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MEMORANDUM

SUBJECT: **MOLINATE** - Data Package Submitted for Consideration by the HED Mechanism of Toxicity SARC - Assessment of Mode of Action Data for Reproductive Effects

TO: Karl Baetcke, Ph.D.
Chairman, Mechanism Committee
Science Advisory Branch, HED (7509C)

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Registrant:	Zeneca
Chemical:	S-ethyl hexahydro-1H-azepine-1-carbothioate
Synonym:	Molinate, Ordram
Caswell No.:	444
PC Code:	041402
Use/Site:	pre-emergence herbicide for use on rice

Attached is the proposed mechanism of action data presentation document on Molinate for consideration by the Mechanism of Toxicity SARC, along with the DATA EVALUATION REPORTS of the critical studies. Additionally, copies of other papers, either published or pre-publication papers submitted by the Registrant, are appended.

1. Statement of **Proposed Mode of Toxicity**: Based on the nature of the Molinate-induced intra-testicular lesion and the dependence of spermatid release by the Sertoli cell on testosterone, it is hypothesized by the Registrant that the male reproductive impairment caused by Molinate is the result of a block in the production of testosterone by the Leydig cell.

2. Below is a list of the relevant studies used in this document.

a. **[MRID 44918001]** Ellis, M. K. and Farnworth, M. J. (1999). First Revision to MOLINATE: Effect of Molinate and Molinate Metabolites Following Seven Day Administration on Testis and Sperm Morphology in the Rat.
b. **[MRID 43158202]** Hadge, MCE. (1993). Molinate: Sperm Morphology in the Rat.
c. **[MRID 44521004]** Wickramaratne. A. (1997). The morphological effects of the thiocarbamate herbicide, molinate, on the ovary, adrenal and testis of the Sprague-Dawley Rat.
d. **[MRID 44918002]** Ellis. M. K. and Farnworth. M. J. (1999). MOLINATE: Investigation into the Mode of Action in the Rat Leydig Cell *In Vitro*.
e. **[MRID 44765003]** Foster, J. R. (1999). Neutral Cholesterol Ester Hydrolase: A Key Enzyme in the Control of Steroidogenesis in Rodents.
f. **[MRID 44918003]** Ellis. M. K. and Farnworth. M. J. (1999). First Revision to MOLINATE: Effect of Molinate and Molinate Metabolites on Plasma and Testicular Interstitial Fluid Hormone Concentrations in the Rat *In Vivo*.
g. **[MRID 44521003]** Ellis. M.K.; Coutts. C.T.; Lovatt, C.A.; Richardson, A.G.; Laird, W.J.D.; Cooper. G.K.; Moore, R.B.; Pitts, M.R.; Dunn, D.S.: and Wickramaratane, G.A. (1997). Species Comparison in the Metabolism of the Herbicide Molinate.
h. **[MRID 42361308]** Horner, J. M. (1992). Molinate: Mechanistic Study in the Pregnant Rat.
I. **[MRID 44373601]** Williams. J. (1997). MOLINATE: An Evaluation of Vaginal Opening in Rat Pups.
j. **ABSTRACT**. Jewell. W. T. And Miller. M.G. (1999). Comparison of Human and Rat Metabolism of Molinate in Liver microsomes and slices.
k. **PUBLICATION**. Jewell, W.T. and Miller, M.G. (1998). Identification of a Carboxylesterase as the Major Protein Bound by Molinate. Toxicology and Applied Pharmacology **149**, 226-234.
l. **PUBLICATION.** Jewell, W.T. Hess, R.A.. and Miller, M.G. (1998). Testicular Toxicity of Molinate in the Rat: Metabolic Activation *via* Sulfoxidation. Toxicology and Applied Pharmacology **149**, 159-166.
m. **PUBLICATION.** Ellis, M.K.; Richardson, A.G.; Foster, J. R.; *et al.* (1998). The Reproductive Toxicity of Molinate and Metabolites to the Male Rat: Effects on Testosterone and Sperm Morphology. Toxicology and Applied Pharmacology **151**, 22-32.
n. **[Accession No. 00245675]** Killinger, J.M.; Downs, C.R.; Minor, J.L.; *et al.* (1981). Ordram Fertility Study in Male Rats: Mechanism/Site of Action.
o. **[MRID 44521002]** Wickramaratne, A. (1997). MOLINATE: Rodent Reproductive Toxicity and Its Relevance to Humans: A Review.
p. **[MRID 44521005]** Wickramaratne, A. (1997). MOLINATE: Elucidation of the Processes Underlying the Reproductive Effects in the Male Rat.

3. **INTRODUCTION**. Molinate is a reproductive toxicant, and the rat is the most sensitive species for this effect. The Registrant's position is that the reproductive effect of Molinate "requires the production of molinate sulfoxide and the dependence on the enzyme cholesterol ester hydrolase for steroid sex hormone production." Additionally, the Registrant concludes that the reproductive toxicity to the rat is induced by a mechanism that is specific to rodents.

