(h) Specimens, samples, or other nondocumentary materials need not be retained after EPA has notified in writing the sponsor or testing facility holding the materials that retention is no longer required by EPA. Such notification normally will be furnished upon request after EPA or FDA has completed an audit of the particular study to which the materials relate and EPA has concluded that the study was conducted in accordance with this part.

(i) Records required by this part may be retained either as original records or as true copies such as photocopies, microfilm, microfiche, or other accurate reproductions of the original records.

PART 795—PROVISIONAL TEST GUIDELINES

Subpart A [Reserved]

Subpart B—Provisional Chemical Fate Guidelines

Subpart C—Provisional Environmental Effects Guidelines

Subpart D—Provisional Health Effects Guidelines

AUTHORITY: 15 U.S.C. 2603.

Subpart A [Reserved]

Subpart B—Provisional Chemical Fate Guidelines

§ 795.70 Indirect photolysis screening test: Sunlight photolysis in waters containing dissolved humic substances.

(a) *Introduction.* (1) Chemicals dissolved in natural waters are subject to two types of photoreaction. In the first case, the chemical of interest absorbs sunlight directly and is transformed to products when unstable excited states of the molecule decompose. In the second case, reaction of dissolved chemical is the result of chemical or electronic excitation transfer from light-absorbing humic species in the natural water. In contrast to direct photolysis, this photoreaction is governed initially by the spectroscopic properties of the natural water.

(2) In general, both indirect and direct processes can proceed simultaneously. Under favorable conditions the measurement of a photoreaction rate constant in sunlight (K_{pE}) in a natural water body will yield a net value that is the sum of two first-order reaction rate constants for the direct (k_{DE}) and indirect (k_{IE}) pathways which can be expressed by the relationship

Equation 1

$$k_{pE} = k_{DE} + k_{IE}.$$

This relationship is obtained when the reaction volume is optically thin so that a negligible fraction of the incident light is absorbed and is sufficiently dilute in test chemical; thus the direct and indirect photoreaction processes become first-order.

(3) In pure water only, direct photoreaction is possible, although hydrolysis, biotransformation, sorption, and volatilization also can decrease the concentration of a test chemical. By measuring k_{pE} in a natural water and k_{DE} in pure water, k_{IE} can be calculated.

(4) Two protocols have been written that measure k_{DE} in sunlight or predict k_{DE} in sunlight from laboratory measurements with monochromatic light (USEPA (1984) under paragraph (f)(14) and (15) of this section; Mill et al. (1981) under paragraph (f)(9) of this section; Mill et al. (1982) under paragraph (f)(10) of this section; Mill et al. (1983) under paragraphs (f)(11) of this section). As a preface to the use of the present protocol, it is not necessary to know k_{DE}; it will be determined under conditions that definitively establish whether k_{IE} is significant with respect to k_{DE}.

(5) This protocol provides a cost effective test method for measuring k_{IE} for test chemicals in a natural water

(synthetic humic water, SHW) derived from commercial humic material. It describes the preparation and standardization of SHW. To implement the method, a test chemical is exposed to sunlight in round tubes containing SHW and tubes containing pure water for defined periods of time based on a screening test.

(6) To correct for variations in solar irradiance during the reaction period, an actinometer is simultaneously insolated. From these data, an indirect photoreaction rate constant is calculated that is applicable to clear-sky, near-surface, conditions in fresh water bodies.

(7) In contrast to k_{DE}, which, once measured, can be calculated for different seasons and latitudes, k_{IE} only applies to the season and latitude for which it is determined. This condition exists because the solar action spectrum for indirect photoreaction in humic-containing waters is not generally known and would be expected to change for different test chemicals. For this reason, k_{pE}, which contains k_{IE}, is likewise valid only for the experimental data and latitude.

(8) The value of k_{pE} represents an atypical quantity because k_{IE} will change somewhat from water body to water body as the amount and quality of dissolved aquatic humic substances change. Studies have shown, however, that for optically-matched natural waters, these differences are usually within a factor of two (Zepp et al. (1981) under paragraph (f)(17) of this section).

(9) This protocol consists of three separate phases that should be completed in the following order: In Phase 1, SHW is prepared and adjusted; in Phase 2, the test chemical is irradiated in SHW and pure water (PW) to obtain approximate sunlight photoreaction rate constants and to determine whether direct and indirect photoprocesses are important; in Phase 3, the test chemical is again irradiated in PW and SHW. To correct for photobleaching of SHW and also solar irradiance variations, tubes containing SHW and actinometer solutions are exposed simultaneously. From these data k_{pE} is calculated that is the sum of k_{IE} and k_{DE} (Equation 1) (Winterle and Mill (1985) under paragraph (f)(12) of this section).

(b) *Phase 1—Preparation and standardization of synthetic natural water*—(1) *Approach.* (i) Recent studies have demonstrated that natural waters can promote the indirect (or sensitized) photoreaction of dissolved organic chemicals. This reactivity is imparted by dissolved organic material (DOM) in the form of humic substances. These materials absorb sunlight and produce reactive intermediates that include singlet oxygen ($^1O_2$) (Zepp et al. (1977) under paragraph (f)(20) of this section, Zepp et al. (1981) under paragraph (f)(17) of this section, Zepp et al. (1981) under paragraph (f)(18) of this section, Wolff et al. (1981) under paragraph (f)(16) of this section, Haag et al. (1984) under paragraph (f)(6) of this section, Haag et al. (1984) under paragraph (f)(7) of this section); peroxy radicals (RO_2-) (Mill et al. (1981) under paragraph (f)(9) of this section; Mill et al. (1983) under paragraph (f)(8) of this section); hydroxyl radicals ($HO-$) (Mill et al. (1981) under paragraph (f)(9) of this section, Draper and Crosby (1981, 1984) under paragraphs (f)(3) and (4) of this section); superoxide anion ($O_2^{-}-$) and hydroperoxy radicals ($HO-$). (Cooper and Zika (1983) under paragraph (f)(1) of this section, Draper and Crosby (1983) under paragraph (f)(2) of this section); and triplet excited states of the humic substances (Zepp et al. (1981) under paragraph (f)(17) of this section, Zepp et al. (1985) under paragraph (f)(21) of this section). Synthetic humic waters, prepared by extracting commercial humic or fulvic materials with water, photoreact similarly to natural waters when optically matched (Zepp et al. (1981) under paragraphs (f)(17) and (18) of this section).

(ii) The indirect photoreactivity of a chemical in a natural water will depend on its response to these reactive intermediates, and possibly others yet unknown, as well as the ability of the water to generate such species. This latter feature will vary from water-to-water in an unpredictable way, judged by the complexity of the situation.

(iii) The approach to standardizing a test for indirect photoreactivity is to use a synthetic humic water (SHW) prepared by water-extracting commercial humic material. This material is

inexpensive, and available to any laboratory, in contrast to a specific natural water. The SHW can be diluted to a dissolved organic carbon (DOC) content and uv-visible absorbance typical of most surface fresh waters.

(iv) In recent studies it has been found that the reactivity of SHW mixtures depends on pH, and also the history of sunlight exposure (Mill et al. (1983) under paragraph (f)(11) of this section). The SHW solutions initially photobleach with a time-dependent rate constant. As such, an SHW test system has been designed that is buffered to maintain pH and is pre-aged in sunlight to produce, subsequently, a predictable bleaching behavior.

(v) The purpose of Phase 1 is to prepare, pre-age, and dilute SHW to a standard mixture under defined, reproducible conditions.

(2) *Procedure.* (i) Twenty grams of Aldrich humic acid are added to a clean 2-liter Pyrex Erlenmeyer flask. The flask is filled with 2 liters of 0.1 percent NaOH solution. A stir bar is added to the flask, the flask is capped, and the solution is stirred for 1 hour at room temperature. At the end of this time the dark brown supernatant is decanted off and either filtered through coarse filter paper or centrifuged and then filtered through 0.4)m microfilter. The pH is adjusted to 7.0 with dilute H_2SO_4 and filter sterilized through a 0.2)m filter into a rigorously cleaned 2-liter Erlenmeyer flask. This mixture contains roughly 60 ppm DOC and the absorbance (in a 1 cm path length cell) is approximately 1.7 at 313 nm and 0.7 at 370 nm.

(ii) Pre-aging is accomplished by exposing the concentrated solution in the 2-liter flask to direct sunlight for 4 days in early spring or late fall; 3 days in late spring, summer, or early fall. At this time the absorbance of the solution is measured at 370 nm, and a dilution factor is calculated to decrease the absorbance to 0.50 in a 1 cm path length cell. If necessary, the pH is readjusted to 7.0. Finally, the mixture is brought to exact dilution with a precalculated volume of reagent-grade water to give a final absorbance of 0.500 in a 1-cm path length cell at 370 nm. It is tightly capped and refrigerated.

(iii) This mixture is SHW stock solution. Before use it is diluted 10-fold with 0.010 M phosphate buffer to produce a pH 7.0 mixture with an absorbance of 5.00×10^{-2} at 370 nm, and a dissolved organic carbon of about 5 ppm. Such values are characteristic of many surface fresh waters.

(3) *Rationale.* The foregoing procedure is designed to produce a standard humic-containing solution that is pH controlled, and sufficiently aged that its photobleaching first-order rate constant is not time dependent. It has been demonstrated that after 7 days of winter sunlight exposure, SHW solutions photobleached with a nearly constant rate constant (Mill et al. (1983) under paragraph (f)(11) of this section).

(c) *Phase 2—Screening test*—(1) *Introduction and purpose.* (i) Phase 2 measurements provide approximate solar photolysis rate constants and half-lives of test chemicals in PW and SHW. If the photoreaction rate in SHW is significantly larger than in PW (factor of >2X) then the test chemical is subject to indirect photoreaction and Phase 3 is necessary. Phase 2 data are needed for more accurate Phase 3 measurements, which require parallel solar irradiation of actinometer and test chemical solutions. The actinometer composition is adjusted according to the results of Phase 2 for each chemical, to equalize as much as possible photoreaction rate constants of chemical in SHW and actinometer.

(ii) In Phase 2, sunlight photoreaction rate constants are measured in round tubes containing SHW and then mathematically corrected to a flat water surface geometry. These rate constants are not corrected to clear-sky conditions.

(2) *Procedure.* (i) Solutions of test chemicals should be prepared using sterile, air-saturated, 0.010 M, pH 7.0 phosphate buffer and reagent-grade (or purer) chemicals.[1] Reaction mixtures should be prepared with chemicals at concentrations at less than one-half their solubility in pure water and at concentrations such that, at any wavelengths above 290 nm, the absorbance in a standard quartz sample cell with a

[1] The water should be ASTM Type IIA, or an equivalent grade.

1-cm path length is less than 0.05. If the chemicals are too insoluble in water to permit reasonable handling or analytical procedures, 1-volume percent acetonitrile may be added to the buffer as a cosolvent.

(ii) This solution should be mixed 9.00:1.00 by volume with PW or SHW stock solution to provide working solutions. In the case of SHW, it gives a ten-fold dilution of SHW stock solution. Six mL aliquots of each working solution should then be transferred to separate 12 × 100 mm quartz tubes with screw tops and tightly sealed with Mininert valves.[2] Twenty four tubes are required for each chemical solution (12 samples and 12 dark controls), to give a total of 48 tubes.

(iii) The sample tubes are mounted in a photolysis rack with the tops facing geographically north and inclined 30° from the horizontal. The rack should be placed outdoors over a black background in a location free of shadows and excessive reflection.

(iv) Reaction progress should be measured with an analytical technique that provides a precision of at least ±5 percent. High pressure liquid chromatography (HPLC) or gas chromatograph (GC) have proven to be the most general and precise analytical techniques.

(v) Sample and control solution concentrations are calculated by averaging analytical measurements for each solution. Control solutions should be analyzed at least twice at zero time and at other times to determine whether any loss of chemical in controls or samples has occurred by some adventitious process during the experiment.

(vi) Whenever possible the following procedures should be completed in clear, warm, weather so that solutions will photolyze more quickly and not freeze.

(A) Starting at noon on day zero, expose to sunlight 24 sample tubes mounted on the rack described above. Tape 24 foil-wrapped controls to the bottom of the rack.

(B) Analyze two sample tubes and two unexposed controls in PW and SHW for chemical at 24 hours. Calculate the round tube photolysis rate constants $(k_p)_{SHW}$ and $(k_p)_W$ if the percent conversions are J 20 percent but F 80 percent. The rate constants $(k_p)_{SHW}$ and $(k_p)_W$ are calculated, respectively, from Equations 2 and 3:

Equation 2

$$(k_p)_{SHW} = (1/t)Pn(C_o/C_t)_{SHW} \text{ (in } d^{-1})$$

Equation 3

$$(k_p)_W = (1/t)Pn(C_o/C_t)_W \text{ (in } d^{-1}),$$

where the subscript identifies a reaction in SHW or PW; t is the photolysis time in calendar days; C_o is the initial molar concentration; and C_t is the molar concentration in the irradiated tube at t. In this case t = 1 day.

(C) If less than 20 percent conversion occurs in SHW in 1 day, repeat the procedure for SHW and PW at 2 days, 4 days, 8 days, or 16 days, or until 20 percent conversion is reached. Do not extend the experiment past 16 days. If less than 20 percent photoreaction occurs in SHW at the end of 16 days the chemical is ''photoinert''. Phase 3 is not applicable.

(D) If more than 80 percent photoreaction occurs at the end of day 1 in SHW, repeat the experiment with eight each of the remaining foil-wrapped PW and SHW controls. Divide these sets into four sample tubes each, leaving four foil-wrapped controls taped to the bottom of the rack.

(*1*) Expose tubes of chemical in SHW and PW to sunlight starting at 0900 hours and remove one tube and one control at 1, 2, 4, and 8 hours. Analyze all tubes the next day.

(*2*) Extimate $(k_p)_{SHW}$ for the first tube in which photoreaction is J 20 percent but F 80 percent. If more than 80 percent conversion occurs in the first SHW tube, report: ''The half-life is less than one hour'' and end all testing. The chemical is ''photolabile.'' Phase 3 is not applicable.

(*3*) The rate constants $(k_p)_{SHW}$ and $(k_p)_W$ are calculated from equations 2 and 3 but the time of irradiation must be adjusted to reflect the fact that day-averaged rate constants are approximately one-third of rate constants averaged over only 8 daylight hours.

[2] Mininert Teflon sampling vials are available from Alltech Associates, Inc., 202 Campus Dr., Arlington Heights, IL 60004.

For 1 hour of insolation enter t = 0.125 day into equation 2. For reaction times of 2, 4, and 8 hours enter 0.25, 0.50 and 1.0 days, respectively. Proceed to Phase 3 testing.

