AN ABSTRACT OF THE THESIS OF

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Title: BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF

CARBON TETRACHLORIDE (CCl$_4$) TOXICITY IN THE

RAINBOW TROUT (Salmo gairdneri)

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Abstract approved: _____
Lavern J. Weber

Plasma enzyme activities are established parameters of estimating liver dysfunction or damage in clinical medicine and experimental mammalian toxicology. Aquatic toxicology needs to develop these and other biochemical tests for aquatic organisms. The application of diagnostic procedures to fishes can be used to evaluate the hepatotoxic effects of potential waterborne chemicals. Carbon tetrachloride, a model hepatotoxicant, produces consistent pathological changes in the livers of laboratory mammals. Toxicological studies with fishes indicated that this compound can produce a "similar pathology" in the livers of these vertebrates.

The initial objectives of this study were to demonstrate plasma and liver activities for alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) in the rainbow trout, Salmo gairdneri, and to develop precise enzyme assay conditions for the

enzyme which best evaluated the hepatotoxic response of CCl_4 given by intraperitoneal injection. Plasma GPT activity was less variable than GOT activity in control and CCl_4-treated fish, and, therefore, optimum assay temperature, pH and alanine concentration were determined for GPT.

The next objective was to determine the temporal pattern for plasma GPT activity after treatment with CCl_4. Significant elevation in plasma enzyme activity was measured as early as 3 hours post-treatment. The diet appeared to influence the hepatotoxic response and resultant plasma GPT activity. Histological examination of liver tissue indicated there was no correlation between liver damage and plasma GPT activity.

Concurrent with the increased plasma GPT activity was a decrease in total plasma protein concentration and an increase in body weight. The subsequent objective was to explain the 50% decrease in plasma protein concentration as early as 24 hours post-treatment. The decrease in plasma albumin concentration did not account for the entire reduction in plasma proteins; therefore, another plasma protein fraction may have been lost from the vascular compartment.

The nephrotoxic effect of CCl_4 was evaluated to determine if plasma proteins were excreted in the urine. CCl_4 produced an anuria as early as 1 hour post-treatment; however, histological

examination of kidney tissue indicated no consistent morphological damage from CCl_4. These findings, and measurement of urinary protein, established that significant quantities of plasma proteins were not excreted in the urine. Plasma proteins may have been lost through inflammation and hemorrhages in the peritoneal cavity.

The final objective was to explain the increase in body weight experienced by CCl_4-treated fish. There was a significant negative correlation between the changes in relative body weight and urine flow; therefore, the inability of the kidney to remove body water appeared to be responsible for the weight gain by these fish.

The results of this study indicate that plasma GPT activity appears to be a sensitive indicator of CCl_4 toxicity in the rainbow trout and that measurements of plasma enzymes can be useful tools to assess chemically-induced organ damage in fishes. The results show that diet may be a significant variable in fish toxicological studies. In addition, other biochemical and physiological parameters can prove beneficial in the overall assessment of chemical toxicity to fish.

Biochemical and Physiological Aspects of Carbon
Tetrachloride (CCl_4) Toxicity in the Rainbow
Trout (<u>Salmo gairdneri</u>)

by

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BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF CARBON TETRACHLORIDE (CCl_4) TOXICITY IN THE RAINBOW TROUT (<u>Salmo</u> <u>gairdneri</u>)

I. NON-TOXICOLOGICAL EXPERIMENTS

Introduction

The measurement of plasma enzyme activities has become an important tool in clinical medicine and experimental toxicology (Cornish, 1971; Wilkinson, 1970b). Because of the current interest in basic and applied research in fish physiology and toxicology, there have been attempts to use enzyme diagnostic techniques in the assessment of the hepatotoxic response of fishes treated with carbon tetrachloride (CCl_4) (Bell, 1968; Inui, 1969; Racicot <u>et al.</u>, 1975; Statham <u>et al.</u>, 1977). If these procedures are to be interpretive in pathological conditions, it is first necessary to determine "normal" ranges for plasma and tissue enzyme activities in healthy animals and to establish physiological factors that can affect plasma activities under pathological conditions.

Alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) activities were measured in the liver of rainbow trout; however, appreciable activities of these transaminases occurred in erythrocytes and kidney tissue (Gaudet <u>et al.</u>, 1975; Smith <u>et al.</u>, 1974). Gingerich <u>et al.</u> (1978) reported that CCl_4

produced considerable hemolysis in rainbow trout treated by intra-peritoneal (i.p.) injection. Gaudet et al. (1975) determined that GOT activity in rainbow trout erythrocytes was eight times greater than in an equivalent volume of plasma; therefore, CCl_4-induced hemolysis may erroneously elevate plasma GOT activity. Measurable plasma transaminase activity may also occur from CCl_4-induced kidney damage. Moon (1950) and Stricker et al. (1968) reported that CCl_4 was nephrotoxic to humans and laboratory mammals. No information is currently available regarding the nephrotoxic effect of CCl_4 in fishes.

In preparation for toxicological investigations with CCl_4, the objectives of this study were: 1) to determine "normal" activity ranges for alanine aminotransferase (GPT) and aspartate amino-transferase (GOT) in plasma and liver tissue from a population of rainbow trout; 2) to select the enzyme that would best define the hepatotoxic response in these fish; 3) to establish optimum assay conditions for this enzyme; and 4) to compare the liver and kidney activities and to determine the apparent Michaelis constants (Km) in these tissues for this enzyme. In addition, this study determined and compared "normal" GPT and GOT activities in plasma and liver from three other species in the Family Salmonidae: the steelhead trout, Salmo gairdneri gairdneri; the brook trout, Salvelinus fontinalis and the kokanee salmon, Oncorhynchus nerka kennerlyi.

Materials and Methods

Animals and Holding Conditions

All fishes were purchased from the Oregon Department of Fish and Wildlife. Rainbow trout and steelhead trout (Skamania River strain) were from Roaring River Hatchery, Scio, Oregon; brook trout and kokanee salmon were obtained from other hatcheries. Laboratory fishes were held in aquaria (200 gal) or artificial streams (brook trout) supplied with continuously flowing well water.[1] In an attempt to determine any variation in plasma and liver GPT activities between steelhead held in the laboratory and those in the natural environment, one-half of these fish were kept in a "live box" cage (6' x 3' x 3') in the Willamette River for 60 days. All fish were fed a commercial fish diet,[1] Purina Trout Chow^R[2] or Donaldson-Ore-Aqua,^{R}[3] every day, but food was withheld for 24 hours prior to an experiment. Rainbow trout, laboratory steelhead and kokanee salmon were maintained on a controlled photoperiod, 12L:12D. River steelhead and brook trout had a natural photoperiod, approximately 14L:10D at that time of year. All fishes were acclimated to the holding conditions for at least two

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[1] See Results section for acclimation temperature and diet.

[2] Ralston Purina Co., St. Louis, Missouri.

[3] Oregon-Aqua Foods, Newport, Oregon.

weeks and were in apparently good health. The time of the year in which individual experiments were performed is indicated in the Results section.

Blood Collection

Blood was obtained from the caudal peduncle using a heparinized syringe and needle (23G x 3/4"). The plasma was separated from blood cells by centrifugation and kept at $0-4^{\circ}C$ in test tubes rinsed with a 2.5% (w/v) solution of potassium oxalate.

Blood and liver samples from the Willamette River steelhead were obtained at the river site and kept in ice until assayed at the laboratory (approximately 6 hours).

Hemolysate Preparation

In the CCl_4-induced hemolysis experiment, pooled blood from eight fish was divided into 10 2-ml samples. Undiluted CCl_4 was added to five of the samples starting with 10 μl and increasing the volume by 10 μl for each sample, i.e. 10-50 μl. The five control samples received equivalent volumes of Cortland saline (Wolfe, 1963). All samples were gently mixed and centrifuged after a 15 minute "incubation" period. The plasma from each sample was analyzed for GPT and GOT activities and hemoglobin concentration.

In the physically-induced hemolysis experiment, 2 ml of blood from a fish were divided into 1 ml samples. One sample was rapidly forced through a 26 gauge hypodermic needle one to four times to produce varying degrees of hemolysis. The remaining 1 ml sample served as a control. The centrifuged plasma was analyzed for enzyme activities and plasma hemoglobin concentration.