4. **BACKGROUND**. Decreased fertility, abnormal sperm, decreased percent motile sperm, decreased sperm numbers, decreased litter size, decreased % born live, decreased pup viability, increased incidence of microscopic lesions in the ovary, testes, and adrenals, delayed vaginal opening, and reproductive organ weight effects are consistent findings in the rat following exposure to Molinate. Molinate causes a distinctive sperm lesion, and the morphological changes in the testes are considered by the Registrant to be consistent with a delayed release of the late spermatids to the seminiferous tubular lumen, a process controlled by the release of testosterone. The hypothesis, based on the nature of the Molinate-induced, intra-testicular lesion and the dependence of spermatid release by the Sertoli cell on testosterone, is that male reproductive impairment caused by Molinate is the result of a block in the production of testosterone by the Leydig cell. The Registrant contends that there is a requirement for metabolic activation for the impairment of reproduction and concludes that the results of the studies strongly suggest that Molinate sulphoxide is the proximate metabolite responsible for the block in steroid production following Molinate exposure to the rat.

5. **MECHANISM OF ACTION ISSUES**

A. PHYSIOLOGICAL MARKER: altered sperm morphology

In a 13-week inhalation study [MRID 00241965] in rats on Molinate, abnormal spermatozoa were found in the epididymides. In a rat fertility study [MRID 00245675] designed specifically to identify the stage of the spermatogenic cycle affected by Molinate, there was an increase in the number of abnormal sperm and an increase in the number of seminiferous tubules with degenerating spermatids/spermatocytes per testis. In a 4-week inhalation study [MRID 41589203] in the rat, there was an increase in the percent of abnormal sperm [detached heads, sperm with broken membranes between head and midpiece, or between midpiece and tail sections]. The data suggested to the Registrant that the mid- to late stages were affected, with the major effect being on the late spermatid stage. NOTE: In several preliminary studies in rabbits, sperm abnormalities were evident also.

1. In a study designed specifically to assess sperm morphology [MRID 44918001], Molinate and Molinate sulphoxide displayed an increase in abnormal sperm [detached heads, mid-piece lesion, tail abnormalities] following the 7-day exposure period in rats.

> Ellis, M. K. and Farnworth, M. J. (1999). First Revision to MOLINATE: Effect of Molinate and Molinate Metabolites Following Seven Day Administration on Testis and Sperm Morphology in the Rat. EXECUTIVE SUMMARY: In a special study [MRID 44918001] to establish whether the sperm abnormalities observed following Molinate exposure are due to Molinate or its metabolites, 4 Sprague-Dawley CD male rats/group were administered Molinate (96.9% a.i.) at 40 or 140 mg/kg/day, or Molinate sulphoxide at 10 or 20 mg/kg/day, or 4-Hydroxymolinate at 10 mg/kg/day, or Hexamethyleneimine at 10 mg/kg/day *via* subcutaneously-implanted osmotic mini-pumps for 7 days. Both light microscopy and

scanning electron microscopy were used to examine sperm samples obtained at necropsy.

None of the test materials displayed any apparent adverse effect on the weight of the testes. Light microscopy examination revealed an increased incidence of detached sperm heads at both dose levels of Molinate [26%-36%] and Molinate sulphoxide [=25%]. Mid-piece lesions were observed at a comparable incidence [=10%] in both dose groups of Molinate and in the high-dose group of Molinate sulphoxide. The highest incidence [=10%] of tail abnormalities was observed in the high-dose Molinate group. In general, 4-Hydroxymolinate and Hexamethyleneimine did not show an effect on sperm morphology, although a slight increase in the incidence of mid-piece lesions and tail abnormalities above control was noted in the 4-Hydroxymolinate group [an apparent increase in tail abnormalities was displayed in one of the 3 rats examined under the light microscope and the slight increase in mid-piece lesions was noted compared to the lack of this finding in the control].

2. In another study [MRID 43158202], the administration of Molinate *via* gavage for 35 days resulted in a dose-related increase in sperm abnormalities in the rat.

Hadge, MCE. (1993). Molinate: Sperm Morphology in the Rat. Zeneca Central Toxicology Laboratory. EXECUTIVE SUMMARY: In a special study [MRID 43158202], male Crl:CD(SD)BR rats [12/group] were exposed to Molinate [96.8% a.i.] *via* gavage for 35 consecutive days at dose levels of 0, 0.5, 1, 2, 3, 4, and 8 mg/kg/day.

There were no deaths or clinical signs that could be attributed to treatment, and body weights and body-weight gains were comparable among the groups. The objective of the study was to define more precisely the NOEL for changes in sperm morphology observed in the rat following exposure to Molinate. All rats displayed headless sperm, but the percent abnormal was greater at all dose levels of Molinate [not explicitly dose-related] compared to the controls. There was a dose-related increase in the incidence of sperm midpiece abnormalities, and the number of sperm affected was increased with increasing dose. None of the concurrent controls displayed this type of abnormality. No NOEL for sperm morphology was determined.

3. In a paper entitled "The morphological effects of the thiocarbamate herbicide, molinate, on the ovary, adrenal and testis of the Sprague-Dawley Rat" [MRID 44521004; no individual data submitted], the results of a study in which male rats were dosed for 35 days *via* gavage at dose levels of 0, 10, 30, and 60 mg/kg/day are reported.

