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TREATMENT OF ANIMALS

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Subject: Public Comments on the HPV Challenge Program Test Plan for 2-Pentanamine, 2,4,4-trimethyl- (Primene™ TOA; CAS No. 107-45-9) by Rohm and Haas Chemicals, LLC.

The following comments on the HPV Challenge Program test plan for Primene™ TOA by Rohm and Haas Chemicals are submitted on behalf of People for the Ethical Treatment of Animals, the Physicians Committee for Responsible Medicine, the Humane Society of the United States, the Doris Day Animal League, and Earth Island Institute. These health, animal protection, and environmental organizations have a combined membership of more than ten million Americans.

Primene™ TOA is used primarily as an intermediate for making salts and derivatives. It is used in the fuel and lubricants, agricultural, pharmaceutical and metals industries.

Rohm and Haas Chemicals is proposing to conduct a combined repeated dose with reproduction/developmental toxicity screening test (OECD 422), acute toxicity to fish test (OECD 203) and mouse micronucleus test (OECD 474). Together, these tests will cause the suffering and death of 875 animals. Primene™ TOA is a C8 primary amine. This structure suggests that a search using QSAR may identify chemical analogs with existing toxicity data that could potentially reduce the need for new animal testing including the proposed OECD 422. In this regard, we cite a QSAR study of the toxicity of amines to fish below.

We are very concerned that Rohm and Haas Chemicals is proposing new *in vivo* genetic toxicity testing, in clear contradiction of the principles laid out for the HPV Program in both the EPA's October 1999 letter to chemical sponsors and its December 2000 *Federal Register* notice on the program, which clearly state that *in vivo* genotoxicity testing should be conducted only when known chemical properties preclude the use of *in vitro* testing and justification for doing so is provided by the sponsor. Since the sponsor's test plan cites an *in vitro* genotoxicity test – that we note yielded exclusively negative results, it is clear that Primene™ TOA's chemical properties do not preclude the use of *in vitro* testing. No justification for the proposed *in vivo* test is offered. An *in vitro* chromosomal aberration test, OECD 473, should be conducted – per the *Federal Register* instructions – rather than the *in vivo* micronucleus test which will cause the suffering and death of 80 animals.

Rohm and Haas Chemicals is also proposing to conduct an acute toxicity to fish test, which will kill approximately 120 animals. No ecotoxicity data for aquatic plants or invertebrates exist for Primene™ TOA. The fish test is intended to show whether exposure to the test chemical will result in large-scale fish death thereby predicting economic loss and ecologic damage. If this exposure kills the food on which fish subsist, it could deplete fish populations even without direct fish toxicity. Since the toxicity of Primene™ TOA to aquatic plants and invertebrates is still unknown, tests on fish are premature.

The EPA guidance document “The Use of Structure-Activity Relationships (SAR) in the High Production Volume Chemicals Challenge Program” notes that if a QSAR model is available, it may be used with the appropriate rationale for its applicability to the HPV candidate chemical. We note the study entitled *Validation and Upgrade of a QSAR Study of the Toxicity of Amines to Freshwater Fish* by Newsome, L. D., et al. (1991)¹ that includes Primene™ TOA among the CAS Registry Numbers listed and urge Rohm and Haas Chemicals and EPA to consider the applicability of this study as well as ECOSAR to satisfy the acute fish toxicity endpoint for Primene™ TOA.

In a recent publication, the Ecotoxicology Task Force of the European Center for the Validation of Alternative Methods (ECVAM) described a method with the potential to reduce the number of fish used in ecotoxicity testing for chemical substances by 55%-70%.² Noting that fish are less sensitive than algae or daphnia in acute aquatic toxicity tests roughly 85% of the time³, a fish acute threshold (step-down) method was developed. An upper threshold concentration (UTC) is set at the lowest EC₅₀ value observed in the algae and daphnia tests. An acute test is carried out at the UTC using five test and five control fish. If no toxicity is observed, no further tests are carried out and the acute fish toxicity result (LC₅₀) is reported as greater than the UTC value. If toxicity is observed, a second test is performed at a step-down concentration using a dilution factor of 3.2, based on a semi-logarithmic concentration series. The testing continues to lower concentrations until no toxicity is observed. The LC₅₀ 96-hour value can be obtained from all step-down threshold test data by applying the binominal method of interpolation. An additional refinement could be obtained by terminating the test after 24 hours of exposure, when lethality and/or serious morbidity are observed in two out of five fish. We strongly urge the use of this new testing strategy when no replacement for the acute fish toxicity test is perceived to be applicable.

