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2,4-D Butoxyethyl Ester Kinetics in Embryos of *Xenopus laevis:* The Role of the Embryonic Jelly Coat in Reducing Chemical Absorption

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Abstract. The role of the jelly coat in providing a protective barrier to chemical absorption was studied using the embryos of the amphibian, *Xenopus laevis.* Embryos with or without a jelly coat were water exposed to the butoxyethyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D BEE) and the rates of uptake, metabolism, distribution, and excretion were determined. The water uptake clearance rates were slower for embryos with a jelly coat (1.5–4.5 $ml_{water} \cdot g_{embryo}^{-1} \cdot h^{-1}$ or 0.040–0.022 $ml_{water} \cdot h^{-1}$ per embryo) in comparison to dejellied embryos (14–21 $ml_{water} \cdot g_{embryo}^{-1} \cdot h^{-1}$ 0.0066–0.021 $ml_{water} \cdot h^{-1}$ per embryo). This accounted for the much lower residues in embryos with a jelly coat than in dejellied embryos during 8 h of exposure. Despite quantitative differences in uptake, once 2,4-D BEE had entered the embryos, metabolism and distribution were similar between the two test groups. 2,4-D BEE was metabolized to 2,4-dichlorophenoxyacetic acid (2,4-D) with half-lives ranging from 35 to 42 minutes. The radioactive residues, as determined by whole body autoradiography, appeared throughout the embryo with a slight accumulation in the blastocoel. Furthermore, 35% of the radioactive residues were located in the jelly coat and 65% in the developing embryo. Based on a slower 2,4-D elimination in embryos with a jelly coat, the diffusive properties that decreased 2,4-D BEE uptake appeared to similarly decrease elimination of its metabolite. The common practice of removing jelly coats prior to embryonic amphibian toxicity studies, as in the widely used Frog Embryo Teratogenesis Assay–*Xenopus* (FETAX), is discouraged based on the kinetic differences observed in this study.

Introduction

The jelly coat surrounding amphibian embryos is required for anchoring egg masses to aquatic vegetation, providing protection from mechanical disturbance, sperm binding and capacitation, and prevention of both polyspermy and cross-species fertilization (Delplace *et al.* 2002). Furthermore, the jelly coat provides protection to the embryo by attenuating UVB radiation (Licht 2003) and decreases the likelihood of anoxia by reducing ''respiratory density'' of an embryo mass (Seymour and Bradford 1995). The presence of the jelly coat may also have importance in toxicology and especially for the Frog Embryo Teratogenesis Assay–*Xenopus* (FETAX) where removal of the jelly coat is a component of this toxicity assay. Unhatched embryos tended to be less sensitive to xenobiotic chemicals than their hatched counterparts, and researchers have hypothesized that the jelly coat provides some protection to the developing embryo (Berrill *et al.* 1998; Pauli *et al.* 1999). In this study, the toxicokinetics of the butoxyethyl ester derivative of 2,4-dichlorophenoxyacetic acid (2,4-D BEE) in embryos with or without an intact jelly coat were compared. This provided an indication of the protective capacity of the jelly coat of *Xenopus laevis* embryos.

The herbicidal auxin hormone mimic, 2,4-dichlorophenoxyacetic acid (2,4-D), is widely used for the control of broadleaf weeds in forestry, landscaping, industrial, and agricultural situations. In forestry, the aerial application of herbicides is used to reduce competing vegetation in replant areas. Small wetlands receive spray drift or are oversprayed during this procedure, increasing exposure to resident organisms such as amphibians (Thompson *et al.* 2004). The movement of 2,4-D through the cuticle of the target plants is inhibited by its water solubility (900 mg/L). To increase efficacy, the 2,4-D acid can be esterified, which increases its lipophilicity and subsequent movement through plant cuticular waxes. Esterification of auxinic herbicides also increases absorption into other organisms (Rodgers and Stalling 1972; Barron *et al.* 1990). In the case of 2,4-D BEE, the parent molecule has been shown to be quickly de-esterified in fish (Rodgers and Stalling 1972).

Materials and Methods

Animal Culturing

X. laevis adults were maintained in the Hagen Aqualab, University of Guelph, Guelph, ON, Canada under flow-through conditions using

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filtered, irradiated well water at 18°C under a light:dark cycle of 12:12 h. Adults were fed a rotation of beef or chicken liver and Frog Brittle (Nasco, Fort Atkinson, WI) twice per week. Breeding was induced by the injection of 600 and 800 IU of human chorionic gonadotrophin (Sigma-Aldrich Canada, Oakville, ON, Canada) into the dorsal lymph sac of males and females, respectively. Amplexus, egg laying, and fertilization occurred within 12 h. Culture water used in all breeding tanks, controls, and treatments conformed to the American Society of Testing and Materials guideline for the performance of the Frog Embryo Teratogenesis Assay–*Xenopus* (ASTM 1992). It comprised 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_42H_2O$, and 75 mg $MgSO_4$/L of deionized water. The pH of the culture water was adjusted to 7.2−7.4 using 1N HCl or 1N NaOH and was 21°C.