Testicular atrophy and necrosis of the spermatid cell population, primarily in Stage VIII tubules, were observed at 30 and 60 mg/kg/day and sperm, taken from the epididymis, showed a distinct abnormality of the head-midpiece junction. No effect was reported at 10 mg/kg/day. In a published paper that refers to this study, it is stated that at high doses of Molinate, the development of spermatogonia and spermatocytes was normal, but on passing through the division processes to develop into spermatids, an abnormality consisting of multinucleation and nuclear and cytoplasmic degeneration was seen. At the stage of spermatid release, large residual bodies formed and the lumen contained an increased quantity of extracellular debris. In addition, mature sperm heads without tails were observed

at the base of seminiferous tubules, apparently in the process of being reabsorbed by the Sertoli cells. The lesion was said to be consistent with a disruption of the normal process of spermatogenesis causing a delayed release of the late spermatids from the epithelium of the Sertoli cell into the seminiferous tubular lumen. It further states that, in the rat, this process progresses in a highly synchronized manner. The spermatids are released at a specific stage of the spermatogenic cycle [Stage VIII, as classified by LeBlonde and Clermont, 1952] in a process that is tightly controlled by testosterone [Steinberger and Duckett, 1967]. The association of the initial lesion to the stage of spermatogenesis that is dependent upon appropriate concentrations of testosterone within the seminiferous epithelium [Stage VIII] suggested to the Registrant that Molinate was causing a perturbation of testosterone synthesis, release, or action within the testes.

4. In one of the first mechanistic studies performed on Molinate in the rat [Accession No. 00245675], Molinate exposure to male rats resulted in a decrease in male fertility at dose levels of 4, 12, 30, and 60 mg/kg/day for periods from 5 days to 5 and 10 weeks.

Sperm abnormalities were observed following 5 and 10 weeks of treatment at dose levels of 4 mg/kg/day and above and included detached sperm heads and tails, heads and tails bent at abnormal angles, and rupture of sperm membranes at head-midpiece and midpiece-tail junctions. The **NOAEL** is 0.2 mg/kg/day, and the **LOAEL** is 4 mg/kg/day, based on decreases in the % viable sperm, % motile sperm, % normal sperm, sperm counts, numbers of implants, number of viable fetuses, and increased pre-implantation loss.

5. Ellis, M.K.; Richardson, A.G.; Foster, J.R.; *et al.* (1998). The Reproductive Toxicity of Molinate and Metabolites to the Male Rat: Effects on Testosterone and Sperm Morphology. Toxicology and Applied Pharmacology **151**, 22-32. NOTE: This paper reports the results of several studies; not all have been submitted to the Agency; i.e., there are no individual data for review. The data reported in this paper on sperm morphology are apparently from MRID 44918001, discussed above under A1.

A distinctive sperm lesion was observed following 7 days oral and i.p. administration of Molinate to male rats. At a higher dose level [140 mg/kg/day for 7 days], this lesion was accompanied by morphological changes to the testis that were said to be consistent with a delayed release of late spermatids to the seminiferous tubular lumen, a process said to be controlled by the release of testosterone.

6. Jewell, W.T. Hess, R.A., and Miller, M.G. (1998). Testicular Toxicity of Molinate in the Rat: Metabolic Activation *via* Sulfoxidation. Toxicology and Applied Pharmacology **149**, 159-166.

Testicular damage was evaluated histopathologically in Sprague-Dawley rats 48 hours, and 1, 2, and 3 weeks after single i.p. injections of Molinate [>99% a.i.] at dose levels of 100-400 mg/kg. The results were reported as follows: Testicular damage, characterized by Sertoli cell vacuolation, failed spermiation, and phagocytosis of spermatids particularly evident at Stages X and XI were observed following 1 week at 200 mg/kg and after 48 hours at 400 mg/kg. With increasing time, damage progressed until disorganization of the seminiferous epithelium was extensive, multinucleated giant cells were numerous, and neither spermatozoa nor late step spermatids were present. At 3 weeks at the 200 and 400 mg/kg

dose levels, germ cells in the seminiferous tubules were almost completely absent. No damage was observed at 100 mg/kg at any time point. Testicular damage similar to that observed at 400 mg/kg Molinate were reported for Molinate sulfoxide administered at 200 mg/kg. It was noted in the paper that the direct intratesticular administration of Molinate sulfoxide into the testis did not result in a lesion, suggesting to the authors that either Molinate sulfoxide is not the final toxic metabolite responsible for Molinate's testicular effects or that intratesticular injection does not ensure adequate delivery of toxicant to the testis.

B. **BIOCHEMICAL MARKER:** perturbation of testosterone biosynthesis

Based on the nature of the sperm lesion observed following Molinate exposure to male rats and the morphological changes in the testes, the Registrant hypothesized that the effects were the result of a block in the production of testosterone by the Leydig cell. The effect [modulation of testosterone production] in the rat is hypothesized to be a consequence of an inhibition of cholesterol ester hydrolase [n-CEH], which in turn is said to be a consequence of metabolism of Molinate to Molinate sulfoxide.

1. In a special ***in vivo*** study [MRID 44918003] on steroid hormone concentrations in plasma and testicular interstitial fluid in male rats, decreases in the precursor steroids were observed following oral and i.p. administration of Molinate and i.p. administration of Molinate metabolites.

