DEVELOPMENT OF AVIAN GENOTOXICITY ASSAYS BASED ON NON-LETHAL TECHNIQUES

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PREFACE

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

CHAPTER II

CHAPTER I

A NON-LETHAL TECHNIQUE FOR MEASURING GENOTOXIC EFFECTS IN BIRDS

Abstract - Data derived from mammalian, plant, and microbial models of genotoxicity may not be applicable to birds due to differences in avian genetic structure and physiology. The objective of this study was to develop a standardized, non-lethal genotoxicity assay for use with birds based on modification of a mammalian assay, flow cytometric measurement of variation in nuclear DNA content. Blood samples were collected from brachial veins of juvenile mallards (Anas platyrhynchos) before and after they were administered an oral dose of either methyl parathion (7.5, 15.0, or 30.0 mg/kg body weight), triethylenemelamine (0.25, 0.50, 1.0 mg/kg body weight), or a solvent control. Cells were examined for nine parameters of DNA content and cell cycle kinetics. Results from blood samples were compared with results from spleen tissue, which is more commonly used in flow cytometric assays. Results were divided into three analysis groups: pre-dose, post-dose, and difference between pre- and post-dose endpoints. Within triethylenemelamine dose groups, significant variation was seen only in the pre-dose G_2/G_1 ratio. Methyl parathion groups varied significantly in two parameters: post-dose coefficient of variation of the G_2 peak and post-dose G_2/G_1 ratio. Dose levels of positive control groups may have been too low to elicit a definite genotoxic response.

Despite the limited response in the positive control, evidence of disturbance of normal cell cycle kinetics suggests flow cytometry is a viable alternative for genotoxicity analyses in birds.

Keywords - Genotoxicity Birds Flow Cytometry Biomarker

INTRODUCTION

The time-consuming and expensive nature of classical toxicology testing makes the use of biomarkers highly desirable. Biomarkers can provide a rapid means to test for the presence or damaging effects of toxicants. Genetic damage has been suggested as a useful biomarker for the presence and action of environmental contaminants [1] because many contaminants are mutagenic [2]. Generally, taxa other than birds are used to determine genotoxic potential of contaminants in both laboratory and field settings [3,4]. The reason may lie in difficulties associated with application of standard genetics techniques to chromosomal aberration analysis of the avian genome. Examination of avian chromosomes can be difficult due to high diploid numbers that can exceed 100 [5] and the small size of numerous microchromosomes ($< 1\mu$m) [6] that are not clearly resolved under light microscopy and can be lost completely or obscured by other chromosomes [6].

Extrapolation of data derived from other taxa to possible effects in birds may be questionable because of genetic and metabolic differences among taxa. Birds have the most conservative genomic size among vertebrate classes [7]. Despite large diploid numbers, birds possess about half the amount of DNA of most mammals and two-thirds

that of reptiles [8]. Unlike mammals, birds do not possess large blocks of repetitive DNA [9], resulting in a smaller genome which may make them more vulnerable to damage in functional DNA sequences. Birds also may respond faster than mammals to genotoxicants because they possess a higher metabolic rate and a relatively larger liver compared with mammals [10]. Response may be in the form of rapid production of genetic damage as compounds are rapidly converted into genotoxic metabolites or quick metabolization of genotoxicants into harmless constituents.

Microbes, such as Escherichia coli and Salmonella, which are used extensively in toxicity testing, lack a nuclear membrane to provide an additional barrier to genotoxins. Instead of consisting of multiple chromosomes, the prokaryotic genome is a circular molecule of DNA that is not linked so extensively with proteins and never exhibits the extensive coiling characteristic of eukaryotic chromosomes. Finally, prokaryotes often are not able to break down toxicants into their equally or more toxic metabolites. Although metabolic activation systems have been developed that adequately mimic mammalian physiological systems, no such system for avian species has been developed for use with prokaryotic models of genotoxicity. Therefore, accurate extrapolation from mammalian and microbial genotoxicity data to probable effects in birds may be severely hampered. Developing a genetic toxicity assay for birds would eliminate the need to extrapolate from toxicology data derived from other taxa.

Flow cytometry is used commonly to assess levels of genetic damage in other taxa. Flow cytometry (FCM) is a rapid and accurate means of detecting chromosomal

aberrations in large numbers of cells and is proven to detect mutagenic and clastogenic insult in mammalian species [11-12]. It also has been used in reptilian and avian field experiments [13-15], but laboratory analyses have not been conducted to verify the effectiveness of flow cytometry for use with birds in toxicological studies. Exposure to a chemical mutagen typically increases the coefficient of variation (CV) in the G_1 peak [16], shifts position of the G_1 peak, or produces a shoulder or separate peak [13, 17]. A dose-response relationship between a known mutagen and the CV of treated cells also has been demonstrated in bone marrow cells of mice (Mus sp.) exposed *in vivo* to cyclophosphamide [16].

Several studies have used flow cytometry to detect chromosomal aberrations in wildlife caused by environmental mutagens. McBee and Bickham [12] detected higher CVs in white-footed mice (Peromyscus leucopus) at a site contaminated with petrochemicals than in animals at a reference site. Slider turtles (Pseudemys scripta) exposed to radioactive and non-radioactive contaminants at a site in South Carolina exhibited elevated CV values compared to turtles from an uncontaminated farm pond [13]. Two studies used flow cytometry to examine effects of environmental toxicants on avian species. Adult mallards housed at an abandoned cooling reservoir for a nuclear reactor developed aneuploidy and/or an increase in CV [14]. Custer et al. [15] attributed significantly higher and lower CVs in embryos and 10-day-old chicks of black-crowned night-herons (Nycticorax nycticorax) to genetic damage caused by exposure to petrochemicals. Based on those studies, flow cytometry seems to be a promising tool for use in assessing genetic damage in birds exposed to environmental contaminants.

A secondary goal of this study was to develop an assay that does not require killing the animal. A non-lethal assay would facilitate research on endangered species and could provide information on temporal effects in the same individuals [6]. Several studies examining the usefulness of various tissues in flow cytometric analysis among several taxa have found that spleen was consistently sensitive to toxicant exposure [15, 18, 19], but use of blood could eliminate the need to take biopsies of spleen tissue. Birds possess nucleated erythrocytes [20] that enable adequate quantities of DNA to be extracted from a minimal volume of blood, thereby reducing stress to the bird. Blood has been used successfully in various kinds of studies of avian genetics [5, 21, 22] and should provide accurate, consistent results in flow cytometric analysis of genotoxicity in birds.

To test this technique, triethylenemelamine (TEM) and microencapsulated methyl parathion (MP) were used. TEM is a nitrogen mustard commonly used as a positive control in genetic toxicity research because it has been shown to be clastogenic and mutagenic in mammals at doses far below lethal levels [4, 23]. In mammals, occurrence of chromosomal fragments typically peaks within one day of treatment with TEM and decreases thereafter [24]. TEM has been tested as a sterilant in avian pest species because of the mutagenic effect on spermatozoa in chickens [25-27]. Bickham et al. [23] determined that CV values of affected tissues eventually returned to normal with some treated animals producing a lower CV than that of reference animals. This may indicate that some cells are hypersensitive to TEM and do not recover, so that only the cells resistant to damage from TEM are being measured [23].

Despite heavy regulation, MP ((0,0-dimethyl 0-p-nitrophenyl) phosphorothioate) is one of the most widely-used agricultural insecticides across the U.S. [28] and therefore a compound that many avian species are likely to encounter. Habitat requirements and nesting schedules bring many avian species into the vicinity of agriculture crops during growing seasons, exposing adults and juveniles to pesticide use. MP has been linked to chromosomal lesions in cultured rat cells (Rattus sp.) [29], sister-chromatid exchanges in ovary cells of hamsters (Cricetus sp.), and mutations in lymphoma cells of mice (Mus sp.) [3]. MP belongs to the organophosphate group of pesticides that are highly toxic to birds through affects on cholinesterase levels [30, 31]. Exposure to organophosphates alters behavior, reduces reproductivity, and increases vulnerability to predation in birds [31-33]. Cholinesterase levels can recover [30], but possible long-term genetic effects are unknown.

Most toxicology studies are conducted using pure compounds to demonstrate causality between administered dose and observed effect. However, most pesticides sold for use on crops contain complex mixtures that can be more toxic than the active ingredient [34]. Kale et al. [35] used field-ready mixtures to examine several widely-used pesticides for which government approval had been based on laboratory analyses using pure compounds. All compounds originally reported as not mutagenic in assays of pure compounds tested positive as field formulations. Results of the study by Kale et al. [35] emphasize the need to carefully evaluate goals of laboratory toxicity studies because studies conducted with purified substances can be misleading.

Flow cytometric analysis of nuclear DNA content variation was tested using two compounds and two tissues. Assuming the positive control (TEM) has the same effect in avian systems as it does in mammalian systems, exposure to TEM should result in increased genetic damage as indicated by an increase in the CV of birds. Additionally, if blood is a satisfactory substitute for spleen tissue, the degree of damage to both tissues should be similar. Because avian and mammalian systems differ in many aspects, variables for cell cycle kinetics as well as DNA damage were examined. Disruption of normal cell cycle processes could produce responses such as impaired initiation of DNA synthesis, increased or decreased rates of DNA synthesis, or inhibited division of replicated cells. If either of the test compounds affects cell cycle kinetics, we would expect to see differences in percentages of cells in the S and G_2 regions of DNA histograms compared to references [12]. We might also expect to see differences in proliferation indices [14].

MATERIALS AND METHODS

Fifty-six mallard ducklings were purchased at six-weeks of age from Free Flight Game Bird Farm in Denton, Texas. Mallards were selected as the model species because they have low genetic variability [36], have met EPA approval as a model species for other types of toxicity assessments [37], and have been used in several toxicology studies investigating non-genetic endpoints [33, 38]. Ducklings were weighed to equally distribute sizes among seven dose groups. Individuals in a weight class were assigned randomly to dose groups. Each dose group was housed outside in 3 by 4 by 1.2-m pens located at the Oklahoma Cooperative Fish and Wildlife Research Unit's Wildlife Annex.