Tissue Homogenates

Fish were killed by a blow to the head. The liver and kidney section were immediately removed and placed in an isotonic solution of ice-cold 0.25M sucrose TKM[4] buffer (pH 7.4) (Cousins et al., 1970). Using the whole liver or kidney section, a 1:9 (10% w/v) homogenate was prepared using the sucrose-TKM buffer according to the method of Potter (1955). The homogenate was centrifuged for 15 minutes (600 x g) at $0-4^{\circ}C$ to remove blood cells and cellular debris. The supernatant was drawn off and diluted with the sucrose buffer to a final concentration of 20 mg tissue/ml for measurement of enzyme activities. Protein concentration was determined using the undiluted supernatant which contained 100 mg tissue/ml. The middle (trunk) portion of the kidney was used for the determination

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[4]TKM: 0.05M Tris-HCl; 5 mM $MgCl_2$; 0.025M KCl.

of GPT activity. The "crude" supernatant portion of the homogenate was used to determine liver and kidney GPT activity for the approximation of the alanine-Km values.

Analytical Procedures

Enzyme Assays and Nomenclature. Enzyme activities were measured with a Gilford[5] 2400 recording spectrophotometer fitted with a Haake[6] constant temperature circulating water bath. The assay methods were those of Wroblewski and LaDue (1956) and Karmen (1955) for GPT and GOT, respectively. Tissue assays were modified to account for glutamate dehydrogenase (GDH) activity as described by Bergmeyer and Bernt (1974) (see Appendix for enzyme assay reactions). Unless otherwise noted, the concentrations of reactants in the assay mixture were the same as those in Sigma Bulletin No. 410-U.V. (see below). Chemicals were obtained from Sigma Chemical Co.,[7] except for the lactate dehydrogenase (LDH), which was ammonia-free in glycerol and purchased from Boehringer-Mannheim.[8] All plasma and tissue enzyme activities were determined on the same day that the fish

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[5] Gilford Instrument Labs, Oberlin, Ohio.

[6] Polyscience Corp., Evanston, Illinois.

[7] Sigma Chemical Co., St. Louis, Missouri.

[8] Boehringer-Mannheim, Indianapolis, Indiana.

was killed. Enzyme activities were measured at a wavelength of 340 nm, a light path of 1 cm and an assay temperature of $25^{\circ}C \pm 0.5^{\circ}C$. The final volume of the reaction mixture was 3.0 ml with a pH of 7.5. One International Unit (U) of enzyme activity is that quantity which will form 1 μmol of product per minute under the assay conditions.

Concentrations of compounds in the assay mixture:

Phosphate buffer (pH 7.5)	65 mM
α ketoglutarate	7 mM
Alanine	67 mM
NADH	.085 mM
Lactate dehydrogenase	960 U/l

The following abbreviations will be used:

GPT: alanine aminotransferase or glutamate-pyruvate transaminase (EC 2.6.1.2)

GOT: aspartate aminotransferase or glutamate-oxaloacetate transaminase (EC 2.6.1.1)

LDH: lactate dehydrogenase (EC 1.1.1.27)

GDH: glutamate dehydrogenase (EC 1.4.1.3)

NADH: nicotinamide-adenine dinucleotide (reduced)

Protein and Hemoglobin Concentrations. Tissue protein concentration was determined by the biuret method (Gornall et al., 1949). Plasma hemoglobin concentration was used as an index of

erythrocyte hemolysis and was determined by the method outlined
by Richterich (1969).

Alanine-Km Determination. In the alanine-Km experiment
the α ketoglutarate concentration in the assay mixtures was 7 mM,
which is 10-20 times the known Km concentration for α ketoglutarate
in the measurement of GPT activity in human plasma or pig heart
(Bergmeyer, 1978). The apparent Km for alanine was determined
for GPT in liver and kidney tissue from a Lineweaver-Burke
representation of the experimental data.

Statistical Methods

Mean values for the two steelhead groups were compared by
the Student's t-test for independent sample means (Simpson et al.,
1960).

Results

Alanine Aminotransferase (GPT) and Aspartate
Aminotransferase (GOT) Activities in Plasma
and Liver from Rainbow Trout

In this experiment fish (10-200 g) were held at $11.0^{\circ}C \pm 1.0^{\circ}$
and fed Purina Trout Chow [R] every day. Experiments were
carried out during the fall and winter. Plasma GOT activity was
12 times greater than plasma GPT activity; conversely, liver GPT
activity was greater than liver GOT activity (Table 1).

