B.35. TWO-GENERATION REPRODUCTION TOXICITY STUDY

1. METHOD

This method is a replicate of the OECD TG 416 (2001).

1.1 INTRODUCTION

This method for two-generation reproduction testing is designed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the oestrus cycle, mating behaviour, conception, gestation, parturition, lactation, and weaning, and the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests. In addition to studying growth and development of the F1 generation, this test method is also intended to assess the integrity and performance of the male and female reproductive systems as well as growth and development of the F2 generation. For further information on developmental toxicity and functional deficiencies, either additional study segments can be incorporated into this protocol, consulting the methods for developmental toxicity and/or developmental neurotoxicity as appropriate, or these endpoints could be studied in separate studies, using the appropriate test methods.

1.2 PRINCIPLE OF THE TEST METHOD

The test substance is administered in graduated doses to several groups of males and females. Males of the P generation should be dosed during growth and for at least one complete spermatogenetic cycle (approximately 56 days in the mouse and 70 days in the rat) in order to elicit any adverse effects on spermatogenesis. Effects on sperm are determined by a number of sperm parameters (e.g., sperm morphology and motility) and in tissue preparation and detailed histopathology. If data on spermatogenesis are available from a previous repeated dose study of sufficient duration, e.g. a 90-day study, males of the P generation need not be included in the evaluation. It is recommended, however, that samples or digital recordings of sperm of the P generation are saved, to enable later evaluation. Females of the P generation should be dosed during growth and for several complete oestrus cycles in order to detect any adverse effects on oestrus cycle normality by the test substance. The test substance is administered to parental (P) animals during their mating, during the resulting pregnancies, and through the weaning of their Fl offspring. At weaning the administration of the substance is continued to Fl offspring during their growth into adulthood, mating and production of an F2 generation, until the F2 generation is weaned.

Clinical observations and pathological examinations are performed on all animals for signs of toxicity with special emphasis on effects on the integrity and performance of the male and female reproductive systems and on the growth and development of the offspring.

1.3 DESCRIPTION OF THE TEST METHOD

1.3.1 Selection of animal species

The rat is the preferred species for testing. If other species are used, justification should be given and appropriate modifications will be necessary. Strains with low fecundity or well-known high incidence of developmental defects should not be used. At the commencement of the study, the weight variation of animals used should be minimal and not exceed 20 % of the mean weight of each sex.

1.3.2 Housing and feeding conditions

The temperature in the experimental animal room should be 22 °C (± 3°). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test substance when administered by this method.

Animals may be housed individually or be caged in small groups of the same sex. Mating procedures should be carried out in cages suitable for the purpose. After evidence of copulation, mated females shall be single-caged in delivery or maternity cages. Mated rats may also be kept in small groups and separated one or two days prior to parturition. Mated animals shall be provided with appropriate and defined nesting materials when parturition is near.

1.3.3 Preparation of animals

Healthy young animals, which have been acclimated to laboratory conditions for at least 5 days and have not been subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, source, sex, weight and/or age. Any sibling relationships among the animals should be known so that mating of siblings is avoided. The animals should be randomly assigned to the control and treated groups (stratification by body weight is recommended). Cages should be arranged in such a way that possible effects due to cage placement are minimised. Each animal should be assigned a unique identification number. For the P generation, this should be done before dosing starts. For the F1 generation, this should be done at weaning for animals selected for mating. Records indicating the litter of origin should be maintained for all selected F1 animals. In addition, individual identification of pups as soon after birth as possible is recommended when individual weighing of pups or any functional tests are considered.

Parental (P) animals shall be about 5 to 9 weeks old at the start of dosing. The animals of all test groups shall, as nearly as practicable, be of uniform weight and age.

1.4 PROCEDURE

1.4.1 **Number and sex of animals**

Each test and control group should contain a sufficient number of animals to yield preferably not less than 20 pregnant females at or near parturition. For substances that cause undesirable treatment related effects (e.g. sterility, excessive toxicity at the high dose), this may not be possible. The objective is to produce enough pregnancies to assure a meaningful evaluation of the potential of the substance to affect fertility, pregnancy and maternal behaviour and suckling, growth and development of the F1 offspring from conception to maturity, and the development of their offspring (F2) to weaning. Therefore, failure to achieve the desired number of pregnant animals (i.e. 20) does not necessarily invalidate the study and should be evaluated on a case-by-case basis.

