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Metabolism of Diazinon in Rainbow Trout Liver Slices

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Abstract

Introduction—Understanding biotransformation pathways in aquatic species is an integral part of ecological risk assessment with respect to the potential bioactivation of chemicals to more toxic metabolites. The long-range goal is to gain sufficient understanding of fish metabolic transformation reactions to be able to accurately predict fish xenobiotic metabolism. While some metabolism data exist, there are few fish *in vivo* exposure studies where metabolites have been identified and the metabolic pathways proposed. Previous biotransformation work has focused on *in vitro* studies which have the advantage of high throughput but may have limited metabolic capabilities, and *in vivo* studies which have full metabolic capacity but are low throughput. An aquatic model system with full metabolic capacity in which a large number of chemicals could be tested would be a valuable tool.

Materials and Methods—The current study evaluated the *ex vivo* rainbow trout liver slice model, which has the advantages of high throughput as found *in vitro* models and non-differentiated cells and cell to cell communication found in *in vivo* systems. The pesticide diazinon, which has been previously tested both *in vitro* and *in vivo* in a number of mammalian and aquatic species including rainbow trout, was used to evaluate the *ex vivo* slice model as a tool to study biotransformation pathways.

Results/Discussion—While somewhat limited by the analytical chemistry method employed, results of the liver slice model, mainly that hydroxypyrimidine was the major diazinon metabolite, are in line with the results of previous rainbow trout *in vivo* studies.

Conclusion—Therefore, the rainbow trout liver slice model is a useful tool for the study of metabolism in aquatic species.

Keywords

metabolism; trout; liver slices; diazinon; biotransformation

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Introduction

There are many challenges involved in predicting chemical environmental exposure pathways, such as mechanism of chemical uptake into the organism, accounting for environmental degradation and biotransformation, and understanding effects of metabolism. In particular biotransformation has been less studied than some of these other challenges in part due to the difficulty of working with such a complex phenomenon. Biotransformation focuses not only on parent chemical uptake but metabolite formation and metabolic pathways of biotransformation reactions. Metabolic transformation data is of value to inform toxicological risk assessors where a metabolite may be equally toxic, or more toxic, than the parent chemical form. Likewise, a thorough understanding of metabolic biotransformation across species and chemical classes is essential in ecological risk assessments.

While some fish metabolism data exists, there are relatively few *in vivo* exposure studies where metabolites have been identified and fish metabolic pathways proposed.¹ *In vivo* studies can be costly in time, space, and expense to perform. More often fish studies look at microsomal metabolism or other *in vitro* metabolism assays to identify and characterize metabolites, but these systems lack many pharmacokinetic aspects of the *in vivo* system that govern chemical uptake and distribution.^{2,3} Rainbow trout have been used for *in vivo* and *in vitro* chemical kinetics and metabolism studies but many of these studies focused on loss of parent chemical and not biotransformation.^{4,5,6,7}

There is a need for *ex vivo* models that resemble the *in vivo* environment in that they have the complex intercellular interaction as *in vivo* systems and cells which have not dedifferentiated as often found in *in vitro* systems. The *ex vivo* precision cut liver slice model has proven to be useful for the study of mammalian drug metabolism in part because within the slice intercellular interactions are maintained and cells have not dedifferentiated.^{8,9} While many slice metabolism studies have focused on drug metabolism in mammalian systems this technique has been applied to study other chemicals such as pesticides and with other species such as fish.^{10,11,12,13,14} An additional benefit of the liver slice model is one fish can supply 100 to 200 slices, sufficient for an entire dose-response test, thus greatly reducing the number of fish used relative to a similar *in vivo* test.¹⁵ This eliminates the problem of fish-to-fish variability and leads to reduced animal use which in turn leads to reduced costs. Alternatively, the same model could be used to assess the variability between fish within a species if needed by running multiple experiments, or to address differences between species. Previous work by this lab using the rainbow trout liver slice model to study chemically-induced vitellogenin gene expression found liver slices were capable of metabolizing an inactive chemical methoxychlor to a gene inducing chemical dihydroxymethoxychlor.¹⁴

Use of the rainbow trout liver slice model as a tool to study metabolism requires the evaluation of the system with a chemical which has been tested in rainbow trout *in vivo* and *in vitro* systems. Of interest to this research group is the metabolism of pesticides. The organophosphate insecticide diazinon (DZ) has been tested *in vivo* and *in vitro* in both fish and mammalian species.^{1,2,16,17} Identified DZ metabolites in fish include hydroxypyrimidine (PYR), dehydrodiazinon (DH-DZ, isopropenyl diazinon), diazoxon

(DZO), isopropenyl diazinon, hydroxymethyl diazinon and hydroxy diazinon.^{1,2,3} Seguchis and Asaka, in an *in vivo* study of rainbow trout, loach (*Misgurnus anguillicaudatus*), and carp (*Cyprinus carpio*) found PYR was the main DZ metabolite in all species.¹ They also found some species specific differences such as DH-DZ was found in carp and rainbow trout but not loach. In a related *in vitro* study, Fuji and Asaka², using subcellular liver fractions, identified PYR as the major rainbow trout DZ metabolite but PYR was not found in exposures to yellowfin (*Seriola quinqueradiata*) liver fractions. Unlike the *in vivo* study the *in vitro* study found DZO in all species tested as previously observed by Hogan and Knowles.^{2,3} The amount of both *in vivo* and *in vitro* DZ metabolism data along with the differences in DZ metabolism seen between assay systems and among different species makes DZ a good candidate to evaluate the rainbow trout liver slice assay.