Table 1. Alanine aminotransferase (GPT) and aspartate amino-
transferase (GOT) activities in plasma and liver from
rainbow trout.

Enzyme	Plasma Activity[a]		Tissue Activity[b]		
	N	(U/l)	N	(U/g)	(U/mg x 10^{-2})
GPT	12	9.3 ± 1.3[c]	12	28.8 ± 3.1	16.0 ± 1.5
GOT	7	113.4 ± 27.4	12	14.3 ± 0.7	8.2 ± 0.6

[a]International Units of activity per liter at $25^{\circ}C$, pH 7.5.

[b]International Units of activity per g liver or per mg liver protein
at $25^{\circ}C$, pH 7.5.

[c]Values are the mean $\pm$ S.E.M. for N number of fish.

Effect of in vitro Hemolysis on Alanine
Aminotransferase (GPT) and Aspartate
Aminotransferase (GOT) Activities in
Plasma from Rainbow Trout

Fish (150-300 g) were held at $15.0^{o}C \pm 1.0^{o}$ and fed the

Donaldson diet every day. Experiments were done in the spring.

Plasma GPT and GOT activities did not increase after physically-

induced hemolysis; however, the corresponding plasma hemoglobin

concentrations increased approximately 7- and 16-fold in the GPT

and GOT samples, respectively (Table 2).

At the highest in vitro CCl_4 concentration, i.e. 2.5% (v/v),

plasma GPT activity did not increase, even though the hemoglobin

concentration was 430 times higher than the control concentration.

Plasma GPT activity did increase by 1.5 times, i.e. from 80 U/l

to 12.0 U/l, at a CCl_4 concentration of 1% (v/v) (Table 2). Plasma

GOT activity increased 1.4 times, i.e. from 120.6 U/l to 162.8 U/l,

at 2.5% (v/v) CCl_4. GPT activity actually decreased at 1.5% and

2.0% CCl_4, but GOT activity was higher than control values at all

CCl_4 concentrations.

A regression analysis of GPT and GOT activities and plasma

hemoglobin concentrations in physically-induced samples indicated

that there was some correlation between the degree of hemolysis

and plasma enzyme activities. The correlation coefficients (r) of

GPT and GOT were 0.29 and 0.36, respectively. A similar analysis

Table 2. Ratio[a] of plasma alanine aminotransferase (GPT), aspartate aminotransferase (GOT) activities, and plasma hemoglobin concentration after in vitro CCl_4-induced or physically-induced hemolysis of blood from rainbow trout.

| | Plasma Enzyme and Hemoglobin Ratio | | | |
	GPT	Hb	GOT	Hb
Physically Induced	0.93[b]	6.9	1.0[c]	16.6
	(6)		(7)	
CCl_4 Induced				
10 μl[d]	1.2	261	1.2	261
20 μl	1.5	328	1.3	328
30 μl	0.8	386	1.3	386
40 μl	0.8	233	1.1	233
50 μl	1.0	430	1.4	430

[a]Ratio was determined as follows:

Physically induced: $\dfrac{\text{Mean hemolyzed value}}{\text{Mean non-hemolyzed value (control)}}$

CCl_4 induced: $\dfrac{CCl_4 \text{ value}}{\text{Cortland control}}$

[b]Ratio is mean of 6 fish.

[c]Ratio is mean of 7 fish.

[d]Volume of CCl_4 added to 2 ml whole blood.

of the CCl_4-treated samples produced correlation coefficients of 0.13 for GPT and 0.89 for GOT.

Effects of Assay Temperature and Hydrogen Ion Concentration (pH) on Alanine Aminotransferase (GPT) Activity in Liver Tissue from Rainbow Trout

Fish (150-200 g) were held at $11.0^{\circ}C \pm 1.0^{\circ}C$ and fed Purina Trout Chow [R] every day. The values were determined in the fall. Liver GPT activities were measured at $7.5^{\circ}C$, $15^{\circ}C$, $25^{\circ}C$, $30^{\circ}C$ and $37^{\circ}C$ (pH 7.5) and at pH values of 6.5, 7.0, 7.5, 8.0 and 8.5 $(25.0^{\circ}C)$.