> Ellis, M. K. and Farnworth, M. J. (1999). First Revision to MOLINATE: Effect of Molinate and Molinate Metabolites on Plasma and Testicular Interstitial Fluid Hormone Concentrations in the Rat *In Vivo*. EXECUTIVE SUMMARY: In a special study [MRID 44918003] to test the hypothesis that the toxicity of Molinate is due to metabolic biotransformation, 6 Sprague-Dawley CD male rats/group/time point and test material were administered single oral [Molinate (96.9% a.i.) at 50, 100, 200 mg/kg] or single intraperitoneal (i.p.) [Molinate at 40 mg/kg; Molinate sulphoxide at 1, 10, 20 mg/kg; Molinate sulphone at 1, 2, 5, 10 mg/kg; 4-Hydroxymolinate at 1, 5, 10, 43 mg/kg; and Hexamethyleneimine at 5, 40 mg/kg] doses and sacrificed at 2 hours, 6 hours, or 24 hours post dose. Male steroid hormone/precursor [testosterone, androstenedione, 17α-hydroxyprogesterone, progesterone, and cholesterol] concentrations in the plasma and testicular interstitial fluid were monitored.
>
> Decreased plasma testosterone and androstenedione levels were observed following oral and i.p. administration of Molinate, i.p. administration of Molinate sulphoxide, and i.p. administration of hexamethyleneimine. Decreased interstitial fluid testosterone, androstenedione, and progesterone levels were observed in these same groups. Decreased interstitial fluid 17α-hydroxyprogesterone levels were observed following oral and i.p. administration of Molinate and i.p. administration of Molinate sulphoxide. Slightly elevated plasma cholesterol levels were noted in the Molinate [oral and i.p.] and Molinate sulphoxide [i.p.] groups.

2. In an ***in vitro*** study [MRID 44918002], the effect of the addition of precursor steroids on the inhibition of testosterone synthesis by Molinate and Molinate sulphoxide in Leydig cell cultures

from male rats was investigated.

Ellis, M. K. and Farnworth, M. J. (1999). MOLINATE: Investigation into the Mode of Action in the Rat Leydig Cell *In Vitro*. EXECUTIVE SUMMARY: In a special study [MRID 44918002] to examine the effects of Molinate [96.9% a.i.] and Molinate sulphoxide on steroid hormone production in isolated rat Leydig cell culture, Leydig cell cultures were treated with non-cytotoxic concentrations of Molinate [400 μM] and Molinate sulphoxide [400 μM], prior to the supplementation with testosterone steroid precursors [androstenedione, 17α-hydroxyprogesterone, progesterone, 22-hydroxycholesterol, cholesterol oleate, pregnenolone, and cholesterol (latter two only in the Molinate cultures)]. The control Leydig cell cultures were treated with dimethyl formamide [DMF]. A positive control, Ketoconizole [0.5 μM], was also included in this study. Additionally, the effects of Molinate [3.125 μM to 400 μM], Molinate sulphoxide [0.008 μM to 10 μM], and Molinate sulphone [0.30 nM to 50 nM] on cholesterol ester hydrolase [n-CEH] activity in the Leydig cell cultures were investigated.

Decreased testosterone levels were observed in Leydig cell cultures treated with Molinate, Molinate sulphoxide, and Ketoconizole compared to the DMF control cultures. Ketoconizole displayed the lowest testosterone values. There was a dose-related increase in testosterone production following addition of increased levels of the precursor steroids progesterone, 17α-hydroxyprogesterone, and pregnenolone, androstenedione in the Molinate, Molinate sulphoxide, and Ketoconizole Leydig cell cultures, but only slight increases following addition of cholesterol, 22-hydroxycholesterol, and cholesterol oleate.

Also in this study, both Molinate sulphoxide and Molinate sulphone were shown to inhibit cholesterol ester hydrolase [the enzyme that converts cholesterol esters to cholesterol], ***in vitro***. This enzyme was shown to be only slightly inhibited by Molinate. NOTE: In a published paper [Ellis, *et al., (1998);* below], Molinate was shown to inhibit this enzyme ***in vivo***.

3. Ellis, M.K.; Richardson, A.G.; Foster, J.R.; *et al.* (1998). The Reproductive Toxicity of Molinate and Metabolites to the Male Rat: Effects on Testosterone and Sperm Morphology. Toxicology and Applied Pharmacology **151**, 22-32, cited above under A5.

Molinate caused a marked decrease in the concentration of circulating and testicular testosterone at dose levels [≥40 mg/kg/day in corn oil for 7 days] where a distinctive sperm lesion was observed. Additionally, esterase activity in the Leydig cells was inhibited following Molinate exposure, in contrast to an *in vitro* study where it was shown to be a poor inhibitor of esterase activity.

The following was also discussed in this paper: The control of spermatogenesis is directed by the production of testosterone but is intimately controlled by the Sertoli cells that lie within the seminiferous epithelium. The effect of Molinate may result from a direct action upon the Sertoli cell or indirectly by perturbing Leydig cell biochemistry that ultimately results in the inhibition of testosterone synthesis. An effect on esterase activity was used as a marker of perturbed Leydig cell biochemistry *in vivo*. It is stated that esterases are exquisitely expressed within the Leydig cells

within the testes and are a sensitive marker for damage [Bingfang, *et al.* (1995); Somkuti, *et al.* (1987)]. Although the roles of these enzymes are uncertain, it is conceivable that they are involved in the release of cholesterol from its storage ester, ultimately for testosterone synthesis [Bingfang, *et al.* (1995); Somkuti, *et al.* (1987)].

Also reported in this paper are the results of a single-dose oral study in which Molinate [40 mg/kg] markedly inhibited esterase activity in the Leydig cell, and this inhibition was said to have been present at a time before morphological damage was apparent within the seminiferous tubules. Following a 10-day administration of Molinate, a significant reduction was observed in esterase activity at dose levels that induced morphological damage within the testes [dose levels not cited].

Additionally, in support of a direct action of Molinate upon the testes, radiolabel derived from [^{3}H] Molinate was said to have localized predominantly within the Leydig cells [no report cited; data have not been submitted for review]; no dose levels were reported and the route of exposure was not reported.