1.4.2 **Preparation of Doses**

It is recommended that the test substance be administered orally (by diet, drinking water or gavage) unless another route of administration (e.g. dermal or inhalation) is considered more appropriate.

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water, the toxic characteristics of the vehicle must be known. The stability of the test substance in the vehicle should be determined.

1.4.3 **Dosage**

At least three dose levels and a concurrent control shall be used. Unless limited by the physical-chemical nature or biological effects of the test substance, the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering. In case of unexpected mortality, studies with a mortality rate of less than approximately 10 percent in the parental (P) animals would normally still be acceptable. A descending sequence of dose levels should be selected with a view to demonstrating any dosage related effect and no-observed-adverse-effects levels (NOAEL). Two to four fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages. For the dietary studies the dose interval should be not more than 3 fold. Dose levels should be selected taking into account any existing toxicity data, especially results from repeated dose studies. Any available information on metabolism and kinetics of the test compound or related materials should also be considered. In addition, this information will also assist in demonstrating the adequacy of the dosing regimen.
The control group shall be an untreated group or a vehicle-control group if a vehicle is used in administering the test substance. Except for treatment with the test substance, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used, the control group shall receive the vehicle in the highest volume used. If a test substance is administered in the diet, and causes reduced dietary intake or utilisation, then the use of a pair-fed control group may be considered necessary. Alternatively data from controlled studies designed to evaluate the effects of decreased food consumption on reproductive parameters may be used in lieu of a concurrent pair-fed control group.

Consideration should be given to the following characteristics of vehicle and other additives: effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

1.4.4 **Limit test**

If an oral study at one dose level of at least 1000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet or drinking water using the procedures described for this study, produces no observable toxic effects in either parental animals or their offspring and if toxicity would not be expected based upon data from structurally and /or metabolically related compounds, then a full study using several dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher oral dose level to be used. For other types of administration, such as inhalation or dermal application, the physical-chemical properties of the test substance, such as solubility, often may indicate and limit the maximum attainable level of exposure.

1.4.5 **Administration of doses**

The animals should be dosed with the test substance on a 7-days-a-week basis. The oral route of administration (diet, drinking water, or gavage) is preferred. If another route of administration is used, justification shall be provided, and appropriate modifications may be necessary. All animals shall be dosed by the same method during the appropriate experimental period. When the test substance is administered by gavage, this should be done using a stomach tube. The volume of liquid administered at one time should not exceed 1 ml/l00 g body weight (0.4 ml/100 g body weight is the maximum for corn oil), except in the case of aqueous solutions where 2 ml/100 g body weight may be used. Except for irritant or corrosive substances, which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimised by adjusting the concentration to ensure a constant volume at all dose levels. In gavage studies, the pups will normally only receive test substance indirectly through the milk, until direct dosing commences for them at weaning. In diet or drinking water studies, the pups will additionally receive test substance directly when they commence eating for themselves during the last week of the lactation period.

For substances administered via the diet or drinking water, it is important to ensure that the quantities of the test substance involved do not interfere with normal nutrition or water balance. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the body weight of the animal may be used; the alternative used must be specified. For a substance administered by gavage, the dose should be given at similar times each day, and adjusted at least weekly to maintain a constant dose level in terms of animal body weight. Information regarding placental distribution should be considered when adjusting the gavage dose based on weight.