The long-range goal of this research is to gain sufficient understanding of fish metabolic transformation, including types of chemical reactions and pathway sequences, in order to accurately predict xenobiotic metabolism in fish. There is a need to know what metabolites are formed to then be able to assess potential toxicity of metabolites and how this may in turn affect vulnerability across taxa. Comparison of this fish metabolism data to mammalian data will help reduce uncertainty regarding conservation of metabolism across taxa, especially for metabolites deemed residues of concern in pesticide risk assessments. The first step in achieving the long range goal is to develop and evaluate a biological model to address the larger questions. Thus, the short term goal and the focus of this paper is to evaluate the rainbow trout liver slice model as a chemical metabolism tool by using it to study the metabolism of the pesticide diazinon.

Materials and Methods

Fish

Immature rainbow trout (*Oncorhynchus mykiss*), Erwin strain, used for production of liver slices were obtained from US Geological Survey, Upper Midwest Environmental Science Center (LaCrosse, WI). Fish were allowed to acclimate for at least two weeks prior to use in Lake Superior water (2- μ m filtered, ultraviolet light treated, 11°C, pH = 7.7, hardness = 45 mg/l) and kept on a constant photoperiod of 16-h light: 8-h dark. Fish body weights ranged from 210 to 873 grams, and liver weights from 2.35 to 7.46 grams. Hepatic-somatic index ranged from 0.9 to 1.1%. Male and female trout were used for this study. Sex was determined by visual inspection of gonads.

Chemicals

Test chemicals and critical assay reagents, along with the acronyms used to identify the chemicals, Chemical Abstract Services Registry Number (CASRN), source, and purity are listed in Table 1. All other chemicals used for biological and chemical analysis were purchased from Sigma Aldrich unless otherwise specified (Sigma Aldrich, St Louis, MO, USA).

Rainbow Trout Liver Slice Metabolism Assay-DZ exposure.

Precision cut trout liver slices were prepared and exposed to test chemical as previously described for various lengths of time at 11°C in a 12-well tissue culture plate.^{13,14} Liver cores (8 mm) were prepared using a modified tissue coring press (Model MD2000; Alabama Research and Development, Munford, AL). Liver cores were cut into 200 mm thick slices using a Krumdiek precision tissue slicer (Alabama Research and Development). DZ stocks prepared in ethanol were diluted to 200 µM in exposure media consisting of phenol red-free L-15 media (Gibco, Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS), and penicillin (100 U/mL)/streptomycin (100 ¼g/mL). A typical test for metabolic activity of a selected chemical consisted of three elements: a) one set of plates for measuring phase I metabolism (PI); b) another set of plates for measuring phase II metabolism (PII); and, c) third set for measuring the effect of exposure media without slice on parent chemical concentration (media only, MO). The potential loss of parent chemical was measured in all three sets of plates. A loss of parent chemical in PI and PII plates would presumably be due to a combination of phase I and phase II metabolism. Phase II samples underwent a beta-glucuronidase enzyme hydrolysis procedure prior to processing for analysis, see section Analytical Chemistry for details. DZ was tested at 200 µM with three replicates at each time point. Media and slice samples for chemical analysis were taken at 0, 1, 3, 6, and 24 hours. Ethanol solvent controls, 0.054% final concentration, were incubated under the same conditions and sampled at the same time points. Chemical test solutions were prepared, then dispensed into the appropriate wells. For example, 200 µM DZ was prepared by the addition of 43.2 µl of 367 mM DZ to 80 ml of exposure media. Then 1.7 ml of this test solution was added to the appropriate wells of the three test elements PI, PII, and MO. Test solutions in the 12 well plates were allowed to equilibrate to incubation temperature before the addition of one liver slice per well.

Assay Media Conditions and Liver Slice Viability.

Media pH and osmolality were monitored using a Radiometer Blood Gas Analyzer ABL800 Flex (Radiometer Medical, Copenhagen, Denmark). Presence of precipitation or chemical insolubility in the slice media was determined by visual inspection.

Toxicity of DZ was measured in a parallel set of plates prepared with the same exposure media and slices as used in the metabolism plates. Samples for toxicity measurements were only taken at 24 hours. The mitochondrial dehydrogenase assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) as substrate was used to assess cytotoxicity in the liver slices.¹⁸ As an additional toxicity measurement, the amount of lactate dehydrogenase enzyme (LDH) leakage from liver slices into media was measured following the 24 or 48 h incubation and compared with total slice LDH activity in the slices at 0 h as previously described.^{13,14,19} Prior to testing for metabolic activity, DZ toxicity to liver slices was assessed at 10, 100, 200, 500, 1000 and 2000 µM.

DZO Stability in Exposure Media

Preliminary tests indicated loss of the potential metabolite DZO in exposure media even in the absence of liver slices. To determine if the enzymes found in serum, which is critical to cell health in culture, were responsible for the loss, DZO 200 µM was incubated under

normal exposure condition in normal exposure media and exposure media without FBS. The [PYR] in media was measured in triplicate samples at 0, 3 and 24 hours. The [DZ] in media was measured in triplicate samples at 3 and 24 hours.