4. In a paper [MRID 44765003: no individual data included] entitled Neutral Cholesterol Ester Hydrolase: A Key Enzyme in the Control of Steroidogenesis in Rodents, two conflicting statements regarding neutral cholesterol ester hydrolase were found. On page 12 in the second paragraph of 3. **Neutral Cholesterol Ester Hydrolase [n-CEH]**, it states that two major forms of nCEH have been reported from rat testes, both of which appear to be specific to the organ. One type is located within the Leydig cell while the other appears to be specific to the Sertoli cell. At the top of page 14, which is the last paragraph under 4. **Regulation of nCEH**, it states that nCEH has been shown to be exclusively localized in the Leydig cells of the testes and not to be expressed in the Sertoli cells or germinal epithelium.

5. Jewell, W.T. and Miller, M.G. (1998). Identification of a Carboxylesterase as the Major Protein Bound by Molinate. Toxicology and Applied Pharmacology **149**, 226-234. The study investigated the nature of the binding reaction of ^{14}C-Molinate, ^{14}C-Molinate sulphoxide, and ^{14}C-Molinate sulphone in liver and testis microsomal preparations. The effect of Molinate administration on *in vivo* esterase activity was assessed both by enzymatic measurement and by histochemical measurement.

> Molinate treatment caused a marked inhibition of nonspecific esterase activity in both liver and testis. In the testis, histochemical staining showed the esterase activity inhibited by Molinate was localized primarily to the Leydig cell, consistent with the localization of Hydrolase A. From these data, it is proposed that Molinate-induced inhibition of esterase activity in the Leydig cell could inhibit the mobilization of cholesterol esters required for testosterone biosynthesis.

C. SPECIES SPECIFICITY

Although previously the Registrant had argued that the rodent-specific reproductive impairment following Molinate exposure could be attributed solely to the difference in metabolism between the rat and man, the recent submission states that, although the metabolic profiles of Molinate differ across species [rat, mouse, rabbit, and man], "this difference is considered insufficient to account

solely for the differential susceptibility of the species examined." The Registrant states that the mechanism of the reproductive impairment has been associated with a **perturbation** of testosterone synthesis; **a consequence of an inhibition of *n*CEH by Molinate sulfoxide**. In the 1998 Ellis, M.K., *et al.* paper cited above, "The Reproductive Toxicity of Molinate and Metabolites to the Male Rat: Effects on Testosterone and Sperm Morphology", it states that Molinate sulfoxide inhibits general ester hydrolysis within the Leydig cells of the rat testis, and such action would also prevent the release of cholesterol from its storage ester within this cell type; a reaction catalyzed by the enzyme neutral cholesterol ester hydrolase, nCEH. The paper continues as follows: The major source of cholesterol in rodents is from high-density lipoproteins [HDLs] in plasma [Gwynne *et al., 1976;* Barter and Lally, 1978; Sigurdsson *et al.* 1979; van't Hoofte *et al.* 1981; Glass *et al.* 1983] that are hydrolyzed within the cell cytosol by nCEH. In rabbit, dog, and man, the majority of the cholesterol is obtained from low-density lipoproteins [LDLs]; the cholesterol being released on acidic hydrolysis in lysosomes [Payne *et al.* 1985; Havel and Hamilton, 1988]. Therefore, it is stated that the inhibition of cytosolic nCEH by Molinate sulfoxide [see below under METABOLISM] in the rabbit, dog, and man is unlikely to significantly affect cholesterol availability in these species as this enzyme is not required for the release of cholesterol from LDLs [Latendresse *et al.* 1993]. Molinate sulfoxide is a major ntetabolite of Molinate in the rat, mouse, and dog and consequently, metabolism alone would suggest that these species would be equally susceptible to the reproductive effects of Molinate. According to the Registrant, the formation of Molinate sulfoxide is a prerequisite for the reproductive impairment caused by Molinate, but the species sensitivity is dependent upon physiological and biochemical differences.

D. METABOLISM

According to the Registrant, the metabolism of Molinate in mammals is primariiy by three routes: **carbon oxidation** [hexahydro-1H-azepineoxidation] to 3- and 4-hydroxymolinate, **sulfur oxidation** to Molinate sulphoxide, and **thiocarbamate cleavage** to hexamethyleneimine [Figure 1, page 28 of MRID 44521005, copy appended]. The sulphur oxidation pathway, *via* a sulfoxide [and possibly sulfone] intermediate, yields a cysteine or mercapturate conjugate. It is stated that the proportion of metabolism through each of these pathways varies in rat, mouse, rabbit, dog, monkey, and man [Table 1, below]. The numbers in [ ] are from a paper [MRID 44521003] entitled "Species comparison in the metabolism of the herbicide molinate" by Ellis, MK; Coutts, CT; Lovatt, CA; *et al.* , submitted by the Registrant previously [discussed below], while the other numbers are those presented by the Registrant in slides at a meeting. The **Registrant's position** is that **sulfur oxidation of Molinate is a prerequisite for the onset of the sperm, testis, ovarian, and adrenal effects**. It is argued that the rat metabolizes Molinate predominately by this route and man does not. However, this is based on data from studies in the rat at high dose levels [72 mg/kg po] and a study in humans at a very low dose [0.03-0.07 mg/kg po].