(*4*) Once $(k_p)_{SHW}$ and $(k_p)_W$ are measured, determine the ratio R from equation 4:

Equation 4

$$R = (k_p)_{SHW}/(k_p)_W.$$

The coefficient R, defined by Equation 4, is equal to $[(k_I + k_D)/k_D]$. If R is in the range 0 to 1, the photoreaction is inhibited by the synthetic humic water and Phase 3 does not apply. If R is in the range 1 to 2, the test chemical is marginally susceptable to indirect photolysis. In this case, Phase 3 studies are optional. If R is greater than 2, Phase 3 measurements are necessary to measure k_{pE} and to evaluate k_{IE}.

(vii) Since the rate of photolysis in tubes is faster than the rate in natural water bodies, values of near-surface photolysis rate constants in natural and pure water bodies, k_{pE} and k_{DE}, respectively, can be obtained from $(k_p)_{SHW}$ and $(k_p)_W$ from Equations 5 and 6:

Equation 5

$$k_{pE} = 0.45(k_p)_{SHW}$$

Equation 6

$$k_{DE} = 0.45(k_p)_W.$$

The factor 0.45 is an approximate geometric correction for scattered light in tubes versus horizontal surfaces. A rough value of k_{IE}, the rate constant for indirect photolysis in natural waters or SHW, can be estimated from the difference between k_{pE} and k_{DE} using Equation 7:

Equation 7

$$k_{IE} = k_{pE} - k_{DE}.$$

(3) *Criteria for Phase 2.* (i) If no loss of chemical is found in dark control solutions compared with the analysis in tubes at zero time (within experimental error), any loss of chemical in sunlight is assumed to be due to photolysis, and the procedure provides a valid estimate of k_{pE} and k_{DE}. Any loss of chemical in the dark-control solutions may indicate the intervention of some other loss process such as hydrolysis, microbial degradation, or volatilization. In this case, more detailed experiments are needed to trace the problem and if possible eliminate or minimize the source of loss.

(ii) Rate constants determined by the Phase 2 protocol depend upon latitude, season, and weather conditions. Note that $(k_p)_{SHW}$ and k_D values apply to round tubes and k_{pE} and k_{DE} values apply to a natural water body. Because both $(k_p)_{SHW}$ and k_D are measured under the same conditions the ratio $((k_p)_{SHW}/k_D)$ is a valid measure of the susceptibility of a chemical to indirect photolysis. However, since SHW is subject to photobleaching, $(k_p)_{SHW}$ will decrease with time because the indirect rate will diminish. Therefore, R >2 is considered to be a conservative limit because $(k_p)_{SHW}$ will become systematically smaller with time.

(4) *Rationale.* The Phase 2 protocol is a simple procedure for evaluating direct and indirect sunlight photolysis rate constants of a chemical at a specific time of year and latitude. It provides a rough rate constant for the chemical in SHW that is necessary for Phase 3 testing. By comparison with the direct photoreaction rate constant, it can be seen whether the chemical is subject to indirect photoreaction and whether Phase 3 tests are necessary.

(5) *Scope and limitations.* (i) Phase 2 testing separates test chemicals into three convenient categories: ''Photolabile'', ''photoinert'', and those chemicals having sunlight half-lives in round tubes in the range of 1 hour to 50 days. Chemicals in the first two categories fall outside the practical limits of the test, and cannot be used in Phase 3. All other chemicals are suitable for Phase 3 testing.

(ii) The test procedure is simple and inexpensive, but does require that the chemical dissolve in water at sufficient concentrations to be measured by some analytical technique but not have appreciable absorbance in the range 290 to 825 nm. Phase 2 tests should be done during a clear-sky period to obtain the best results. Testing will be less accurate for chemicals with half-lives of less than 1 day because dramatic fluctuations in sunlight intensity can arise

from transient weather conditions and the difficulty of assigning equivalent reaction times. Normal diurnal variations also affect the photolysis rate constant. Phase 3 tests should be started as soon as possible after the Phase 2 tests to ensure that the $(k_p)_{SHW}$ estimate remains valid.

(6) *Illustrative Example.* (i) Chemical A was dissolved in 0.010 M pH 7.0 buffer. The solution was filtered through a 0.2)m filter, air saturated, and analyzed. It contained 1.7×10^{-5} M A, five-fold less than its water solubility of 8.5×10^{-5} M at 25 °C. A uv spectrum (1-cm path length) versus buffer blank showed no absorbance greater than 0.05 in the wavelength interval 290 to 825 nm, a condition required for the Phase 2 protocol. The 180 mL mixture was diluted by the addition of 20 mL of SHW stock solution.

(ii) The SHW solution of A was photolyzed in sealed quartz tubes (12 × 100 mm) in the fall season starting on October 1. At the end of 1 and 2 days, respectively, the concentration of A was found to be 1.13×10^{-5} M and 0.92×10^{-5} M compared to unchanged dark controls (1.53×10^{-5} M).

(iii) The tube photolysis rate constant of chemical A was calculated from Equation 2 under paragraph (c)(2)(vi)(B) of this section. The first time point at day 1 was used because the fraction of A remaining was in the range 20 to 80 percent:

$$(k_p)_{SHW} = (1/1d)Pn(1.53 \times 10^{-5}/1.13 \times 10^{-5})$$
$$(k_p)_{SHW} = 0.30\ d^{-1}.$$

(iv) From this value, k_{pE} was found to be 0.14 $d-1$ using equation 5 under paragraph (c)(2)(vii) of this section:

$$k_{pE} = 0.45(0.30\ d^{-1}) = 0.14d^{-1}.$$

(v) From measurements in pure water, k_D for chemical A was found to be 0.085 d^{-1}. Because the ratio of $(k_p)_{SHW}/k_D$(= 3.5) is greater than 2, Phase 3 experiments were started.

(d) *Phase 3—Indirect photoreaction with actinometer: Calculation of* k_{IE} *and* k_{pE}—(1) *Introduction and purpose.*

(i) The purpose of Phase 3 is to measure k_{Io}, the indirect photolysis rate constant in tubes, and then to calculate k_{pE} for the test chemical in a natural water. If the approximate $(k_p)_{SHW}$ determined in Phase 2 is not significantly greater than k_D measured for the experiment date of Phase 2, then Phase 3 is unnecessary because the test chemical is not subject to indirect photoreaction.

(ii) In the case $(k_p)_{SHW}$ is significantly larger than k_D, Phase 3 is necessary. The rate constant $(k_p)_{SHW}$ is used to choose an actinometer composition that matches the actinometer rate to the test chemical rate. Test chemical solutions in SHW and in pure water buffer are then irradiated in sunlight in parallel with actinometer solutions, all in tubes.

(iii) The actinometer used is the *p*-nitroacetophenone-pyridine (PNAP/PYR) system developed by Dulin and Mill (1982) under paragraph (f)(5) of this section and is used in two EPA test guidelines (USEPA (1984) under paragraphs (f) (14) and (15) of this section). By varying the pyridine concentration, the PNAP photolysis half-life can be adjusted over a range of several hours to several weeks. The starting PNAP concentration is held constant.

(iv) SHW is subject to photobleaching that decreases its ability to promote indirect photolysis based on its ability to absorb sunlight. This effect will be significant when the test period exceeds a few days. To correct for photobleaching, tubes containing SHW are irradiated in action to the other tubes above.

(v) At any time, the loss of test chemical is given by Equation 8 assuming actinometric correction to constant light flux:

Equation 8

$$-(d[C]/dt) = k_I[C] + k_D[C].$$

(vi) The indirect photolysis rate constant, k_I, is actually time dependent because SHW photobleaches; the rate constant k_I, after pre-aging, obeys the formula:

Equation 9

$$k_I = k_{Io} \exp(-kt),$$

in which k_{Io} is the initial indirect photoreaction rate constant and k is the SHW photobleaching rate constant. After substituting equation 9 for k_I in Equation 8 under paragraph (d)(1)(v) of

this section, and rearranging, one obtains

$-(d[C]/[C] = k_{Io}[\exp(-kt)]dt + k_D\,dt.$

This expression is integrated to give Equation 10:

Equation 10

$Pn(C_o/C)_{SHW} = (k_{Io}/k)[1-\exp(-kt)] + k_D\,t.$

The term (k_{Io}/k) can now be evaluated. Since in pure water, $Pn(C_o/C)_W = k_D\,t$, then subtracting this equation from Equation 10 gives

Equation 11

$Pn(C_o/C)_{SHW}\text{-}Pn(c_o/C)_W = (k_{Io}/k)[1\text{-}\exp(\text{-}kt)].$

The photobleaching fraction, [1-exp(-kt)], is equivalent to the expression $[1-(A_{370}/A°_{370})]$, where $A°_{370}$ and A_{370} are the absorbances at 370 nm, and are proportional to humic sensitizer content at times zero and t. Therefore, (k_{Io}/k) is derived from the slope of a linear regression using $[Pn(C_o/C)_{SHW}\text{-}Pn(C_o/C)_W]$ as the dependent variable and $[1-(A_{370}/A°_{370})_{SHW}]$ as the independent variable.

(vii) To evaluate k_{Io}, the parameter k has to be evaluated under standard sunlight conditions. Therefore, the photolysis rate constant for the PNAP/PYR actinometer (k_A) is used to evaluate k by linear regression on Equation 12:

Equation 12

$Pn(A°_{370}/A_{370}) = (k/k_A)Pn(C_o/C)_{PNAP},$

where the slope is (k/k_A) and the value of k_A is calculated from the concentration of pyridine and the absorption of light by PNAP: $k_A = 2.2(0.0169)[PYR]k_a$. Values of k_a are listed in the following Table 1.

TABLE 1—DAY AVERAGED RATE CONSTANT (K_a) [1] FOR SUNLIGHT ABSORPTION BY PNAP AS A FUNCTION OF SEASON AND DECADIC LATITUDE [2]

Latitude	Season			
	Spring	Summer	Fall	Winter
20° N	515	551	409	327
30° N	483	551	333	232
40° N	431	532	245	139
50° N	362	496	154	6

[1] k_a = @ e_{ga} L_g in the units of day^{-1}, (Mill et al. (1982) under paragraph (f)(10) of this section).

[2] For use in Equation 15 under paragraph (d)(2)(i) of this section.

The value of k_{Io} is then given by Equation 13:

Equation 13

$k_{Io} = (k_{Io}/k)(k/k_A)k_A.$

(viii) To obtain k_D, determine the ratio (k_D/k_A) from a linear regression of $Pn(C_o/C)_W$ versus $Pn(C_o/C)_{PNAP}$ according to Equation 13a:

Equation 13a

$Pn(C_o/C)_W = (k_D/k_A)Pn(C_o/C)_{PNAP}.$

The slope is (k_D/k_A), and k_D is obtained by multiplication of this slope with the known value of k_A: i.e., $k_D = (k_D/k_A)k_A$.

(ix) Then, $(k_p)_{SHW}$ values in SHW are determined by summing k_D and K_{Io} as follows:

Equation 14

$(k_p)_{SHW} = k_{Io} + k_D.$

(x) Finally, k_{pE} is calculated from the precise relationship, Equation 5a:

Equation 5a

$k_{pE} = 0.455(k_p)_{SHW}.$

(2) *Procedure.* (i) Using the test chemical photoreaction rate constant in round tubes, $(k_p)_{SHW}$, determined in Phase 2 under paragraph (c) of this section, and the absorption rate constant, kα found in Table 1, under paragraph (d)(1)(vii) of this section, calculate the molar pyridine concentration required by the PNAP/PYR actinometer using Equation 15:

Equation 15

$[PYR]/M = 26.9[(k_p)_{SHW}/k_a].$

This pyridine concentration makes the actinometer rate constant match the test chemical rate constant.

(A) The variable k_a (= @ e $_{ga}$ L_g) is equal to the day-averaged rate constant for sunlight absorption by PNAP (USEPA (1984) under paragraph (f)(14) of this section; Mill et al. (1982) under paragraph (f)(10) of this section, Zepp and Cline (1977) under paragraph (f)(19) of this section) which changes with season and latitude.

(B) The variable k_a is selected from Table 1 under paragraph (d)(1)(vii) of this section for the season nearest the mid-experiment date of Phase 2 studies and the decadic latitude nearest the experimental site.

(ii) Once [PYR] is determined, an actinometer solution is prepared by adding 1.00 mL of 1.0×10^{-2} M (0.165 gms/100 mL) PNAP stock solution (in CH_3 CN solvent) and the required volume, V, of PYR to a 1 liter volumetric flask. The flask is then filled with distilled water to give 1 liter of solution. The volume V can be calculated from Equation 16:

Equation 16

V/mL=[PYR]/0.0124.

The PNAP/PYR solutions should be wrapped with aluminum foil and kept out of bright light after preparation.

(iii) The following solutions should be prepared and individually added in 6.00 mL aliquots to 12/100 mm quartz sample tubes; 8 tubes should be filled with each solution:

(A) PNAP/PYR actinometer solution.

(B) Test chemical in pH 7.0, 0.010 M phosphate buffer.

(C) Test chemcial in pH 7.0, 0.010 M phosphate buffer/SHW.

(D) pH 7.0, 0.010 M phosphate buffer/SHW. Four tubes of each set are wrapped in foil and used as controls.

(iv) The tubes are placed in the photolysis rack (Phase 2, Procedure) at 0900 hours on day zero, with the controls taped to the bottom of the rack. One tube of each composition is removed, along with their respective controls, according to a schedule found in Table 2, which categorizes sampling times on the basis of $(k_p)_{SHW}$ determined in Phase 1.

TABLE 2—CATEGORY AND SAMPLING PROCEDURE FOR TEST AND ACTINOMETRY SOLUTIONS

Category	k_p $(d^{-1})_{SHW}$	Sampling procedure
A	5.5 J K_p J 0.69	Sample at 0, 1, 2, 4, and 8h.
B	0.69>k_p J 0.017	Sample at 0, 1, 2, 4, and 8d.
C	0.17>k_p J 0.043	Sample at 0, 4, 8, 16, and 32d.

(v) The tubes containing PNAP, test chemical, and their controls are analyzed for residual concentrations soon after the end of the experiment. PNAP is conveniently analyzed by HPLC, using a 30 cm C_{18} reverse phase column and a uv detector set at 280 nm. The mobile phase is 2 percent acetic acid, 50 percent acetonitrile and 48 percent water (2 mL/min flow rate). Tubes containing only SHW (solution D) should be analyzed by absorption spectroscopy at 370 nm after storage at 4 °C in the dark. The absorbance range to be measured is 0.05 to 0.01 AU (1 cm).

