample to be investigated	Maximal tolerated germ liter
Coliforms	100 ml	0
E.coli	100 ml	0
Streptococcus fecalis	100 ml	0
Sulphite reducing clostridium	20 ml	0

Total number of colonies in 1 ml drinking water should not exceed 100
(incubation temperature 20 ± 2 °C and 36 ± 1 °C)

Calculation of FOOD CONSUMPTION and ACTIVE INGREDIENT INTAKE

Body weights and the initial and final weights of diet are measured in grams for the calculation.

1 FOOD CONSUMPTION**1.1 Food Consumption per Animal per Day**

$$= \frac{H - R}{nT}$$

H = Weight of administered food (if necessary, plus weight of food container) at time of weighing (initial weight)

R = Weight of unconsumed food (if necessary, plus weight of food container) at time of weighing back (final weight)

nT = Number of days between weighing and weighing back

1.2 Mean Food Consumption per Animal per Day (Date-Related)

$$= \frac{\text{Sum of All Values Available at a Specific Date}}{\text{No. of Values}}$$

All food consumption values existing at a specific date (per animal per day, see 1.1) are totaled up. This total is divided by the number of values existing at that date.

1.3 Mean Food Consumption per Animal per Day

$$= \frac{\text{Sum of All Values}}{\text{No. of Values}}$$

All existing food consumption values (per animal per day, see 1.1) are totaled up. This total is divided by the number of existing values.

1.4 Cumulative Food Consumption per Animal

$$= (\text{Mean Food Consumption per Animal per Day}) \times n\text{Days}$$

For mean food consumption per animal per day, see 1.3. nDays is established from the total number of food consumption days.

1.5 Food Consumption per kg Body Weight per Day

$$= \frac{\text{Feed Consumption per Animal per Day}}{\text{Body Weight of the Animal}} \times 1000$$

For food consumption per animal per day, see 1.1. The value that was obtained within the time interval from the day of weighing back (final wt.) to the day of weighing back minus 7 is taken as the basis for the body weight. If no determination of the body weight of the animals within his time interval was planned, the time interval from the day of weighing back to the day of weighing back plus 6 is taken as the basis. If no body weight value is available within either of these two time intervals, no food consumption is calculated.

1.6 Mean Food Consumption per kg Body Weight per day

$$= \frac{\text{Sum of all Values Available at a Specific Date}}{\text{No. of Values}}$$

All food consumption values existing at a specific date (per kg body weight per day, see 1.5) are totaled up. This total is divided by the number of values existing at that date.

1.7 Mean Food Consumption per kg Body Weight per Day

$$= \frac{\text{Sum of All Values}}{\text{No. of Values}}$$

All existing food consumption values (per kg body weight per day, see 1.5) are totaled up. This total is divided by the number of existing values.

1.8 Cumulative Food Consumption per kg Body Weight

= (Mean Food Consumption per kg Body Weight per Day) x nDays
For mean food consumption per kg body weight per day, see 1.7.
nDays is established from the total number of food consumption days.

2. ACTIVE INGREDIENT (AI) INTAKE

The active ingredient (AI) intake is calculated from the food consumption data by using a "Dose Factor".

where: Dose in ppm, Food consumption in g, AI intake in mg

$$\text{Dose Factor} = \frac{\text{Dose}}{1000}$$

2.1 Mean AI Intake per Animal per Day

= (Mean Food Consumption per Animal per Day) x Dose Factor
For mean food consumption per animal per day (see 1.3).

2.2 Cumulative AI Intake per Animal

= (Cumulative Food Consumption per Animal) x Dose Factor
For cumulative food consumption per animal (see 1.4).

2.3 AI Intake per kg Body Weight per Day

= (Food Consumption per kg Body Weight per Day) x Dose Factor
For food consumption per kg body weight per day (see 1.5).

2.4 Mean AI Intake per kg Body Weight per Day

= (Mean Food Consumption per kg Body Weight per Day at a Specific Date) x Dose Factor
For mean food consumption per kg body weight per day at a specific date (see 1.6).

2.5 Mean AI Intake per kg Body Weight per Day

= (Mean Food Consumption per kg Body Weight per Day) x Dose Factor
For mean food consumption per kg body weight per day (see 1.7).

2.6 Cumulative AI Intake per kg Body Weight

= (Cumulative Food Consumption per kg Body Weight) x Dose Factor.
For cumulative food consumption per kg body weight (see 1.8.).