Inhibition of Serum Enzyme Activity

Activity of serum enzyme paraoxonase 1(PON-1), which is known to hydrolyze the organophosphates parathion and diazoxon, can be inhibited by 2-hydroxyquinoline (HQU) .^{20,21} Exposure media was prepared as described earlier and divided into 3 equal parts. To two of these parts HQU was added to final concentrations of 10 and 20 μ M. Then DZO, 200 μ M final concentration, was added to all three exposure media types; no HQU, 10 μ M HQU and 20 μ M HQU. These solutions were added to incubation plates and held under normal incubation conditions. Chemical analysis was performed on triplicate media samples at 0, 3, 24, and 48 hours.

Enzyme Inhibition Study-DZ.

An enzyme inhibition test was used to maximize the potential detection of DZO in slice and media samples exposed to DZ by inhibiting the activity of DZO metabolizing enzymes found in the serum of the exposure media and within the slices. Exposure media was prepared as described in section Rainbow Trout Liver Slice Metabolism Assay-DZ exposure, with the exception of the addition of the HQU 20 μ M prior to the addition of DZ. Livers slice preparation and exposure to DZ was performed as described earlier with the exception of two sets of exposures were prepared one with and without HQU.

Analytical Chemistry.

Exposure media and slice when present were analyzed for parent chemical and possible metabolites. Three potential DZ metabolites hydroxypyrimidine (PYR), diazoxon (DZO), and dehydrodiazinon (DH-DZ) were commercially available and used as standards for chemical analysis. The PI and MO test element samples were removed from the wells then processed for direct analysis. These media samples, 500 μ L, were treated with 25 μ L of ZnSO₄ (25%) and 25 μ L saturated Ba(OH)₂ at room temperature to stop any reactions and precipitate proteins.²² Acetonitrile (ACN, 150 μ L) was then added to each sample, followed by vortexing (10 s) and centrifugation for 12 min at 20,800 rcf (4°C). Supernatants were transferred to amber 1.5 ml LC vials and diluted to 10% ACN prior to analysis. To measure chemical concentration within the slices, they were blotted dry, hydrated in 300 μ L of water (3 min), sonicated and processed as described for the media samples.

Samples for the third test element, phase II metabolism (PII), underwent a beta-glucuronidase enzyme hydrolysis procedure prior to processing for analysis. This enzyme hydrolysis cleaves off the glucuronide group from the undetectable phase II metabolite leaving the detectable phase I metabolite. Initial processing of PII samples was the same as the other sample types, 500 μ L media were taken and slices were hydrated in 300 μ L of water and sonicated. Then unlike the other sample types, PII media and slice samples were treated with beta-glucuronidase (β Gluc; 60 and 30 units for media and slices respectively in 100 mM potassium phosphate buffer pH 6.8), and incubated for 12 h at 37°C in capped 1.5 ml microfuge tubes on an orbital shaker. These PII samples were then processed for analysis

as previously described for the first two test elements using a modified procedure to compensate for sample volume change due to addition of hydrolysis reagents (buffer + enzyme). Specifically, media and slice enzymatic lysates were treated with sufficient ZnSO_4 , Ba(OH)_2 and ACN to keep the dilution factor constant with non-enzymatic samples prior to precipitation and LC. Activity of β Gluc preparations prior to use was confirmed by high pressure liquid chromatography (HPLC) using a Phenyl β -d Glucuronide STD (p-Gluc) (Serrano manuscript in preparation). Chemical concentrations measured in PII hydrolyzed samples represent a total of phase I and phase II metabolites. The mean measured PI concentration from non-enzymatically treated samples were subtracted from the mean PII totals, resulting in the quantity of phase II conjugated metabolites.

Chemical analysis was performed by HPLC on an Ultimate 3000 Thermo Electron-Dionex focused ultra-high pressure LC (uHPLC) system (Ft. Lauderdale, FL) consisting of the following modules: solvent delivery pump, refrigerated autosampler (6°C), heated column compartment and diode array detector (DAD). PYR was analyzed at 229 nm and all other chemicals at 206 nm. Injections were made onto a Synergi Hydro-RP 4u, 75×2.0 mm column kept at 40°C. Mobile phases consisted of 2.5 % (A) and 95% (B) ACN/ 20 mM acetate/ 8.3 mM trimethylamine (TEA; 33.4 $\mu\text{L/mL}$)/11.5 mM glacial acetic acid (GAA; 0.066 $\mu\text{L/mL}$). Mobile phase A was extracted twice with methylene chloride and hexane to eliminate GAA impurities eluting at PYR retention time (RT). A steep solvent program (6.4 min; 0.750 mL/min) was used to separate chemicals of interest as follows: 0-60 % B in 2 min; 60-100 % B in 0.25 min; 100 % B held for 1.25 min; 100-0 % B in 0.1 min and equilibration for 3.3 min.

Qualitative analysis of parent chemical (DZ) and three possible metabolites (PYR, DZO and DH-DZ) was performed with LC software by comparison of retention times (RT) and absorbance properties to commercially-available standards. DZ, DZO and DH-DZ all were contaminated with < 1% with PYR. Diazinon and metabolite concentrations in media and slices were determined by HPLC using standards and calibration curves as previously described.¹⁴

Data Analysis.