(vi) If controls are well-behaved and show no significant loss of chemical or absorbance change, then k_I can be calculated. In tabular form (see Table 4 under paragraph (d)(6)(iii)(A) of this section) arrange the quantities $Pn(C_o/C_t)_{SHW}$, $Pn(C_o/C_t)_{SHW}$, $[1-(A_{370}/A^o_{370})]$, $Pn(A^o_{370}/A_{370})$, and $Pn(C_o/C)_{PNAP}$ in order of increasing time. According to Equation 11 under paragraph (d)(1)(vi) of this section in the form of Equation 17,

Equation 17

$$Pn(C_o/C)_{SHW} - Pn(C_o/C)_W = (k_{Io}/k)[1-(A_{370}/A^o_{370})],$$

plot the quantities $[Pn(C_o/C_t)_{SHW} - Pn(C_o/C_t)_W]$ versus the independent variable $[1-(A_{370}/A^o_{370})]$. Obtain the slope (S1) by least square linear regression. Under the assumptions of the protocol, $S1 = (k_{Io}/k)$.

(vii) According to Equation 12 under paragraph (d)(1)(vii) of this section, plot the quantities $Pn(A^o_{370}/A_{370})$ versus the independent variable $Pn(C_o/C_t)_{PNAP}$. Obtain the slope (S2) by least squares linear regression on Equation 12 under paragraph (d)(1)(vii) of this section. Under the assumptions of the protocol, $S2 = (k/k_A)$.

(viii) Then, using Equation 13a under paragraph (d)(1)(vii) of this section, determine the slope (S3) by least squares linear regression. Under the assumptions of the protocol, S3 is equal to (k_D/k_A).

(ix) From Equation 18

Equation 18

$$k_A = 0.0372[PYR]k_a,$$

calculate k_A using k_a values found in Table 1 under paragraph (d)(1)(vii) of this section. The value of k_a chosen must correspond to the date closest to the mid-experiment date and latitude closest to that of the experimental site.

(x) The indirect photoreaction rate constant, k_{Io}, is determined using Equation 19,

Equation 19

$$k_{Io} = (S1)(k_A)(S2),$$

by incorporating the quantities k_A, S1, and S2 determined as described in paragraphs (d)(2) (ix), (vi), and (vii) of this section, respectively.

(xi) The rate constant k_D is calculated from Equation 20,

Equation 20

$$k_D = (S3)(k_A),$$

using the quantities S3 and k_A determined as described above.

(xii) Then, $(k_p)_{SHW}$ is obtained by summing k_D and k_{Io}, as described by Equation 14 in paragraph (d)(1)(ix) of this section:

Equation 14

$$(k_p)_{SHW} = k_{Io} + k_D.$$

(xiii) Finally, k_{pE} is obtained by multiplying $(k_p)_{SNW}$ by the factor 0.455, as described by Equation 5a in paragraph (d)(1)(x) of this section:

Equation 5a

$$k_{pE} = 0.455\ (k_p)_{SHW}$$

As determined, k_{pE} is the net environmental photoreaction rate constant. It applies to clear sky conditions and is valid for predicting surface photoreaction rates in an average humic containing freshwater body. It is strictly valid only for the experimental latitude and season.

(3) *Criteria for Phase 3.* As in Phase 2, Phase 3 tests are assumed valid if the dark controls are well behaved and show no significant loss of chemical. In such a case, loss of test chemical in irradiated samples is due to photoreaction.

(4) *Rationale.* Simultaneous irradiation of a test chemical and actinometer provide a means of evaluating sunlight intensities during the reaction period. Parallel irradiation of SHW solutions allows evaluation of the extent of photobleaching and loss of sensitizing ability of the natural water.

(5) *Scope and limitations of Phase 3 protocol.* Test chemicals that are classified as having half-lives in SHW in the range of 1 hour to 50 days in Phase 2 listing are suitable for use in Phase 3 testing. Such chemicals have photoreaction half-lives in a range accommodated by the PNAP/PYR actinometry in sunlight and also accommodate the persistence of SHW in sunlight.

(6) *Illustrative example.* (i) From Phase 2 testing, under paragraph (c)(6)(iii) of this section, chemical A was found to have a photolysis rate constant, $(k_p)_{SHW}'$ of 0.30 d^{-1} in fall in round tubes at latitude 33° N. Using Table 1 under paragraph (d)(1)(vii) of this section for 30° N, the nearest decadic latitude, a fall value of k_a equal to 333 d^{-1} is found for PNAP. Substitution of $(k_p)_{SHW}$ and k_a into Equation 15 under paragraph (d)(2)(i) of this section gives [PYR] = 0.0242 M. This is the concentration of pyridine that gives an actinometer rate constant of 0.30 d^{-1} in round tubes in fall at this latitude.

(ii) The actinometer solution was made up by adding a volume of pyridine (1.95 mL) calculated from equation 16 under paragraph (d)(2)(ii) of this section to a 1 liter volumetric flask containing 1.00 mL of 1.00×10^{-2} M PNAP in acetonitrile. The flask was filled to the mark with distilled water to give final concentrations of [PYR] = 0.0242 M and [PNAP] = 1.00×10^{-5} M. Ten tubes of each of the following solutions were placed in the photolysis rack at 1,200 hours on day zero:

(A) Chemical A (1.53×10^{-5} M) in standard SHW (0.010 M, pH 7 phosphate buffer).

(B) Chemical A (1.53×10^{-5}), in 0.010 M, pH 7 phosphate buffer.

(C) SHW standard solution diluted with water 0.90 to 1.00 to match solution A.

(D) PNAP/PYR actinometer solution. Ten additional foil-wrapped controls of each mixture were taped to the bottom of the rack.

(iii) The test chemical had been placed in category B, Table 2 under the paragraph (d)(2)(iv) of this section, on the basis of its Phase 2 rate constant under paragraph (c) of this section. Accordingly, two tubes of each irradiated solution and two tubes of each blank solution were removed at 0, 1, 2, 4, and 8 days at 1,200 hours. The averaged analytical results obtained at the end of the experiment are shown in the following Table 3.

TABLE 3—CHEMICAL ANALYTICAL RESULTS FOR ILLUSTRATIVE EXAMPLE, PHASE 3

Day	$10^5[C]^{SHW}$, M	$10^5[C]^{W}$, M	A^{SHW}_{370}	10^5 [PNAP], M
0	1.53	1.53	0.0500	1.00
1	1.03	1.40	0.0470	0.810
2	0.760	1.30	0.0440	0.690
4	0.300	1.01	0.0370	0.380
8	0.130	0.800	0.0320	0.220

Data for solutions A through D are given in column 2 through 5, respectively. No significant chemical loss was found in the dark controls.

(A) From these items the functions $Pn(C_o/C)_{SNW}$′ $Pn(C_o/C)_W$′ [1—$(A_{370}/A^o_{370})_{SNW}$], $Pn(A^o_{370}/A_{370})$, and $Pn(C_o/C)_{PNAP}$ were calculated, as shown in the following Table 4 which was derived from Table 3 under paragraph (d)(6)(iii) of this section:

TABLE 4—PHOTOREACTION FUNCTION FOR ILLUSTRATIVE EXAMPLES, PHASE 3, DERIVED FROM TABLE 3

Day	$Pn(C_o/C)_{SHW}$	$Pn(C_o/C)_W$	$1–(A_{370}/A^o_{370})$	$Pn(A^o_{370}/A_{370})$	$Pn(C_o/C)_{PNAP}$
0	0	0	0	0	0
1	0.396	0.0888	0.0600	0.0618	0.211
2	0.700	0.163	0.120	0.128	0.371
4	1.629	0.415	0.260	0.301	0.968
8	2.465	0.648	0.360	0.446	1.514

(B) Slope S1 = (k_{Io}/k) was calculated according to Equation 17 under paragraph (d)(2)(vi) of this section and was found to be 4.96 by a least squares regression with a correlation coefficient equal to 0.9980. The following Figure 1 shows a plot of Equation 17 under paragraph (d)(2)(vi) of this section and its best-fit line.

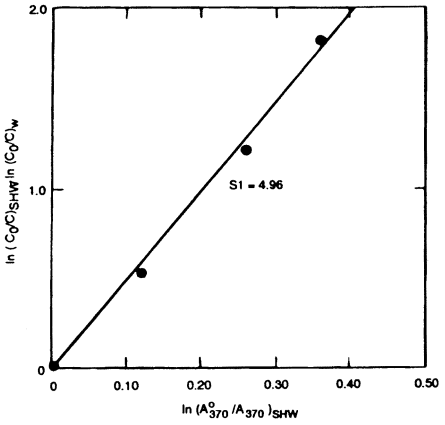

FIGURE 1—GRAPHIC DETERMINATION OF S1 = (K_{Io}/k) BASED ON EQUATION 17 UNDER PARAGRAPH (D)(2)(VI) OF THIS SECTION.

(C) Slope S2 = (k/k_a) was also derived from Table 4 under paragraph (d)(6)(iii)(A) of this section by a fit of $Pn(A^{o}_{370}/A_{370})_{SHW}$ and $Pn(C_o/C)_{PNAP}$ to Equation 12 under paragraph (d)(l)(vii) of this section. This plot is displayed in the following Figure 2; the slope S2 was found to be 0.295 and the correlation coefficient was equal to 0.9986.

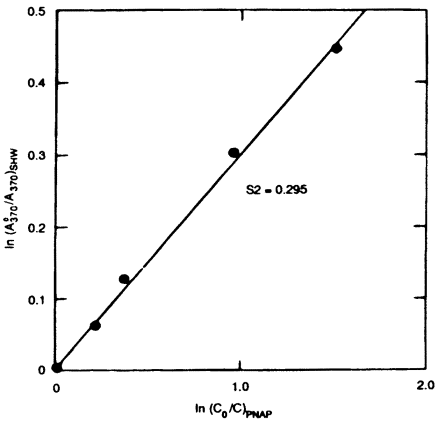

FIGURE 2—GRAPHIC DETERMINATION OF S2 = (K/K_A) BASED ON EQUATION 12 UNDER PARAGRAPH (D)(1)(VII) OF THIS SECTION.

(D) Using the data in columns 3 and 6 in Table 4 under paragraph (d)(6)(iii)(A) of this section, slope S3 was calculated by regression from Equation 13a under paragraph (d)(1)(viii) of this section and was found to be 0.428 with correlation coefficient equal to 0.99997.

(E) Using Equation 18 under paragraph (d)(2)(ix) of this section, k_A was found to be = $0.300d^{-1}$.

(F) The values of S1, S2, and k_A were then combined in Equation 19 under paragraph (d)(2)(x) of this section to give k_{Io} as follows:

Equation 19

$k_{Io} = (4.96)(0.300)(0.295) = 0.439d^{-1}$.

(G) The rate constant k_D was calculated from the product of S3 and k_A as expressed in Equation 20 under paragraph (d)(2)(xi) of this section as follows:

Equation 20

$k_D = (0.428)(0.300) = 0.128d^{-1}$.

(H) The sum of k_D and k_{Io} was multiplied by 0.455 to obtain k_{pE} as follows:

Equation 21

$k_{pE} = (0.455)(0.439 + 0.128)d^{-1} = 0.258\ d^{-1}$.

(I) Since k_{pE} is a first-order rate constant, the half-life, $t_{1/2E}$, is given by Equation 22:

Equation 22

$t_{1/2E} = 0.693/k_{pE}$.

Substituting the value of k_{pE} from Equation 21 under paragraph (d)(6)(iii)(H) of this section in Equation 22 yielded

Equation 23

$t_{1/2E} = 0.693/0.258 d^{-1} = 2.7 d$.

(e) *Data and reporting*—(1) *Test conditions*—(i) *Specific analytical and recovery procedures.* (A) Provide a detailed description or reference for the analytical procedures used, including the calibration data and precision.

(B) If extraction methods were used to separate the solute from the aqueous solution, provide a description of the extraction method as well as the recovery data.

(ii) *Other test conditions.* (A) Report the site and latitude where the photolysis experiments were carried out.

(B) Report the dates of photolysis, weather conditions, times of exposure, and the duration of exposure.

(C) If acetonitrile was used to solubilize the test chemical, report the volume percent.

(D) If a significant loss of test chemical occurred in the control solutions for pure water and SHW, indicate the causes and how they were eliminated or minimized.

(2) *Test data report*—(i) Phase 2 Screening Test under paragraph (c) of this section. (A) Report the initial molar concentration of test chemical, C_o, in pure water and SHW for each replicate and the mean value.

(B) Report the molar concentration of test chemical, C_t, in pure water and SHW for each replicate and the mean value for each time point t.

(C) Report the molar concentration of test chemical for each replicate control sample and the mean value for each time point.

(D) Report the values of $(k_p)_{SHW}$ and $(k_p)_W$ for the time point t in which the fraction of test chemical photoreacted is in the range 20 to 80 percent.

(E) If small losses of test chemical were observed in SHW and pure water, report a first-order rate constant loss, $(k_p)_{loss}$. Calculate and report $(k_p)_{obs}$ for SHW and/or pure water. Calculate and report the corrected first-order rate constant for SHW and/or pure water using the relationship expressed in Equation 24:

Equation 24

$k_p = (k_p)_{obs} - (k_p)_{loss}$.

(F) Report the value of R calculated from Equation 4 under paragraph (c)(2)(vi)(D)(*4*) of this section.

(G) Report the values of k_{pE} and k_{DE} obtained from Equations 5 and 6, respectively under paragraph (c)(2)(vii) of this section; report the corresponding half-life calculated from Equation 22 under paragraph (d)(6)(iii)(I) of this section.

(ii) Phase 3—Indirect photoreaction with actinometer. (A) Report the initial molar concentration of test chemical, C_o, in pure water and in SHW for each replicate and the mean value.

(B) Report the initial absorbance A^o_{370} of the SNW solution.

(C) Report the initial molar concentration of PNAP of each replicate and the mean value in the actinometer. Report the concentration of pyridine used in the actinometer which was obtained from Equation 15 under paragraph (d)(2)(i) of this section.

(D) Report the time and date the photolysis experiments were started, the time and date the experiments were completed, and the elapsed photolysis time in days.

(E) For each time point t, report the separate values of the absorbance of the SHW solution, and the mean values.

(F) For each time point for the controls, report the separate values of the molar concentrations of test chemical in pure water and SHW, and the absorbance of the SHW solution, and the mean values.

(G) Tabulate and report the following data: t, $[C]^{SHW}$, $[C]^W$, A^{SNW}_{370}, [PNAP].

(H) From the data in (G), tabulate and report the following data: t, $Pn(C_o/C)_{SNW}$, $Pn(C_o/C)_W$, $[1-(A_{370}/A^o_{370})SNW]$, $Pn(A^o_{370}/A_{370})$, $Pn(C_o/C)_{PNAP}$.

(I) From the linear regression analysis of the appropriate data in step (H) in Equation 17 under paragraph (d)(2)(vi) of this section, report the slope S1 and the correlation coefficient.