1.4.6 **Experimental schedules**

Daily dosing of the parental (P) males and females shall begin when they are 5 to 9 weeks old. Daily dosing of the F1 males and females shall begin at weaning; it should be kept in mind that in cases of test substance administration via diet or drinking water, direct exposure of the F1 pups to the test substance may already occur during the lactation period. For both sexes (P and F1), dosing shall be continued for at least 10 weeks before the mating period. Dosing is continued in both sexes during the 2 week mating period. Males should be humanely killed and examined when they are no longer needed for assessment of reproductive effects. For parental (P) females, dosing should continue throughout pregnancy and up to the weaning of the F1 offspring. Consideration should be given to modifications in the dosing schedule based on available information on the test substance, including existing toxicity data, induction of metabolism or bioaccumulation. The dose to each animal should normally be based on the most recent individual body weight determination. However, caution should be exercised when adjusting the dose during the last trimester of pregnancy.
Treatment of the P and F1 males and females shall continue until termination. All P and F1 adult males and females should be humanely killed when they are no longer needed for assessment of reproductive effects. F1 offspring not selected for mating and all F2 offspring should be humanely killed after weaning.

1.4.7 **Mating procedure**

1.4.7.1 *Parental (P) mating*

For each mating, each female shall be placed with a single male from the same dose level (1:1 mating) until copulation occurs or 2 weeks have elapsed. Each day, the females shall be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm are found. In case pairing is unsuccessful, re-mating of females with proven males of the same group could be considered. Mating pairs should be clearly identified in the data. Mating of siblings should be avoided.

1.4.7.2 *F1 mating*

For mating the F1 offspring, at least one male and one female should be selected at weaning from each litter for mating with other pups of the same dose level but different litter, to produce the F2 generation. Selection of pups from each litter should be random when no significant differences are observed in body weight or appearance between the litter mates. In case these differences are observed, the best representatives of each litter should be selected. Pragmatically, this is best done on a body weight basis but it may be more appropriate on the basis of appearance. The F1 offspring should not be mated until they have attained full sexual maturity.

Pairs without progeny should be evaluated to determine the apparent cause of the infertility. This may involve such procedures as additional opportunities to mate with other proven sires or dams, microscopic examination of the reproductive organs, and examination of the oestrous cycles or spermatogenesis.

1.4.7.3 *Second mating*

In certain instances, such as treatment-related alterations in litter size or the observation of an equivocal effect in the first mating, it is recommended that the P or F1 adults be remated to produce a second litter. It is recommended to remate females or males, which have not produced a litter with proven breeders of the opposite sex. If production of a second litter is deemed necessary in either generation, animals should be remated approximately one week after weaning of the last litter.

1.4.7.4 *Litter size*

Animals shall be allowed to litter normally and rear their offspring to weaning. Standardisation of litter sizes is optional. When standardisation is done, the method used should be described in detail.

1.5 OBSERVATIONS

1.5.1 **Clinical observations**

A general clinical observation should be made each day and, and in the case of gavage dosing its timing should take into account the anticipated peak period of effects after dosing. Behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity should be recorded. An additional, more detailed examination of each animal should be conducted on at least a weekly basis and could conveniently be performed on an occasion when the animal is weighed. Twice daily, during the weekend once daily when appropriate, all animals should be observed for morbidity and mortality.

1.5.2 **Body weight and food/water consumption of parent animals**

Parental animals (P and Fl) shall be weighed on the first day of dosing and at least weekly thereafter. Parental females (P and F1) shall be weighed at a minimum on gestation days 0, 7, 14, and 20 or 21, and during lactation on the same days as the weighing of litters and on the day the animals are killed. These observations should be reported individually for each adult animal. During the premating and gestation periods food consumption shall be measured weekly at a minimum. Water consumption shall be measured weekly at a minimum if the test substance is administered in the water.

1.5.3 **Oestrus cycle**

Estrous cycle length and normality are evaluated in P and F1 females by vaginal smears prior to mating, and optionally during mating, until evidence of mating is found. When obtaining vaginal/cervical cells, care should be taken to avoid disturbance of mucosa and subsequently, the induction of pseudopregnancy (1).

1.5.4 **Sperm parameters**

For all P and F1 males at termination, testis and epididymis weight shall be recorded and one of each organ reserved for histopathological examination (see section 1.5.7, 1.5.8.1). Of a subset of at least ten males of each group of P and F1 males, the remaining testes and epididymides should be used for enumeration of homogenisation-resistant spermatids and cauda epididymal sperm reserves, respectively. For this same subset of males, sperm from the cauda epididymides or vas deferens should be collected for evaluation of sperm motility and sperm morphology. If treatment-related effects are observed or when there is evidence from other studies of possible effects on spermatogenesis, sperm evaluation should be conducted in all males in each dose group; otherwise enumeration may be restricted to control and high-dose P and F1 males.