Mean liver GPT activity was four times greater at $37^{\circ}C$ than at $7.5^{\circ}C$ (Table 3) and increased linearly from $7.5^{\circ}C$ to $25^{\circ}C$ (Fig. 1). The Q_{10}[9] for the specific activity was 2.4 for the temperature interval from $7.5^{\circ}C$ to $17.5^{\circ}C$. In general, Q_{10} values can range from 2 to 3 for thermochemical, i.e. enzymatic, reactions while photochemical reactions have Q_{10} values less than 1.5 (Hoar, 1975). The greatest variability in activity occurred at assay temperatures above $25^{\circ}C$.

A transformation of the data to an Arrhenius Plot gave the relationship in Fig. 2. The point at which the experimental curve deflects from the hypothetical straight line is considered indicative

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[9]The Q_{10} is the change in reaction velocity (activity) caused by a $10^{\circ}C$ increase in assay temperature.

Table 3. Alanine aminotransferase (GPT) activity in liver tissue from rainbow trout at increasing assay temperature.

	Assay Temperature ($\pm$ 0.5°C)				
	7.5	15.0	25.0	30.0	37.0
Tissue Activity[a] (U/g)	9.0[b] $\pm$1.0	19.0 $\pm$1.0	31.1 $\pm$1.1	39.2 $\pm$1.9	41.2 $\pm$2.2
Specific Activity (U/mg protein x 10^{-2})	5.0 $\pm$0.9	10.3 $\pm$0.8	17.1 $\pm$2.0	21.4 $\pm$2.4	22.5 $\pm$2.5

[a] International Units of activity at pH 7.5.

[b] Values are mean $\pm$ S.E.M. of 3 fish.

Figure 1.   Specific alanine aminotransferase (GPT) activity in
rainbow trout liver at increasing assay temperature.
Values are the mean ± SEM for three fish.   Assay pH
= 7.5.
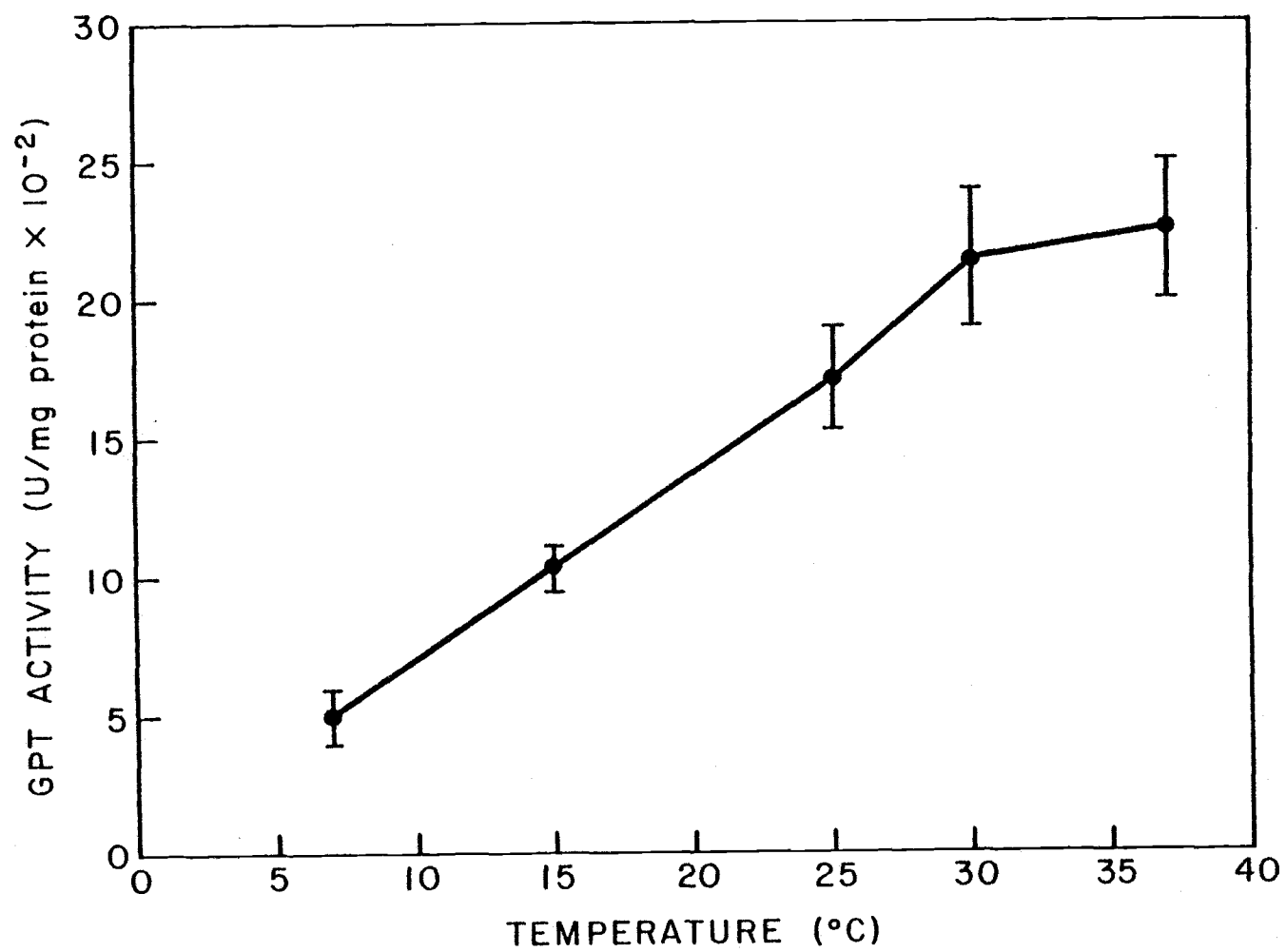
Figure 1.