(J) From the linear regression analysis of the appropriate data in step (H)

in Equation 12 under paragraph (d)(1)(vii) of this section, report the slope S2 and the correlation coefficient.

(K) From the linear regression analysis of the appropriate data in step (H) in Equation 13a under paragraph (d)(1)(viii) of this section, report the slope S3 and the correlation coefficient.

(L) If loss of chemical was observed during photolysis in pure water and SHW, then report the data $Pn(C_o/C)_{corr}$, $Pn(C_o/C)_{obs}$, $Pn(C_o/C)_{loss}$ as described in paragraph (e)(2)(E) of this section. Repeat steps (H), (I), (J), (K) where applicable and report S1, S2, S3 and the corresponding correlation coefficients.

(M) Report the value of the actinometer rate constant obtained from Equation 18 under paragraph (d)(2)(ix) of this section.

(N) Report the value of k_{Io} obtained from Equation 19 under paragraph (d)(2)(x) of this section.

(O) Report the value of k_D obtained from Equation 20 under paragraph (d)(2)(xi) of this section.

(P) Report the value of $(k_{pE})_{SHW}$, obtained from Equation 14 under paragraph (d)(1)(ix) of this section, and the value of k_{pE} obtained from Equation 5a under paragraph (d)(1)(x) of this section.

(Q) Report the half-life, $t_{1/2E}$, obtained from Equation 22 under paragraph (d)(6)(iii)(I) of this section.

(f) *References.* For additional background information on this test guideline the following references should be consulted.

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Research and Development, EPA, Athens, GA, and Office of Pollution Prevention and Toxics, EPA, Washington, DC. (July 1983).

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[53 FR 34522, Sept. 7, 1988; 53 FR 37393, Sept. 26, 1988]

Subpart C—Provisional Environmental Effects Guidelines

§ 795.120 Gammarid acute toxicity test.

(a) *Purpose.* This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures subject to environmental effects test regulations under the Toxic Substances Control Act (TSCA) (Pub. L. 94–469, 90 Stat. 2003 (15 U.S.C. 2601 *et seq.*)). This guideline describes a test to develop data on the acute toxicity of chemicals to gammarids. The United States Environmental Protection Agency (EPA) will use data from this test in assessing the hazard of a chemical to aquatic organisms.

(b) *Definitions.* The definitions in section 3 of TSCA and in part 792 of this chapter, Good Laboratory Practice Standards, apply to this test guideline. The following definitions also apply to this guideline:

Death means the lack of reaction of a test organism to gentle prodding.

Flow-through means a continuous or an intermittent passage of test solution or dilution water through a test chamber or a holding or acclimation tank, with no recycling.

LC50 means the median lethal concentration, i.e., that concentration of a chemical in air or water killing 50 percent of the test batch of organisms within a particular period of exposure (which shall be stated).

Loading means the ratio of the biomass of gammarids (grams, wet weight) to the volume (liters) of test solution in either a test chamber or passing through it in a 24-hour period.

Solvent means a substance (e.g., acetone) which is combined with the test substance to facilitate introduction of the test substance into the dilution water.

Static system means a test chamber in which the test solution is not renewed during the period of the test.

(c) *Test procedures*—(1) *Summary of the test.* In preparation for the test, test chambers are filled with appropriate volumes of dilution water. If a flow-through test is performed, the flow of dilution water through each chamber is adjusted to the rate desired. In a static test, the test substance is introduced into each test chamber. In a flow-through test, the rate in which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The test is started by randomly introducing gammarids, which have been acclimated to the test conditions, into the test chambers. Gammarids in the test chambers are observed periodically during the test; the dead gammarids are removed and the findings recorded. Dissolved oxygen concentration, pH, temperature, and the concentration of test substance in test chambers are measured at specified intervals. Data collected during the test are used to develop concentration—response curves and LC50 values for the test substance.

(2) [Reserved]

(3) *Range-finding test.* (i) A range-finding test should be conducted to establish test substance concentrations to be used for the definitive test.

(ii) The gammarids shall be exposed to a wide-range of concentrations of the test substance (e.g., 1, 10, 100 mg/1, etc.), usually under static conditions.

(iii) A minimum of five gammarids should be exposed to each concentration of test substance for a period of 96 hours. The exposure period may be shortened if data suitable for determining concentrations in the definitive test can be obtained in less time. Nominal concentrations of the test substance may be acceptable.

(4) *Definitive test.* (i) The purpose of the definitive test is to determine the 24, 48, 72, and 96—hour LC50 values and the concentration-response curves.

(ii) A minimum of 20 gammarids per concentration shall be exposed to five or more concentrations of the test substance chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, 64 mg/L). The range and number of concentrations to which the organisms are exposed shall be such that in 96 hours there is at least one concentration resulting in mortality greater than 50 and less than 100 percent, and one concentration causing greater than zero and less than 50 percent mortality. An equal number of gammarids may be placed in two or more replicate test chambers. Solvents should be avoided, if possible. If solvents have to be used, a solvent control, as well as a dilution control, shall be tested at the highest solvent concentration employed in the treatments. The solvent should not be toxic or have an effect on the toxicity of the test substance. The concentration of solvent should not exceed 0.1 ml/L.

(iii) Every test shall include a concurrent control using gammarids from the same population or culture container. The control group shall be exposed to the same dilution water, conditions and procedures, except that none of the test substance shall be is added to the chamber.

(iv) The dissolved oxygen concentration, temperature and pH of the test solution shall be measured at the beginning of the test and at 24, 48, 72 and 96 hours in at least one replicate each of the control, and the highest, lowest and middle test concentrations.

(v) The test duration is 96 hours. The test is unacceptable if more than 10 percent of the control organisms die during the test.

(vi) In addition to death, any abnormal behavior or appearance shall also be reported.

(vii) Gammarids shall be randomly assigned to the test chambers. Test chambers shall be positioned within the testing area in a random manner or in a way in which appropriate statistical analyses can be used to determine whether there is any variation due to placement.

(viii) Gammarids shall be introduced into the test chambers after the test substance has been added.

(ix) Observations on compound solubility shall be recorded. The investigator should record the appearance of surface slicks, precipitates, or material adhering to the sides of the test chambers.

(5) [Reserved]

(6) *Analytical measurements*—(i) *Water quality analysis.* The hardness, acidity, alkalinity, pH, conductivity, TOC or

COD, and particulate matter of the dilution water shall be measured at the beginning of each definitive test.

(ii) *Collection of samples for measurement of test substance.* Each sample to be analyzed for the test substance concentrations shall be taken at a location midway between the top, bottom, and sides of the test chamber. Samples should not include any surface scum or material dislodged from the bottom or sides. Samples shall be analyzed immediately or handled and stored in a manner which minimizes loss of test substance through microbial degradation, photogradation, chemical reaction, volatilization, or sorption.

(iii) *Measurement of test substance.* (A) For static tests, the concentration of dissolved test substance (that which passes through a 0.45 micron filter) shall be measured in each test chamber at least at the beginning (zero-hour, before gammarids are added) and at the end of the test. During flow-through tests, the concentration of dissolved test substance shall be measured in each test chamber at least at 0 and 96-hours and in at least one chamber whenever a malfunction of the test substance delivery system is observed.

(B) The analytical methods used to measure the amount of test substance in a sample shall be validated before beginning the test. This involves adding a known amount of the test substance to each of three water samples taken from a chamber containing dilution water and the same number of gammarids as are placed in each test chamber. The nominal concentrations of the test substance in these samples should span the concentration range to be used in the test. Validation of the analytical method should be performed on at least two separate days prior to starting the test.

(C) An analytical method is not acceptable if likely degradation products of the test substance give positive or negative interferences, unless it is shown that such degradation products are not present in the test chambers during the test.

(D) Among replicate test chambers, the measured concentrations shall not vary more than 20 percent. The measured concentration of the test substance in any chamber during the test shall not vary more than plus or minus 30 percent from the measured concentration in that chamber at zero time.

(E) The mean measured concentration of dissolved test substance shall be used to calculate all LC50's and to plot all concentration-response curves.

(d) *Test conditions for definitive test*—(1) *Test species*—(i) *Selection.* (A) The amphipods, *Gammarus fasciatus, G. pseudolimnaeus,* and *G. lacustris* are specified for this test.

(B) Gammarids can be cultured in the laboratory or collected from natural sources. If collected, they must be held in the laboratory for at least 14 days prior to testing.

(C) Gammarids used in a particular test shall be of similar age and/or size and from the same source or culture population.

(ii) *Acclimation.* If the holding water is from the same source as the dilution water, acclimation to the dilution water shall be done gradually over a 48-hour period. The gammarids then shall be held at least 7 days in the dilution water prior to testing. Any changes in water temperature should not exceed 2 °C per day. Gammarids should be held for a minimum of 7 days at the test temperature prior to testing.

(iii) *Care and handling.* Gammarids shall be cultured in dilution water under similar environmental conditions to those used in the test. Organisms shall be handled as little as possible. When handling is necessary it should be done as gently, carefully and quickly as possible. During culturing and acclimation, gammarids shall be observed carefully for signs of stress and mortality. Dead and abnormal individuals shall be discarded.

(iv) *Feeding.* The organisms shall not be fed during testing. During culturing, holding, and acclimation, a sufficient quantity of deciduous leaves, such as maple, aspen, or birch, should be placed in the culture and holding containers to cover the bottom with several layers. These leaves should be aged for at least 30 days in a flow-through system before putting them in aquaria. As these leaves are eaten, more aged leaves should be added. Pelleted fish food may also be added.

(2) *Facilities*—(i) *Apparatus*—(A) Facilities needed to perform this test include:

(*1*) Containers for culturing, acclimating and testing gammarids;

(*2*) Containers for aging leaves under flow-through conditions;

(*3*) A mechanism for controlling and maintaining the water temperature during the culturing, acclimation and test periods;

(*4*) Apparatus for straining particulate matter, removing gas bubbles, or aerating the dilution water, as necessary; and

(*5*) An apparatus for providing a 16-hour light and 8-hour dark photoperiod with a 15- to 30-minute transition period.

(B) Facilities should be well ventilated and free of fumes and disturbances that may affect the test organism.

(C) Test chambers shall be covered loosely to reduce the loss of test solution or dilution water due to evaporation and to minimize the entry of dust or other particulates into the solutions.

(ii) *Construction materials.* Construction materials and equipment that may contact the stock solution, test solution or dilution water should not contain substances that can be leached or dissolved into aqueous solutions in quantities that can alter the test results. Materials and equipment that contact stock or test solutions should be chosen to minimize sorption of test substances. Glass, stainless steel, and perfluorocarbon plastic should be used wherever possible. Concrete, fiberglass, or plastic (e.g., PVC) may be used for holding tanks, acclimation tanks, and water supply systems, but they should be aged prior to use. Rubber, coopper, brass, galvanized metal, and lead should not come in contact with the dilution water, stock solution, or test solution.

(iii) *Test substance delivery system.* In flow-through tests, diluters, metering pump systems or other suitable devices shall be used to deliver the test substance to the test chambers. The system used shall be calibrated before each test. The general operation of the test substance delivery system shall be checked twice daily during a test. The 24-hour flow shall be equal to at least five times the volume of the test chamber. During a test, the flow rates should not vary more than 10 percent from one test chamber to another.

(iv) *Test chambers.* Test chambers shall contain at least one liter of test solution. Test chambers made of stainless steel should be welded, not soldered. Test chambers made of glass should be glued using clear silicone adhesive. As little adhesive as possible should be left exposed in the interior of the chamber. A substrate, such as a bent piece of stainless steel screen, should be placed on the bottom of each test chamber to provide cover for the gammarids.

(v) *Cleaning of test system.* Test substance delivery systems and test chambers should be cleaned before each test. They should be washed with detergent and then rinsed sequentially with clean water, pesticide-free acetone, clean water, and 5-percent nitric acid, followed by two or more changes of dilution water.

(vi) *Dilution water.* (A) Clean surface or ground water, reconstituted water, or dechlorinated tap water is acceptable as dilution water if gammarids will survive in it for the duration of the culturing, acclimating, and testing periods without showing signs of strees. The quality of the dilution water should be constant enough that the month-to-month variation in hardness, acidity, alkalinity, conductivity, TOC or COD, and particulate matter is not more than 10 percent. The pH should be constant within 0.4 unit. In addition, the dilution water should meet the following specifications measured at least twice a year:

Substance	Maximum concentration
Particulate matter	20 mg/L
Total organic carbon (TOC) or	2 mg/L
chemical oxygen demand (COD)	5 mg/L
Boron, fluoride	100 ug/L
Un-ionized ammonia	1 ug/L
Aluminum, arsenic, chromium, cobalt, copper, iron, lead, nickel, zinc.	1 ug/L
Residual chlorine	3 ug/L
Cadmium, mercury, silver	100 ng/L
Total organophosphorus pesticides	50 ng/L
Total organochlorine pesticides plus:	
polychlorinated biphenyls (PCBs) or	50 ng/L
organic chlorine	25 ng/L

(B) If the dilution water is from a ground or surface water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) shall be measured. Reconstituted water can be made by adding specific amounts of reagent-grade chemicals to deionized or distilled water. Glass-distilled or carbon-filtered deionized water with a conductivity less than 1 micromho/cm is acceptable as the diluent for making reconstituted water.

(C) The concentration of dissolved oxygen in the dilution water shall be between 90 and 100 percent saturation. If necessary, the dilution water can be aerated before the addition of the test substance. All reconstituted water should be aerated before use.

(3) *Test parameters.* Environmental parameters during the test shall be maintained as specified below:

(i) Water temperature of 18 ±1 °C.

(ii) Dissolved oxygen concentration between 60 and 105 percent saturation.

(iii) The number of gammarids placed in a test chamber shall not be so great as to affect the results of the test. Ten gammarids per liter is the recommended level of loading for the static test. Loading requirements for the flow-through test will vary depending on the flow rate of dilution water. The loading should not cause the dissolved oxygen concentration to fall below the recommended levels.

(iv) Photoperiod of 16 hours light and 8 hours darkness.

(e) *Reporting.* The sponsor shall submit to the EPA all data developed by the test that are suggestive or predictive of toxicity. In addition, the test report shall include, but not necessarily be limited to, the following information:

(1) Name and address of the facility performing the study and the dates on which the study was initiated and completed.

(2) Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

(3) Statistical methods employed for analyzing the data.

(4) The test substance identified by name, Chemical Abstracts (CAS) number or code number, source, lot or batch number, strength, purity, and composition, or other appropriate characteristics.

(5) Stability of the test substance under the conditions of the test.

(6) A description of the methods used, including:

(i) The source of the dilution water, its chemical characteristics (e.g., hardness, pH, etc.) and a description of any pretreatment.