All analytical data were plotted or reported as mean $\pm$ standard deviation (SD). Chromeleon® Thermo-Dionex LC software (v2.0; Thermo Electron-Dionex, Ft Lauderdale, FL) was used for data acquisition, peak integration, and limits of detection (LOD) determination. Accuracy was calculated as % difference between nominal and measured chemical concentrations. Limit of detection for LC was established as the analyte concentration corresponding to a signal to noise ratio method in Chromeleon® of at least 3x by peak height. Lower limits of quantification (LLOQ), were measured at a S/N of at least 10:1 for each chemical. Experimental detection limits for all chemicals are reported in Table 2.

GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA) was used for curve fitting and statistical analysis. Linear regressions were generated from peak areas of chemical standards in 10% acetonitrile. These regressions were then used to interpolate concentrations from peak areas of samples. ANOVA analysis with a Dunnett's multiple

comparison test and two-tailed t-tests were used to determine significant toxicity responses in the slice assay ($P < 0.05$).

Results

DZ and DZO Toxicity

Significant DZ toxicity was detected by both MMT and LDH assays at 2000 μM DZ (Fig. 1). Furthermore, while not significant there was a suggestion of toxicity at 1000 μM DZ. Therefore, DZ metabolism tests were conducted at 200 μM , which avoided toxic concentrations but maximized the possibility of metabolite detection. (Fig. 1). No visible precipitate was observed in exposure media a [DZ] of 200 μM . Also, no pH difference was observed between solvent control and 200 μM DZ exposure media.

Effect of Fetal Bovine Serum on DZO

While DZ was stable in exposure media in the absence of slices, preliminary test results indicated losses of DZO in exposure media over time even in the absence of liver slices tissue. To determine if the loss was due to a biotic or abiotic mechanism DZO was incubated in media with and without fetal bovine serum under normal incubation conditions (Fig. 2). When incubated in L-15 media containing penicillin and streptomycin (L-15 P/S) no change in DZO concentration was observed over the duration of the 24-hour incubation. If fetal bovine serum was added to L-15 P/S to form the normal exposure media, DZO concentrations decreased significantly by 3 hours of incubation at 11°C and was completely absent at 24 hours. Conversely, there was significant accumulation of PYR in media containing FBS by 3 hours which by 24 hours accounted for 83% of the initial parent chemical. Only a trace amount of PYR, 1.1% of the initial parent chemical, was observed following incubation in the absence of FBS.

Inhibition of DZO Metabolism.

DZO has been established in the literature as a metabolite of DZ.^{23,24} It was shown from media testing in the current set of experiments that DZO was quickly metabolized to PYR even in the absence of liver tissue. Therefore, if DZO formed as a metabolite of DZ in the liver slice tissue was to be detected, further enzymatic metabolism of DZO to PYR would need to be stopped or at least inhibited. The enzyme inhibition effect of HQU was determined in exposure media at two concentrations 10 and 20 μM (Fig. 3). In the absence of inhibitor, the concentration of DZO in exposure media was reduced by 3 hours and was completely absent by 24 hours. Significant accumulation of PYR by 3 hours was seen which by 24 hours represented 92% of the initial DZO concentration. The addition of 10 μM HQU slowed the rate of DZO metabolism but did not completely inhibit PYR formation (Fig. 3). Addition of 20 μM HQU resulted in more inhibition of DZO conversion than seen with 10 μM HQU but was not complete. With a 10 μM HQU concentration the [PYR] was 41% of the initial [DZO] at 24 hours and 64% at 48 hours. The 20 μM HQU treatment sample at 24 hours had even less conversion with PYR accounting for only 30% at 24 and 48% at 48 hours of the initial [DZO] (Fig. 3).

DZ Uptake and Metabolism

DZ was tested in the rainbow trout liver slice assay on three separate occasions. In two of the three tests the phase II metabolite concentrations were measured. Phase II metabolites could not be measured in the third test due to the presence of the enzyme inhibitor HQU in the exposure media. HQU likely interfered with the glucuronidase reaction used in the technique for detection of the phase II conjugates.

In the absence of liver slices no loss of media DZ occurred and no measurable PYR or DZO accumulated during incubation at 11°C for 24 hours. With the addition of liver slice tissue, a nearly 55% loss of DZ in the media was seen at 1 hour in one test (Fig. 4). The other two test confirmed these result having similar amount of DZ loss, 48% and 42% at 3 hours, the earliest time point for these two tests (Table 3). The total loss of DZ in media in the presence of slice relative to media with no slice was consistent in the three tests ranging from 122 to 176 nmoles at 3 hours (Fig. 5). The depletion of DZ was initially rapid with over 90% of the total depletion of DZ occurring within the first three hours. The remaining roughly 10% depletion occurred during the following 21 hours.

The loss of [DZ] from media was accompanied by a rapid uptake into liver slices. Within one hour of slice exposure to DZ measurable concentrations of DZ had accumulated within the slice (Fig. 6). DZ slice concentrations increased at 3 hours and then appeared to plateau. Maximum DZ slice concentrations were relatively consistent among the three tests ranging from 43 to 69 nmoles/slice (Tables 3, 4). The general trend of DZ accumulation within the slice was also consistent among all tests. Loss of [DZ] in the media and uptake into slices also resulted in the appearance of PYR and PYR-glucuronide (PYR-gluc) metabolites in slices. In all three tests there was a detectable PYR peak in slice digests although it was not quantifiable (Table 3), whereas there was sufficient [PYR-gluc] to quantify, albeit at low concentrations. Measurable levels of PYR-gluc were detectable at one hour and increased out to 24 hours in one test, while in the other test concentrations peaked at six hours then decrease to near detection levels by 24 hours. Maximum [PYR-gluc] in slices were, Test 1, 0.8 and Test 2, 1.8 nmoles/slice, much lower than slice [DZ] (Fig. 7).