Separation of groups prevented ingestion of feces from birds given different doses. Pens were constructed of poultry netting with tarpaulin draped over the top to provide shade and protection from inclement weather and predators. Purina Game Bird Chow and fresh water were provided ad libitum in feed troughs and buckets. Ducks were given two weeks to adjust to their new surroundings before experiments were initiated.

Because genetic background of ducks was unknown, blood samples were collected 2 weeks after the animals arrived. Heparinized vacuum tubes were used to draw 2 ml of blood from the brachial vein of each animal. Each animal was assigned a code number used to identify all samples from that animal and keep the identity of dose groups unknown to the investigator until data were analyzed. After collecting pre-dose samples, a 1 month rest period enabled animals to recover from lowered blood levels and stress. Twenty-four hours after dosing, blood samples were collected again in the same manner. Feather pulp, liver, spleen, heart, kidney, and brain samples also were taken after animals were killed by CO_2 asphyxiation. Of these tissues, only spleen was used for flow cytometric analyses; other organ tissues were preserved for future research. Separate aliquots of two drops of blood were placed in cryotubes containing 1.5 ml of freezing media and stored at -80°C [15]. All organ samples were stored in 1.5 ml cryotubes, placed in liquid nitrogen for transport to the laboratory, and maintained at -80°C until analysis.

Route of exposure for both test compounds was based on an attempt at a realistic route of exposure for the pesticide. Driver et al. [39] found that dermal exposure and

preening were major routes of organophosphate uptake for bobwhite quail (Colinus virginianus). Inhalation was the major factor for only one hour after spraying. In mallards, percutaneous applications of methyl parathion are more toxic than oral doses (LD_{50} of an oral dose is 60.5 mg methyl parathion/kg body weight; dermal dose is 53.6 mg methyl parathion/kg body weight), so doses were administered orally [40]. Because mallards can detect contaminants in their food [38], doses were dispensed in gelatin capsules. TEM dosages were determined from previous experiments using this compound on avian species [25, 27]. Spermatogenesis was completely inhibited in yellow-throated sparrows (Petronia xanthocollis) injected daily with 1 mg TEM/kg body weight for five days [27]. An antifertility study in which male Japanese quail (Coturnix japonica) were given a single oral dose of 5 mg TEM/kg body weight [25] showed that fertility was reduced after several days. No information on mortality was provided in either study. Because the intent was to deliver a non-lethal dose to birds, dosages for TEM were 0, 0.25, 0.50, and 1.00 mg/kg body weight. For microencapsulated MP, doses were 0, 7.5, 15.0, and 30.0 mg/kg body weight. Individual doses were calculated from weights of ducks measured the day prior to dosing. Each dose was pipetted into a capsule and the capsule topped off with corn oil. Reference groups were given a capsule containing only corn oil.

Methods for flow cytometric preparation and analyses were based on those used by McBee and Bickham [12], McBee [4], and Otto [41]. Chicken erythrocyte nuclei were used as an external reference standard and to check calibration of the flow cytometer after every fifth individual was analyzed. Samples of solid tissue were disrupted using a Tissue

Tearor® and then incubated in pretreatment solution (4.2 g citric acid, 0.5 ml Tween 20, 100 ml ddH_2O) for 20 min. Samples were centrifuged for 10 min at 100x g, supernatant was removed, and pellets were fixed by adding cold 70% ethanol. Samples were filtered using 35-micron nylon cloth. Blood samples were prepared by placing 0.25 ml of freezing media-blood solution directly into cold 70% ethanol for 10 min and then filtering. Filtered samples were kept at 4°C at least 24 h before staining. For staining, five drops from each sample were placed into glass culture tubes with 0.5 ml of pretreatment solution. After incubation for 10 min at room temperature, 2.5 ml of 4'-6-diaminido-2-phenylindole (DAPI) staining solution were added to each sample. Stained samples were stored for approximately 18 h in the dark before being examined. Three replicate samples were processed for each animal. New standard solutions were checked against old solutions to maintain machine consistency.

The flow cytometric endpoints inspected most commonly in studies of environmental genotoxicity are relative position of G_1 peak, coefficient of variation of the G_1 peak (CVG_1), proliferation index (PI), and DNA index (DI). Position of the G_1 peak is a relative measure of DNA content of the cells in the G_1 stage of the cell cycle (Fig.1). CVG_1 is the coefficient of variation around the G_1 peak (one standard deviation on each side). PI is an indication of production of new cells in treated animals [14] and is calculated by combining percentage of cells in the S and G_2 stages for each sample and dividing by the average of the same endpoints for the reference group. DI is calculated by dividing percentage of cells in G_1 stage by the average of the same endpoint for the reference group. This analysis indicates if the relative proportion of cells in G_1 stage of

treated animals has been affected [14]. Additional endpoints included in this analysis were position of G_2 peak and CVG_2, which are measurements of relative DNA content and coefficient of variation in the G_2 stage of the cell cycle. Percent G_2 refers to the proportion of cells within the sample that is in the G_2 stage of the cell cycle. Percent S (synthesis) provides information regarding the proportion of cells in the process of replicating DNA. The G_2/G_1 ratio compares quantity of DNA in replicated cells to that in unreplicated cells. Replicated cells should contain twice the quantity of DNA, resulting in a ratio of 2.0 for healthy cell populations.

Results for blood tissues were separated into three analysis groups: pre-dose, post-dose, and difference between the two dose states. Pre-dose data were examined to define genetic conditions prior to dosing. We expected endpoint values to conform to a normal distribution. Inspection of post-dose data from spleen tissue enabled comparison between tissue types. The use of change in pre- and post-dose endpoints could nullify the effect of pre-existing abnormalities in DNA.

All analyses were conducted using SAS© [42]. Assumptions for parametric testing were not met ($p < 0.05$), so the Kruskal-Wallis test based on rank sums was employed [43]. Each chemical was analyzed separately. Dose groups that differed significantly for a variable were subjected to a pairwise comparison for Kruskal-Wallis rank sums. Numbers of individuals per dose group and tissue varied between 6 and 8, because of normal attrition and difficulties in collecting tissue.

RESULTS

Prior to dosing, no parameters differed within dose groups assigned to TEM exposure, but mean values of G_2/G_1 ratio varied significantly ($p = 0.0235$) among groups designated for MP exposure (Table 1). Pairwise comparisons revealed that animals designated for the middle dose group for MP had significantly higher G_2/G_1 ratios than all other dose groups, although maximum variation among mean values was only 0.01.

After exposure, two endpoints differed significantly in blood samples for each compound (Table 1). Pairwise comparisons of G_2/G_1 indicated a significantly lower ratio ($p = 0.0454$) in the low-dose group for TEM compared with the reference group. For animals exposed to MP, the low dose group produced a significantly smaller ($p = 0.0274$) CVG_2 than the middle and reference dose groups. When comparing the change between pre- and post-dose blood, reference and high dose groups had lower CVG_1 among TEM dosed animals. No significant differences were found among any variables examined from spleen tissues.

Several p-values derived from Kruskal-Wallis analyses were only slightly greater than the alpha value of 0.05 selected at the beginning of the study. If the confidence interval is reduced to 90%, the null hypothesis (all dose groups are the same) was rejected eleven times. No pre-dose variables differed within dose groups assigned to TEM exposure, and no additional variables differed within dose groups assigned to MP exposure. Reducing the confidence interval to 90% did not change between-group

relationships for the G_2/G_1 ratio derived from blood samples collected prior to dosing with MP. For blood samples of animals dosed with TEM, $\%G_2$ and PI were significantly higher (respectively $p = 0.0958$ and 0.0814) in the middle dose group compared with the reference and high dose groups, and DI was significantly lower ($p = 0.0979$) in the middle dose group than the other groups. No additional differences were found among variables when comparing the change between pre- and post-dose.

Within spleen samples, two variables differed within dose groups. For ducks exposed to TEM, the high dose group was significantly lower than the reference group for CVG_2 and $\%G_2$ (respectively $p = 0.0770$ and 0.0975). For animals treated with MP, CVG_2 values in spleen tissue again differed with the reference group significantly lower ($p = 0.0645$) than the low and high dose group but statistically similar to the mid-dose.

A sub-diploid peak (Fig. 1) was observed in post-dose blood samples of several individuals; therefore, post-dose blood samples were reanalyzed. Percentages varied among dose groups, but there were fewer occurrences of subdiploid peaks in the higher dose groups (Fig. 2). The middle dose group for MP showed a higher percentage of individuals with the sub-diploid peak than the other two MP dose groups. Chi-square analysis indicated no significant difference in occurrence of peaks between dose groups, but samples were small ($n < 9$). A trend response could not be evaluated statistically because of low sample sizes. DNA was examined using agarose gel electrophoresis (DNA was loaded into a 1.0% agarose gel set at 96V for 1 h and stained with ethidium bromide)

to explore the possibility of an apoptotic population, but the DNA laddering characteristic of apoptosis was not observed. The subdiploid peak was not observed in spleen samples.

DISCUSSION

The lack of a definite dose response in positive control groups was unexpected. Doses and exposure duration selected for this study were based on those used for quail and sparrows [25, 26] and on those previously used with Rattus [18]. None of the investigators reported mortalities although doses given to quail and sparrows were several times that which would be lethal to a rat [44]. A longer duration of exposure may be necessary to elicit a dose response in spleen and blood tissue of mallards. More likely, concentrations used were not high enough.