Table 1. Proposed Species Differences in Metabolism of Molinate

species	Primary Biotransformation Pathways		
	carbon oxidation	sulfur oxidation	thiocarbamate cleavage

rat	30 [32]	46 [29]	24 [33]
mouse	30 [50]	28 [21]	42 [23]
rabbit	82 [63]	9 [7]	9 [5]
dog	37 [36]	19 [33]	40 [28]
monkey	43	19	1
man	39	1	not determined

1. Ellis, M.K.; Coutts, C.T.; Lovatt, C.A.; Richardson, A.G.; Laird, W.J.D.; Cooper, G.K.; Moore, R.B.; Pitts, M.R.; Dunn, D.S.; and Wickramaratane, G.A. (1997). Species Comparison in the Metabolism of the Herbicide Molinate. [Submitted to Drug Metab. Dispos.]. A study report with individual data from this study has not been submitted to the Agency for review to date. STUDY DESIGN: In a comparative metabolism study [MRID 44521003; no individual data submitted], a single dose of Molinate {40 mg [^{14}C]Molinate/kg} was administered *via* gavage to Sprague-Dawley rats, CD-1 mice, pure bred beagle dogs, and New Zealand rabbits, and urine and feces were collected over a 72-hour period post dose. Blood samples were collected at termination only from the rats and mice.

All four species rapidly excreted the radiolabel, and Molinate was extensively metabolized to more polar products in all species. At a comparative oral dose of 40 mg/kg, the proportion of the administered dose recovered in the urine was: rat (male 74±6%; female 73±3%), mouse (male 65±15%; female 63±19%), dog (male 82±6%), and rabbit (male 83±8%). Radiolabel recovered in the feces of rats and mice accounted for 16±8% and 24±14%, respectively [similar data for dogs and rabbits not provided]. The % of the administered dose found as the urinary metabolite mercapturate conjugate (metabolite **XVI**) was 25% (rat), not detected (mouse), 7% (dog), and 2% (rabbit).

Molinate was not identified in any of the analyzed urine samples. Sulfur oxidation of Molinate: Sulfur oxidation alone generates principally Molinate sulfoxide, which may further oxidize to Molinate sulfone. Neither of these compounds was identified in the urine of any species, but the author states that "their formation is inferred from the identification of both a molinate cysteine conjugate (metabolite XII) and mercapturate (metabolite XVI)." These two metabolites are derived from a glutathione conjugation of Molinate sulfoxide (and sulfone) followed by sequential loss of glutamatic acid and glycine. **The glutathione conjugation reaction is expected to release ethane sulfenic and ethane sulfinic acids from Molinate sulfoxide and sulfone, respectively; however, these products were not identified in this study.** A minor metabolite of this sulfoxidation pathway is 4-hydroxymolinate mercapturate (metabolite V). Hydroxylation of the S-ethyl moiety: It is proposed that S-carboxymethyl molinate (metabolite XVIII) may be derived from two independent pathways [Figure 3, appended]: (1) hydroxylation of the S-ethyl moiety to yield compound XX, followed by sequential oxidation to aldehyde (XXI) and acid (metabolite XVIII); (2) transamination of the cysteine conjugate to the pyruvic acid analogue (XVII) followed by oxidative decarboxylation. It is stated that the metabolite profile for [^{14}C]Molinate sulfoxide in the rat and mouse would favor pathway 1 and preclude pathway 2 [there was no analogous statement regarding the rabbit or dog]. Hydroxylation of the S-ethyl moiety at the carbon adjacent to sulfur with loss of acetaldehyde yields hexahydro-1H-azapine-1-carbothioate that was identified as the glucuronide (metabolite VII).

Hydroxylation of the hexahydro-1H-azepine (hexamethyleneimine) moiety: At least six glucuronides of hydroxylated molinate were found, which were converted to the aglycone upon glucuronidase treatment. Analysis of the most abundant of these metabolites established the S-ethyl group was intact and hence hydroxylation had occurred about the hexahydro-1H-azepine ring. The major products of hexahydro-1H-azepine ring hydroxylation in the rat, mouse, and dog are 3- and 4-hydroxymolinate glucuronide (metabolites XIV and XI, respectively), whereas in the rabbit, 4-hydroxymolinate sulfate predominates (metabolite VIII). It is stated that it is likely that the six glucuronides are diasterioisomers derived from hexahydro-1H-azepine hydroxylation at C-2, C-3, and C-4.

Overall, it was concluded that sulfur oxidation was a major route of metabolism in the rat, mouse, and dog, but carbon oxidation predominated in the rabbit.

Metabolism of molinate sulfoxide in the rat: Administration of [14C]Molinate sulfoxide [40 mg/kg] to rats resulted in the rapid appearance of three metabolites in the urine, with ≈72% of the administered dose being eliminated in the urine within 24 hours. The major metabolite was the mercapturate metabolite XVI, which accounted for 86% of the recovered radiolabel. Also identified was 4-hydroxymolinate mercapturate (metabolite V, 9% of recovered dose). It is stated that the identification of metabolite V is supportive of hexahydro-1H-azepine oxidation being secondary to sulfur oxidation. The third metabolite (metabolite XIX, 5%) was unidentified but thought to be the glucuronide conjugate of metabolite V.

Following single, oral [gavage] doses of [^{14}C]Molinate [1, 40, and 200 mg/kg] to rats, "the proportion of the oral dose excreted as the mercapturate (metabolite **XVI**) decreased with decreasing dose." "At a dose of 200 mg/kg molinate about 32% of the urinary radiolabel was excreted as metabolite **XVI**, whereas, at doses of 40, 16 and 1 mg/kg molinate, metabolite **XVI** accounted for about 29 [25 in table], 22 and 11% of urinary radiolabel, respectively." It is stated that the "Data suggests that carbon-oxidation predominates at low doses of molinate; this pathway saturates on increasing dose and the metabolism switches to sulfur oxidation."