Significant concentrations of phase I metabolite PYR and phase II metabolite PYR-gluc were observed in the slice exposure media at one hour (Fig. 8). These metabolites were assumed to have been formed within the liver cells and exported into the media. At three hours the concentrations of PYR and PYR-gluc were roughly equal but beyond this point the accumulation rate of PYR was greater than that of PYR-gluc leading to higher phase I metabolite media concentrations at 6 and 24 hours.

DZ – Metabolism and Mass Balance

Comparison of the mass balance between wells with and without a slice present revealed a significant gap. As stated earlier the [DZ] in exposure media alone under normal incubation conditions did not change over a 24 hour period. In the presence of slice, chemical concentrations changed drastically with the loss of DZ and the formation of PYR and PYR-gluc. The sum of the PYR and PYR-gluc formed was less than the loss of DZ. In the first test roughly 40% of the 1 hour MO [DZ] cannot be accounted for in wells where slice was

present. In other words, only 60% of the MO concentration is in the form of DZ, PYR or PYR-gluc in wells, media and slice, which contained a liver slice (Table 3). This roughly 60% number holds up for all time points but the ratios of DZ, PYR and PYR-gluc change over time with DZ contribution decreasing and PYR metabolites increasing. The mass balance of the second test was more complete with 80% of the MO [DZ] accounted for in wells with a slice present. The remaining 20 to 40% of the parent chemical cannot be accounted for but some may be in the form of low concentrations of DZO.

DZ – HQU Inhibitor Experiment

DZO has been identified in the literature as a metabolite of DZ. From tests described above DZO was found to be metabolized to PYR even in the absence of liver tissue. It is possible DZO was formed in slices, but was quickly converted by enzymes within the liver tissue and the serum to PYR which did not allow DZO to achieve measurable concentrations. Also working against detection of DZO is the detection limits are higher for DZO than the other chemicals (Table 2). The enzyme inhibitor HQU slowed metabolism of DZO to PYR but did not completely inhibit metabolism. Using HQU maximized the potential to detect DZO, it did not guarantee the accumulation of measurable quantities of DZO.

Even in the presence of HQU, DZO was not detectable in any media or slice sample when slices were exposed to DZ. There was one significant difference between samples treated with HQU and those without HQU, a difference in PYR media concentrations at 24 hours (Table 4). While media [PYR] at three and six hours were roughly equal between samples with and without HQU, there was significantly less PYR in media samples with HQU than those without HQU at 24 hours. The [PYR] in media samples without HQU behaved similar to two other tests in that PYR continued to accumulate. Unlike the without HQU samples, the samples treated with HQU had no change in [PYR] between 6 and 24 hour samples (Table 4). This resulted in an additional 12 nmoles of PYR at 24 hours in the without HQU relative to the with HQU samples.

Discussion

DZ Biotransformation Pathways

Arguments can be made for two DZ biotransformation pathways in rainbow trout liver slice. There is direct evidence for the DZ to PYR to PYR-gluc pathway. The evidence for the second pathway which has a DZO intermediate to PYR is indirect and involves assumptions. In the DZ to PYR pathway, DZ from the media is taken up by the slice and converted to PYR, some of which undergoes phase II metabolism to form PYR-gluc. These metabolites are then excreted back into the media. The data directly supports this pathway in that upon exposure to slices [DZ] in media rapidly decreased while simultaneously increasing in slices (Fig. 6). Additionally, both PYR and PYR-gluc were detected in slices along with larger quantities of both the metabolites being found in the media (Fig. 7, 8).

Neither DZO or DH-DZ were detected in any slice or media samples. Furthermore, no additional peaks, indicative of metabolite formation, were detected in the HPLC chromatograms. While there was no direct evidence of a DZO intermediate, there was

indirect evidence it formed but was quickly converted to PYR within the slice or exposure media (Fig 4). Unlike DZ, DZO was not stable in the slice exposure media. Even in the absence of liver tissue DZO was quickly metabolized to PYR in exposure media at assay temperatures (Fig. 2). The metabolism of DZO in the absence of liver tissue was likely the result of enzymes, in particular paraoxonase 1(PON-1), found in the fetal bovine serum of the exposure media. The addition of enzyme inhibitor HQU to the exposure media slowed the rate of conversion of DZO to PYR but did not completely stop conversion (Fig. 3). Even with the addition of HQU to the exposure media of a DZ liver slice exposure, DZO was not detected in any slice or media samples. Unfortunately, using the current analytical methods low quantities of DZO, below 11 μM , could have been present but not seen. Thus, it is possible DZO may have been formed in the slices and some of the PYR measured could be from an alternative pathway of DZ to DZO to PYR.