Figure 2.   Arrhenius Plot of alanine aminotransferase (GPT)
            activity in rainbow trout liver.   Values are the mean of
            three fish.
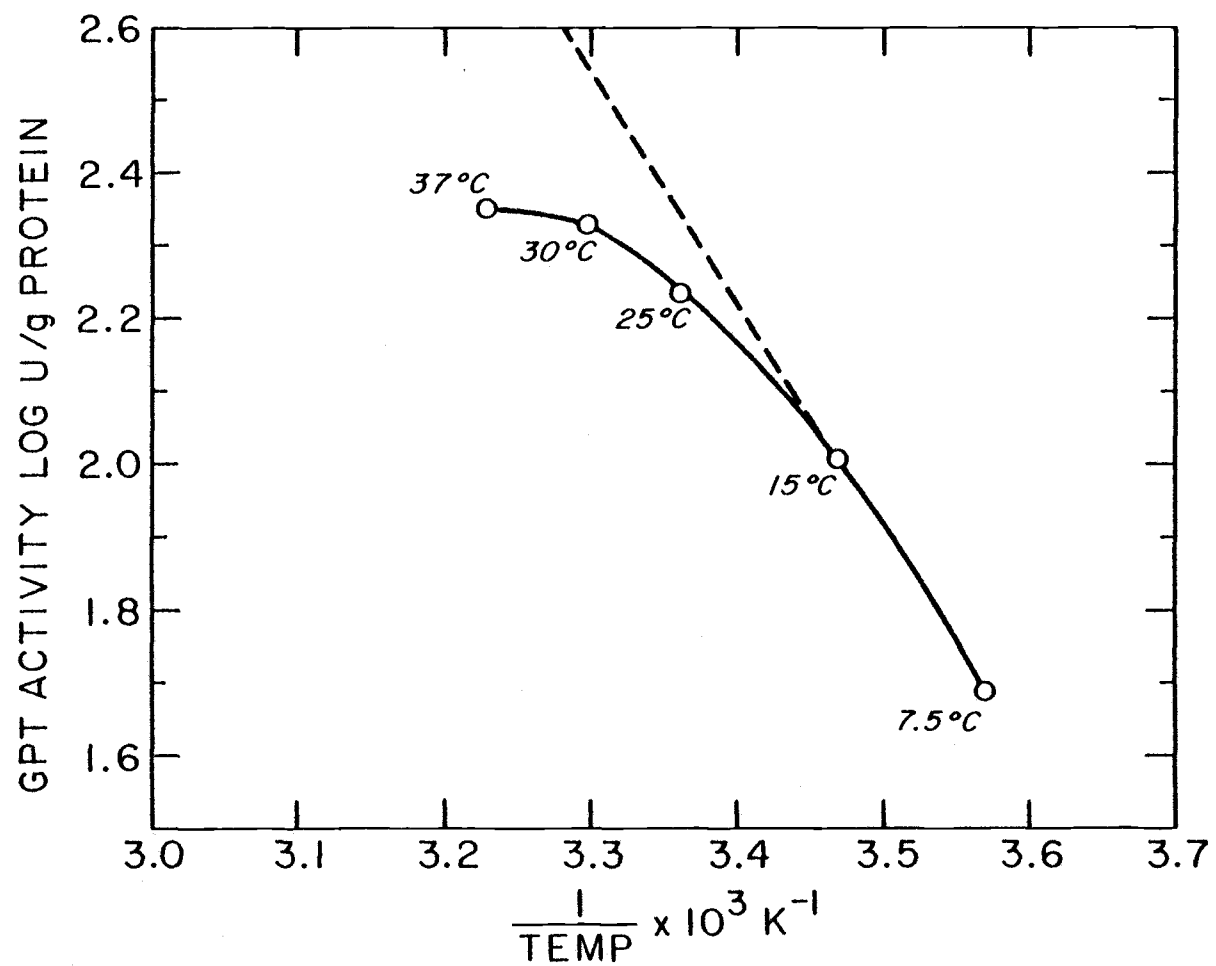
Figure 2.