Test Substances and Analytical Chemistry

2,4-D acid (2,4-D) (99.5%; Lot # Moriss/1710) and the butoxyethyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D BEE) (99.5%; Lot # 1999C970153336) standards were supplied by Dow (Dow AgroSciences, Indianapolis, IN). ^{14}C-ring labeled 2,4-D was purchased from Sigma (Sigma-Aldrich Canada, Oakville, ON, Canada). ^{14}C 2,4-D had a formula weight of 221.0 g/mol, a specific activity of 2.9 mCi/mmol, and a radiochemical purity of 97.2%. Labeled 2,4-D BEE was prepared by acid-catalyzed esterification from the reagents of 2-butoxyethanol (Sigma-Aldrich Canada) and ^{14}C 2,4-D. First, the following chemicals were added to a 50-ml round-bottom flask: 125 µCi of ^{14}C 2,4-D dissolved in 6 ml of toluene + 2 ml 2-butoxyethanol + 1 ml concentrated HCl. The flask plus an attached Dean-Stark trap with a condenser was placed on a heating mantle with a multivariate temperature control and gently boiled for 2 h. The temperature was increased slightly for 30 min wherein 6 ml of additional toluene, 1 ml of HCl, and 1 ml of 2-butoxyethanol was added to the flask. After an additional 1 h of boiling, the flask was cooled. The contents, containing unreacted alcohol, ^{14}C 2,4-D, toluene, HCl, and ^{14}C 2,4-D BEE, was loaded onto a C_{18} separatory column. The column was eluted with 3 × 10 ml of water, and 3 × 10 ml of CH_3CN. The CH_3CN fraction contained 95% of the ^{14}C 2,4-D BEE produced. Using a rotary evaporator, the water and CH_3CN fractions were concentrated and resuspended in 1 ml acetonitrile. Eighty percent of the radioactivity was recovered. ^{14}C 2,4-D BEE was further purified by high performance liquid chromatography (HPLC) using an Agilent Series 1100 pump, autosampler, UV detector, and ChemStation software. The system used an Ultracarb 5 ODS (C18) 4.6 mm × 25 cm column and the following solvent system at a flow of 1 ml/min: 25% CH_3CN: 75% 0.1% formic acid in H_2O for 10 min; 75% CH_3CN: 25% 0.1% formic acid in H_2O for the remainder of the 22-min run. The radiolabel was quantified using an in-line EG&G Berthold Radioflow LB508 detector (Berthold GmbH & Co., Bad Wildbad, Germany) containing a 1000-µl flow cell and RadioStar software ver. 3.0. Fraction collection and liquid scintillation counting (LSC) were used when lower detection limits were needed. Retention time comparisons with standards were used to verify ^{14}C 2,4-D BEE and ^{14}C 2,4-D presence.

Experimental Protocol

Experiments began when embryos reached the blastula stages 8 to 9 (Gosner 1960). Minutes prior to exposure, half of the embryos were gently swirled in a 2% w/v cysteine solution at pH 8.1 for about 4 minutes (ASTM 1992). This removed the jelly coat while leaving the vitelline membrane and the developing embryo intact. Test units consisted of 200-ml glass beakers containing 100 ml of culture water spiked with 0.8 µmol/L to 1.2 µmol/L ^{14}C 2,4-D BEE. Twelve experimental units represented 6 time points for the uptake phase (0.5, 1, 2, 4, 6, 8 h) with two repetitions. Fifty embryos, representing a density of approximately 1.5 g/L, were added to each experimental unit; 25 with an intact jelly coat, and 25 that had been dejellied. The embryos were separated by placing one group in a small mesh basket suspended about 1 cm from the bottom of the beaker. The other group was placed on the bottom of the beaker. The group placed in the basket was alternated for the two experiments. Two additional experimental units contained 350 embryos at the same animal/water/chemical ratio as in the uptake phase. At 8 h, these embryos were moved to two 40-L aquaria each containing 20 L of clean culture water. Sample time points for the excretion phase were 1, 2, 4, 8, 14, and 22 h post-transfer for the first experiment, and 1, 2, 4, 8, 16, 40, and 65 h post-transfer for the second experiment.

Two beakers containing no ^{14}C 2,4-D BEE, one for dejellied embryos and one containing embryos without a jelly coat, were included in the experiment. At every sample time point, five embryos were removed from each unit, placed on a small piece of tin foil, the excess water drawn off, and subsequently weighed.

At each sample time point, two animal and two water samples were taken, one for HPLC and one for LSC. The first animal sample was prepared for analytical work using a modified method based on that of Barron *et al.* (1990). Twenty embryos were homogenized using a Polytron® (Brinkmann Instruments, Mississauga, ON, Canada) tissue homogenizer in 10 ml acetone/1% H_3PO_4. The homogenate was shaken at 4°C for 30 min, centrifuged at 6,000 *g* at 4°C for 30 min, and the supernatant was collected. After one additional 10-ml acetone/1% H_3PO_4 extraction, the pooled supernatant was evaporated to near dryness under vacuum at 45°C using a rotary evaporator. The contents were filtered through a 0.45-µm filter and samples were brought to 1 ml with deionized water (pH 3). The sample was loaded onto a C_8 column under vacuum where 2 ml of methanol was loaded and the elutant was collected. The sample was then brought to 1 ml under a nitrogen stream. HPLC was used to separate 2,4-D BEE and its metabolite, 2,4-D (the only metabolite observed). The mean recovery ± SEM for spiked embryo samples (with jelly coat) was 93.8 4.2% (n = 8) for ^{14}C 2,4-D BEE and 94.5 ± 4.3% (n = 6) for ^{14}C 2,4-D acid. No 2,4-D was observed in the 2,4-D BEE samples, demonstrating that 2,4-D BEE was not hydrolyzed to 2,4-D during the extraction procedure. For the second animal sample, total radioactive residues in the embryos were determined by placing five embryos in a 7-ml glass vial containing 180 µl of tissue solubilizer (Hydamine Hydroxide®; ICN Biomedicals, Irvine, CA). Samples were placed in a 60°C oven for 2–5 h until the tissue was digested. CytoScinct ES (ICN Biomedicals) was added and the radioactivity was measured using a Beckman LS6K-SC scintillation counter (Beckman Instruments Inc., Fullerton, CA). Limits of detection and quantification for radioactivity in embryo samples were 0.24 ηmol/g and 0.44 ηmol/g, respectively. To measure total radioactivity in the water, a 500-µl sample was added to EcoLite (+) (ICN Biomedicals) and measured using LSC. Water samples were directly injected into the HPLC.