(ii) A description of the test substance delivery system, test chambers, the depth and volume of solution in the chamber, the way the test was begun (e.g., test substance addition), the loading, the lighting, and the flow rate.

(iii) Frequency and methods of measurements and observations.

(7) The scientific name, weight, length, source, and history of the organisms used, and the acclimation procedures and food used.

(8) The concentrations tested, the number of gammarids and replicates per test concentration. The reported results should include:

(i) The results of dissolved oxygen, pH and temperature measurements.

(ii) If solvents are used, the name and source of the solvent, the nominal concentration of the test substance in the stock solution, the highest solvent concentration in the test solution and a description of the solubility determination in water and solvents.

(iii) The measured concentration of the test substance in each test chamber just before the start of the test and at all subsequent sampling periods.

(iv) In each test chamber at each observation period, the number of dead and live test organisms, the percentage of organisms that died, and the number of test organisms that showed any abnormal effects in each test chamber at each observation period.

(v) The 48, 72 and 96-hour LC50's and their 95 percent confidence limits. When sufficient data have been generated, the 24-hour LC50 value also. These calculations should be made using the mean measured test substance concentrations.

(vi) The observed no-effect concentration (the highest concentration tested at which there were no mortalities or abnormal behavioral or physiological effects), if any.

(vii) Methods and data for all chemical analyses of water quality and test substance concentrations, including method validations and reagent blanks.

(9) A description of all circumstances that may have affected the quality or integrity of the data.

(10) The names of the sponsor, study director, principal investigator, names of other scientists or professionals, and the names of all supervisory personnel involved in the study.

(11) A description of the transformations, calculations, or operations performed on the data, a summary and analysis of the data, and a statement of the conclusions drawn from the analysis. Results of the analysis of data should include the calculated LC50 value, 95 percent confidence limits, slope of the transformed concentration-response line, and the results of a goodness-of-fit test (e.g., chi-square test).

(12) The signed and dated reports prepared by any individual scientist or other professional involved in the study, including each person who, at the request or direction of the testing facility or sponsor, conducted an analysis or evaluation of data or specimens from the study after data generation was completed.

(13) The locations where all specimens, raw data, and the final report are stored.

(14) The statement prepared and signed by the quality assurance unit.

[52 FR 24462, July 1, 1987]

Subpart D—Provisional Health Effects Guidelines

§ 795.225 Dermal pharmacokinetics of DGBE and DGBA.

(a) *Purpose.* The purpose of these studies is to determine:

(1) The absorption of diethylene glycol butyl ether (DGBE) after administration by the dermal route.

(2) The biotransformation of DGBE administered dermally.

(3) The dermal absorption of DGBE and diethylene glycol butyl ether acetate (DGBA).

(b) *Test procedures*—(1) *Animal selection*—(i) *Species.* The species utilized for investigating DGBE and DGBA shall be the rat, a species for which historical data on the toxicity and carcinogenicity of many compounds are available and which is used extensively in percutaneous absorption studies.

(ii) *Animals.* Adult female Sprague Dawley rats shall be used. The rats shall be 7 to 8 weeks old and weigh 180 to 220 grams. Prior to testing, the animals shall be selected at random for each group. Animals showing signs of ill health shall not be used.

(iii) *Animal care.* (A) The animals should be housed in environmentally controlled rooms with 10 to 15 air changes per hour. The rooms should be maintained at a temperature of 25 ±2 °C and humidity of 50 ±10 percent with a 12-hour light/dark cycle per day. The rats should be isolated for at least 7 days prior to use.

(B) During the acclimatization period, the rats should be housed in cages on hardwood chip bedding. All animals shall be provided with conventional laboratory diets and water ad libitum.

(2) *Administration of DGBE and DGBA*—(i) *Test substances.* These studies require the use of ^{14}C-labeled DGBE and DGBA. The use of ^{14}C-DGBE and ^{14}C-DGBA is required for the determinations in paragraphs (a) (1), (2), and (3) of this section because they will facilitate the work and improve the reliability of quantitative determinations.

(ii) *Dosage and treatment.* (A) Two doses of DGBA shall be used in the study, a ''low'' dose and a ''high'' dose. Three doses of DGBE shall be used in the study, a neat ''low'' dose, an aqueous ''low'' dose, and neat ''high'' dose. When administered dermally, the ''high'' dose level should ideally induce some overt toxicity such as weight loss. The ''low'' dose level should correspond to a no observed effect level.

(B) For dermal treatment, the doses shall be applied in a volume adequate to deliver the prescribed doses. The backs of the rats should be lightly shaved with an electric clipper shortly before treatment. The dose shall be applied with a micropipette on a specific area (for example, 2 cm^2) on the freshly shaven skin.

(iii) *Washing efficiency study.* Before initiation of the dermal absorption studies described in paragraph (b)(2)(iv)(A) of this section, an initial washing efficiency experiment shall be

performed to assess the extent of removal of the applied DGBE and DGBA by washing with soap and water. Groups of four rats should be lightly anesthetized with sodium pentobarbital. These animals shall then be treated with dermal doses of test substance at the low dose level. Soon after application (5 to 10 minutes) the treated animals shall be washed with soap and water then housed in individual metabolism cages for excreta collection. Urine and feces shall be collected at 8, 24, and 48 hours following dosing. Collection of excreta shall continue every 24 hours if a significant amounts of DGBE, DGBA, or metabolites continue to be eliminated.

(iv) *Determination of absorption, biotransformation, and excretion.* (A) Eight animals shall be dosed once dermally with the low dose of ^{14}C-DGBE.

(B) Eight animals shall be dosed once dermally with the high dose of ^{14}C-DGBE.

(C) Eight animals shall be dosed once dermally with the low dose of ^{14}C-DGBA.

(D) Eight animals shall be dosed once dermally with the high dose of ^{14}C-DGBA.

(E) The high and low doses of ^{14}C-DGBE and ^{14}C-DGBA shall be kept on the skin for 24 hours. After application, the animals shall be placed in metabolism cages for excreta collection. After 24 hours, any test material remaining on the skin will be washed off and the containment cell removed. Radiolabeled material in the wash will be accounted for in the total recovery. Urine and feces shall be collected at 8, 24, 48, 72, and 96 hours after dosing, and if necessary, daily thereafter until at least 90 percent of the dose has been excreted or until 7 days after dosing, whichever occurs first.

(3) *Observation of animals*—(i) *Urinary and fecal excretion.* The quantities of total ^{14}C excreted in urine and feces by rats dosed as specified in paragraph (b)(2)(iv) of this section shall be determined at 8, 24, 48, 72 and 96 hours after dosing, and if necessary, daily thereafter until at least 90 percent of the dose has been excreted or until 7 days after dosing (whichever occurs first). Four animals from each group shall be used for this purpose.

(ii) *Biotransformation after dermal dosing.* Appropriate qualitative and quantitative methods shall be used to assay urine specimens collected from rats dosed with DGBE as specified in paragraph (b)(2)(iv) of this section. Any metabolite which comprises greater than 10 percent of the dose shall be identified.

(c) *Data and reporting*—(1) *Treatment of results.* Data shall be summarized in tabular form.

(2) *Evaluation of results.* All observed results, quantitative or incidental, shall be evaluated by an appropriate statistical method.

(3) *Test report.* In addition to the reporting requirements as specified in the TSCA Good Laboratory Practice Standards, in part 792, subpart J of this chapter, the following specific information shall be reported:

(i) Species, strain, and supplier of laboratory animals.

(ii) Information on the degree (i.e., specific activity for a radiolabel) and sites of labeling of the test substances.

(iii) A full description of the sensitivity and precision of all procedures used to produce the data.

(iv) Relative percent absorption by the dermal route for rats administered low and high doses of ^{14}C-DGBE and ^{14}C-DGBA.

(v) Quantity of isotope, together with percent recovery of the administered dose, in feces and urine.

(vi) Biotransformation pathways and quantities of DGBE and metabolites in urine collected after administering single high and low dermal doses to rats.

[53 FR 5946, Feb. 26, 1988, as amended at 54 FR 41834, Oct. 12, 1989]

§ 795.228 Oral/dermal pharmacokinetics.

(a) *Purpose.* The purposes of these studies are to:

(1) Ascertain whether the pharmacokinetics and metabolism of a chemical substance or mixture (''test substance'') are similar after oral and dermal administration.

(2) Determine bioavailability of a test substance after oral and dermal administration.

(3) Examine the effects of repeated dosing on the pharmacokinetics and metabolism of the test substance.

(b) *Definitions.* (1) *Bioavailability* refers to the rate and relative amount of administered test substance which reaches the systemic circulation.

(2) *Metabolism* means the study of the sum of the processes by which a particular substance is handled in the body and includes absorption, tissue distribution, biotransformation, and excretion.

(3) *Percent absorption* means 100 times the ratio between total excretion of radioactivity following oral or dermal administration and total excretion following intravenous administration of test substance.

(4) *Pharmacokinetics* means the study of the rates of absorption, tissue distribution, biotransformation, and excretion.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species.* The rat shall be used for pharmacokinetics testing because it has been used extensively for metabolic and toxicological studies. For dermal bioavailability studies, the rat and the mini-pig shall be used.

(ii) *Test animals.* For pharmacokinetics testing and dermal studies, adult male and female Sprague-Dawley rats, 7 to 9 weeks of age, shall be used. For dermal studies, young adult mini-pigs shall also be used. The animals should be purchased from a reputable dealer and shall be identified upon arrival at the testing laboratory. The animals shall be selected at random for the test groups and any animal showing signs of ill health shall not be used. In all studies, unless otherwise specified, each test group shall contain at least 4 animals of each sex for a total of at least 8 animals.

(iii) *Animal care.* (A) The animals shall be housed in environmentally controlled rooms with at least 10 air changes per hour. The rooms shall be maintained at a temperature of 24 ±2 °C and humidity of 50 ±20 percent with a 12-hour light/dark cycle per day. The animals shall be kept in a quarantine facility for at least 7 days prior to use and shall be acclimated to the experimental environment for a minimum of 48 hours prior to administration of the test substance.

(B) During the acclimatization period, the animals shall be housed in suitable cages. All animals shall be provided with certified feed and tap water *ad libitum.* The mini-pig diet shall be supplemented with adequate amounts of ascorbic acid in the drinking water.

(2) *Administration of test substance*—(i) *Test substance.* The use of a radioactive test substance is required for all studies. Ideally, the purity, radioactive and nonradioactive, is greater than 99 percent. The radioactive and nonradioactive test substances shall be chromatographed separately and together to establish purity and identity. If the purity is less than 99 percent or if the chromatograms differ significantly, EPA should be consulted.

(ii) *Dosage and treatment*—(A) *Intravenous.* The low dose of test substance, in an appropriate vehicle, shall be administered intravenously to groups of rats and mini-pigs of each sex. If feasible, the same low dose should be used for intravenous, oral, and dermal studies.

(B) *Oral.* Two doses of text substance shall be used in the oral study, a low dose and a high dose. The high dose should ideally induce some overt toxicity, such as weight loss. The low dose should correspond to a no-observed effect level. The oral dosing shall be accomplished by gavage or by administering the encapsulated test substance. If feasible, the same high and low doses should be used for oral and dermal studies.

(C) *Dermal.* (*1*) *Dermal treatment.* For dermal treatment, two doses, comparable to the low and high oral doses, shall be dissolved in a suitable vehicle and applied in volumes adequate to deliver comparable doses. The backs of the animals should be lightly shaved with an electric clipper 24 hours before treatment. The test substance shall be applied to the intact shaven skin (approximately 2 cm^2 for rats, 5 cm^2 for mini-pigs). The dosed areas shall be protected with a suitable porous covering which is secured in place, and the animals shall be housed separately.

(*2*) *Washing efficacy study.* Before initiation of the dermal absorption studies, an initial washing efficacy experiment shall be conducted to assess the removal of the applied low dose of the test substance by washing the exposed skin area with soap and water and an

appropriate organic solvent. The low dose shall be applied to 4 rats and 4 mini-pigs in accordance with paragraph (c)(2)(ii)(C)(*1*) of this section. After application (5 to 10 minutes), the treated areas of 2 rats and 2 mini-pigs shall be washed with soap and water and the treated areas of the remaining rats and pigs shall be washed with an appropriate solvent. The amounts of test substance recovered in the washings shall be determined to assess efficacy of its removal by washing.

(iii) *Dosing and sampling schedule*—(A) *Rat studies.* After administration of the test substance, each rat shall be placed in a metabolic unit to facilitate collection of excreta. For the dermal studies, excreta from the rats shall also be collected during the 6 hour exposure periods. At the end of each collection period, the metabolic units shall be cleaned to recover any excreta that might adhere to them. All studies, except the repeated dosing study, shall be terminated at 7 days or after at least 90 percent of the radioactivity has been recovered in the excreta, whichever occurs first.

(*1*) *Intravenous study.* Group A shall be dosed once intravenously at the low dose of test substance.

(*2*) *Oral study.* (*i*) Group B shall be dosed once per os with the low dose of test substance.

(*ii*) Group C shall be dosed once per os with the high dose of test substance.

(*3*) *Dermal studies.* Unless precluded by corrosivity, the test substance shall be applied and kept on the skin for a minimum of 6 hours. At the time of removal of the porous covering, the treated area shall be washed with an appropriate solvent to remove any test substance that may be on the skin surface. Both the covering and the washing shall be assayed to recover residual radioactivity. At the termination of the studies, each animal shall be sacrificed and the exposed skin area removed. An appropriate section of the skin shall be solubilized and assayed for radio-activity to ascertain if the skin acts as a reservoir for the test substance. Studies on the dermal absorption of corrosive test substances should be discussed with EPA prior to initiation.

(*i*) Group D shall be dosed once dermally with the low dose of test compound.

(*ii*) Group E shall be dosed once dermally with the high dose of the test substance.

(*4*) *Repeated dosing study.* Group F shall receive a series of single daily oral low doses of nonradioactive test substance over a period of at least 7 days. Twenty-four hours after the last nonradioactive dose, a single oral low dose of radioactive test substance shall be administered. Following dosing with the radioactive substance, the rats shall be placed in individual metabolic units as described in paragraph (c)(2)(iii) of this section. The study shall be terminated at 7 days after the last dose, or after at least 90 percent of the radioactivity has been recovered in the excreta, whichever occurs first.

(B) *Mini-Pig studies.* For all mini-pig studies, the test groups shall consist of four young adult animals. After administration of the test substance, each mini-pig shall be kept in a metabolic unit to facilitate collection of excreta. At the end of each collection period, the metabolic units are to be cleaned to recover any excreta that might adhere to them. All studies shall be terminated at 7 days, or after at least 90 percent of the radio-activity has been recovered in the excreta, whichever occurs first.