2. In a cited study in human volunteers exposed [route not indicated] to Molinate [amount not stated] at "concentrations above the permitted occupational exposure concentration", sulfur oxidation only accounted for ≈1-5% of the administered dose, the principal route of metabolism being to 4-hydroxymolinate [Krieger, R., *et al.* (1992). The Toxicologist **12**, 126].

3. In another human study [MRID 42582302] in which a single oral dose of 5 mg Molinate [body weights 70-103 kg] was tested, 4-hydroxymolinate was also demonstrated to be the major urinary metabolite [≈39% of the dose] in man.

4. In a recent published article by Jewell, WT and Miller, MG [Drug Metab Dispos (1999), July 27 (7): 842-847], a comparison was made of the metabolic capability of rat and human liver microsomes and slices to form either nontoxic ring-hydroxylated metabolites of Molinate or the toxic metabolites derived from the sulfoxidation of Molinate. It is stated that **sulfoxidation would be the preferred high-dose pathway** whereas **hydroxylation would predominate at low dose** levels **in both species.**

SUMMARY of DATA in SUPPORT of MECHANISM of TOXICITY [males]: From the mechanistic studies submitted to date that include individual data, the following have been demonstrated: (1) Molinate, Molinate sulphoxide, and hexamethyleneimine exposure *via* the i.p. route and Molinate exposure *via* the oral route result in decreases in testosterone and other steroid hormones in both the plasma and the testicular interstitial fluid; (2) Molinate sulphoxide and Molinate sulphone inhibit nCEH ***in vitro***, Molinate only slightly; (3) an increase in testosterone synthesis is observed ***in vitro*** following addition of testosterone steroid precursors to Leydig cell cultures exposed to Molinate and its metabolites.

E. FEMALE RAT EFFECTS

Historically, the male rat has been the focus of studies on Molinate. However, in several routine studies; i.e., the 2-year rat, 18-month mouse, 2-generation reproduction, and chronic dog, effects were observed in the ovaries in female animals and the adrenal glands of both sexes. Since the ovarian interstitial and the adrenocortical tissues have a primary role in the production of steroid hormones, and the production and maturation of sperm within the testes is absolutely dependent upon the appropriate concentrations of testosterone at the appropriate time, a link between the three tissues suggested to the Registrant a common pathogenesis for the observed effects of Molinate in the rat, namely a perturbation of the steroidogenic pathway. All three tissues synthesize their respective steroid hormones *via* common pathways using cholesterol as the precursor chemical. Both the adrenocortical cells and the ovarian interstitial cells store cholesterol, in the form of droplets, within their cytoplasm, and the storage involves esterification of free cholesterol by the enzyme acyl CoA cholesterol acyltransferase.

1. To further investigate what the Registrant considers "rodent-specific" reproductive toxicity following high doses of Molinate, a study was performed to investigate the effects of Molinate in the female reproductive organs and adrenals. Female Sprague-Dawley rats were dosed with Molinate *via* gavage at 0, 10, 40, 100, and 150 mg/kg/day for 7 days [individual data not submitted; MRID 44521004].

ADRENAL GLAND: In the control, it was stated that in oil red O-stained frozen sections, the adrenal cortex stained "strongly for the presence of lipid". Adrenal glands in the Molinate females at 100 and 150 mg/kg/day were visibly enlarged compared to the control and showed a hypertrophied adrenal cortex. The cells lying within the adrenal cortex, particularly those of the zona fasciculata, were enlarged with centrally-located nuclei and a grossly vacuolated cytoplasm. The oil red O-stained sections showed that the cells had accumulated large amounts of positive-staining lipid in their cytoplasm. OVARY: Histological appearance of the control ovarian tissue was said to be normal, while in the frozen sections, stained with oil rad O, the interstitial tissue showed that the majority of the oil red O positive cells were present in the interstitial tissue, with lesser amounts being found in the corpora lutea. Marked hypertrophy of the interstitial compartment was observed in the Molinate females at 100 and 150 mg/kg/day, with the hypertrophied cells exhibiting a vacuolated appearance. In the oil red O-stained frozen sections, the hypertrophied interstitial tissue showed a markedly increased accumulation of lipid material. Previously, these effects had been noted in the chronic rat, mouse carcinogenicity, and two-generation reproduction studies.

2. In a mechanistic study [MRID 42361308], Sprague-Dawley female rats were administered Molinate at dose levels of 75, 135, and 200 mg/kg/day on days 7-9 of gestation.

> There was a dose-related increase in the incidence and severity of fatty vacuolation of corpora luteal cells, a dose-related increase in adrenal weight [significant at all dose levels], a dose-related increase in the incidence and severity of cellular swelling and vacuolation in the zonae fasciculata and reticularis of the adrenal cortex, multifocal degeneration with necrosis and loss of cells in the zona fasciculata, and increased neutral lipid content in cells of these two regions of the adrenal cortex at all dose levels. NOTE: In a 2-generation reproduction study [MRID 41333402] in which only the females were dosed with Molinate, the NOAEL for ovarian lesions was 6 ppm [0.34 mg/kg/day] and the LOAEL was 50 ppm [2.9 mg/kg/day]. In the definitive 2-generation reproduction study, the NOAEL for ovary and adrenal lesions was 20 ppm [1.9 mg/kg/day] and the LOAEL was 50 ppm [4.7 mg/kg/day]. In the 2-year rat study, the incidence of ovarian lesions was increased significantly over control at 300 ppm [15 mg/kg/day] and was above that of the historical control at 40 ppm [2 mg/kg/day]. There was no apparent increase in ovarian lesions at 7 ppm [0.4 mg/kg/day].