Radiolabel Distribution Experiments

Whole-body autoradiography (WBARG), as described by Ullberg *et al.* (1982), was performed to determine the distribution of radioactive residues in embryos. About 80 embryos, at the blastula stages, were water exposed to 3.6 ηmol/ml ^{14C}-2,4-D BEE in 2 × 100 ml of culture water. The first exposure unit contained embryos with a jelly coat inclusive of 3 clumps (10–20 embryos/clump) plus 20 single embryos. The other exposure unit contained 40 embryos with their jelly coats removed just prior to exposure. After a 4-h exposure, embryos were removed from the experimental units by pipette and rinsed for 5 s in de-ionized water. Embryos were embedded in a 5%

carboxymethylcellulose gel and rapidly frozen to –80°C in a hexane/dry ice slurry. Frozen blocks were sectioned on tape (10-µm sections) at –25°C using a specially designed cryomicrotome (Jung Cryomacrocut, Leica, San Diego, CA). Sections were freeze dried at –25°C for 16 h and placed on flexible storage phosphor screens for 90 h. The digital scans of the phosphor screens were performed using a Cyclone Storage Phosphor System and were then visualized and quantified based on Digital Light Units (DLU)/mm^2 using the Optiquant software (Optiquant ver. 3.1, Packard Instrument Co., IL). To determine total radioactive residues, three samples of five embryos each were counted by LSC, as previously described.

To further characterize the distribution of 2,4-D in embryos, we examined the percentage of radiolabel found in the jelly coat compared to that found in the remaining embryo. Conditions were equivalent to the kinetic experiments in which 50 embryos, all with an intact jelly coat, were placed in one of three experimental units representing the sample time points of 2, 4, and 8 h. At each time point, the embryos were split into two groups of 25. One group had their jelly coats removed following the cysteine protocol as described above and the other group, with intact jelly coats, was swirled in culture water for an equivalent amount of time. Embryos in each group were then spilt into groups of five and the radioactive residue concentration was determined using LSC.

Data Analyses

The concentrations of 2,4-D BEE and its metabolite 2,4-D in embryos over time were fit to a multicompartmental model as represented in Figure 1. Differential equations were fit to the data using the nonlinear regression software WinNonlin (ver 3.1; Pharsight, Mountain View, CA) such that from 0 to 8 h:

$$\frac{dC_{TOTAL}}{dt} = (Cl_U * C_W) - (k_{EM} * C_{ACID})$$

$$\frac{dC_{ESTER}}{dt} = (Cl_U * C_W) - (k_M * C_{ESTER})$$

$$\frac{dC_{ACID}}{dt} = (k_M * C_{ESTER}) - (k_{EM} * C_{ACID})$$

and from 8 h onward:

$$\frac{dC_{TOTAL}}{dt} = -(k_{EM} * C_{ACID})$$

$$\frac{dC_{ESTER}}{dt} = -(k_M * C_{ESTER})$$

$$\frac{dC_{ACID}}{dt} = (k_M * C_{ESTER}) - (k_{EM} * C_{ACID})$$

where Cl_U was the water uptake clearance rate of 2,4-D BEE ($ml_{water} \cdot g^{-1}_{embryo} \cdot h^{-1}$), C_w = concentration of 2,4-D BEE in exposure water (ηmol/ml), C_{TOTAL} = concentration of total radioactive residues in embryos (ηmol/g), C_{ESTER} = concentration of 2,4-D BEE in embryos (ηmol/g), C_{ACID} = concentration of 2,4-D in embryos (ηmol/g),

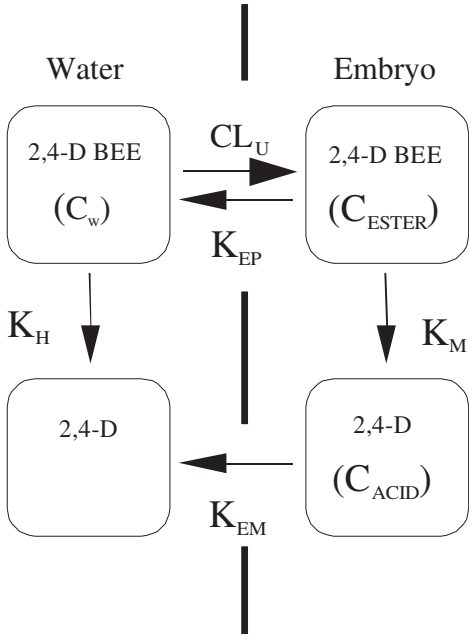

Fig. 1. Schematic representation of a 2,4-D butoxyethyl ester (BEE) kinetic model in *Xenopus laevis* embryos. Cl_U = water uptake clearance rate of 2,4-D BEE, k_{EP} = rate of 2,4-D BEE excretion, C_w = concentration of 2,4-D BEE in exposure water, C_{ESTER} = concentration of 2,4-D BEE in embryos, C_{ACID} = concentration of 2,4-D in embryos, k_M = rate of 2,4-D BEE metabolism, k_{EM} = rate of 2,4-D excretion, and k_H = rate of 2,4-D BEE removal from water due to chemical hydrolysis and/or volatilization

k_M = rate of 2,4-D BEE metabolism (h^{-1}), and k_{EM} = rate of 2,4-D excretion (h^{-1}). Minimization was performed using the Gauss-Newton (Levenberg and Hartley) process with a maximum of 50 iterations and a convergence criterion of 0.0001.