In vitro test methods to replace the acute fish toxicity test are also available. The validated DarT Test⁴ uses fertilized zebrafish eggs as a surrogate for living fish. Since the eggs do not hatch during the test period, the DarT is classified as a non-animal test. The exposure period is 48 hours, and assessed endpoints include coagulation, development of blastula, gastrulation, termination of gastrulation, development of somites, movements, extension of the tail, development of eyes, heartbeat, circulation, heart rate, pigmentation, and edema. Endpoints comparable to lethality in vivo include failure to complete gastrulation after 12 hours, no somites after 16 hours, no heartbeat after 48 hours, and coagulated eggs. The other endpoints provide further insight for a more detailed assessment of the effects of test substances. The reliability and relevance of the DarT test have been confirmed through an international, multi-laboratory validation study coordinated and financed by the German Environmental Protection Agency. Predictions of acute toxicity from the DarT test were highly concordant with in vivo reference

data.⁵ This *in vitro* test has been accepted in Germany as a replacement for the use of fish in the assessment of wastewater effluent⁶, and has since been nominated for development into an OECD test guideline. It is clearly suitable for immediate use as a replacement for the use of fish in SIDS screening studies.

Another promising *in vitro* assay is TETRATOX. In this assay, the protozoan *Tetrahymena pyriformis* is used as a biomarker for acute lethality in fish.⁷ The biochemistry and physiology of *T. pyriformis* have been thoroughly investigated since the 1950s, and this assay has been used, in various forms, for aquatic toxicity testing since the 1970s.⁸ In this test, a range-finding study followed by three replicate definitive tests is performed for each test substance. Each treatment replicate consists of a minimum of five different concentrations per substance tested. Thus, at least 30 data points make up each analysis. The current, standardized protocol is for a 40 hour static test, which provides for multigenerational exposure. Range-finding tests are also included to allow an accurate approximation of both the highest concentration with no observed effect on population growth and the lowest concentration with total inhibition of cell replication. Output measures from the TETRATOX assay are the 50% inhibitory growth concentration (IGC50, mmol/L) and the 95% fiducial interval. The current TETRATOX database includes more than 2,000 industrial organic chemicals, including over 800 aliphatic chemicals, 900 aromatic chemicals, 400 neutral narcotics, and 400 direct-acting electrophiles, among others. The TETRATOX protocol has now been standardized and has undergone a preliminary ring test.⁹ The German EPA is currently funding a second, more elaborate ring test, with the goal of establishing an OECD test guideline. In the meantime, data generated by TETRATOX demonstrate a consistently high degree of concordance with data from *in vivo* acute studies in fish, which supports the use of this assay as a replacement for toxicity studies in fish.¹⁰

In summary, a search using QSAR should be conducted to identify chemical analogs with existing toxicity data that could potentially reduce the need for new animal testing. Also, an *in vitro* chromosomal aberration test following OECD 473, using human lymphocytes or an established cell line, should be conducted – per the *Federal Register* instructions – rather than an *in vivo* genotoxicity test which will cause the suffering and death of approximately 80 animals. Finally, the QSAR study of the toxicity of amines to freshwater fish by Newsome, L. D., et al. (1991) should be reviewed and the applicability of the suggested alternatives to the acute toxicity to fish test should be considered. Thank you for your attention to these comments. I may be reached at 610-586-3975, or via e-mail at josephm@peta.org.

Sincerely,

Joseph Manuppello
Research Associate
Research & Investigations

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- ¹ Newsome, L. D., et al. 1991. Validation and Upgrade of a QSAR Study of the Toxicity of Amines to Freshwater Fish. ASTM Special Technical Publication, No. 1179. Landis, W. G., et al. eds. 1993. 413-426.
- ² Jerama, S., et al. 2005. A strategy to reduce the use of fish in acute ecotoxicity testing of new chemical substances notified in the European Union. *Regulatory Toxicology and Pharmacology* 42, 218-224.
- ³ Hutchinson, T.H., et al. 2003. A strategy to reduce the numbers of fish used in acute ecotoxicity testing of pharmaceuticals. *Environ. Toxicol. Chem.* 22, 3031-3036.
- ⁴ Nagel, R. 2002. DarT: the embryo test with the zebrafish *Danio rerio*: A general model in ecotoxicology and toxicology. *ALTEX* 19 (Suppl. 1), 38-48.
- ⁵ Schulte, C., et al. 1996. Testing acute toxicity in the embryo of zebrafish (*Brachydanio rerio*): An alternative to the fish acute toxicity test. *Proceedings of the 2nd World Congress on Alternatives and Animal Use in the Life Sciences*. Utrecht.
- ⁶ Friccius, T., et al. 1995. Der Embryotest mit dem Zebrabarbling: Eine neue Möglichkeit zur Prüfung und Bewertung der Toxizität von Abwasserproben. *Vom Wasser* 84, 407-418.
- ⁷ Schultz, T.W. 1997. TETRATOX *Tetrahymena pyriformis* population growth impairment endpoint: A surrogate for fish lethality. *Toxicological Methods* 7, 289-309.
- ⁸ Sinks, G.D. 2001. Correlation of *Tetrahymena* and *Pimephales* toxicity: Evaluation of 100 additional compounds. *Environmental Toxicology and Chemistry* 20, 917-921.
- ⁹ Larsen, J. 1997. "Progress in an ecotoxicological standard protocol with protozoa: results from a pilot ring test with *Tetrahymena pyriformis*", *Chemosphere* 35, 1023-1041.
- ¹⁰ Seward, J.R., et al. 2001. Reproducibility of toxicity across mode of toxic action in the *Tetrahymena* population growth impairment assay. *Aquatic Toxicology* 53, 33-47.