(*1*) *Intravenous study.* Group G is to be dosed once intravenously at the low dose of the test substance.

(*2*) *Dermal studies.* Following the experimental guidance described in (c)(2)(iii)(A)(*3*) of this section:

(*i*) Group H shall be dosed once dermally with the low dose of test substance.

(*ii*) Group I shall be dosed once dermally with the high dose of the test substance.

(3) *Types of studies*—(i) *Pharmacokinetics studies*—(A) *Rat studies.* Groups A through F shall be used to determine the kinetics of absorption of the test substance. In the group administered the test substance by intravenous routes, (i.e., Group A), the concentration of radioactivity in blood and excreta shall be measured following administration. In groups administered

the test substance by the oral and dermal route (i.e., Groups B, C, D, E and F), the concentration of radioactivity in blood and excreta shall be measured at selected time intervals during and following the exposure period.

(B) *Mini-Pig studies.* Groups G, H, and I shall be used to determine the extent of dermal absorption of the test substance. The amount of radioactivity in excreta shall be determined at selected time intervals.

(ii) *Metabolism studies—Rat studies.* Groups A through F shall be used to determine the metabolism of the test substance. Urine, feces, and expired air shall be collected for identification and quantification of the test substance and metabolites.

(4) *Measurements*—(i) *Pharmacokinetics.* Four animals from each group shall be used for these purposes.

(A) *Rat studies—(1) Bioavailability.* The levels of radioactivity shall be determined in whole blood, blood plasma or blood serum at 15 and 30 minutes and at 1, 2, 8, 24, 48, and 96 hours after initiation of dosing.

(*2*) *Extent of absorption.* The total quantities of radioactivity shall be determined for excerta collected daily for 7 days or until at least 90 percent of the radioactivity has been recovered in the excreta.

(*3*) *Excretion.* The quantities of radioactivity eliminated in the urine, feces, and expired air shall be determined separately at appropriate time intervals. The collection of carbon dioxide may be discontinued when less than one percent of the dose is found to be exhaled as radioactive carbon dioxide in 24 hours.

(*4*) *Tissue distribution.* At the termination of each study, the quantities of radioactivity in blood and in various tissues, including bone, brain, fat, gastrointestinal tract, gonads, heart, kidney, liver, lungs, muscle, skin, and residual carcass of each animal shall be determined.

(*5*) *Changes in pharmacokinetics.* Results of pharmacokinetics measurements (i.e., bioavailability and extent of absorption, tissue distribution, and excretion) obtained in rats receiving the single low oral dose of the test substance (Groups B and C) shall be compared to the corresponding results obtained in rats receiving repeated oral doses of the test substance (Group F).

(B) *Mini-Pig studies—Extent of absorption.* The total quantities of radioactivity shall be determined for excreta daily for 7 days or until at least 90 percent of the test substance has been excreted.

(ii) *Metabolism.* Four animals from each group shall be used for these purposes.

(A) *Rat studies—(1) Biotransformation.* Appropriate qualitative and quantitative methods shall be used to assay urine, feces, and expired air collected from rats. Efforts shall be made to identify any metabolite which comprises 5 percent or more of the administered dose and the major radioactive components of blood.

(*2*) *Changes in biotransformation.* Appropriate qualitative and quantitative assay methodology shall be used to compare the composition of radioactive compounds in excreta from rats receiving a single oral dose (Groups B and C) with those in the excreta from rats receiving repeated oral doses (Group H).

(d) *Data and reporting.* The final test report shall include the following:

(1) *Presentation of results.* Numerical data shall be summarized in tabular form. Pharmacokinetic data shall also be presented in graphical form. Qualitative observations shall also be reported.

(2) *Evaluation of results.* All quantitative results shall be evaluated by an appropriate statistical method.

(3) *Reporting results.* In addition to the reporting requirements as specified in 40 CFR part 792, the following specific information shall be reported:

(i) Species and strains of laboratory animals.

(ii) Chemical characterization of the test substance, including:

(A) For the radioactive test substances, information on the site(s) and degree of radiolabeling, including type of label, specific activity, chemical purity, and radiochemical purity.

(B) For the nonradioactive compound, information on chemical purity.

(C) Results of chromatography.

(iii) A full description of the sensitivity, precision, and accuracy of all procedures used to generate the data.

(iv) Percent of absorption of test substance after oral and dermal exposures to rats and dermal exposure to minipigs.

(v) Quantity and percent recovery of radioactivity in feces, urine, expired air, and blood. In dermal studies on rats and mini-pigs, include recovery data for skin, skin washings, and residual radioactivity in the covering as well as results of the washing efficacy study.

(vi) Tissue distribution reported as quantity of radioactivity in blood and in various tissues, including bone, brain, fat, gastrointestinal tract, gonads, heart, kidney, liver, lung, muscle, skin and in residual carcass of rats.

(vii) Materials balance developed from each study involving the assay of body tissues and excreta.

(viii) Biotransformation pathways and quantities of test substance and metabolites in excreta collected after administering single high and low doses to rats.

(ix) Biotransformation pathways and quantities of the test substance and metabolites in excreta collected after administering repeated low doses to rats.

(x) Pharmacokinetics model(s) developed from the experimental data.

[54 FR 33411, Aug. 14, 1989; 54 FR 49844, Dec. 1, 1989; 55 FR 25392, June 21, 1990]

§ 795.231 Pharmacokinetics of isopropanal.

(a) *Purpose.* The purposes of these studies are to:

(1) Ascertain whether the pharmacokinetics and metabolism of the ''test substance'' are similar after oral and inhalation administration.

(2) Determine bioavailability of the test substance after oral and inhalation administration.

(3) Examine the effects of repeated dosing on the pharmacokinetics and metabolism of the test substance.

(b) *Definitions.* (1) ''*Bioavailability*'' refers to the rate and relative amount of administered test substance which reaches the systemic circulation.

(2) ''*Metabolism*'' means the study of the sum of the processes by which a particular substance is handled in the body, and includes absorption, tissue distribution, biotransformation, and excretion.

(3) ''*Pharmacokinetics*'' means the study of the rates of absorption, tissue distribution, biotransformation, and excretion.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species.* The rat shall be used because it has been used extensively for metabolic and toxicological studies.

(ii) *Test animals.* For pharmacokinetics testing, adult male and female rats (Fischer 344 or strain used for major toxicity testing), 7 to 9 weeks of age, shall be used. The animals should be purchased from a reputable dealer and shall be identified upon arrival at the testing laboratory. The animals shall be selected at random for the testing groups and any animal showing signs of ill health shall not be used. In all studies, unless otherwise specified, each test group shall contain at least four animals of each sex for a total of at least eight animals.

(iii) *Animal care.* (A) Animal care and housing should be in accordance with DHEW Publication No. (NIH)-85-23, 1985, entitled ''Guidelines for the Care and Use of Laboratory Animals.''

(B) The animals should be housed in environmentally controlled rooms with at least 10 air changes per hour. The rooms shall be maintained at a temperature of 22 ±2 °C and humidity of 50 ±20 percent with a 12-hour light/dark cycle per day. The animals shall be kept in a quarantine facility for at least 7 days prior to use and shall be acclimated to the experimental environment for a minimum of 48 hours prior to treatment.

(C) During the acclimatization period, the animals should be housed in suitable cages. All animals shall be provided with certified feed and tap water *ad libitum.*

(2) *Administration of test substance*—(i) *Test substance.* The use of radioactive test substance is required for all materials balance and metabolite identification requirements of the study. Ideally, the purity of both radioactive and nonradioactive test substance should be greater than 99 percent. The radioactive and nonradioactive substances shall be chromatographed separately and together to establish purity and identity. If the purity is less than 99

percent or if the chromatograms differ significantly, EPA should be consulted.

(ii) *Dosage and treatment*—(A) *Intravenous.* The low dose of test substance, in an appropriate vehicle, shall be administered intravenously to four rats of each sex.

(B) *Oral.* Two doses of test substance shall be used in the oral portion of the study, a low dose and a high dose. The high dose should ideally induce some overt toxicity, such as weight loss. The low dose level should correspond to a no-observed effect level. The oral dosing shall be accomplished by gavage or by administering an encapsulated test substance. If feasible, the same high and low doses should be used for oral and dermal studies.

(C) *Inhalation.* Two concentrations of the test substance shall be used in this portion of the study, a low concentration and a high concentration. The high concentration should ideally induce some overt toxicity, while the low concentration should correspond to a no observed level. Inhalation treatment should be conducted using a ''nose-cone'' or ''head only'' apparatus to prevent ingestion of the test substance through ''grooming''.

(iii) *Dosing and sampling schedule.* After administration of the test substance, each rat shall be placed in a separate metabolic unit to facilitate collection of excreta. For the inhalation studies, excreta from the rats shall also be collected during the exposure periods. At the end of each collection period, the metabolic units shall be cleaned to recover any excreta that might adhere to the cages. All studies, except the repeated dose study, shall be terminated at 7 days, or after at least 90 percent of the radioactivity has been recovered in the excreta, whichever occurs first.

(A) *Intravenous study.* Group A shall be dosed once intravenously at the low dose of test substance.

(B) *Oral studies.* (*1*) Group B shall be dosed once *per os* with the low dose of the test substance.

(*2*) Group C shall be dosed once *per os* with the high dose of the test substance.

(C) *Inhalation studies.* A single 6-hour exposure period shall be used for each group.

(*1*) Group D shall be exposed to a mixture of the test substance in air at the low concentration.

(*2*) Group E shall be exposed to a mixture of test substance in air at the high concentration.

(D) *Repeated dosing study.* Group F shall receive a series of single daily oral low doses of nonradioactive test substance over a period of at least 7 consecutive days. Twenty four hours after the last nonradioactive dose, a single oral low dose of radioactive test substance shall be administered. Following dosing with radioactive substance, the rats shall be placed in individual metabolic units as described in paragraph (c)(2)(iii) of this section. The study shall be terminated 7 days after the last dose, or after at least 90 percent of the radioactivity has been recovered in the excreta, whichever occurs first.

(3) *Types of studies*—(i) *Pharmacokinetics studies.* Groups A through F shall be used to determine the kinetics of absorption of the test substance. In groups administered the substance by intravenous or oral routes, (i.e., Groups A, B, C, F), the concentration of radioactivity in blood and excreta including expired air shall be measured following administration. In groups administered the substance by the inhalation route (i.e., Groups D and E), the concentration of radioactivity in blood shall be measured at selected time intervals during and following the exposure period. In the groups administered the substance by inhalation (i.e., Groups D and E), the concentration of radioactivity in excreta (including expired air) shall be measured at selected time intervals following the exposure period. In addition, in the groups administered the substance by inhalation, the concentration of test substance in inspired air shall be measured at selected time intervals during the exposure period.

(ii) *Metabolism studies.* Groups A through F shall be used to determine the metabolism of the test substance. Excreta (urine, feces, and expired air) shall be collected for identification and quantification of test substance and metabolites.

(4) *Measurements*—(i) *Pharmacokinetics.* Four animals from each group shall be used for these purposes.

(A) *Bioavailability*. The levels of radioactivity shall be determined in whole blood, blood plasma or blood serum at 15 minutes, 30 minutes, 1, 2, 3, 6, 9, and 18 hours after dosing; and at 30 minutes, 3, 6, 6.5, 7, 8, 9, 12, and 18 hours after initation of inhalation exposure.

(B) *Extent of absorption*. The total quantities of radioactivity shall be determined for excreta collected daily for 7 days, or after at least 90 percent of the radioactivity has been recovered in the excreta, whichever occurs first.

(C) *Excretion*. The quantities of radioactivity eliminated in the urine, feces, and expired air shall be determined separately at appropriate time intervals. The collection of the intact test substance or its metabolites, including carbon dioxide, may be discontinued when less than 1 percent of the administered dose is found to be exhaled as radioactive carbon dioxide in 24 hours.

(D) *Tissue distribution*. At the termination of each study, the quantities of radioactivity in blood and in various tissues, including bone, brain, fat, gastrointestinal tract, gonads, heart, kidney, liver, lungs, muscle, skin, spleen, and residual carcass of each animal shall be determined.

(E) *Changes in pharmacokinetics*. Results of pharmacokinetics measurements (i.e., biotransformation, extent of absorption, tissue distribution, and excretion) obtained in rats receiving the single low oral dose of test substance (Group B) shall be compared to the corresponding results obtained in rats receiving repeated oral doses of test substance (Group F).

(F) *Biotransformation*. Appropriate qualitative and quantitative methods shall be used to assay urine, feces, and expired air collected from rats. Efforts shall be made to identify any metabolite which comprises 5 percent or more of the dose eliminated.

(G) *Changes in biotransformation*. Appropriate qualitative and quantitative assay methodology shall be used to compare the composition of radioactive substances in excreta from the rats receiving a single oral dose (Groups B and C) with those in the excreta from rats receiving repeated oral doses (Group F).

(ii) [Reserved]

(d) *Data and reporting*. The final test report shall include the following:

(1) *Presentation of results*. Numerical data shall be summarized in tabular form. Pharmacokinetics data shall also be presented in graphical form. Qualitative observations shall also be reported.

(2) *Evaluation of results*. All quantitative results shall be evaluated by an appropriate statistical method.

(3) *Reporting results*. In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards (40 CFR 792.185), the following specific information shall be reported:

(i) Species and strains of laboratory animals.

(ii) Chemical characterization of the test substance, including:

(A) For the radioactive test substance, information on the site(s) and degree of radiolabeling, including type of label, specific activity, chemical purity, and radiochemical purity.

(B) For the nonradioactive substance, information on chemical purity.

(C) Results of chromatography.

(iii) A full description of the sensitivity, precision, and accuracy of all procedures used to generate the data.

(iv) Extent of absorption of the test substance as indicated by: percent absorption of the administered oral dose; and total body burden after inhalation exposure.

(v) Quantity and percent recovery of radioactivity in feces, urine, expired air, and blood.

(vi) Tissue distribution reported as quantity of radioactivity in blood and in various tissues, including bone, brain, fat, gastrointestinal tract, gonads, heart, kidney, liver, lung, muscle, skin, spleen and in residual carcass of each rat.

(vii) Biotransformation pathways and quantities of the test substance and metabolites in excreta collected after administering single high and low doses to rats.

(viii) Biotransformation pathways and quantities of the test substance and metabolites in excreta collected after administering repeated low doses to rats.

(ix) Pharmacokinetics model(s) developed from the experimental data.

[54 FR 43261, Oct. 23, 1989]

§ 795.232 Inhalation and dermal pharmacokinetics of commercial hexane.

(a) *Purposes.* The purposes of these studies are to:

(1) Determine the bioavailability of the test substances after dermal and inhalation administration.