A parameter for 2,4-D BEE excretion (k_{EP}, Figure 1) was not included in the model, although the validity of the models, inclusive of k_{EP}, was tested. Although the Akaike's Information Criterion (Yamaoka *et al.* 1978) values slightly decreased in all of the curve fits, thus suggesting an increased correctness of the models, the parameter estimates were either nonsensical or had coefficients of variation greater than 100%. Therefore, the more parsimonious model was chosen that did not include 2,4-D BEE excretion. This was in accordance with research using a similar compound, triclopyr BEE, in fish where the assumption was made that ester excretion did not occur (Barron *et al.* 1990).

The concentration of 2,4-D BEE in the water, C_W, was represented by a linear equation of 2,4-D BEE in the water over time as measured by HPLC and thus represented the decline of 2,4-D BEE in the water due to ester hydrolysis to 2,4-D and/or volatilization (k_H; Figure 1) and uptake by embryos (CL_U; Figure 1). Therefore, through each iteration of the model, the 2,4-D BEE water concentration was recalculated to account for the decline of the highly absorbable 2,4-D BEE form. Assumptions of the model were that 2,4-D uptake was insignificant as compared to 2,4-D BEE uptake [Barron *et al.* 1990 (assumed), Rodgers and Stalling 1972 (tested)] and that 2,4-D BEE deesterification to 2,4-D was irreversible.

To determine the effect of using weight-normalized concentrations versus per-embryo concentrations, half-lives for the embryos with jelly coats were also calculated (8 h to final time point) using the noncompartmental approach in WinNonlin, (ver. 3.1).

Table 1. Kinetic values and experimental conditions for water-exposed *Xenopus laevis* embryos, with or without the embryonic jelly coat, to the butoxyethyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D BEE)

	Repetition 1		Repetition 2	
Parameter	Jelly coat removed	Jelly coat intact	Jelly coat removed	Jelly coat intact
CL_u ($ml_{water} \cdot g_{embryo}^{-1} \cdot h^{-1}$)	21.8 ± 1.18	4.5 ± 0.30	14.4 ± 0.99	1.5 ± 0.24
CL_u ($ml_{water} \cdot h^{-1}$) per embryo	0.040 ± 0.002	0.021 ± 0.001	0.022 ± 0.002	0.0066 ± 0.001
k_M (h^{-1})	1.2 ± 0.35	1.2 ± 0.38	1.0 ± 0.29	1.2 ± 0.48
k_{EM} (h^{-1})	0.066 ± 0.0092	0.086 ± 0.013	0.078 ± 0.012	0.27 ± 0.061
C_{max} total (ηmol/g)	91.4	18.0	77.3	4.7
C_{max} total (ηmol/embryo)	0.17	0.085	0.12	0.021
^{14}C 2,4-D BEE curve R^2	0.63	0.77	0.73	0.63
^{14}C 2,4-D curve R^2	0.82	0.32	0.87	0.49
^{14}C total curve R^2	0.70	0.71	0.81	0.38
Concentration of 2,4-D BEE in water at test start (ηmol/ml)	0.86	0.86	1.21	1.21
Embryo weight per 5 embryos (mg ± SEM)	9.2 ± 0.18 (0–30 h)	23.7 ± 0.73 (0–22 h) 42.0 ± 0.25 (30 h)	7.5 ± 0.35 (0–72 h)	22.0 ± 0.45 (0–24 h) 64.9 ± 2.0 (48 h) 9.8 ± 0.4 (72 h)

Note. The embryonic jelly coat was either left intact or removed by swirling in a 2% w/v cysteine solution at pH 8.1. Cl_U water uptake clearance rate of 2,4-D BEE, k_M rate of 2,4-D BEE metabolism, k_{EM} rate of 2,4-D excretion from embryos, C_{max} maximum total concentration of radioactive residues in embryos. Standard deviations were model estimated using the individual measurements from the two repetitions at each sample time point. Coefficients of determination are presented for each model-derived curve.

Generated curves were compared using *F*-tests and mean comparisons were performed using *t*-tests. The Type I error rate was set at 0.05.

Results

Embryos went through a number of life stages during the tests. The timing of embryonic development was as follows: test start = blastula (Gosner stage 8–9), 6–9 h = large yolk plug (Gosner stage 11), 16 h = neural plate visible (Gosner stage 13), 24 h = tail bud to heartbeat (Gosner stage 17–19). Embryos that did not have a jelly coat were considerably more advanced at this point than those with a jelly coat. Dejellied embryos were almost all hatched out of the vitelline membrane by 36 h, whereas those with a jelly coat hatched after 48 h but before 72 h. Furthermore, the weight of the dejellied embryos remained constant throughout both experiments. The embryos with a jelly coat were 2.5 times heavier than dejellied embryos from 0 to 24 h (Table 1). At 30 h until at least 48 h, these embryos doubled or tripled in weight, likely due to water absorption in the jelly coat. Once hatched out of the jelly coat and vitelline membrane, the embryos were of similar weight to the dejellied embryos, suggesting that the previous weight difference was due only to the jelly coat. By hatching, total radioactive residues in the embryos were close to background levels (dejellied embryos) or not detectable (embryos with jelly coat).