Based on mammalian data, a small change in CVG_1 was not surprising for the high dose group of TEM. However, the observed change in the reference group was not, and makes the results of this analysis suspect. The significant difference for G_2/G_1 ratio in pre-dose samples of blood may be merely a random difference occurring among the many variables examined and did not appear to affect pose dose results. No dose response-like pattern was detected within all other parameters at either $\propto = 0.05$ or $\propto = 0.10$ although many more variables differed among dose groups at the $\propto = 0.10$ level. Seven of 11 of the variables differing at $\propto = 0.10$ were in the post-dose data for blood, that could indicate blood tissue responds faster or at lower doses than spleen. Although statistical significance was limited, several cytometric parameters were altered. Some of these are

not normally examined in biomarker studies and have not been validated as biomarkers of exposure; however, these data may provide valuable details about cell cycle disruption and should not be excluded from toxicological analyses. For example, mean values for %S of TEM-dosed animals showed a similar but more exaggerated pattern than in pre-dose values. Mean values increased from reference to the middle dose group, but the mean for the high dose dropped below the reference. This pattern appears similar to that in Bickham's study [23]. However, closer examination reveals that for four individuals in the low dose group, two in the middle dose group, and one in the high dose group, no cells were detected in the synthesis stage of the cell cycle. All animals in the reference group had detectable S-regions indicating normal levels of synthesis. The proportion of cells in the synthesis stage appeared to have been altered in animals exposed to TEM, but a clear dose response was not apparent.

Conducting a dosing experiment on birds allowed us to collect baseline genetic data for each individual and examine the change after dosing. With small sample sizes the probability increases that randomly selected groups will be inherently different. The absolute change between pre- and post-dose samples was analyzed (Table 1,2) with one parameter, G_2/G_1 ratio for blood of MP-exposed animals, differing at the $\propto = 0.10$ level. This indicates that variables did not change significantly after treatment and supports the use of blood as a sensitive tissue for non-lethal studies. These results stress the importance of collecting pre-exposure data whenever possible, especially when sample sizes will be small.

Presence of sub-diploid peaks such as those observed in histograms of blood tissue can be indicative of aneuploidy or apoptosis [14, 17]. Frequency of occurrence of sub-diploid peaks from ducks exposed to TEM (Fig. 2) indicates a reverse dose trend similar to that seen by Bickham et al. [23]. They observed that high doses of TEM in Rattus produced a narrower CV in the G_1 peak than in reference animals and suggested that sensitive cells are being damaged to the point of necrosis and have been removed from the population. Remaining cells produce a narrower peak than normally observed because inherent variation within the cell population has been reduced. Similarly, low doses of TEM may slightly disrupt cellular kinetics resulting in the production of subdiploid peaks. With increasing dose, occurrence of subdiploid peaks decreases as sensitive cells die in response to increased disruption of cellular kinetics.

Aneuploidy is an unlikely explanation for the sub-diploid peak because of the length of time for exposure. George et al. [14] also observed subdiploid peaks in mallards exposed to radiation, but only 2 out of 14 ducks in their study develop aneuploid peaks after nine months exposure to ^{137}Cs. Such a high frequency of aneuploidy as detected in my study is unlikely after only a 24-h exposure period. Apoptosis may have been induced, but examination of DNA extracted from the same animals did not produce the characteristic ladder in agarose gels indicative of apoptosis.

This study supports the feasibility of using flow cytometry as a standard biomarker of exposure to mutagens for avian species. Cell-cycle kinetics were disrupted in the positive control groups although a definite dose response was not observed. Interference

with normal cell cycling would be lethal to developing embryos and chicks and would reduce normal replacement and repair mechanisms of tissues in both juveniles and adults. If the level of significance are relaxed slightly ($\propto = 0.10$), 6 parameters are significantly different. TEM appears to be an adequate positive control, but dose levels should be doubled or tripled from those that we administered. The high dose of MP was half the LD50 for mallards. At this dose, one individual exhibited toxic symptoms of disorientation, trembling, and inability to stand. Increasing the dose may have defeated the intent of the study by killing several individuals. Unfortunately, because the positive control failed to produce a distinct dose response, we are unable to make definite statements about the genotoxicity of MP. Occurrence of subpeaks in both chemicals may indicate a similar affect on DNA.

Avian populations are exposed regularly to genotoxins that may alter genetic configuration and ultimately affect long-term survival. Birds nesting within and around agricultural crops and forests are particularly susceptible to pesticide exposure [45]. In North Dakota, 13 of 16 of the most common pesticides used were toxic to waterfowl or aquatic invertebrates [32]. Neotropical migrants may be receiving additional pesticide exposure in South America. With further development, FCM has the potential to facilitate identification of xenobiotic damage in wild populations of birds, and its development as a possible genetic toxicity assay for birds should be pursued.

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Table 1. Mean values for flow cytometric parameters measured from blood samples of ducks before and after treatment with either TEM or MP. Standard deviations are in italics underneath each corresponding mean. Statistical analyses of TEM and MP dose groups were conducted separately.

			TEM	TEM	TEM	MP	MP	MP
		Reference	0.25	0.50	1.00	7.50	15.00	30.00
		(*n*=8)	(*n*=7)	(*n*=7)	(*n*=8)	(*n*=6)	(*n*=7)	(*n*=7)
DI	Pre	1.00	1.00	0.99	1.01	0.99	1.00	0.97
		0.04	*0.04*	*0.03*	*0.05*	*0.04*	*0.04*	*0.05*
	Post *	0.96	1.02	0.95	1.01	0.96	1.01	1.00
		0.04	*0.09*	*0.04*	*0.04*	*0.02*	*0.04*	*0.07*
	Diff	0.04	0.03	0.04	0.00	0.03	0.02	0.03
		0.05	*0.11*	*0.05*	*0.06*	*0.05*	*0.08*	*0.06*
PI	Pre	1.00	1.02	1.03	0.93	1.03	1.06	1.15
		0.23	*0.20*	*0.16*	*0.24*	*0.21*	*0.22*	*0.27*
	Post *	1.00	1.07	1.27	0.93	1.22	1.02	0.98
		0.21	*0.24*	*0.25*	*0.25*	*0.09*	*0.17*	*0.37*
	Diff	0.00	0.05	0.24	0.00	0.19	0.04	0.17
		0.29	*0.39*	*0.27*	*0.33*	*0.24*	*0.33*	*0.35*
G_1	Pre	57.10	57.54	58.61	56.63	56.28	56.96	55.96
		3.15	*3.04*	*3.54*	*3.36*	*3.74*	*2.72*	*3.21*
	Post	54.38	53.22	54.44	56.63	52.97	55.92	55.47
		2.25	*3.00*	*1.87*	*2.60*	*1.86*	*2.49*	*2.70*

	Diff	2.81	4.32	4.18	0.00	3.31	1.03	0.49
		4.91	*3.44*	*3.30*	*3.40*	*4.31*	*3.40*	*5.09*
CVG_1	Pre	3.97	4.49	4.48	4.25	3.85	3.75	4.27
		0.55	*0.49*	*0.95*	*0.95*	*0.48*	*0.21*	*0.37*
	Post	3.98	3.84	3.79	3.70	3.59	3.65	3.69
		0.51	*1.01*	*0.64*	*0.71*	*0.47*	*0.46*	*0.32*
	Diff ‡	0.01	0.65	0.69	0.55	0.26	0.10	0.57
		0.39	*0.68*	*1.03*	*0.76*	*0.80*	*0.65*	*0.46*
G_2	Pre	83.90	83.59	83.46	84.95	83.38	82.91	81.55
		3.65	*3.26*	*2.55*	*3.80*	*3.34*	*3.57*	*4.39*
	Post	84.82	86.77	80.74	85.82	81.41	85.44	85.14
		3.22	*7.76*	*3.80*	*3.72*	*1.40*	*4.05*	*5.63*
	Diff	4.82	8.42	7.52	0.13	5.99	2.59	0.44
		9.29	*6.71*	*6.73*	*6.98*	*8.51*	*6.72*	*9.86*
CVG_2	Pre	3.27	3.15	3.53	3.38	2.95	2.91	3.31
		0.69	*0.24*	*0.89*	*0.89*	*0.50*	*0.42*	*0.51*
	Post †	3.30	3.60	3.10	3.10	2.60	3.20	3.00
		0.30	*0.50*	*0.30*	*0.60*	*0.20*	*0.40*	*0.60*
	Diff	0.03	0.46	0.40	0.19	0.27	0.31	0.27
		0.65	*0.51*	*1.15*	*0.81*	*0.74*	*0.57*	*0.83*
$\%G_2$	Pre	15.63	16.02	16.04	14.56	15.99	16.74	18.10
		3.67	*3.18*	*2.52*	*3.89*	*3.16*	*3.55*	*4.47*
	Post *	14.60	15.63	18.66	13.86	18.21	15.27	14.31
		2.91	*3.67*	*3.74*	*3.61*	*1.53*	*2.59*	*5.67*

	Diff	1.03	0.38	2.61	0.66	2.21	1.46	3.79
		4.40	*5.61*	*4.39*	*5.27*	*3.68*	*5.27*	*5.41*
%S	Pre	0.48	0.39	0.49	0.49	0.62	0.35	0.35
		0.41	*0.32*	*0.24*	*0.21*	*0.36*	*0.10*	*0.15*
	Post	0.58	0.56	0.61	0.32	0.37	0.26	0.55
		0.46	*0.85*	*0.59*	*0.20*	*0.16*	*0.29*	*0.11*
	Diff	0.10	0.16	0.12	0.17	0.25	0.09	0.20
		0.75	*0.95*	*0.52*	*0.27*	*0.33*	*0.33*	*0.22*
G_2/G_1	Pre †	2.00	1.99	2.00	2.01	2.00	2.01	2.00
		0.01	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*
	Post ‡§	2.02	2.00	2.01	2.01	2.02	2.00	2.01
		0.01	*0.01*	*0.02*	*0.01*	*0.00*	*0.02*	*0.00*
	Diff	0.02	0.00	0.01	0.00	0.01	0.01	0.01
		0.01	*0.01*	*0.02*	*0.01*	*0.01*	*0.02*	*0.01*

‡ = Indicates significant difference in triethylenemelamine data at $\propto = 0.05$.