(2) Compare the pharmacokinetics and metabolism of the test substances after intravenous, dermal, and inhalation administration.

(3) Examine the effects of repeated doses on the pharmacokinetics and metabolism of the test substances.

(b) *Definitions.* (1) *Bioavailability* refers to the relative amount of administered test substance which reaches the systemic circulation and the rate at which this process occurs.

(2) *Metabolism* means the sum of the enzymatic and nonenzymatic processes by which a particular substance is handled in the body.

(3) *Pharmacokinetics* means the study of the rates of absorption, tissue distribution, biotransformation, and excretion.

(4) *Low dose* should correspond to 1/10 of the high dose.

(5) *High dose* shall not exceed the lower explosive limit (LEL) and ideally should induce minimal toxicity.

(6) *Test substance* refers to the unlabeled and both radiolabeled mixtures (^{14}C-*n*-hexane and ^{14}C-methylcyclopentane) of commercial hexane used in the testing.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species.* The rat shall be used for pharmacokinetics testing because it has been used extensively for metabolic and toxicological studies.

(ii) *Test animals.* Adult male and female rats shall be used for testing. The rats shall be 7 to 9 weeks old and their weight range should be comparable from group to group. The animals shall be purchased from a reputable dealer and shall be permanently identified upon arrival. The animals shall be selected at random for the testing groups, and any animal showing signs of ill health shall not be used.

(iii) *Animal care.* (A) Animal care and housing shall be in accordance with DHHS/PHS NIH Publication No. 86–23, 1985, ''*Guidelines for the Care and Use of Laboratory Animals.*''

(B) The animals shall be housed in environmentally controlled rooms with at least 10 air changes per hour. The rooms shall be maintained at a temperature of 18 to 26 degrees centigrade and humidity of 40 to 70 percent with a 12–hour light/dark cycle per day. The animal subjects shall be kept in a quarantine facility for at least 7 days prior to use, and shall be acclimated to the experimental environment for a minimum of 48 hours prior to treatment.

(C) During the acclimatization period, the rats shall be housed in suitable cages. All animals shall be provided with certified feed and tap water *ad libitum.*

(2) *Administration of test substances*—(i) *Test substances.* The study will require he use of both radiolabeled and unlabeled test substances. All unlabeled commercial hexane shall be from the same lot number.Two kinds of radiolabeled test substances will be tested. ^{14}C-*n*-hexane shall be the only radiolabeled component of one, and ^{14}C-MCP shall be the only radiolabeled component of the other test substance. The use of both radiolabeled test substances is required for all pharmacokinetics and metabolism studies described in this rule, except for the bioavailability measurements required in (c)(4)(i)(A) of this section.The bioavailability measurements need only be conducted with the test substance containing ^{14}C-*n*-hexane or an unlabeled test substance may be used if it can be demonstrated that the analytical sensitivity of the method used with the unlabeled test substance is equal to or greater than the sensitivity which could be obtained with the radiolabeled test substance. If an unlabeled test substance is used for bioavailability measurements, these measurements shall be extended to include relevant metabolites of *n*-hexane. These test substances shall contain at least 40 liquid volume percent but no more than 55 liquid volume percent *n*-hexane and no less than 10 liquid volume percent methylcyclopentane (MCP) and otherwise conform to the specifications prescribed in the American Society for Testing and Materials

Designation D 1836–83 (ASTM D 1836), ''Standard Specification for Commercial Hexanes'', published in the *1986 Annual Book of ASTM Standards: Petroleum Products and Lubricants,* ASTM D 1836–83, pp. 966–967, 1986, which is incorporated by reference in accordance with 5 U.S.C. 552(a).ASTM D 1863–83 is available for public inspection at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202–741–6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.* Copies are available at the addresses in §700.17(b)(1) and (2) of this chapter. This incorporation by reference was approved by the Director of the Office of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. This material is incorporated as it exists on the date of approval, and a notice of any change in this material will be published in the FEDERAL REGISTER.

(ii) *Dosage and treatment*—(A) *Intravenous.* An appropriate dose of the test substance shall be administered intravenously. The intravenous data obtained in this portion of the study shall be suitable for the determination of absorption, distribution, and excretion parameters of the test substance. Factors that should be considered in the selection of the intravenous doses are: The acute toxicity of the test substance, the availability of a suitable vehicle (if saline is unsuitable) and the solubility of the test substance in the vehicle.

(B) *Inhalation.* Two concentrations of each test substance shall be used in this portion of the study, a low concentration and a high concentration. The high concentration should induce minimal toxicity, but shall not exceed the lower explosive limit (LEL). The low concentration shall correspond to 1/10 of the high concentration. Inhalation treatment shall be conducted using a ''nose-cone'' or ''head only'' apparatus to reduce ingestion of the test substance through ''grooming'' or dermal absorption.

(C) *Dermal.* Dermal absorption studies should be conducted by the methodology of Susten, A.S., Dames, B.L. and Niemeier, R.W., ''*In vivo* percutaneous absorption studies of volatile solvents in hairless mice. I. Description of a skin depot'', In: *Journal of Applied Toxicology* 6:43–46, (1986), or by some other suitable method because the test substances have significant volatility. The high and low doses shall be tested in rats.

(iii) *Dosing and sampling schedule.* Each experimental group shall contain at least four animals of each sex. After administration of the test substance, each rat shall be placed in an individual metabolic unit for collection of urine, feces, and expired air. For the dermal studies, excreta from the rats shall also be collected during the exposure periods. At the end of each collection period, the metabolic units shall be cleaned to recover any excreta that might adhere to the units. All studies, except the repeated dose studies, shall be terminated at 7 days, or after at least 90 percent of the administered radioactivity has been recovered in the excreta, whichever occurs first. All studies described below shall be conducted separately with each radiolabeled test substance.

(A) *Intravenous study.* Group A shall be given a single intravenous dose of the radiolabeled test substance to result in a level of commercial hexane in the blood that approximates the level from the other routes of exposure so that the data can be used to determine absorption and excretion parameters.

(B) *Inhalation studies.* A single 6–hour exposure period shall be used for each group.

(*1*) Group B shall be exposed to a mixture of the radiolabeled test substance in air at the low concentration.

(*2*) Group C shall be exposed to a mixture of the radiolabeled test substance in air at the high concentration.

(C) *Dermal studies.* The test substance shall be applied and kept on the skin for a minimum of 6 hours. The covering apparatus components shall be assayed to recover residual radioactivity. At the termination of the studies, each animal shall be sacrificed and the exposed skin area removed. An appropriate section of the skin shall be solubilized and assayed for radioactivity to ascertain whether the skin acts as a reservoir for the test substance.

(*1*) Group D shall be given one dermal, low dose of the radiolabeled test substance.

(*2*) Group E shall be given one dermal, high dose of the radiolabeled test substance.

(D) *Repeated dosing study*. Group F shall receive a series of single daily 6–hour inhalation exposures to unlabeled test substance at the low dose over a period of at least 7 days. A single 6–hour inhalation exposure to the radiolabeled test substance at the low dose shall be administered 24 hours after the last unlabeled exposure. Following administration of the radiolabeled substance, the rats shall be placed in individual metabolic units and excreta collected. The study shall be terminated 7 days after the last exposure, or after at least 90 percent of the radioactivity has been recovered in the excreta, whichever occurs first.

(3) *Types of studies*—(i) *Pharmacokinetics studies*. Groups A through F shall be used to determine the kinetics of absorption of the test substance. In animal subjects administered the test substance intravenously (i.e., Group A), the concentration of test substance in blood and excreta shall be measured following administration. In animal subjects administered the test substance by the inhalation and dermal routes (i.e., Groups B through F), the concentration of test substance in blood shall be measured at selected time intervals during and following the exposure period. In animal subjects administered the test substance by the inhalation route (i.e., Groups B, C, and F) the concentration of test substance in excreta shall be measured following exposure. In animal subjects administered the test substance by the dermal route (i.e., Groups D and E) the concentration of test substance in excreta shall be measured during and following exposure. These measurements allow calculation of uptake, half lives, and clearance. In addition, in the groups administered the test substance by inhalation (i.e., Groups B, C, and F), the concentration of test substance in the exposure chamber air shall be measured at selected time intervals during the exposure period.

(ii) *Metabolism studies*. Groups A through F shall be used to determine the metabolism of the test substance. Excreta (urine, feces, and expired air) shall be collected for identification and measurement of the quantities of test substance and metabolites.

(4) *Measurements*—(i) *Pharmacokinetics*. At least four animals from each group shall be used for these purposes.

(A) *Bioavailability*. The levels of test substance and relevant metabolites, as appropriate, shall be determined in whole blood, blood plasma or blood serum at appropriate intervals after initiation of intravenous, dermal, and inhalation exposure. The sampling intervals should be compatible with the exposure route under study. The determinations need only be done on animals administered the test substance containing ^{14}C-*n*-hexane or, if the analytical sensitivity is equal or greater, unlabeled test substance may be used.

(B) *Extent of absorption*. The total quantities of radioactivity shall be determined for excreta collected daily for 7 days, or until at least 90 percent of theradioactivity has been recovered in the excreta, whichever occurs first.

(C) *Excretion*. The quantities of radioactivity eliminated in the urine, feces, and expired air shall be determined separately at time intervals that provide accurate measurement of clearance and excretory rates. The collection of carbon dioxide may be discontinued when less than one percent of the dose is found to be exhaled as radioactive carbon dioxide in 24 hours.

(D) *Tissue distribution*. At the termination of each study, the quantities of radioactivity shall be determined in blood and in various tissues, including bone, brain, fat, gastrointestinal tract, gonads, heart, kidney, liver, lungs, muscle, skin, spleen, thymus, and residual carcass of each animal.

(E) *Change in pharmacokinetics*. Results of pharmacokinetics measurements (i.e., biotransformation, extent of absorption, tissue distribution, and excretion) obtained in rats receiving the single inhalation exposure to the low dose of the test substance (Group B) shall be compared to the corresponding results obtained in rats receiving repeated inhalation exposures to the low dose of the test substance (Group F).

(ii) *Metabolism.* At least four animals from each group shall be used for these purposes.

(A) *Biotransformation.* Appropriate qualitative and quantitative methods shall be used to assay urine, feces, and expired air collected from rats. Efforts shall be made to identify any metabolite which comprises 5 percent or more of the dose administered.

(B) *Changes in biotransformation.* Appropriate qualitative and quantitative assay methods shall be used to compare the composition of radioactive compounds in excreta from rats receiving a single inhalation exposure (Groups B and C) with that from rats receiving repeated inhalation exposures (Group F).

(d) *Data and reporting.* The final test report shall include the following:

(1) *Presentation of results.* Numerical data shall be summarized in tabular form. Pharmacokinetics data shall also be presented in graphical form. Qualitative observations shall also be reported.

(2) *Evaluation of results.* All data shall be evaluated by appropriate statistical methods.

(3) *Reporting results.* In addition to the reporting requirements as specified in 40 CFR part 792, the following information shall be reported.

(i) Strain of laboratory animals.

(ii) Chemical characterization of the test substances, including:

(A) For the radiolabeled test substances, information on the sites and degree of radiolabeling, including type of label, specific activity, chemical purity prior to mixing with the unlabeled hexane mixture, and radiochemical purity.

(B) For the unlabeled test substance, information on lot number and the percentage of MCP and *n*-hexane.

(C) Results of chromatography.

(iii) A full description of the sensitivity, precision, and accuracy of all procedures used to obtain the data.

(iv) Percent and rate of absorption of the test substance after inhalation and dermal exposures.

(v) Quantity and percent recovery of radioactivity in feces, urine, expired air, and blood. For dermal studies, include recovery data for skin and residual radioactivity in the covering apparatus.

(vi) Tissue distribution reported as quantity of radioactivity in blood, in various tissues including bone, brain, fat, gastrointestinal tract, gonads, heart, kidney, liver, lung, muscle, skin, spleen, thymus, and in residual carcass.

(vii) Biotransformation pathways, to the extent possible, and quantities of the test substances and metabolites in excreta collected after administering single high and low doses.

(viii) Biotransformation pathways, to the extent possible, and quantities of test substances and metabolites in excreta collected after administering repeated low doses.

(ix) Pharmacokinetics models to the extent they can be developed from the experimental data.

[55 FR 632, Jan. 8, 1990, as amended at 58 FR 34205, June 23, 1993; 60 FR 34466, July 3, 1995; 69 FR 18803, Apr. 9, 2004; 77 FR 46293, Aug. 3, 2012]

§ 795.250 Developmental neurotoxicity screen.

(a) *Purpose.* In the assessment and evaluation of the toxic characteristics of a chemical, it is important to determine when acceptable exposures in the adult may not be acceptable to a developing organism. This test is designed to provide information on the potential functional and morphologic hazards to the nervous system which may arise in the offspring from exposure of the mother during pregnancy and lactation.

(b) *Principle of the test method.* The test substance is administered to several groups of pregnant animals during gestation and lactation, one dose level being used per group. Offspring are randomly selected from within litters for neurotoxicity evaluation. The evaluation includes observation to detect gross neurological and behavioral abnormalities, determination of motor activity, neuropathological evaluation, and brain weights. Measurements are carried out periodically during both postnatal development and adulthood.

(c) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* Testing should be performed in the Sprague Dawley rat.

(ii) *Age.* Young adult animals (nulliparous females) shall be used.

(iii) *Sex.* Pregnant females shall be used at each dose level.

(iv) *Number of animals.* The objective is for a sufficient number of pregnant rats to be exposed to ensure that an adequate number of offspring are produced for neurotoxicity evaluation. At least 20 litters are recommended at each dose level. This number assumes a coefficient of variation of 20 to 25 percent for most behavioral tests. If, based upon experience with historical control data or data for positive controls in a given laboratory, the coefficient of variation for a given task is higher than 20 to 25 percent, then calculation of appropriate sample sizes to detect a 20 percent change from control values with 80 percent power would need to be done. For most designs, calculations can be made according to Dixon and Massey (1957) under paragraph (e)(5) of this section, Neter and Wasserman (1974) under paragraph (e)(10) of this section, Sokal and Rohlf (1969) under paragraph (e)(11) of this section, or Jensen (1972) under paragraph (e)(8) of this section.

(A) On day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, 4 males and 4 females per litter. Whenever the number of male or female pups prevents having 4 of each sex per litter, partial adjustment (for example, 5 males and 3 females) is permitted. Adjustments are not appropriate for litters of less than 8 pups. Elimination of runts only is not appropriate. Individual pups should be identified uniquely after standardization of litters. A method that may be used can be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(B) After standardization of litters, males and females shall be randomly assigned to one of each of three behavioral tasks. Alternatively, more than one of the behavioral tasks may be conducted in the same animal. In the latter case, a minimum of 1 to 2 days should separate the tests when conducted at about the same age.