Comparisons of curves generated from the embryos with and without the jelly coat were significantly different for the first ($P_{210,\ 2,\ 70} < 0.001$) and second ($P_{188,\ 2,\ 66} < 0.001$) experimental runs (Figure 2). The dejellied embryos had water uptake clearance rates greater than their jellied counterparts, meaning that they absorbed much more of the 2,4-D BEE from the water (Table 1). This is also demonstrated by a comparison of the maximum concentrations achieved within each experiment on both a per-gram and a per-embryo basis (C_{max}, Table 1). The rates of metabolism of 2,4-D BEE to 2,4-D between experimental runs and between embryos with or without a jelly coat were very consistent with half-lives of 2,4-D BEE metabolism ranging from 35 to 42 min. 2,4-D excretion rates, based on concentrations related to wet weight, were generally consistent with half-lives ranging from 8.1 to 10.5 h, except in the second experiment using embryos with a jelly coat where the model estimated half-life of excretion was 2.5 h. The excretion phase was not well represented by the model for this group, resulting in a faster elimination than was actually observed. Using noncompartmental analysis of total radioactive residues during the elimination phase for embryos with a jelly coat, the half-life using concentrations per embryo was greater than that if the analysis was done on a per wet weight basis [Rep #1, $t_{1/2}$ = 7.4 h (per wet weight; R^2 = 0.90) and $t_{1/2}$ = 9.5 h (per embryo; R^2 = 0.77); Rep #2, $t_{1/2}$ = 16.2 h (per wet weight; R^2 = 0.68) and $t_{1/2}$ = 24.7 h (per embryo; R^2 = 0.42). Radioactivity was not detectable in water samples taken during the excretion phase of the experiments.

WBARG was used to observe the distribution of radioactive residues in the embryos. The jelly coats were not clearly visible in either the cryomicrotome sections or their phosphor screen images (Figure 3). For quantification using the Optiquant software, only the embryo within the jelly coat was counted if the jelly coat was present. Total radioactive residues, as determined using Optiquant, were 93,393 ± 4278 (DLU/mm^2/embryo ± SEM; n = 40 sections from 9 embryos) for embryos with a jelly coat and 185,778 ± 4283 (n = 120 sections from 20 embryos) for dejellied embryos. These were significantly different (t = 11.8, df = 158, $p < 0.001$) and well represented the total radioactive residues, as determined by LSC, where embryos with a jelly coat contained 50% of that which was observed in dejellied embryos [0.365 ± 0.056 ηmol/embryo with a jelly coat (n = 3), and 0.758 ± 0.034 ηmol/embryo without a jelly coat (n = 3)]. Three clumps of embryos with intact jelly coats were also examined and compared to single embryos with a jelly coat. Total radioactive

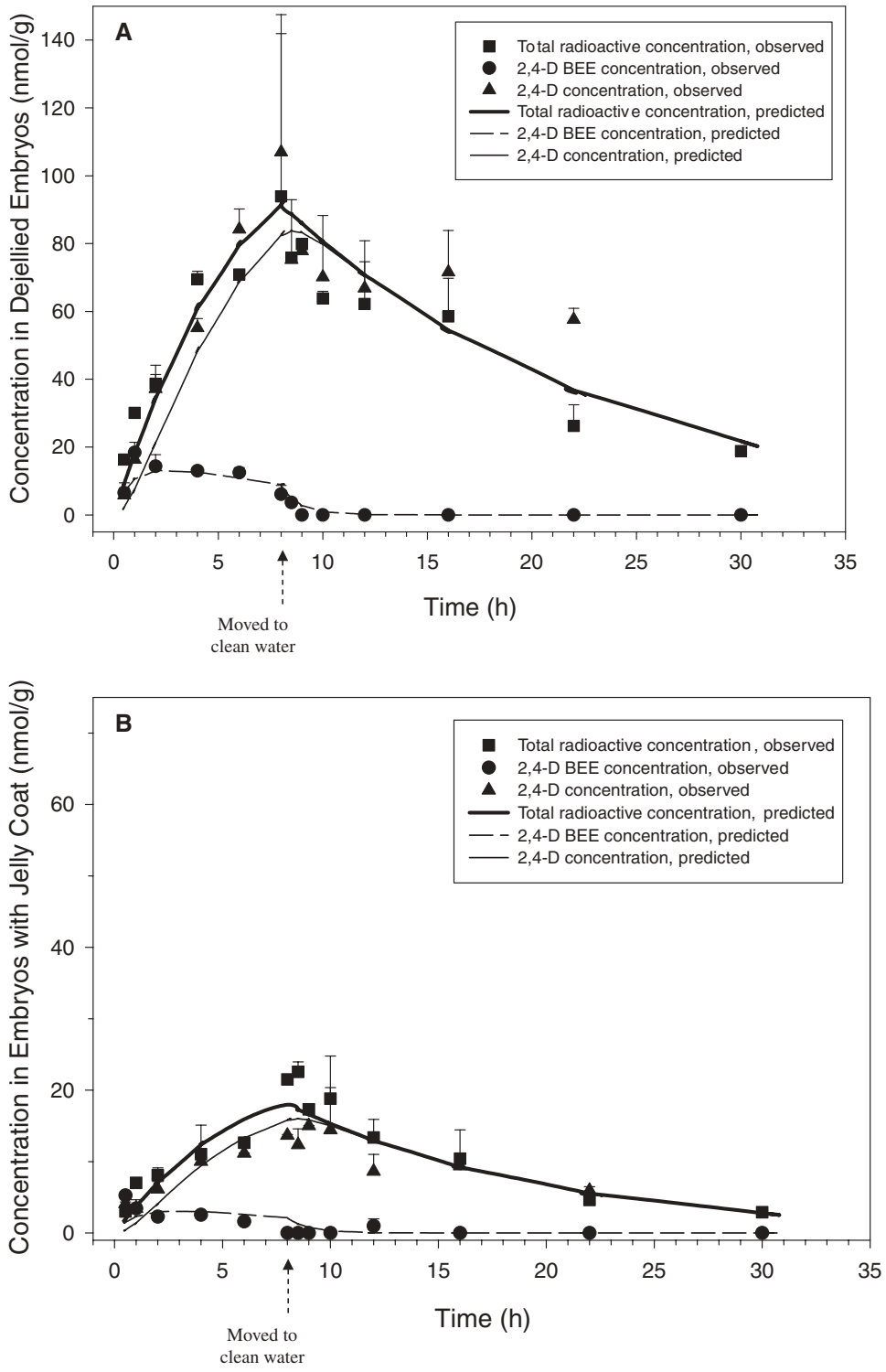

Fig. 2. Radioactive residues in dejellied (**A**) and jellied (**B**) *Xenopus laevis* embryos during an 8-h static water exposure to 0.86 ηmol/ml ^{14}C 2,4-D butoxyethyl ester (BEE) followed by a 22-h excretion period in clean water (experimental repetition 1). Observed values are the mean and standard error of total radioactive 2,4-D BEE and 2,4-D concentrations in embryos and were based on two observations at each time point. Notice the scale difference on the *y*-axis where the jellied embryo scale is half that of the dejellied embryo scale