Mass Balance

It does not matter to the PYR portion of mass balance calculation whether PYR came from DZ or DZO. However, undetected DZO may account for a small portion of the mass balance gap. At 24 hours there were 12 nmoles more PYR in the media without HQU treatment than in the media with HQU inhibitor treatment (Table 4). It is possible these 12 nmoles were DZO, which due to the presence of HQU had not been converted to PYR (Table 2). In 1.7 ml of exposure media this results in a concentration of 7.05 μM [DZO] which was below the 11 μM detection limit. If 12 nmoles DZO were converted to PYR it would make up over half of the total PYR concentration found in the media at 24 hours. While representing a significant portion of the total [PYR], 12 nmoles of DZO would represent only ~4% of the 304 nmoles/well measured in the MO wells at 24 hours. Therefore, any unmeasured DZO would account for at most 10 to 20 % of the 20 to 40% unaccounted portion of the mass balance equation (Table 3). The remainder, or at least a significant portion of it could potentially be linked to the formation of glutathione conjugates.^{25,26} An important pesticide detoxification route involves glutathione S-transferase conjugation. Both DZ and DZO may form the same proposed conjugate. (fig, 9). The resulting glutathione conjugate has a calculated LogKow of -0.03 as the neutral and -2.53 for the ionic species which makes it unlikely that these conjugates would be observed under the chromatographic conditions used in this study. For comparison the reference structures DZ, DZO, DH-DZ and PYR have calculated LogKow's of 4.19, 3.38, 3.55 and 2.29 respectively.

Phase II sulfate conjugates could also be part of the unaccounted portion of the mass balance equation. Under the analysis conditions used the phase II sulfate conjugates would not be detected. Sulfate conjugate levels at 24 hours could have been equal to glucuronide conjugate levels as previously observed when using this same slice assay system with different chemicals (Serrano manuscript in preparation). At 24 hours, 7 to 8 nmoles/well of PYR-gluc were found in the media and slice. If PYR-sulfate was at equivalent concentrations it would represent 5 to 10 percent of the unaccounted portion of the mass balance equation.

Slice Assay Evaluation

Diazinon has been previously tested in both rainbow trout *in vivo* and *in vitro* assays. Seguchi and Asaka using whole fish extracts and GC detection in a rainbow trout *in vivo* system found PYR to be the major metabolite.¹ They also found DH-DZ at much lower concentration than PYR but did not detect DZO. Fuji and Asaka used the same GC detection methods as the Seguchi study but an *in vitro* method. They exposed rainbow trout subcellular liver fractions to DZ and also determined PYR was the major metabolite.^{1,2} In addition, moderate concentrations of DZO and low concentrations of DH-DZ were detected. The Asaka studies using a GC method reported DH-DZ and DZO detection limits, 0.05uM and 0.09 respectively, lower than detection limits for the current work which used a HPLC method (Table 2). The *ex vivo* slice data presented here more closely resembled the *in vivo* rainbow trout results than the *in vitro* results in that PYR was the major metabolite seen and no DZO was detected. While Asaka detected DH-DZ in rainbow trout liver fractions exposed to DZ, it was at low concentrations, 20 to 30 times lower than the [PYR]. Therefore, it may have been present in the trout liver slice but below the detection limits.

DZ metabolism has been studied in a number of different fish and mammalian species in both *in vivo* and *in vitro* systems with differences in metabolites seen among fish species and between fish and mammals. While some of the differences could be attributed to different analytical methods, a pair of papers by Asaka, and a pair of papers by Keizer, using the same analytical methods in both *in vivo* and *in vitro* exposures of the same fish species found both similarities and differences between the two assay systems. Asaka found PYR to be the major metabolite in both systems but PYR and DZO was detected in the *in vitro* system. Similarly, Keizer detected only PYR in guppy and zebrafish *in vivo* exposures to DZ but PYR and DZO in hepatic S-13 fraction of guppy, zebrafish, and rainbow trout. Only PYR was found in carp S-13 fractions. These findings provide credible evidence that while there are some metabolites seen in *in vitro* preparations that are not found *in vivo*, many of the differences seen are not just artifacts of lab to lab or method to method variability.^{27,28}

The main characteristic separating fish *in vivo* and *in vitro* DZ metabolism is the lack of the metabolite DZO in the *in vivo* assays. It is known, DZO not DZ is the chemical responsible for the observed toxicity. Therefore, DZO must be present at some low concentration in the fish and this concentration is sufficient to bind acetylcholine esterase inhibiting the enzymes activity resulting in toxicity. The *in vitro* mean ratios of PYR to DZO found in the Asaka and Keizer articles was 3:1.^{2,28} The highest combined concentrations of PYR and PYR-gluc measured in the slice assay was 26 nmoles/well. Using the mean ratio of the *in vitro* study to estimate the [DZO] in a slice resulted in a value of 8.7 nmoles/well which is well below the detection limit of DZO for this study (Table 2). Therefore, it is possible DZO was produced in the slice but due to limitations of the analytical method was not detected. The same phenomenon could also be occurring in other fish *in vivo* studies cited.

Unlike the fish *in vivo* studies many of the mammalian *in vivo* studies used a C¹⁴ labeled DZ/ GC analytical method.^{17,29} The rat and dog studies identified PYR as the most abundant metabolite. They also detected DZO and a number of additional metabolites at low concentrations. These finding may illustrate the importance of the analytical methods sensitivity, because there is a strong possibility that DZO was present at concentrations that

have biological effect in the trout liver slices and fish *in vivo* but was not detected due to limitations of the analytical method. Use of a radiolabeled parent chemical may have resulted in better sensitivity which in turn may lead to a more complete understanding of the metabolic pathways.