(C) One male and one female shall be randomly selected from each litter for sacrifice at weaning as specified in paragraph (c)(8) of this section.

(2) *Control group.* A concurrent control group shall be used. This group shall be a sham treated group, or, if a vehicle is used in administering the test substance, a vehicle control group. Animals in the control groups shall be handled in an identical manner to test group animals. The vehicle shall neither be developmentally toxic nor have effects on reproduction.

(3) *Dose levels and dose selection.* (i) At least 3 dose levels plus a control (vehicle control, if a vehicle is used) shall be used.

(ii) If the substance has been shown to be developmentally toxic either in a standard developmental toxicity study or a pilot study, the highest dose level shall be the maximum dose which will not induce *in utero* or neonatal deaths or malformations sufficient to preclude a meaningful evaluation of neurotoxicity.

(iii) In the absence of standard developmental toxicity, unless limited by the physicochemical nature or biologicial properties of the substance, the highest dose level shall induce some overt maternal toxicity but shall not result in a reduction in weight gain exceeding 20 percent during gestation and lactation.

(iv) The lowest dose should not produce any grossly observable evidence of either maternal or developmental neurotoxicity.

(v) The intermediate dose(s) shall be equally spaced between the highest and lowest dose.

(4) *Dosing period.* Day 0 in the test is the day on which a vaginal plug and/or sperm are observed. The dose period shall cover the period from day 6 of gestation through weaning (21 days postnatally).

(5) *Administration of test substance.* The test substance or vehicle should be administered orally by intubation. The test substance shall be administered at the same time each day. The animals shall be weighed periodically and the dosage based on the most recent weight determination.

(6) *Observation of dams.* (i) A gross examination of the dams shall be made at least once each day, before daily treatment. The animals shall be observed by

trained technicians who are blind with respect to the animal's treatment, using standardized procedures to maximize inter-observer reliability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of inter-observer reliability is required.

(ii) During the treatment and observation periods, cage-side observations shall include:

(A) Any responses with respect to body position, activity level, coordination of movement, and gait.

(B) Any unusual or bizarre behavior including, but not limited to headflicking, head searching, compulsive biting or licking, self-mutilation, circling, and walking backwards.

(C) The presence of:

(*1*) Convulsions.

(*2*) Tremors.

(*3*) Increased levels of lacrimation and/or red-colored tears.

(*4*) Increased levels of salivation.

(*5*) Piloerection.

(*6*) Pupillary dilation or constriction.

(*7*) Unusual respiration (shallow, labored, dyspneic, gasping, and retching) and/or mouth breathing.

(*8*) Diarrhea.

(*9*) Excessive or diminished urination.

(*10*) Vocalization.

(iii) Signs of toxicity shall be recorded as they are observed, including the time of onset, the degree and duration.

(iv) Animals shall be weighed at least weekly.

(v) The day of delivery of litters shall be recorded.

(7) *Study conduct*—(i) *Observation of offspring.* (A) All offspring shall be examined cage-side daily for gross signs of mortality and morbidity.

(B) All offspring shall be examined outside the cage for gross signs of toxicity whenever they are weighed or removed from their cages for behavioral testing. The offspring shall be observed by trained technicians, who are blind with respect to the animal's treatment using standardized procedures to maximize inter-observer reliability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of inter-observer reliability is required. At a minimum, the end points outlined in paragraph (c)(6)(ii) of this section shall be monitored as appropriate for the developmental stage being observed.

(C) Any gross signs of toxicity in the offspring shall be recorded as they are observed, including the time of onset, the degree, and duration.

(ii) *Developmental landmarks.* Live pups should be counted and litters weighed by weighing each individual pup at birth, or soon thereafter, and on days 4, 7, 13, 17, and 21, and biweekly thereafter. The age of the pups at the time of the appearance of the following developmental landmarks shall be determined:

(A) *Vaginal opening.* General procedure for this determination may be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(B) *Testes descent.* General procedure for this determination may be found in Adams et al. (1985) under paragraph (e)(1) of this section.

(iii) *Motor activity.* (A) Motor activity shall be monitored specifically on days 13, 17, 21, 45 (±2 days), and 60 (±2 days). Motor activity shall be monitored by an automated activity recording apparatus. The device used shall be capable of detecting both increases and decreases in activity, i.e., baseline activity as measured by the device shall not be so low as to preclude decreases nor so high as to preclude increases. Each device shall be tested by standard procedures to ensure, to the extent possible, reliability of operation across devices and testing of animals within dose groups shall be balanced across devices.

(B) Each animal shall be tested individually. The test session shall be long enough to demonstrate habituation of motor activity in control animals, i.e., to approach asymptotic levels by the last 20 percent of the session. Animals' activity counts shall be collected in equal time periods of no greater than 10 minutes duration. All sessions shall have the same duration. Treatment groups shall be counter-balanced across test times.

(C) Efforts shall be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the

variables which can affect motor activity are sound level, size, and shape of the test cage, temperature, relative humidity, lighting conditions, odors, use of home cage or novel test cage, and environmental distractions.

(D) Additional information on the conduct of a motor activity study may be obtained in the TSCA motor activity guideline, in § 798.6200 of this chapter.

(iv) *Auditory startle test.* An auditory startle habituation test shall be performed on the offspring on days 22 and 60. Details on the conduct of this testing may be obtained in Adams et al. (1985) under paragraph (e)(1) of this section. In performing the auditory startle task, the mean response amplitude on each block of 10 trials (5 blocks of 10 trials per session on each day of testing) shall be made. While use of prepulse inhibition is not a requirement, it may be used at the discretion of the investigator. Details on the conduct of this testing may be obtained from Ison (1984) under paragraph (e)(7) of this section.

(v) *Active avoidance test.* Active avoidance testing shall be conducted beginning at 60 to 61 days of age. Details on the apparatus may be obtained in Brush and Knaff (1959) and on the conduct of testing from Brush (1962), under paragraphs (e)(2) and (e)(4) of this section, respectively; reviews on active avoidance conditioning by Brush (1971) and McAllister and McAllister (1971) can be found under paragraphs (e)(3) and (e)(9) of this section, respectively. In performing the active avoidance task, the following measures should be made:

(A) Mean number of shuttles during the adaptation period preceding each daily session.

(B) Mean number and latency of avoidances per session, presented in blocks of 10 trials (2 blocks of 10 trials per session across 5 sessions).

(C) Mean number and latency of escapes per session, presented in blocks of 10 trials as above.

(D) Mean duration of shocks per session, presented in blocks of 10 trials as above.

(E) Mean number of shuttles during the inter-trial intervals.

(8) *Post-mortem evaluation*—(i) *Age of animals.* One male and one female per litter shall be sacrificed at weaning and the remainder following the last behavioral measures. Neuropathology and brain weight determinations shall be made on animals sacrificed at weaning and after the last behavioral measures.

(ii) *Neuropathology.* Details for the conduct of neuropathology evaluation may be obtained in the TSCA neuropathology guideline, in § 798.6400 of this chapter. At least 6 offspring per dose group shall be randomly selected from each sacrificed group (weaning and adulthood) for neuropathologic evaluation. These animals shall be balanced across litters, and equal numbers of males and females shall be used. The remaining sacrificed animals shall be used to determine brain weight. Animals shall be perfused *in situ* by a generally recognized technique. After perfusion, the brain and spinal cord shall be removed and gross abnormalities noted. Cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum and midbrain, the cerebellum and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swelling; Gasserian ganglia, dorsal root ganglia, dorsal and ventral root fibers, proximal sciatic nerve (mid-thigh and sciatic notch), sural nerve (at knee), and tibial nerve (at knee). Tissue samples from both the central and peripheral nervous system shall be further immersion-fixed and stored in appropriate fixative for further examination. After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast except for the sural nerve which should be embedded in plastic. A method for plastic embedding is described by Spencer et al. under paragraph (e)(12) of this section. Tissue sections shall be prepared from the tissue blocks. The following general testing sequence is recommended for gathering histopathological data:

(A) *General staining.* A general staining procedure shall be performed on all tissue specimens in the highest treatment group. Hematoxylin and eosin (H&E) shall be used for this purpose. The staining shall be differentiated

properly to achieve bluish nuclei with pinkish background.

(B) *Special stains.* Based on the results of the general staining, selected sites and cellular components shall be further evaluated by use of specific techniques. If H&E screening does not provide such information, a battery of stains shall be used to assess the following components in all appropriate required samples: Neuronal body (e.g., Einarson's gallocyanin), axon (e.g., Kluver's Luxol Fast Blue), and neurofibrils (e.g., Bielchosky). In addition, nerve fiber teasing shall be used. A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If lesions are observed, the special techniques shall be repeated in the next lower treatment group until no further lesions are detectable.

(C) *Alternative technique.* If the anatomical locus of expected neuropathology is well-defined, epoxy-embedded sections stained with toluidine blue may be used for small sized tissue samples. This technique obviates the need for special stains.

(iii) *Brain weight.* At least 10 animals that are not sacrificed for histopathology shall be used to determine brain weight. The animals shall be decapitated and the brains carefully removed, blotted, chilled, and weighed. The following dissection shall be performed on an ice-cooled glass plate: First, the rhombencephalon is separated by a transverse section from the rest of the brain and dissected into the cerebellum and the medulla oblongata/pons. A transverse section is made at the level of the ''optic chiasma'' which delimits the anterior part of the hypothalamus and passes through the anterior commissure. The cortex is peeled from the posterior section and added to the anterior section. This divides the brain into four sections, the telencephalon, the diencephalon/midbrain, the medulla oblongata/pons, and the cerebellum. Sections shall be weighed as soon as possible after dissection to avoid drying. Detailed methodology is available in Glowinski and Iversen (1966) under paragraph (e)(6) of this section.

(d) *Data reporting and evaluation.* In addition to the reporting requirements specified in part 792, subpart J of this chapter, the final test report shall include the following information.

(1) *Description of system and test methods.* (i) A detailed description of the procedures used to standardize observation and operational definitions for scoring observations.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. These data do not have to be from studies using prenatal exposures. However, the laboratory must demonstrate competence in testing neonatal animals perinatally exposed to chemicals and establish test norms for the appropriate age group.

(iii) Procedures for calibrating and assuring the equivalence of devices and balancing treatment groups.

(iv) A short justification explaining any decisions where professional judgement is involved such as fixation technique and choice of stains.

(2) *Results.* The following information shall be arranged by test group dose level.

(i) In tabular form, data for each animal shall be provided showing:

(A) Its identification number and litter from which it came.

(B) Its body weight and score on each developmental landmark at each observation time; total session activity counts and intrasession subtotals on each day measured; auditory startle response magnitude session counts and intrasession subtotals on each day measured; avoidance session counts and intrasession counts on each day measured; time and cause of death (if appropriate); locations, nature or frequency, and severity of the lesions; total brain weight; absolute weight of each of the four sections; and weight of each section as a percentage of total brain weight. A commonly used scale such as 1 + , 2 + , 3 + , and 4 + for degree of severity of lesions ranging from very slight to extensive may be used for morphologic evaluation. Any diagnoses derived from neurologic signs and lesions, including naturally occurring diseases or conditions, shall also be recorded.

(ii) Summary data for each group shall include:

(A) The number of animals at the start of the test.

(B) Body weights of the dams during gestation and lactation.

(C) Litter size and mean weight at birth.

(D) The number of animals showing each observation score at each observation time.

(E) The percentage of animals showing each abnormal sign at each observation time.

(F) The mean and standard deviation for each continuous end point at each observation time. These will include body weight, motor activity counts, acoustic startle responses, performance in active avoidance tests, and brain weights (both absolute and relative).

(G) The number of animals in which any lesion was found.

(H) The number of animals affected by each different type of lesion, the average grade of each type of lesion, and the frequency of each different type and/or location of lesions.

(3) *Evaluation of data.* An evaluation of the test results shall be made. The evaluation shall include the relationship between the doses of the test substance and the presence or absence, incidence, and severity of any neurotoxic effect. The evaluation shall include appropriate statistical analyses. The choice of analyses shall consider tests appropriate to the experimental design and needed adjustments for multiple comparisons.

(e) *References.* For additional background information on this test guideline, the following references should be consulted:

(1) Adams, J., Buelke-Sam, J., Kimmel, C.A., Nelson, C.J., Reiter, L.W., Sobotka, T.J., Tilson, H.A., and Nelson, B.K. ''Collaborative behavioral teratology study: Protocol design and testing procedure.'' *Neurobehavioral Toxicology and Teratology*. 7: 579–586. (1985).

(2) Brush, F.R. ''The effects of intertrial interval on avoidance learning in the rat.'' *Journal of Comparative Physiology and Psychology*. 55: 888–892. (1962).

(3) Brush, F.R. ''Retention of aversively motivated behavior.'' In: ''Adverse Conditioning and Learning.'' Brush, F.R., ed., New York: Academic Press. (1971).

(4) Brush, F.R. and Knaff, P.R. ''A device for detecting and controlling automatic programming of avoidance-conditioning in a shuttle-box.'' *American Journal of Psychology*. 72: 275–278 (1959).

(5) Dixon, W.J. and Massey, E.J. ''Introduction to Statistical Analysis.'' 2nd ed. New York: McGraw-Hill. (1957).

(6) Glowinski, J. and Iversen, L.L. ''Regional studies of catecholamines in the rat brain-I.'' *Journal of Neurochemistry*. 13: 655–669. (1966).

(7) Ison, J.R. ''Reflex modification as an objective test for sensory processing following toxicant exposure.'' *Neurobehavioral Toxicology and Teratology*. 6: 437–445. (1984).

(8) Jensen, D.R. ''Some simultaneous multivariate procedures using Hotelling's T2 Statistics.'' *Biometrics*. 28: 39–53. (1972).

(9) McAllister, W.R. and McAllister, D.E. ''Behavioral measurement of conditioned fear.'' In: ''Adverse Conditioning and Learning.'' Brush, F.R., ed., New York: Academic Press (1971).

(10) Neter, J. and Wasserman, W. ''Applied Linear Statistical Models.'' Homewood: Richard D. Irwin, Inc. (1974).

(11) Sokal, R.P. and Rohlf, E.J. ''Biometry.'' San Francisco: W.H. Freeman and Co. (1969).

(12) Spencer, P.S., Bischoff, M.C., and Schaumburg, H.H., ''Neuropathological methods for the detection of neurotoxic disease.'' In: ''Experimental and Clinical Neurotoxicology.'' Spencer, P.S. and Schaumburg, H.H., eds., Baltimore, MD: Williams & Wilkins, pp. 743–757. (1980).

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PART 796—CHEMICAL FATE TESTING GUIDELINES

Subpart A [Reserved]

Subpart B—Physical and Chemical Properties

Sec.