* = Indicates significant difference in triethylenemelamine data at $\propto = 0.10$.

† = Indicates significant difference in methyl parathion data at $\propto = 0.05$.

§ = Indicates significant difference in methyl parathion data at $\propto = 0.10$.

Table 2. Mean values for flow cytometric parameters measured from spleen samples of ducks treated with either TEM or MP. Standard deviations are in italics underneath each corresponding mean. Statistical analyses of TEM and MP (mg/kg body weight) dose groups were conducted separately.

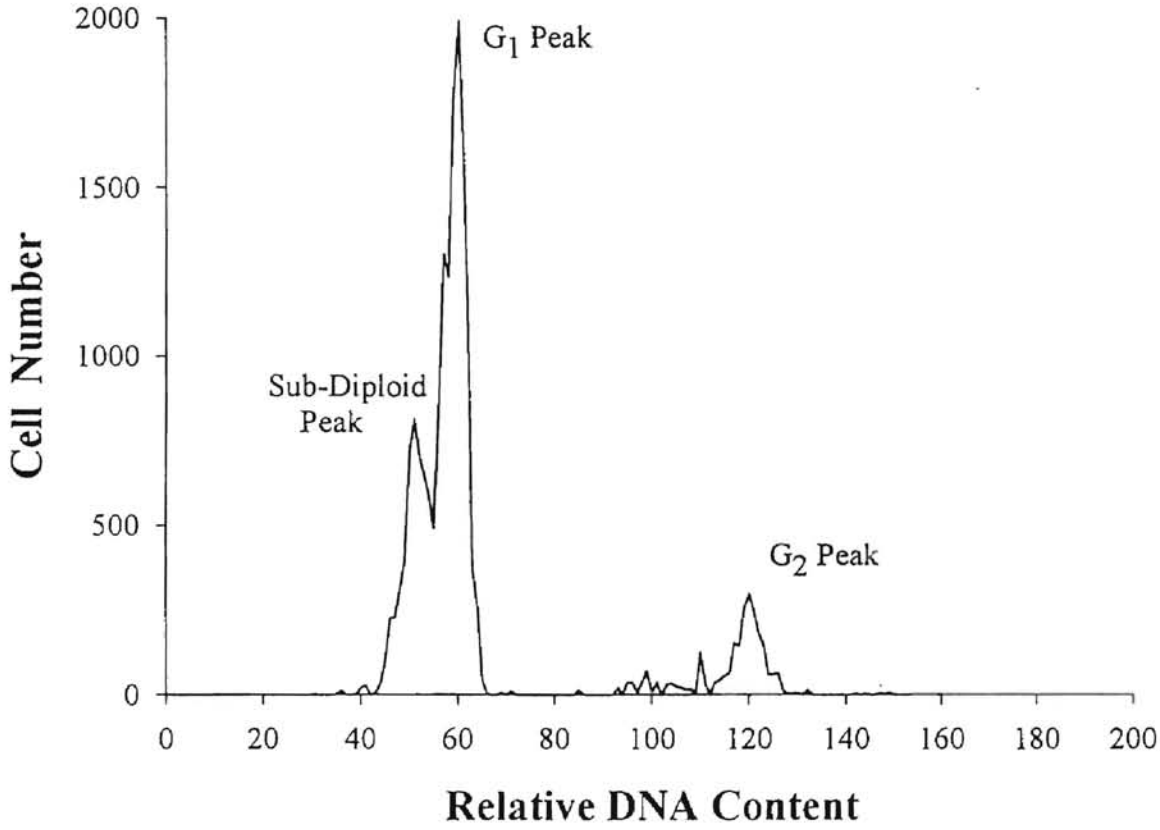
Dose	DI	PI	G_1	CVG_1	G_2	CVG_2 *	$\%G_2$ ‡	%S	G_2/G_1
Reference	1.00	1.00	55.86	4.41	111.38	3.50	2.73	2.95	2.00
(*n*=8)	*0.03*	*0.48*	*3.49*	*0.60*	*6.56*	*0.73*	*1.60*	*1.81*	*0.03*
TEM 0.25	0.97	1.52	56.82	4.46	114.12	3.01	5.20	3.45	2.01
(*n*=7)	*0.03*	*0.56*	*1.76*	*0.94*	*3.61*	*0.91*	*3.50*	*1.73*	*0.02*
TEM 0.5	0.98	1.35	56.20	4.14	112.40	3.00	4.71	2.98	2.00
(*n*=7)	*0.03*	*0.57*	*2.03*	*0.84*	*3.76*	*0.41*	*2.16*	*1.30*	*0.01*
TEM 1.0	1.01	0.92	56.76	4.10	113.38	2.69	2.43	2.78	2.00
(*n*=8)	*0.03*	*0.42*	*4.98*	*1.09*	*10.27*	*1.23*	*1.85*	*1.07*	*0.01*
MP 7.5	0.98	1.40	57.34	5.18	113.17	5.29	4.28	3.67	1.97
(*n*=6)	*0.03*	*0.54*	*3.43*	*1.25*	*6.19*	*2.11*	*2.59*	*1.49*	*0.02*
MP 15	0.98	1.25	56.89	4.82	113.65	3.68	3.45	3.66	2.00
(*n*=8)	*0.04*	*0.68*	*3.92*	*0.99*	*6.83*	*0.72*	*2.31*	*2.50*	*0.04*
MP 30	0.97	1.45	56.62	4.37	112.82	4.56	4.74	3.48	1.99
(*n*=7)	*0.05*	*0.78*	*1.78*	*0.71*	*3.25*	*1.16*	*3.24*	*1.67*	*0.04*

* = Indicates a significant difference in methyl parathion and triethylenemelamine data at $\propto = 0.10$.

‡ = Indicates a significant difference in triethylenemelamine data at $\propto = 0.10$.

Fig. 1. Histogram produced by flow cytometer depicting location of cell cycle stages and displaying a subdiploid peak.

Fig. 2. Occurrence of subdiploid peaks within dose groups from each chemical. All samples were examined twice. Values above bars indicate number of animals in dose group.


2000
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G1 Peak
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G2 Peak
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20
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60
80
100
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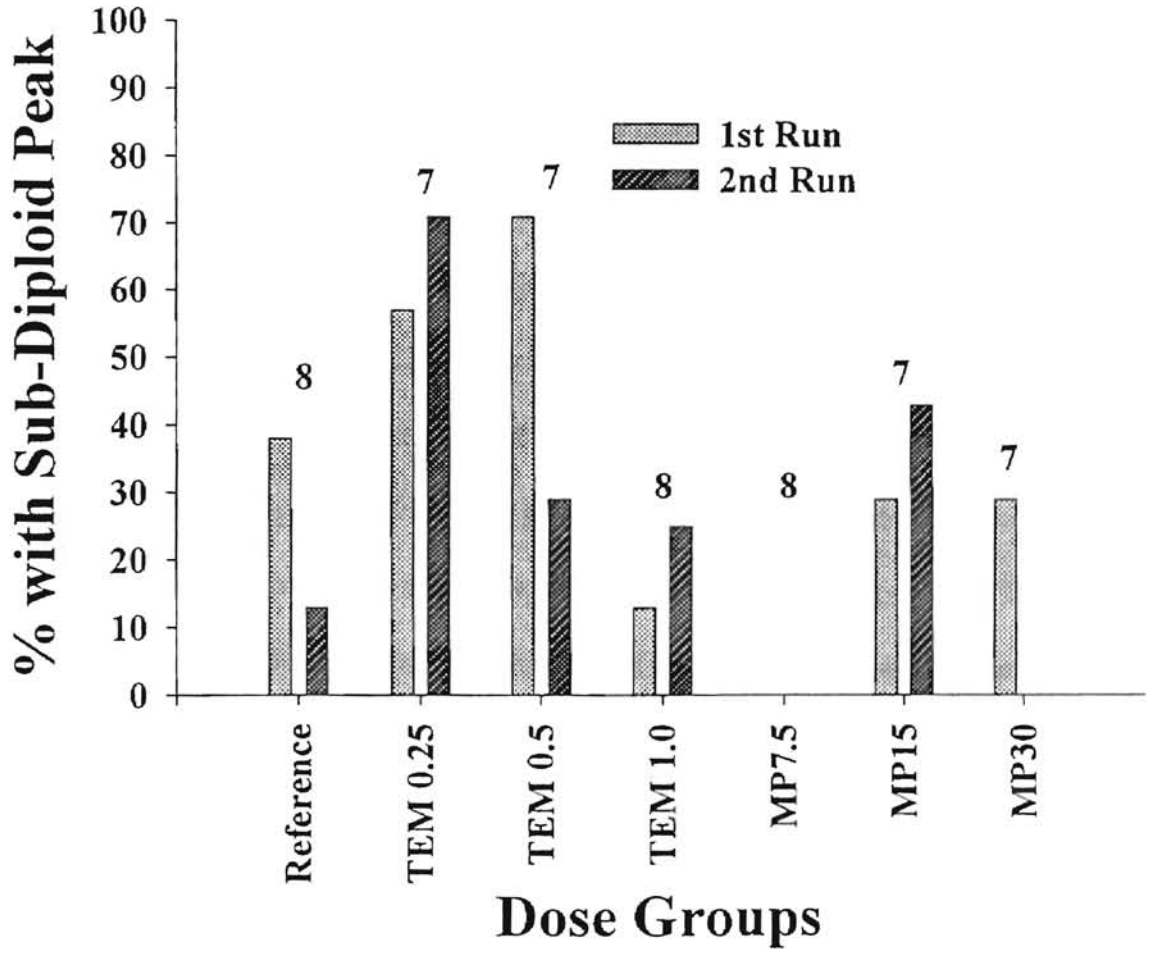
200
Relative DNA Content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1st Run
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7
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8
7
7
Reference
TEM 0.25
TEM 0.5
TEM 1.0
MP7.5
MP15
MP30
Dose Groups