The total number of homogenisation-resistant testicular spermatids and cauda epididymal sperm should be enumerated (2)(3). Cauda sperm reserves can be derived from the concentration and volume of sperm in the suspension used to complete the qualitative evaluations, and the number of sperm recovered by subsequent mincing and/or homogenising of the remaining cauda tissue. Enumeration should be performed on the selected subset of males of all dose groups immediately after killing the animals unless video or digital recordings are made, or unless the specimens are freezed and analysed later. In these instances, the controls and high dose group may be analysed first. If no treatment-related effects (e.g., effects on sperm count, motility, or morphology) are seen the other dose groups need not be analysed. When treatment-related effects are noted in the high-dose group, then the lower dose groups should also be evaluated.

Epididymal (or ductus deferens) sperm motility should be evaluated or video taped immediately after sacrifice. Sperm should be recovered while minimising damage, and diluted for motility analysis using acceptable methods (4). The percentage of progressively motile sperm should be determined either subjectively of objectively. When computer-assisted motion analysis is performed (5)(6)(7)(8)(9)(10) the derivation of progressive motility relies on user-defined thresholds for average path velocity and straightness or linear index. If samples are videotaped (11) or the images are otherwise recorded at the time of necropsy, subsequent analysis of only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated. In the absence of a video or digital image, all samples in all treatment groups should be analysed at necropsy.

A morphological evaluation of an epididymal (or vas deferens) sperm sample should be performed. Sperm (at least 200 per sample) should be examined as fixed, wet preparations (12) and classified as either normal or abnormal. Examples of morphologic sperm abnormalities would include fusion, isolated heads, and misshapen heads and/or tails. Evaluation should be performed on the selected subset of males of all dose groups either immediately after killing the animals, or, based on the video or digital recordings, at a later time. Smears, once fixed, can also be read at a later time. In these instances, the controls and high dose group may be analysed first. If no treatment-related effects (e.g., effects on sperm morphology) are seen the other dose groups need not be analysed. When treatment-related effects are noted in the high-dose group, then the lower dose groups should also be evaluated.

If any of the above sperm evaluation parameters have already been examined as part of a systemic toxicity study of at least 90 days, they need not necessarily be repeated in the two-generation study. It is recommended, however, that samples or digital recordings of sperm of the P generation are saved, to enable later evaluation, if necessary.

1.5.5 **Offspring**

Each litter should be examined as soon as possible after delivery (lactation day 0) to establish the number and sex of pups, stillbirths, live births, and the presence of gross anomalies. Pups found dead on day 0, if not macerated, should preferably be examined for possible defects and cause of death and preserved. Live pups should be counted and weighed individually at birth (lactation day 0) or on day 1, and on regular weigh days thereafter, e.g., on days 4, 7, 14, and 21 of lactation. Physical or behavioural abnormalities observed in the dams or offspring should be recorded.

Physical development of the offspring should be recorded mainly by body weight gain. Other physical parameters (e.g. ear and eye opening, tooth eruption, hair growth) may give supplementary information, but these data should preferably be evaluated in the context of data on sexual maturation (e.g. age and body weight at vaginal opening or balano-preputial separation) (13). Functional investigations (e.g. motor activity, sensory function, reflex ontogeny) of the F1 offspring before and/or after weaning, particularly those related to sexual maturation, are recommended if such investigations are not included in separate studies. The age of vaginal opening and preputial separation should be determined for F1 weanlings selected for mating. Anogenital distance should be measured at postnatal day 0 in F2 pups if triggered by alterations in F1 sex ratio or timing of sexual maturation.

Functional observations may be omitted in groups that otherwise reveal clear signs of adverse effects (e.g., significant decrease in weight gain, etc.). If functional investigations are made, they should not be done on pups selected for mating.