residues were significantly lower ($t = 6.1$, $df = 213$, $p < 0.001$) in the embryos within a clump (55,021 ± 2806, $n = 179$ from 3 clumps, 5 to 8 sections per clump containing 6 to 12 embryos) than in single embryos with a jelly coat (see above). Visually from Figure 3, radioactive residues appeared to be lower in the perivitelline space located between the embryo and the vitelline membrane. It also appeared as if some accumulation occurred within the blastocoels of the embryos (Figure 3).

Because the jelly coats were not easily observable using WBARG, LSC was used to determine the amount of total radioactive residues in the jelly coat in comparison to the embryo. Using this technique, approximately 35% of the radioactivity was counted in the jelly coat and 65% in the

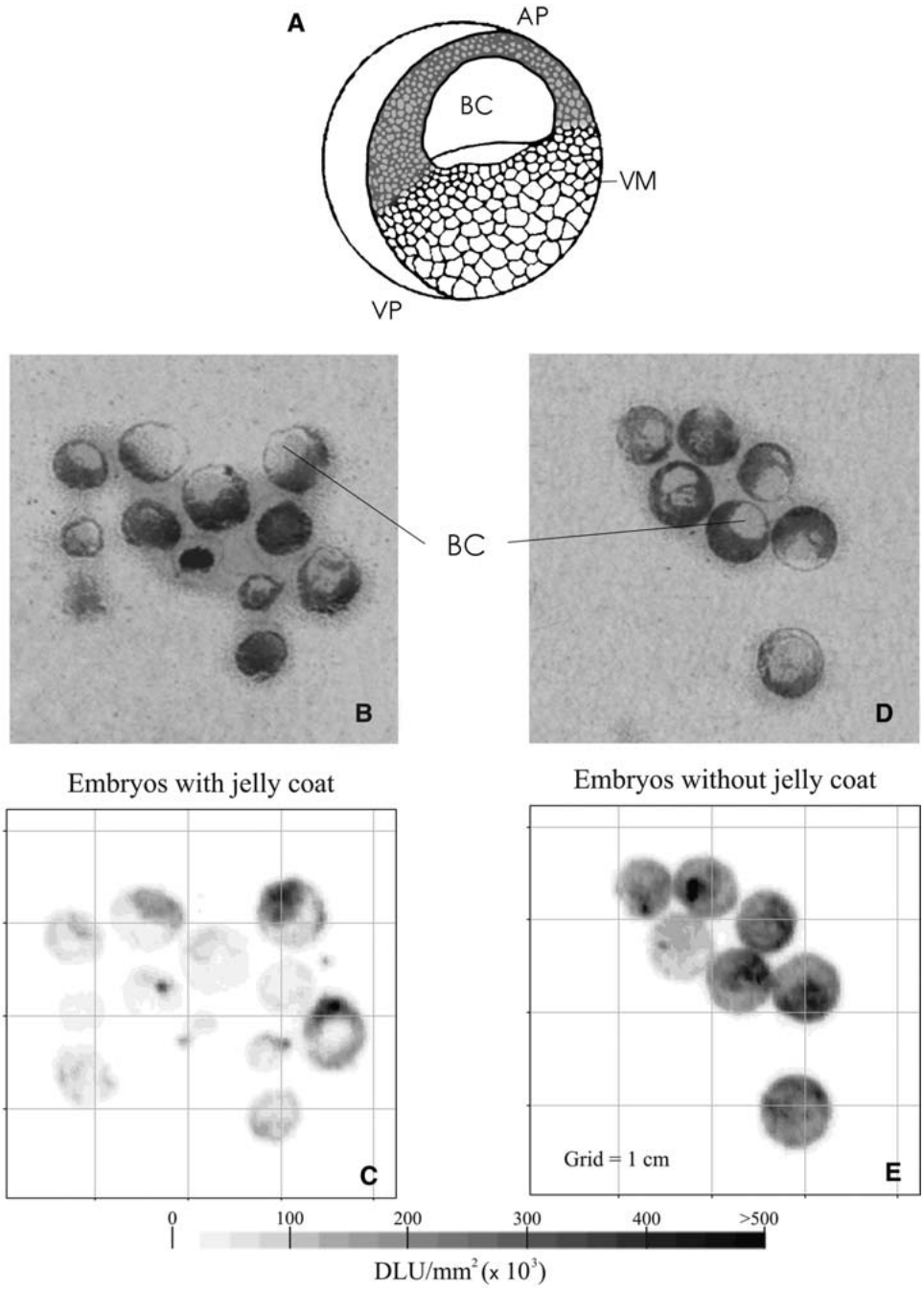

Fig. 3. Whole-body autoradiography of radioactive residues in *Xenopus laevis* blastulae after exposure to 3.6 ηmol/ml ^{14}C 2,4-D BEE for 4 h. (**A**) Cross-sectional schematic of an amphibian blastula inclusive of the animal pole (AP), vegetal pole (VP), vitelline membrane (VM), and blastocoel (BC). The jelly coat is not presented but would surround the embryo entirely. (**B**) Scanned 10-μm cryomicrotome section of a clump of embryos with intact jelly coats and (**C**) the corresponding phosphor screen image. (**D**) Scanned 10-μm cryomicrotome section of dejellied embryos and (**E**) the corresponding phosphor screen image. The highest radiolabel concentrations correspond to red areas. Units on figure are in millimeters. Notice the accumulation of radiolabel in the blastocoel and the lack of radiolabel in the perivitelline space between the vitelline membrane and the cells of the embryo