CHAPTER II

DEVELOPMENT OF AN AVIAN GENOTOXICITY TEST USING AGAROSE GEL ELECTROPHORESIS

Abstract - Testing the capability of xenobiotics to induce genetic lesions generally involves use of microbes, mammals, or plants. Extrapolation to avian species may be unrealistic due to physiological and genetic differences among these taxonomic groups. Assays that permit collection of DNA samples without sacrificing birds will allow researchers to monitor temporal effects, obtain larger sample sizes for field studies, and assess impact on endangered species. My goal was to develop a standardized genotoxicity assay for birds using a non-lethal technique based on electrophoretic analysis of DNA double-strand breaks. Juvenile mallards were dosed orally with either methyl parathion (MP), triethylenemelamine (TEM), or corn oil. Blood and feather pulp samples were collected prior to dosing. At 24-h after dosing, blood, feather pulp, and liver samples were collected. Nonparametric tests of pre-dose samples from TEM dose groups detected significant differences in molecular length (kb) for both blood and pulp tissues and right half percent of blood tissue, but no significant differences occurred in MP dose groups or post-dose TEM dose groups. Results of analyses were inconclusive with no apparent dose response observed in groups administered the positive control. Dose levels of TEM may have been too low to induce a definite response. Analysis techniques need to be

further refined for use of constant field agarose gel electrophoresis of double-strand breaks as a non destructive biomarker in avian species.

Keywords - Genotoxin Avian Double-strand breaks Electrophoresis

INTRODUCTION

Although several prokaryotic and eukaryotic models have been developed to measure genotoxicity of environmental contaminants, few have been designed for avian species. Inconsistent responses within tissues of the same taxonomic group (i.e., mammals, microbes) are not unusual and when crossing phylogenetic lines inconsistencies are even more common. For example, Jenssen and Renberg [1], Mullison [2], and Zetterburg et al. [3] tested the herbicide, 2,4-D, in bone marrow erythrocytes of mice (Mus sp.), brain tissue of rats (Rattus sp.), and Salmonella, respectively. The herbicide did not increase frequency of chromosomal aberrations in erythrocytes of mice, did increase frequency of brain tumors in rats, and had no effect on reversion rate in Salmonella at pH 6.8. The lack of response from Salmonella is particularly striking because this study was conducted with the widely accepted Ames test for bacterial mutagenesis. Accurate extrapolation from genotoxicity data derived from other taxa to probable effects in birds may be hampered severely due to differences in chromosome size, relative proportion of functional DNA, gametic arrangement, metabolic rates, and degree of response from the liver [4-8]. Application of standard techniques for examining chromosomal genotoxicity to the avian genome is difficult because of high diploid numbers [9] and existence of numerous microchromosomes [10]. An avian genotoxicity assay that permits easy

collection of samples under field or laboratory conditions and rapid, inexpensive analysis would help reduce the need to extrapolate from data derived using other taxa. My goal was to develop a standardized genotoxicity assay for birds using a non-lethal technique based on electrophoretic analysis of DNA double-strand breaks.

Exposure to clastogens can result in detectable levels of genetic damage in a short period of time [11]. Because DNA damage such as single-strand breaks (SSBs) and double-strands breaks (DSBs) can occur naturally in an individual, measurements of DNA from exposed animals should be compared with those of a reference group. Although, SSBs are more likely to occur than DSBs, the latter are less likely to be repaired or repaired correctly [12]. Therefore, DSBs have a greater potential to be maintained and are more likely to be lethal to cells than SSBs [11].

Agarose gel electrophoresis appears to be a sensitive technique for detecting DSBs in DNA molecules [13-14]. Migration rates of DNA through an agarose gel are dependent on molecular size, with smaller fragments traveling faster through the gel matrix [14]. Therefore, clastogenic damage will result in greater dispersion of DNA through the gel matrix. Spread of DNA on the gel can be quantified using scanning densitometry [13]. Although pulsed-field electrophoresis is considered very sensitive, especially for high molecular weight DNA, constant-field gel electrophoresis (CFGE) is equally sensitive under optimal conditions and is simpler, faster, and less expensive [13]. A common obstacle with constant-field electrophoresis is the inability of large DNA fragments to exit the loading well. Volume of DNA migrating out of the loading well can

be maximized using a low voltage electrical field as recommended by Wlodek et al. [13] and a low percentage agarose gel. Wlodek et al. [13] did not find that cell concentration or agarose gel concentration altered results significantly, although this is contradictory to other research [15].

Liver is frequently the tissue of choice for electrophoretic analysis of DNA, because it is the primary organ of biotransformation [14, 16]. Unfortunately, collecting this tissue requires sacrificing the animal, and liver DNA can degrade rapidly once isolated. Strand-break analysis using gel electrophoresis has been conducted successfully with DNA extracted from blood of fishes [14]. Like fish, birds possess nucleated erythrocytes [17] enabling adequate quantities of DNA to be extracted from a minimal volume of blood, thereby reducing stress to the bird. Actively proliferating pulp of growing feathers also provides another easily obtained source of nucleated cells. This tissue can be collected readily with minimal stress to the animal. Because of declines in avian populations and interest in long-term monitoring programs, nondestructive biomarkers are becoming increasingly important [18].

CFGE has not been used extensively in genetic toxicology studies for wildlife. Theodorakis et al. [14] conducted a laboratory study in which several genotoxic compounds were investigated using blood cells of bluegill sunfish (Lepomis macrochirus) exposed *in vivo*. Exposure to contaminated sediments were found to be correlated positively with a decrease in median molecular length (mml) of DNA. This measurement, mml, was developed by Freeman et al. [19] to quantify number of single-strand breaks

under the theory that as DNA is fragmented, the mml will decrease. It has been used in field studies by Husby [20] examining DSBs in Peromyscus leucopus induced by exposure to metals and by Theodorakis et al. [21] to correlate DSBs with population changes using mosquitofish (Gambusia affinis). Husby [20] did not find a consistent pattern which correlated well with data derived from flow cytometry and chromosomal analyses of other tissues from the same animals, but Theodorakis et al. [21] found correlative relationships between occurrence of strand breaks, reproductive measurements, and exposure to radionucleotides.

The main objective of this study was to determine the usefulness of agarose gel electrophoresis for analysis of DSBs in avian species. I used one compound (triethylenemelamine, TEM) proven to be clastogenic in mammalian taxa and one compound (methyl parathion, MP) that is an environmental contaminant to which avian species are likely to be exposed. In birds, TEM is an effective sterilant, because of its mutagenic effect on spermatozoa [22-24]. MP (0,0-dimethyl 0-(4-nitrophenyl) phosphorothioate) is a heavily regulated organophosphate insecticide known to produce chromosomal lesions, sister-chromatid exchanges, and mutations in mammals [25, 26]. Furthermore, I examined levels of strand-breakage in three different tissues to determine if non-lethal sampling methods provided similar results to those obtained from tissues requiring death of individuals sampled. For agarose gel electrophoresis, a standard method of data analysis is to calculate median molecular length of DNA samples based on the assumption that damaged DNA will create a broader smear when visualized in an agarose gel and therefore have a lower median molecular length [14, 20]. This does not

take into account the shape of the DNA smear. Two samples could have the same median molecular length, but may have undergone different amounts of genetic damage (Fig. 1). I evaluated the use of three measurements of area covered by each DNA band for providing additional information regarding the extent of strand-breakage (Fig. 2).

Assuming TEM produces clastogenic responses in birds similar to those documented in mammals and DSB analysis using agarose gel electrophoresis is a reliable way to document those clastogenic effect, I would expect DNA samples from animals exposed to TEM to display increasing levels of diffusion in the agarose gel corresponding to increasing dose. Clastogenic damage should be detectable in all tissues examined. If MP is clastogenic in birds similar results also should be obtained for this compound.

MATERIALS AND METHODS

The mallard (Anas platyrhynchos) is designated by the EPA as an acceptable model species [27] for toxicology studies. For genotoxicology studies, the mallard has the additional advantage of low genetic variability [28]. Six-week old ducklings were purchased from Free Flight Game Bird Farm in Denton, Texas. Individuals were weighed and randomly divided among seven dose groups of eight individuals based on weight. Individuals were given a code number used to identify all samples from that animal and mask the identity of dose groups during processing and analysis of tissues. Ducks were housed by dose group in outdoor pens constructed of chicken wire and t-bars. Food and water was given ad libitum. Two weeks after arrival, blood and feather pulp samples were

taken for baseline genetic information. Heparinized vacuum tubes (2 ml) were used to draw blood from the brachial vein of each animal. About 0.2 g of feather pulp was collected from primary feathers by cutting the distal tip off of freshly plucked feathers and scraping the pulp out. Aliquots of each tissue (0.25 ml of blood and 0.2 g pulp) were placed into separate tubes containing 5ml of lysis buffer [29].

Animals were dosed one month after initial samples were collected. Doses were administered orally in gelatin capsules. Dose levels for TEM were set at 0, 0.25, 0.50, 1.00 mg/kg body weight based on previous studies of Jones et al. [22]. MP doses were 0, 7.5, 15.0, and 30.0 mg/kg body weight based on an oral LD_{50} of 60.5 mg/kg body weight for mallards [30]. Ducks were weighed the day before capsules were made. Doses were pipetted into individual capsules and capsules were topped off with corn oil. Reference groups were given a capsule containing only corn oil. Ducks were dosed within 3 h of making the capsules. Post-dose samples were collected 24 h later. Blood and feather pulp were collected as described above. After asphyxiation by CO_2, liver, spleen, heart, kidney, and brain samples also were taken. Organ samples were stored in cryotubes and placed in liquid nitrogen for transport to the laboratory. Only blood, liver, and feather pulp samples were used for electrophoretic analyses; other organ tissues were preserved for future research.