1.5.6 **Gross necropsy**

At the time of termination or death during the study, all parental animals (P and F1), all pups with external abnormalities or clinical signs, as well as one randomly selected pup/sex/litter from both the F1 and F2 generation, shall be examined macroscopically for any structural abnormalities or pathological changes. Special attention should be paid to the organs of the reproductive system. Pups that are humanely killed in a moribund condition and dead pups, when not macerated, should be examined for possible defects and/or cause of death and preserved.

The uteri of all primiparous females should be examined, in a manner which does not compromise histopathological evaluation, for the presence and number of implantation sites.

1.5.7 **Organ weights**

At the time of termination, body weight and the weight of the following organs of all P and F1 parental animals shall be determined (paired organs should be weighed individually):

— Uterus, ovaries;
— Testes, epididymides (total and cauda);
— Prostate;
— Seminal vesicles with coagulating glands and their fluids and prostate (as one unit);
— Brain, liver, kidneys, spleen, pituitary, thyroid and adrenal glands and known target organs.

Terminal body weights should be determined for F1 and F2 pups that are selected for necropsy. The following organs from the one randomly selected pup/sex/litter (see section 1.5.6) shall be weighed: Brain, spleen and thymus.

Gross necropsy and organ weight results should be assessed in context with observations made in other repeated dose studies, when feasible.

1.5.8 **Histopathology**

1.5.8.1 *Parental Animals*

The following organs and tissues of parental (P and F1) animals**,** or representative samples thereof, shall be fixed and stored in a suitable medium for histopathological examination.

— Vagina, uterus with cervix, and ovaries (preserved in appropriate fixative);
— One testis (preserved in Bouin's or comparable fixative), one epididymis, seminal vesicles, prostate, and coagulating gland;
— Previously identified target organ(s) from all P and F1 animals selected for mating.

Full histopathology of the preserved organs and tissues listed above should be performed for all high dose and control P and F1 animals selected for mating. Examination of the ovaries of the P animals is optional. Organs demonstrating treatment-related changes should also be examined in the low- and mid-dose groups to aid in the elucidation of the NOAEL. Additionally, reproductive organs of the low-and mid-dose animals suspected of reduced fertility, e.g., those that failed to mate, conceive, sire, or deliver healthy offspring, or for which oestrus cyclicity or sperm number, motility, or morphology were affected, should be subjected to histopathological evaluation. All gross lesions such as atrophy or tumours shall be examined.

Detailed testicular histopathological examination (e.g. using Bouin's fixative, paraffin embedding and transverse sections of 4-5 μm thickness) should be conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells or sloughing of spermatogenic cells into the lumen (14). Examination of the intact epididymis should include the caput, corpus, and cauda, which can be accomplished by evaluation of a longitudinal section. The epididymis should be evaluated for leukocyte infiltration, change in prevalence of cell types, aberrant cell types, and phagocytosis of sperm. PAS and haematoxylin staining may be used for examination of the male reproductive organs.

The postlactational ovary should contain primordial and growing follicles as well as the large corpora lutea of lactation. Histopathological examination should detect qualitative depletion of the primordial follicle population. A quantitative evaluation of primordial follicles should be conducted for F1 females; the number of animals, ovarian section selection, and section sample size should be statistically appropriate for the evaluation procedure used. Examination should include enumeration of the number of primordial follicles, which can be combined with small growing follicles, for comparison of treated and control ovaries (15)(16)(17)(18)(19).

1.5.8.2 *Weanlings*

Grossly abnormal tissue and target organs from all pups with external abnormalities or clinical signs, as well as from the one randomly selected pup/sex/litter from both the F1 and F2 generation which have not been selected for mating, shall be fixed and stored in a suitable medium for histopathological examination. Full histopathological characterisation of preserved tissue should be performed with special emphasis on the organs of the reproductive system.

2 DATA

2.1 TREATMENT OF RESULTS

Data shall be reported individually and summarised in tabular form, showing for each test group and each generation the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons, the time of any death or humane kill, the number of fertile animals, the number of pregnant females, the number of animals showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the types of parental and offspring observations, the types of histopathological changes, and all relevant litter data.

Numerical results should be evaluated by an appropriate, generally accepted statistical method; the statistical methods should be selected as part of the design of the study and should be justified. Dose-response statistical models may be useful for analysing data. The report should include sufficient information on the method of analysis and the computer program employed, so that an independent reviewer/statistician can re-evaluate and reconstruct the analysis.