actual embryo. The amount of label in the jelly coat was consistent across exposures of 2 h (35.3 ± 2.4%), 4 h (35.1 ± 2.2%), and 8 h (33.4 ± 2.1%), based on five samples at each time point.

Discussion

The major jelly coat components of amphibian embryos are mucin-type glycoproteins that are highly O-glycosylated (Delplace *et al.* 2002). During the egg's passage through the oviduct, layers of these glycoproteins, each with specific morphology and composition, are added in concentric rings. The carbohydrate-rich fiber matrices of the jelly coat are structurally diverse and species specific (Delplace *et al.* 2002). Between the jelly coat and the embryo lies the vitelline membrane, which also consists of glycoproteins (Wolf *et al.* 1976). In *X. laevis*, about 35% of the radioactive residues were located in the jelly coat. Based on the jelly coat composition, especially its lack of lipids, it is not believed that there was preference towards accumulation of the lipophilic parent compound, 2,4-D BEE (log k_{ow} = 4.1) (Syracuse Environmental Research Associates 1999) in the jelly coat. The water-soluble metabolite, 2,4-D (log k_{ow} = –0.75 at pH 7) (Syracuse Environmental Research Associates 1999), may have been bound to jelly coat components, considering that 2,4-D is

principally an anion above pH 4, but was likely present due simply to diffusion into and out of the embryo. Distribution appeared to differ using WBARG because the jelly coat appeared not to contain radioactive residues above background levels (about 7000 DLU/mm^2). The actual amount of radioactive residues present in the jelly coat cannot be reconciled from these two techniques. WBARG effectively showed that the radioactive residues were present throughout the embryo. The apparent slight accumulation of radioactive residues in the water-filled blastocoel in embryos of both groups was likely the more water-soluble metabolite, 2,4-D, whereas the lipophilic parent compound, 2,4-D BEE, would have an affinity for yolk cells in the vegetal pole.

Researchers have hypothesized that the jelly coat provides protection to the embryo during toxicant exposure (Berrill *et al.* 1994; Licht 1985). The vitelline membrane, which is still attached after the dejellying procedure, provides some protection to the embryo (Edginton *et al.* 2003, 2004). For example, the presence of the jelly coat has been found to significantly decrease *Bufo americanus* and *X. laevis* embryo sensitivity by delaying mortality when exposed to the herbicide, Roundup (glyphosate) and the insecticide, diazinon (Griebeling *et al.* 2003). This delay in mortality was likely the result of a slower water uptake clearance rate for embryos with a jelly coat in comparison to dejellied embryos, as was demonstrated for *X. laevis* embryos in this study. This finding may be significant to the performance of FETAX (ASTM 1992), especially when examining a compound for teratogenic effects. This guideline calls for *X. laevis* embryos to be dejellied, using the cysteine procedure as described above. Removal of the jelly coat allows the embryos to be more easily manipulated by preventing the sticky jelly coats from adhering to pipettes or dishes. The faster absorption of compounds, which may increase the rate of mortality, may not necessarily lead to a change in the end result of absolute mortality in the FETAX assay because it is conducted over 4 days. However, teratogenic effects that are developmental stage specific may be overestimated with removal of the jelly coat. It is recommended that the jelly coat remain intact for the performance of FETAX to account for the delay in chemical absorption, particularly if teratogenicity is a major endpoint.

Compared to many native Canadian species, such as Ranids and Bufonids, the jelly coat of *X. laevis* embryos is relatively thin. It is expected that the thickness of the jelly coat affects water uptake clearance rates as well as the elimination rate. Thus, Ranid and Bufonid embryos, having thicker jelly coats, would have lower uptake and elimination rates than was determined for *X. laevis* in this study. To further explain this phenomenon, the model for oxygen diffusion developed for amphibian embryos and embryo masses was examined (Seymour and Bradford 1995). The movement of oxygen into the embryo is in accordance with the Fick diffusion equation. This equation accounts for the size of the egg sphere and demonstrates that, as the thickness of the jelly coat increases, the rate of oxygen uptake by the embryo decreases. Oxygen uptake and the uptake of 2,4-D BEE are both first-order diffusive processes and are driven by concentration differentials. The partial pressure of oxygen is much greater on the outside of the embryo in comparison to the inside where the embryo effectively consumes it. Similarly, during 2,4-D BEE water exposure, the metabolism of 2,4-D BEE within the embryo helps to maintain a gradient that favors diffusion into the embryo. Specifically, the protective role of the jelly coat may be greater in amphibians with thicker jelly coats and has significance for comparative toxicity studies using different amphibian species.