The method of DNA extraction and purification was that described by Longmire et al. [29]. Proteinase-K was added to each tube of lysis buffer and tissue. Tubes were placed on a rotator and incubated overnight at 37°C. To remove proteins, samples were

treated with equilibrated phenol, and the aqueous layer containing DNA was dialyzed against 1X TE for at least 24 h. Because repeated freezing and thawing of DNA can cause some degradation, an aliquot of the purified sample for analysis was maintained at 4°C. The remainder was stored at -80°C.

Although, Wlodek et al. [13] did not find concentration of agarose or DNA concentration to alter results, preliminary tests on excess DNA from reference and dose groups indicated both parameters affected the rate that DNA fragments moved into and through the gel. An aliquot of 10 μl (0.05μg of DNA) was adequate for DNA to exit the well readily while remaining visible when stained with ethidium bromide. All samples were electrophoresed through a 0.4% agarose gel containing 0.5mg/ml ethidium bromide with 1X TBE used as the running buffer.

Using size standards to bracket DNA bands from samples increases precision of measurements (31). After several preliminary experiments with various size standards, a combination of Saccharomyces cerevisiae and High Molecular Weight DNA Marker® (Gibco BRL) loaded in separate wells was selected for this study. The band that emerged from the S. cerevisiae well was assumed to be the smallest band of this size standard, but it could have been several bands together. S. cerevisiae was purchased embedded in agarose blocks. No more than 1/16 of a block of S. cerevisiae was loaded into a well, and 0.3 μl of High Molecular Weight DNA Marker® (Gibco BRL) was loaded into the adjacent well. A maximum of five samples were loaded between sets of standards. To compare variation between gels, four replicates of three samples were run for each tissue. Percent variation

of each replicated sample was calculated by dividing standard deviation by mean for each parameter by tissue and then averaging for the three replicates.

Based on recommendations of Wlodek et al. [13] for low voltage settings when examining large fragments of DNA, gels were run at 24 V. At this voltage, High Molecular Weight DNA Marker® required at least 18 h for bands to separate which was sufficient time for the S. cerevisiae band to become distinct from the well. Gels were photographed under ultraviolet light and negatives were scanned by a laser densitometer into the Molecular Analyst® (Bio-Rad) computer program. Image resolution was adjusted to 300 dpi, pixel depth was set at 12, and filter color was set to grey scale for film. Molecular Analyst® creates a histogram of the optical density (OD) readings versus distance (mm) on a gel for each sample. Background noise was removed manually from the graph produced for each lane. A logistic regression equation was automatically calculated from size standards bordering the sample lanes. Distance along the X axis corresponding to the point of greatest intensity of fluorescence in each sample peak was used to compute molecular length (kilobases). Boundaries of sample peaks were defined manually.

Because small fragments of DNA move through the gel matrix faster than large fragments, the region on the histogram to the right of the highest OD reading should represent smaller strands of DNA (Fig. 2). Right half area of a peak may provide more information about peak shape than whole area under the peak. Clastogenic damage should increase right half area; however, for comparisons among different types of tissue,

absolute area values could produce erroneous results because some tissue types may produce a broader band on a gel (Fig. 3). This problem may be alleviated by examining the proportion of peak area composed of smaller fragments (right half percent, Fig. 2). Therefore, I determined mean molecular length, entire area under the peak, right half area, and right half percent. Data were divided into three categories for each chemical: pre-dose tissues, post-dose tissues, and the difference between post- and pre- dose results. Tissues in each category were analyzed separately by dose compound. The Shapiro-Wilks test for normality was run on all parameters using the SAS© [32] software package. Results indicated that most of the data met assumptions for the Kruskal-Wallis Rank Sum Test, so for consistency it was used for all statistical analyses.

RESULTS

All statistically significant differences occurred in TEM dose groups prior to exposure (Table 1, 2). Those differences were found in molecular lengths of pre-dose blood ($p=0.0420$) and pre-dose pulp ($p=0.0439$), and in right half percent of pre-dose blood ($p=0.0265$). Pairwise comparisons on ranked data indicated that in pre-dose blood samples, the average molecular length of the high dose group was significantly smaller than for the mid- and low dose groups. Right half percent measurements of pre-dose blood were significantly larger in both the reference and high dose than for the low and mid-dose. In pre-dose pulp samples, the molecular length in the mid-dose was significantly smaller than the low and high dose. The pattern of statistical significance was inconsistent among tissues and endpoints. No parameter varied significantly among TEM

dose groups for the liver samples or for any tissues of dose groups exposed to MP. Comparison of proportion of standard deviations for replicated samples indicated that the values obtained for right half percent and molecular length tended to be less variable than whole area and right half area measurements (Table 3). Liver samples were the least variable of all tissues.

DISCUSSION

The scarcity of standardized assays for the study of genotoxicity in birds has been problematic for researchers. Avian species are exposed regularly to environmental contaminants including pesticides, primarily during the nesting season which may coincide with the agricultural growing season [33]. Exposure typically occurs when aerially-applied pesticides are sprayed directly on juveniles and any adults remaining in the nest vicinity. Nests do not necessarily have to be in close proximity to agricultural fields. Under aerial application, pesticide deposits have been detected 85 km from the application site [34]. Many pesticides can directly or indirectly affect immediate survival, but few studies have investigated what long-term genetic damage may occur that could alter reproduction or adult survival. My goal was to develop a technique for detecting effects from sub-lethal doses of genotoxins without having to sacrifice the bird.

For CFGE to become a standardized technique used to measure DSBs in high molecular weight DNA, analytical methods need to be further developed. One problem of using CFGE on high molecular weight DNA is that size standards possessing bands of

sufficient length to bracket large fragments of DNA typically will not exit the well or the portion that does emerge will not separate into distinct bands. Both Husby [20] and Theodorakis et al. [14, 21] used a size standard (λ-phage DNA digested with Hind III) that did not bracket the DNA bands from their samples. The median molecular length that they calculated was extrapolated from a regression equation that did not encompass their sample bands. For those studies an accurate measurement of average DNA strand size may not have been necessary because comparisons were confined within each experiment, but precision was important. The magnitude of error produced by this method remains unknown. Calculating molecular weights will be more precise by deriving a regression line based on size standards that bracket the DNA.

Results from Kruskal-Wallis analysis indicated all significant differences involved pre-dose samples from dose groups exposed to TEM and tissues with high mitotic rates. It should be noted that although feather pulp is a rapidly dividing tissue, it is impossible to collect without some contamination from blood [35]. The differences seen in median molecular lengths of pre-dose feather pulp may be due to the presence of blood that differed also in pre-dose molecular length rather than actual variation in the tissue. Those results did not conform with my predictions. The pattern of response is confusing in that significant differences were found only in pre-dose samples from groups assigned to TEM exposures.

Differences among dose groups in pre-dose results may indicate a bias in assigning ducklings to dose groups; however, assignment was based on weight prior to the first

sampling period in an effort to reduce bias created by a few days difference in age of ducklings. Because ducklings were not separated according to sex, a sex bias may have been created in some groups. Animals were processed by pen (dose group), so another possibility is that during the first round of tissue collection, blood and feather pulp samples were somehow mishandled during collection from particular pens causing damage to the DNA. Samples were stored together and processed in random order for each tissue, starting with pre-dose samples. It is possible, although not plausible, that blood and feather pulp tissues collected prior to dosing from TEM dose groups received rougher handling during extraction and processing. Unfortunately, it is infeasible to test for sex bias and impossible to test for the other potential causes of variation within the scope of this study.

Regardless of the reason for significant differences among pre-dose samples, the lack of a dose response in the animals administered the positive control is likely a result of dose concentrations that were too low. The literature regarding use of TEM as an avian sterilant reported doses several times greater than the lethal dose for rodents [22, 24, 36]. A conservative approach was taken in selecting dose ranges to ensure survival of test subjects. Methyl parathion doses were probably at reasonable levels to prevent killing the ducks. At the highest dose level (MP30), one duck exhibited toxic exposure symptoms of lack of muscle control and trembling.

Due to the lack of response from positive control groups, I cannot verify the usefulness of this technique for avian research. Future research should repeat the study

using higher doses of TEM. This is a critical area of research that needs to be further explored to develop an adequate test for genotoxicity in avian species and to develop an analysis technique that will be used consistently by researchers.

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Table 1. Mean values for parameters measured from blood samples of ducks before and after treatment with either TEM or MP (mg/kg body weight) and the difference between values (Diff). Standard deviations are in italics underneath each corresponding mean. Statistical analyses of TEM and MP dose groups were conducted separately.