The slice model has an advantage over an *in vivo* model when using a radiolabeled chemical in that the slice test apparatus is smaller and more confined. A slice test with appropriate controls may need 72 slices. The entire test could be performed in six twelve well plates generating less than 125 ml of media as opposed to the multiple tanks and large volume of water needed to perform a similar test *in vivo*.

Precision cut liver slices model systems are an important tool in a wide variety of mammalian research. Much of the literature revolves around pharmacology including drug transport, toxicity, and metabolism.^{30,31,32} The *ex vivo* slice model has proven useful in the area of pharmacology because unlike *in vitro* assays the intact intercellular matrix allows for drug transport and the assessment of toxicity. Additionally, the non-differentiated cells of the slice have the full metabolic capabilities of the normal liver tissue. Previous work by this lab confirmed the ability of rainbow trout liver slice to carry out biotransformation as evident by the induction of vitellogenin (Vtg) gene expression in slices exposed to methoxychlor.¹⁴ While methoxychlor does not bind to the rER, it does induce rER mediated Vtg mRNA expression in rainbow trout liver slices. This was possible through the biotransformation by the liver tissue of methoxychlor to monohydroxy and dihydroxy methoxychlor which bound to ER and induced Vtg expression. The biotransformation pathway was determined to be methoxychlor to monohydroxy-methoxychlor to dihydroxy-methoxychlor. Phase II conjugates of both the mono and dihydroxy-methoxychlor were also detected. The fish data presented here confirms the usefulness of the precision cut liver slice model as seen in the mammalian literature. It is a relatively easy and cost effective tool to study chemical metabolism while reducing the number of animals needed to perform tests. One fish can be used to perform an entire test so fish to fish variability of *in vitro* testing is reduced. The intercellular interactions are maintained and cells have not dedifferentiated which means a more *in vivo* like response relative to microsomal or cell line based *in vitro* assays.

Conclusions

A number of conclusions can be drawn from the data presented. The major metabolite of DZ in the *ex vivo* liver rainbow trout liver slice assay was PYR. Smaller amounts of the phase II metabolite PYR-gluc were also found. These data suggest the main biotransformation pathway was directly from DZ to PYR. Additionally, there is indirect evidence of an alternative pathway of DZ to DZO to PYR. Data suggests that while the contribution to total [PYR] from this pathway could be significant, the [DZO] at any given time were low. While some differences existed, the slice model results were similar to previously published *in vivo* and *in vitro* rainbow trout DZ metabolism data. Thus, the rainbow trout liver slice model is a usable tool for future studies of chemical metabolism.

Future research will utilize this *ex vivo* model system to study additional chemicals using a chemical group approach to gain sufficient understanding of biotransformation to predict

fish metabolism. Success of this work will depend upon the sensitivity of the analytical methods employed to detect metabolites.

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FIG. 1.

Toxicity of diazinon (DZ) in rainbow trout liver slices. Determination of MTT activity in slices exposed to carrier control ethanol (●), DZ (■). Determination of LDH leakage into media from slices exposed to ethanol (○) and DZ (□). Symbols represent mean and SD of six replicate slices from six separate wells. *Significant ($p < 0.05$) difference between treatment and control samples. SD, standard deviation. LDH, lactate dehydrogenase enzyme.

FIG. 2.

Concentration of diazoxon (DZO) and hydroxypyrimidine (PYR) in two types of media, one with FBS and the other without FBS. These media were dosed with 200 μ M DZO, but did not contain liver slices. Open symbols for DZO (□) and PYR (▽) represent media without FBS. Filled symbols for DZO (■) and PYR (▼) represent media with FBS. Symbols represent mean and SD of three replicate samples. Error bars are within size of symbols therefore not shown. No 0 hour DZO samples were taken. FBS, fetal bovine serum.

FIG. 3.

Media concentration of diazoxon (DZO) and hydroxypyrimidine (PYR) in normal exposure media and exposure media containing the enzyme inhibitor 2-hydroxyquinoline (HQU). Both exposure media types were dosed with 200 μ M DZO and not exposed to slices. Filled symbols are DZO (■) and PYR (▼) concentrations in normal exposure media. DZO (◆) and PYR (▲) concentrations in exposure media containing 10 μ M HQU. Open symbols are DZO (□) and PYR (▽) concentrations in exposure media containing 20 μ M HQU. Symbols represent mean and SD of three replicate samples. Error bars are within size of symbols therefore not shown.

FIG. 4.

Concentration of diazinon (DZ) in exposure media with no slice present and exposure media incubated in the presence of liver slice. Data are from three separate tests. Symbols for test one are media without slice (Δ) and media with slice ($\blacktriangle$). Symbols for test two are media without slice ($\blacktriangledown$) and media with slice ($\blacklozenge$). Symbols for test three are media without slice ($\circ$) and media with slice ($\square$). Nominal DZ concentration was 340 nmoles/well (200 μ M). Symbols represent mean and SD of three replicate samples.

FIG. 5.

Difference in diazinon (DZ) media concentration between media exposed to slices and media not exposed to slices. Data are from three separate tests. Symbols are test one (▲), two (◆), and three (□). Symbols represent mean and SD of three replicate samples.