Although the jelly coat reduces chemical uptake by individual embryos, the deposition of embryo masses may provide even greater protection. The lower radioactive residues present in our relatively small *X. laevis* embryo clumps demonstrated this. Again, using the oxygen diffusion model, in firm embryo masses, such as those of the amphibian *Ambystoma maculatum*, the partial pressure of oxygen was drastically reduced towards the center because of uptake from neighboring embryos during oxygen diffusion through the mass (Pinder and Friet 1994). Even using the loose embryo masses of *Rana sylvatica*, where water convection occurred through channels between embryos, thus constantly providing oxygen, the partial pressure of oxygen consistently declined towards the center of the mass (Pinder and Friet 1994). Although it appeared from Figure 3 that the outer embryos of the mass absorbed more radioactivity, it was quite difficult to determine which embryos were actually on the outside of the mass because of the two-dimensional (2D) image of a 3D space. Furthermore, the short time of exposure in the experiment did not allow the clump to reach steady state, thus potentially overestimating the protective effect of clumping. Nonetheless, uptake by neighboring embryos on the outer edges of a mass may reduce local concentrations of 2,4-D BEE and this may explain the lower residues within the *X. laevis* embryos clumps as demonstrated in this study. The practice of disrupting clumps and dejellying embryos in the performance of the FETAX assay raises concerns over the environmental relevance of this assay.

Once 2,4-D BEE entered an embryo, metabolism, distribution, and excretion appeared to remain similar. It was not surprising that metabolism and distribution remained constant because the developing embryo was not altered, only its surroundings. Excretion, if examined using concentrations in the embryo based on wet weight, were similar between groups. However, when using an amount per embryo, the half-lives were slower for dejellied embryos than for embryos with a jelly coat, a phenomenon that increased in magnitude as the experimental time increased. Thus, a rapid weight gain appeared to accelerate the elimination of chemical towards the end of the trials with jellied eggs, and an artificial half-life was generated from concentrations based on wet weight. It appeared as if the jelly coat of *X. laevis* embryos delayed the absorption of 2,4-D BEE during water exposure, while also limiting 2,4-D excretion.

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References

American Society for Testing and Materials (1992) Standard guide for conducting the Frog Embryo Teratogenesis Assay–*Xenopus*

(FETAX). E1439-91. In: Annual book of ASTM standards, vol 11.04. Philadelphia, Pennsylvania, pp 1199–1209

Barron MG, Mayes MA, Murphy PG, Nolan RJ (1990) Pharmacokinetics and metabolism of triclopyr butoxyethyl ester in Coho salmon. Aquat Toxicol 16:19–32

Berrill M, Bertram S, McGillivary L, Kolohon M, Pauli B (1994) Effects of low concentrations of forest-use pesticides on frog embryos and tadpoles. Environ Toxicol Chem 13:657–664

Berrill M, Coulson D, McGillivary L, Pauli B (1998) Toxicity of endosulfan to aquatic stages of anuran amphibians. Environ Toxicol Chem 17:1738–1744

Delplace F, Maes E, Lemoine J, Strecker G (2002) Species specificity of O-linked carbohydrate chains of the oviducal mucins in amphibians: structural analysis of neutral oligosaccharide alditols released by reductive B-elimination from the egg-jelly coats of *Rana clamitans*. J Biochem 363:457–471

Edginton AN, Sheridan PM, Stephenson GR, Thompson DG, Boermans HJ (2004) Comparative effects pH and Vision herbicide on two life stages of four anuran amphibian species. Environ Toxicol Chem 23:815–822

Edginton AN, Stephenson GR, Sheridan PM, Thompson DG, Boermans HJ (2003) Effect of pH and Release on two life stages of four anuran amphibians. Environ Toxicol Chem 22:2673–2678

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190

Griebeling KH, Cline GR, Rayburn JR (2003) Role of the jelly coat in the development of two frog species exposed to Roundup and diazinon, using FETAX. PM004. Presented at the 24th Annual Society of Environmental Toxicology and Chemistry Conference, Austin, Texas, USA, November 9–13, 2003

Licht LE (1985) Uptake of ^{14}C DDT by wood frog embryos after short term exposure. Comp Biochem Physiol C: Toxicol Pharmacol 81C:117–119

Licht LE (2003) Shedding light on ultraviolet radiation and amphibian embryos. Bioscience 53:551–561

Pauli B, Coulson D, Berrill M (1999) Sensitivity of amphibian embryos and tadpoles to Mimic 240LV insecticide following single or double exposures. Environ Toxicol Chem 18:2538–2544

Pinder AW, Friet SC (1994) Oxygen transport in egg masses of the amphibians *Rana sylvatica* and *Ambystoma maculatum*: convection, diffusion and oxygen production by algae. J Exp Biol 197:17–30

Rodgers CA, Stalling DL (1972) Dynamics of an ester of 2,4-D in organs of three fish species. Weed Sci 20:101–105

Seymour RS, Bradford DF (1995) Respiration of amphibian eggs. Physiol Zool 68:1–25

Syracuse Environmental Research Associates, Inc. (1999). 2,4-Dichlorophenoxyacetic acid formulations – Human health and ecological risk assessment. SERA TR 95-21-09-01d. www.fs.fed.us/foresthealth/pesticide/risk_assessments/09170224d.pdf Accessed May 10, 2006

Thompson DG, Wojtaszek BF, Staznik B, Chartrand DT, Stephenson GR (2004) Chemical and bio-monitoring to assess potential acute effects of Vision herbicide on native amphibian larvae in forest wetlands. Environ Toxicol Chem 23:843–849

Ullberg S, Larsson B, Tjalve H (1982) Autoradiography. In: Gleen HJ, (ed) Biological applications of radiotracers. CRC Press, Boca Raton, FL, pp 56–108

Wolf DP, Nishihara T, West DM, Wyrick RE, Hedrick JL (1976) Isolation, physicochemical properties, and the macromolecular composition of the vitelline and fertilization envelopes from *Xenopus laevis* eggs. Biochemistry 15:3671–3678

Yamaoka K, Nakagawa T, Uno T (1978) Application of Akaike's Information Criterion (AIC) in the evaluation of linear pharmacokinetic equations. J Pharmacokin Biopharm 6:165–175