2.2 EVALUATION OF RESULTS

The findings of this two-generation reproduction toxicity study should be evaluated in terms of the observed effects including necropsy and microscopic findings. The evaluation will include the relationship, or lack thereof, between the dose of the test substance and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target organs, affected fertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects. The physico-chemical properties of the test substance, and when available, toxicokinetics data should be taken into consideration when evaluating test results.

A properly conducted reproduction toxicity test should provide a satisfactory estimation of a no-effect level and an understanding of adverse effects on reproduction, parturition, lactation, postnatal development including growth and sexual development.

2.3 INTERPRETATION OF RESULTS

A two-generation reproduction toxicity study will provide information on the effects of repeated exposure to a substance during all phases of the reproductive cycle. In particular, the study provides information on the reproductive parameters, and on development, growth, maturation and survival of offspring. The results of the study should be interpreted in conjunction with the findings from subchronic, prenatal developmental and toxicokinetic and other available studies. The results of this study can be used in assessing the need for further testing of a chemical. Extrapolation of the results of the study to man is valid to a limited degree. They are best used to provide information on no-effect-levels and permissible human exposure (20)(21)(22)(23).

3 **REPORTING**

TEST REPORT

The test report must include the following information:

Test substance:

— physical nature and, where relevant, physicochemical properties;
— identification data;
— purity.

Vehicle (if appropriate):

— justification for choice of vehicle if other than water.

Test animals:

— species/strain used;
— number, age and sex of animals;
— source, housing conditions, diet, nesting materials, etc.;
— individual weights of animals at the start of the test.

Test conditions:

— rationale for dose level selection;
— details of test substance formulation/diet preparation, achieved concentrations;
— stability and homogeneity of the preparation;
— details of the administration of the test substance;
— conversion from diet/drinking water test substance concentration (ppm) to the achieved dose (mg/kg body weight/day), if applicable;
— details of food and water quality.

Results:

— food consumption, and water consumption if available, food efficiency (body weight gain per gram of food consumed), and test material consumption for P and F1 animals, except for the period of cohabitation and for at least the last third of lactation;
— absorption data (if available);
— body weight data for P and F1 animals selected for mating;
— litter and pup weight data;
— body weight at sacrifice and absolute and relative organ weight data for the parental animals;
— nature, severity and duration of clinical observations (whether reversible or not);
— time of death during the study or whether animals survived to termination;
— toxic response data by sex and dose, including indices of mating, fertility, gestation, birth, viability, and lactation; the report should indicate the numbers used in calculating these indices;
— toxic or other effects on reproduction, offspring, post-natal growth, etc.;
— necropsy findings;
— detailed description of all histopathological findings;
— number of P and F1 females cycling normally and cycle length;
— total cauda epididymal sperm number, percent progressively motile sperm, percent morphologically normal sperm, and percent of sperm with each identified abnormality;
— time-to-mating, including the number of days until mating ;
— gestation length;
— number of implantations, corpora lutea, litter size;
— number of live births and post-implantation loss;
— number of pups with grossly visible abnormalities, if determined the number of runts should be reported;
— data on physical landmarks in pups and other post natal developmental data; physical landmarks evaluated should be justified;
— data on functional observations in pups and adults, as applicable;
— statistical treatment of results, where appropriate.

Discussion of results.

Conclusions, including NOAEL values for maternal and offspring effects.

REFERENCES

(1) Sadleir, R.M.F.S. (1979). Cycles and Seasons, In: *Reproduction in Mammals:* I. Germ Cells and Fertilization, C.R. Auston and R.V. Short (eds.), Cambridge, New York.

(2) Gray, L.E. et al., (1989). A Dose-Response Analysis of Methoxychlor-Induced Alterations of Reproductive Development and Function in the Rat. *Fundamental and Applied Toxicology* 12:92-108.

(3) Robb, G.W. et al., (1978). Daily Sperm Production and Epididymal Sperm Reserves of Pubertal and Adult Rats. *Journal of Reproduction and Fertility* 54:103-107.