of conformational changes in enzyme structure and possibly

denaturation (Jung and Liese, 1977).

The optimum GPT activity was measured at pH 7.5 (Table 4).

Fig. 3 represents a plot of assay pH and specific GPT activity.

Comparison of Alanine Aminotransferase (GPT) Activity in Liver and Kidney Tissues from Rainbow Trout

Fish (175-300 g) were held at $15.0^{\circ}C \pm 1.0^{\circ}$ and fed Purina

Trout Chow ^{R} every day. Experiments were done in the spring.

Liver tissue from rainbow trout had >30% more GPT activity than

kidney tissue (Table 5). Liver protein concentration was 46%

greater than in kidney tissue. GPT activity in liver and kidney

tissue at increasing alanine concentration is shown in Table 6.

GPT activity in liver tissue was inhibited by alanine concentrations

greater than 100 mM. The specific liver GPT activity of 16.0

$U/mg \times 10^{-2}$ (Table 1) was obtained using an alanine concentration

of 67 mM. Optimum liver GPT activity can be measured with an

alanine concentration of 50 to 100 mM. Transformation of the data

in Table 6 to a Lineweaver-Burke Plot gave the relationship in

Fig. 4. The apparent Km and maximum velocity (Vmax), i.e. the

maximum enzyme activity, for liver and kidney GPT catalyzed

reactions were determined from this representation. The Vmax

activities for GPT were 20 $U/mg \times 10^{-2}$ in the liver and 12.5 U/mg

Table 4. Alanine aminotransferase (GPT) activity in liver from
rainbow trout at increasing assay pH.

	Assay pH				
	6.5	7.0	7.5	8.0	8.5
	(4)	(4)	(4)	(4)	(4)
Tissue Activity[a] (U/g)	17.1[b] $\pm$2.6	21.8 $\pm$2.6	42.7 $\pm$10.4	26.7 $\pm$3.4	34.0 $\pm$5.0
Specific Activity (U/mg protein x 10^{-2})	13.4 $\pm$2.7	15.1 $\pm$0.9	27.2 $\pm$5.8	18.4 $\pm$1.3	19.1 $\pm$3.4

[a] International Units of activity at 25°C.

[b] Values are mean $\pm$ S.E.M. for number of fish in parentheses.