			TEM	TEM	TEM	MP	MP	MP
		Reference	0.25	0.50	1.00	7.50	15.00	30.00
		(*n*=8)	(*n*=6)	(*n*=7)	(*n*=8)	(*n*=6)	(*n*=7)	(*n*=7)
Whole Area	Pre	1.30	1.25	1.10	1.19	1.04	1.03	1.29
		0.46	*0.49*	*0.53*	*0.38*	*0.36*	*0.16*	*0.35*
(OD*mm)	Post	0.85	0.98	0.89	1.09	1.02	0.91	1.01
		0.28	*0.25*	*0.25*	*0.19*	*0.47*	*0.27*	*0.21*
	Diff	0.59	0.45	0.43	0.27	0.62	0.48	0.51
		0.52	*0.16*	*0.32*	*0.13*	*0.30*	*0.33*	*0.31*
Rt Half	Pre	0.84	0.70	0.58	0.80	0.66	0.67	0.78
Area		*0.33*	*0.33*	*0.31*	*0.33*	*0.34*	*0.17*	*0.32*
(OD*mm)	Post	0.37	0.43	0.41	0.50	0.43	0.45	0.45
		0.17	*0.11*	*0.16*	*0.10*	*0.25*	*0.18*	*0.11*
	Diff	0.55	0.37	0.20	0.36	0.79	0.37	0.40
		0.30	*0.19*	*0.19*	*0.22*	*0.20*	*0.23*	*0.21*
Rt Half	Pre *	63.22	54.00	52.00	66.00	60.25	65.14	59.03
Percent		*12.49*	*7.90*	*3.60*	*11.70*	*15.69*	*10.79*	*12.63*
	Post	42.97	44.00	46.00	45.03	40.69	48.86	45.03
		8.92	*9.40*	*7.30*	*3.25*	*6.78*	*12.59*	*8.74*
	Diff	22.47	12.86	11.85	20.97	22.77	27.33	14.53
		11.70	*8.39*	*12.70*	*11.74*	17.31	18.27	*6.80*

Molecular	Pre *	62498	63056	64018	49170	55439	53726	57848
Length (kb)		*24470*	*13581*	*13028*	*5726*	*8395*	*8892*	*13935*
	Post	64216	66564	62988	70511	62086	68800	64116
		8966	*9741*	*7287*	*15972*	*7634*	*18598*	*5882*
	Diff	20105	10793	20914	21341	13043	28644	14191
		14936	*7627*	*23432*	*13539*	*8534*	*24997*	*9826*

* Indicates significant differences between TEM dose groups ($\propto = 0.05$).

Table 2. Mean values for parameters measured from feather pulp samples of ducks before and after treatment with either TEM or MP (mg/kg body weight) and the difference between values (Diff). Standard deviations are in italics underneath each corresponding mean. Statistical analyses of TEM and MP dose groups were conducted separately.

			TEM	TEM	TEM	MP	MP	MP
		Reference	0.25	0.50	1.00	7.50	15.00	30.00
		(*n*=7)	(*n*=7)	(*n*=6)	(*n*=5)	(*n*=5)	(*n*=7)	(*n*=6)
Whole	Pre	1.13	1.03	0.88	1.03	1.11	1.05	1.02
Area		*0.32*	*0.38*	*0.24*	*0.51*	*0.33*	*0.37*	*0.36*
(OD*mm)	Post	1.84	1.51	1.34	1.33	2.42	2.73	1.39
		0.85	*0.76*	*0.46*	*0.55*	*0.84*	*1.68*	*0.67*
	Diff	0.88	0.65	0.42	0.84	1.30	1.62	0.61
		1.03	*0.79*	*0.32*	*0.48*	*0.71*	*1.73*	*0.63*
Rt Half	Pre	0.79	0.69	0.63	0.73	0.79	0.70	0.69
Area		*0.24*	*0.32*	*0.18*	*0.40*	*0.28*	*0.31*	*0.29*
(OD*mm)	Post	1.40	1.19	1.05	1.03	1.83	2.11	1.10
		0.72	*0.63*	*0.39*	*0.46*	*0.73*	*1.40*	*0.53*
	Diff	0.75	0.60	0.39	0.69	1.01	1.38	0.56
		0.83	*0.66*	*0.28*	*0.39*	*0.68*	*1.47*	*0.48*
Rt Half	Pre	69.52	65.00	72.00	70.00	69.68	64.49	66.64
Percent		*5.56*	8.90	*1.90*	*4.80*	*7.97*	*8.69*	*6.52*
	Post	74.94	78.00	77.00	77.00	74.29	76.16	79.40
		10.61	*6.10*	*4.30*	*7.30*	*9.85*	*10.65*	*4.73*
	Diff	14.10	13.72	6.37	7.98	11.92	15.25	12.73
		5.66	*7.81*	*3.62*	*5.82*	*10.88*	*10.00*	*9.47*

Molecular	Pre *	42635	44499	39376	44440	41576	46179	44463
Length (kb)		*4179*	*3977*	*3368*	*2521*	*3227*	*4098*	*2838*
	Post	39147	35221	28981	30647	45132	46051	38078
		12597	*10066*	*2924*	*9183*	*17506*	*11878*	*9941*
	Diff	9782	11701	10152	15392	12988	9428	7611
		9702	*9666*	*5922*	*9458*	*13265*	*7941*	*7461*

* Indicates significant differences between TEM dose groups ($\propto = 0.05$).

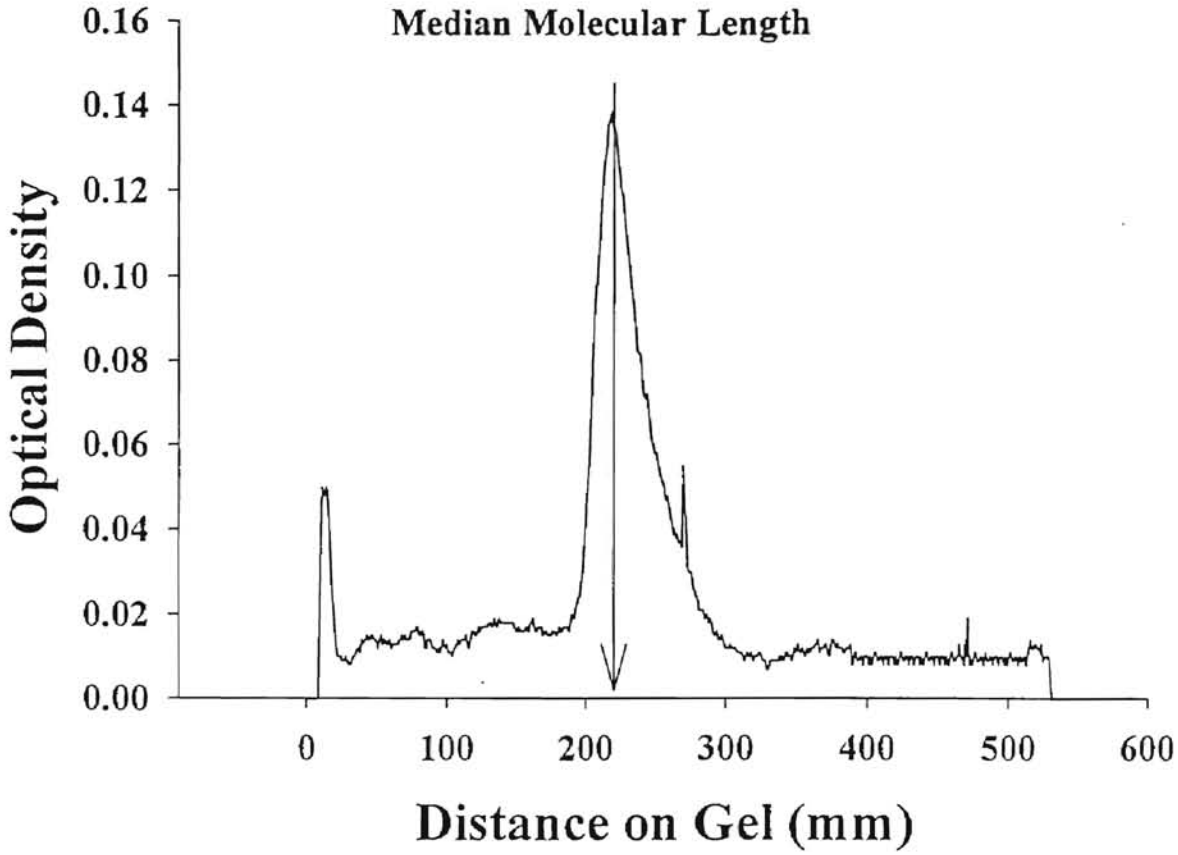
Table 3. Averaged percent variation of standard deviations for replicated samples by tissue type.

Tissue	Dose	Whole Area	Right Half Area	Right Half Percent	Molecular Weight
Blood	Pre	21%	32%	16%	18%
	Post	31%	32%	20%	5%
Pulp	Pre	26%	32%	9%	5%
	Post	23%	24%	4%	3%
Liver	Post	4%	4%	3%	2%

Fig. 1. Depiction of two different peak shapes with similar median molecular lengths.

Fig. 2. Sample histogram produced by Molecular Analyst® (Bio-Rad) computer program depicting separation of peak attributes used to analyze data.

Fig. 3. Comparison of histograms from liver and pulp samples.