(4) Klinefelter, G.R. et al., (1991). The Method of Sperm Collection Significantly Influences Sperm Motion Parameters Following Ethane Dimethanesulfonate Administration in the Rat. *Reproductive Toxicology* 5:39 44

(5) Seed, J. et al. (1996). Methods for Assessing Sperm Motility, Morphology, and Counts in the Rat, Rabbit, and Dog: a Consensus Report. *Reproductive Toxicology* 10(3):237- 244.

(6) Chapin, R.E. et al., (1992).Methods for Assessing Rat Sperm Motility. *Reproductive Toxicology* 6:267-273

(7) Klinefelter**,** G.R. et al., (1992). Direct Effects of Ethane Dimethanesulphonate on Epididymal Function in Adult Rats: an *In Vitro* Demonstration**.** *Journal of Andrology* 13:409-421.

(8) Slott, V.L. et al., (1991). Rat Sperm Motility Analysis: Methodologic Considerations. *Reproductive Toxicology* 5:449-458.

(9) Slott, V.L. and Perreault, S.D., (1993). Computer-Assisted Sperm Analysis of Rodent Epididymal Sperm Motility Using the Hamilton-Thorn Motility Analyzer. In: *Methods in Toxicology*, Part A., Academic, Orlando, Florida. pp. 319-333.

(10) Toth, G.P. et al. (1989). The Automated Analysis of Rat Sperm Motility Following Subchronic Epichlorhydrin Administration: Methodologic and Statistical Considerations. Journal of Andrology 10: 401-415.

(11) Working, P.K. and M. Hurtt, (1987). Computerized Videomicrographic Analysis of Rat Sperm Motility. Journal of Andrology 8:330-337.

(12) Linder, R.E. et al., (1992). Endpoints of Spermatoxicity in the Rat After Short Duration Exposures to Fourteen Reproductive Toxicants. Reproductive Toxicology 6:491-505.

(13) Korenbrot, C.C. et al., (1977). Preputial Separation as an External Sign of Pubertal Development in the Male Rat. Biological Reproduction 17:298303.

(14) Russell, L.D. et al., (1990). Histological and Histopathological Evaluation of the Testis, Cache River Press, Clearwater, Florida.

(15) Heindel, J.J. and R.E. Chapin, (eds.) (1993). Part B. Female Reproductive Systems, Methods in Toxicology, Academic, Orlando, Florida.

(16) Heindel, J.J. et al., (1989) Histological Assessment of Ovarian Follicle Number in Mice As a Screen of Ovarian Toxicity. In: Growth Factors and the Ovary, A.N. Hirshfield (ed.), Plenum, New York, pp. 421-426.

(17) Manson, J.M. and Y.J. Kang, (1989). Test Methods for Assessing Female Reproductive and Developmental Toxicology. In: Principles and Methods of Toxicology, A.W. Hayes (ed.), Raven, New York.

(18) Smith, B.J. et al,. (1991). Comparison of Random and Serial Sections in Assessment of Ovarian Toxicity. Reproductive Toxicology 5:379-383.

(19) Heindel, J.J. (1999). Oocyte Quantitation and Ovarian Histology. In: An Evaluation and Interpretation of Reproductive Endpoints for Human Health Risk Assessment, G. Daston,. and C.A. Kimmel, (eds.), ILSI Press, Washington, DC.

(20) Thomas, J. A. (1991). Toxic Responses of the Reproductive System. In: Casarett and Doull's Toxicology, M.O. Amdur, J. Doull, and C.D. Klaassen (eds.), Pergamon, New York.

(21) Zenick, H. and E.D. Clegg, (1989). Assessment of Male Reproductive Toxicity: A Risk Assessment Approach. In: Principles and Methods of Toxicology, A.W. Hayes (ed.), Raven Press, New York.

(22) Palmer, A.K. (1981). In: Developmental Toxicology, Kimmel, C.A. and J. Buelke-Sam (eds.), Raven Press, New York.

(23) Palmer, A.K. (1978). In Handbook of Teratology, Vol. 4, J.G. Wilson and F.C. Fraser (eds.), Plenum Press, New York.