3. As discussed in MRID 44521004, the Registrant points out that accumulation of lipid droplets within the cells of the ovarian interstitial and adrenal cortical tissue, by high doses of Molinate, has been seen with other compounds that inhibit the production of steroids. The accumulation of excess lipid in the adrenal cortex has been reported following treatment with aminoglutethimide, metapyrone, amphenone, and aniline. These compounds are said to induce lipid accumulation in the adrenal gland by inhibiting one or more of the enzymes involved in the steroidogenic pathway, and morphological changes are seen in the mitochondria and smooth endoplasmic reticulum of affected cells of the adrenal. However, no comparable effects have been observed in the ovarian tissue, according to the Registrant.

Additionally, the Registrant states that lipidosis can be induced in ovarian interstitial and adrenal cortical cells by the organophosphates TOCP [tri-*o*-cresyl phosphate] and TCP [tricresyl phosphate]. The increased accumulation of lipid droplets [cholesterol esters] within the adrenocortical cells and ovarian interstitial cells following Molinate administration to female rats "may reflect a similar mode of action to TOCP and TCP". These compounds produce their effect in rodents *via* different mechanisms to that described for those chemicals that directly inhibit steroidogenesis and the principal mode of action is believed to be *via* a dominant inhibition of neutral cholesterol ester hydrolase, the enzyme responsible for releasing cholesterol from HDL and from intracellular stores. TCP and BTP [butylated triphenyl phosphate] achieve this inhibition in the absence of a prolonged inhibition of steroidogenesis. The toxicity displayed by these two chemicals has significant homology with that induced by Molinate in both the target organ specificity and the morphological changes induced in the sperm, although Molinate did induce a significant reduction in circulating testosterone when given as an acute bolus, whereas neither TCP nor BTP were reported to affect circulating hormone levels on prolonged exposure. NOTE: The Registrant does not indicate if either TCP or BTP affects hormone levels following an acute exposure.

In a discussion of the effects of Molinate on sperm [MRID 44521004], it is stated that at "high doses of molinate the sperm heads become detached from the tails. At lower doses of molinate the sperm lesion becomes more specific and appears to affect primarily the junction between the head and

midpiece with an apparent weakening and rupture of the membrane and a resulting flexure of the head in a backward direction." The Registrant states that this change has been seen with TCP, and the similarity between the morphological effects shown with TCP and that shown with Molinate suggests to the Registrant a link in the way in which both chemicals may be producing their effect. NOTE: There is no discussion in this paper regarding whether TCP results in similar fertility effects as those observed with Molinate in either the male or female rat or whether the effects of TCP are considered specific to the rat.

F. MISSING PIECES

Critical pieces that are missing: (1) data on the effect of Molinate on the acidic hydrolases in lysosomes, which the Registrant states are responsible for releasing cholesterol in non-rodent species; (2) dose required to saturate metabolic pathway; (3) demonstration of an inhibition of n-CEH *in vivo* following oral exposure to Molinate; (4) NOAEL for n-CEH inhibition, in light of the low-dose levels where effects on the adrenal and ovary have been observed [<2 mg/kg/day]; (5) NOAEL for decreased testosterone levels following oral exposure; (6) comparison of the dose levels of Molinate and Molinate sulfoxide used in the mechanistic studies where effects on testosterone, etc. were observed to levels *in vivo* following oral exposure to Molinate where effects on fertility/reproduction were observed. Additionally, other mechanism have not been ruled out; e.g., effect on the pituitary, alterations of LH, etc., and there are no data on testosterone concentrations in the seminiferous tubule fluid. Since the metabolic pathway in the dog appears similar to the metabolic pathway in the rat, a study to investigate possible sperm effects in the dog following Molinate sulfoxide exposure would provide further information on the proposed species specificity.

G. DISCUSSION

Although the mode of action proposed appears reasonable, all of the mechanistic studies have been performed at high-dose levels, and it is known that there is a difference in metabolism between low- and high-dose levels. There does not appear to be a difference in Molinate metabolism between rats and humans. There is no information on the dose level at which the metabolic pathway of ring hydroxylation is saturated and sulfoxidation occurs. Additionally, there is no information regarding the effect of Molinate on the acid hydrolase purported to be involved in cholesterol release in the non-rodent. Since some effects are observed at low-dose levels [<1 mg/kg/day], is there another mode of action at these levels? In the study that demonstrates a decrease in the concentration of the precursor hormones, as well as testosterone, in the plasma and interstitial fluid, no effect was observed on cholesterol levels [only the plasma was monitored for cholesterol]. The purported key event in the block in testosterone synthesis is an inhibition of the enzyme that releases cholesterol from its storage esters within the Leydig cell. Decreased cholesterol levels within the Leydig cell have not been demonstrated. Other sites have not been ruled out by the Registrant; e.g., an effect on the pituitary or hypothalamus by inhibiting the release of LH, and concentrations of testosterone in the seminiferous tubule fluid have not been examined.