Median Molecular Length
Optical Density
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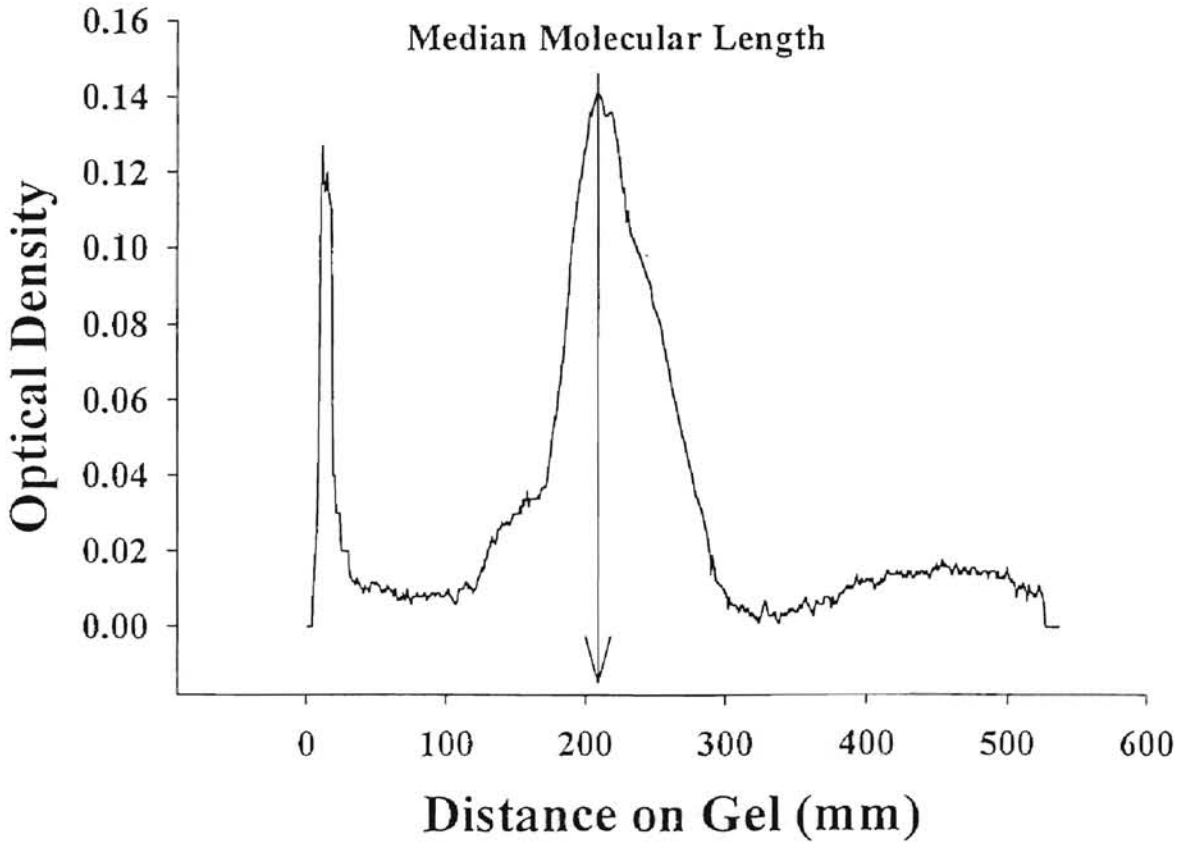
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
100
200
300
400
500
600
Distance on Gel (mm)


Median Molecular Length
Optical Density
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0.14
0.12
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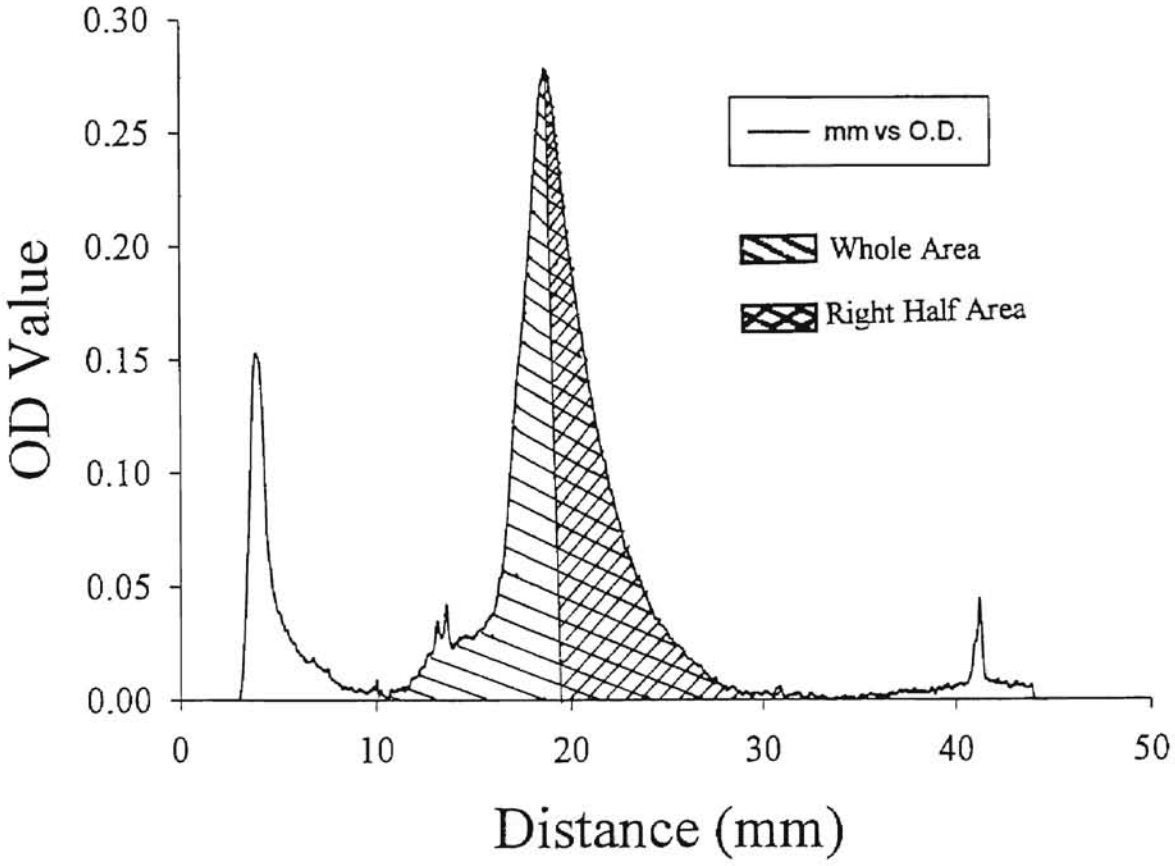
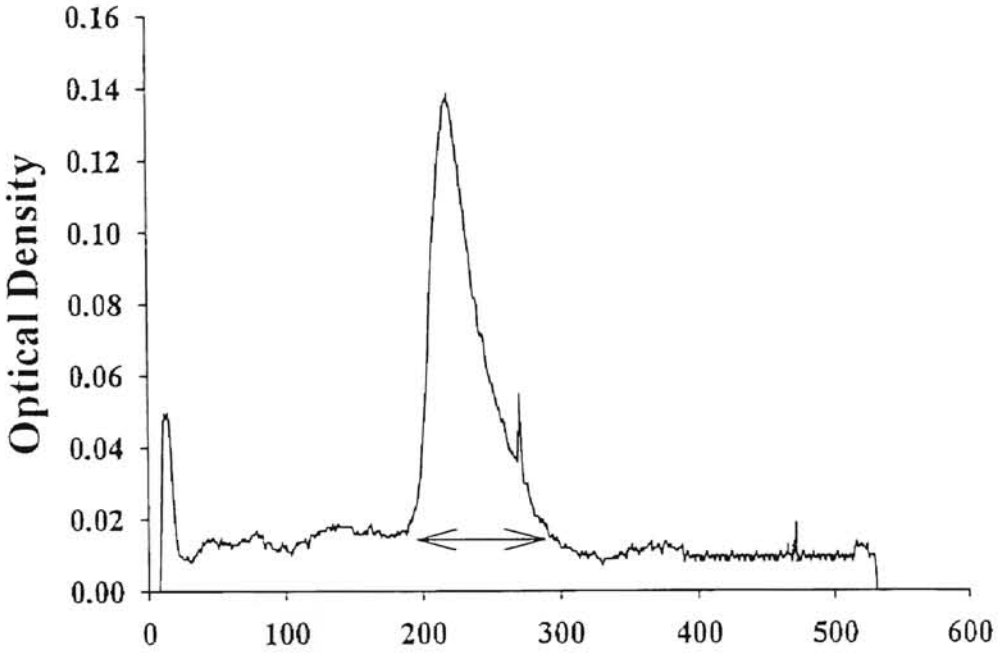
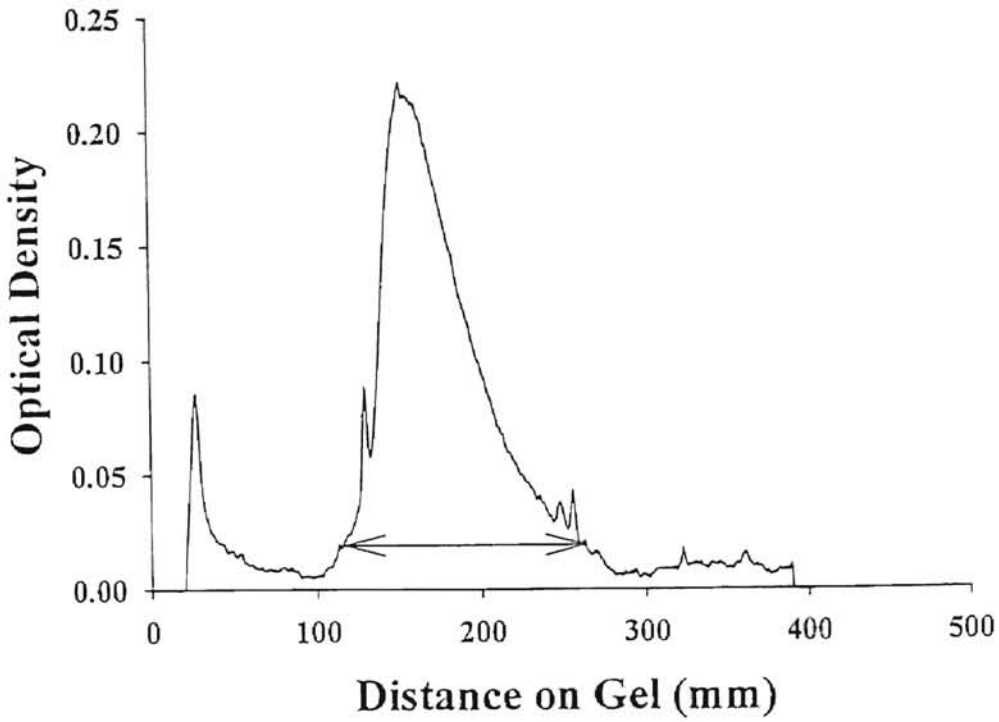
Distance on Gel (mm)


0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD Value
mm vs O.D.
Whole Area
Right Half Area
0
10
20
30
40
50
Distance (mm)

A. Liver



B. Feather Pulp



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