FIG. 6. Concentration of diazinon (DZ) in slices exposed to 200 μ M DZ. Data are from three separate tests. Symbols are test one ($\blacktriangle$), two ($\blacklozenge$), and three ($\square$). Symbols represent mean and SD of three replicate samples.

FIG. 7.

Concentrations of hydroxypyrimidine (PYR) and hydroxypyrimidine-glucuronide (PYR-gluc) in liver slices exposed to 200 μ M diazinon (DZ). There was no measurable PYR detected in any test. Data are from two separate tests. Symbols for test one PYR (▼) and PYR-gluc (▲); test two PYR (▽) and PYR-gluc (△). Symbols represent mean and SD of three replicate samples.

FIG. 8. Concentrations of hydroxypyrimidine (PYR) and hydroxypyrimidine-glucuronide (PYR-gluc) in DZ dosed media incubated in the presence of liver slices. No PYR or PYR-gluc was detected in DZ dosed media in the absence of slices. Data are from two separate tests. Symbols are test one PYR (▼) and PYR-gluc (▲); test two PYR (▽) and PYR-gluc (△). Symbols represent mean and SD of three replicate samples.

FIG 9.

Proposed metabolism pathway of diazinon (DZ) in rainbow trout liver slices. Structures bounded by brackets [] were not detected but were assumed to be present at low concentration or were not detectable by the analytical methods used.

Table 1.

Test chemicals and critical assay reagents.

Chemical Name	Acronym	CASRN	Source ^a	Product #	Lot #	Purity ^b (%)
Diazinon	DZ	333-41-5	CS	11621	4202900	99
Hydroxypyrimidine	PYR	2814-20-2	CS	11621B	4525200	99
Diazoxon	DZO	962-58-3	CS	11624A	4720700	99
Dehydrodiazinon	DH-DZ	32588-20-8	CS	12860	4953000	99
2-Hydroxyquinoline	HQU	59-31-4	SA	270873	01624AAO	98

^aSA=Sigma Aldrich, PO Box 14508, St. Louis, MO 63178, USA; CS=Chem Service, Inc. PO Box 599, West Chester, PA 19381, USA.

^bChemical purity % as reported by supplier.

Table 2.

Chemical detection limits in exposure media.

Chemical Name	Acronym	Limit of Detection nmoles/1.7 ml	Limit of Detection μM	Lower Limit of Quantification μM
Diazinon	DZ	5.1	3	5
Hydroxypyrimidine	PYR	1.7	1	5
Diazoxon	DZO	18.7	11	11
Dehydrodiazinon	DH-DZ	5.1	3	5

Table 3.

Concentration of diazinon (DZ), hydroxypyrimidine (PYR), and hydroxypyrimidine-glucuronide (PYR-gluc) in media and slices exposed to 340 nmoles DZ/well (200 μ M). Media only samples were not incubated with a slice. Media and Slice Phase I samples were taken from the same well. Slice Phase II samples were taken from the separate well. Data is from three separate tests. Values represent mean of three replicate samples.

Time hours	Media only (nmoles/well) DZ	Media (nmoles/well)			Slice (nmoles/slice)			Total nmoles Media+Slice	Total as % Media only
		DZ	Phase I PYR	Phase II PYR-gluc	DZ	Phase I PYR	Phase II PYR-gluc		
TEST 1									
0	312.2	312.2	0	0	0	0	0	312.2	100
1	311.9	138.5	2.7	5.2	22.9	*	0.5	169.8	54
3	312.9	136.7	4.8	5.5	35.5	*	0.8	183.3	59
6	303.0	131.9	10.1	3.8	44.9	*	0.8	191.5	63
24	307.2	119.4	20.9	6.3	40.1	*	0.3	187.0	61
TEST 2									
0	316.9	316.9	0	0	0	0	0	316.9	100
3	283.7	165.5	3.4	1.5	57.2	*	1.5	229.1	81
24	313.7	150.0	20.2	7.4	69.4	*	1.8	248.8	79
TEST 3									
0	339.2	339.2	0	NM	0	0	NM	339.2	100
3	343.2	195.3	7.9	NM	42.6	*	NM	245.8	72**
6	336.3	191.3	13.0	NM	36.0	*	NM	240.3	71**
24	334.7	176.2	22.6	NM	38.8	*	NM	237.6	71**

* peak was detected but not quantifiable (S/N<2)

NM=Not Measured

** value does not include contribution from PYR-gluc

Table 4.

Concentration of diazinon (DZ) and hydroxypyrimidine (PYR) in media and slices exposed to 340 nmoles DZ/well. Media only data samples were not incubated with a slice. Media and Slice samples were taken from the same well. The enzyme inhibitor 2-hydroxyquinoline (HQU) was present in the media of the bottom group of data, and was absent in the media of the top group of data. Values represent mean of three replicate samples.

Time hours	Media only (nmoles/well) DZ	Media (nmoles/well)		Slice (nmoles/slice)	
		DZ	Phase I PYR	DZ	Phase I PYR
Exposure without HQU					
0	339.2	339.2	0	0	0
3	343.2	195.3	7.9	42.6	0
6	336.3	191.3	13.0	36.0	0
24	334.7	176.2	22.6	38.8	0
Exposure with HQU					
0	357.0	357.0	0	0.0	0
3	348.1	219.1	5.2	40.8	0
6	320.0	210.0	10.3	35.8	0
24	304.3	198.2	10.6	32.8	0