Figure 3.   Specific alanine aminotransferase (GPT) activity in
             rainbow trout liver at increasing assay pH.   Values are
             the mean $\pm$ SEM for four individual fish at each pH.
             Assay temperature was 25°C.
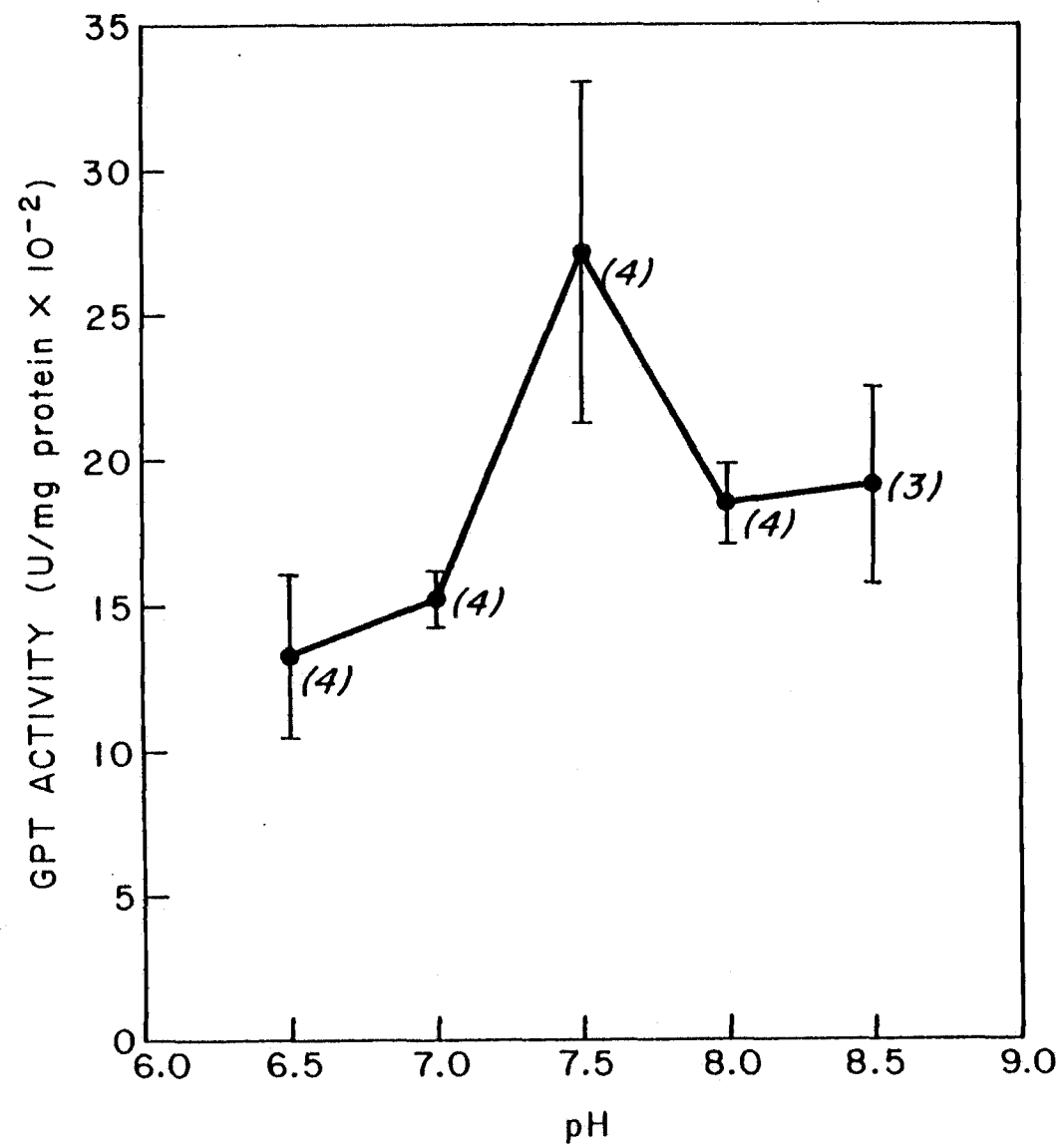
Figure 3.

Table 5. Alanine aminotransferase (GPT) activity and protein concentration in liver and kidney tissue from rainbow trout.

Parameter	Liver	Kidney
Tissue Activity[a] (U/g)	$26.2^{b} \pm 3.5$	14.3 ± 1.5
Protein Concentration (mg/100 mg tissue)	13.6 ± 0.5	9.3 ± 0.3
Specific Activity[a] (U/mg protein x 10^{-2})	19.4 ± 2.8	14.8 ± 1.5 .

[a] International Units of activity at $25^{o}C$, pH 7.5.

[b] Values are mean $\pm$ S.E.M. for 4 fish.

Table 6. Alanine aminotransferase (GPT) activity in liver and kidney tissue from rainbow
trout at increasing alanine concentrations.

	Alanine Concentration (mM)						
	0.5	1.0	5.0	50.0	67.0[a]	100	250
Liver Activity[b] (U/mg protein x 10^{-2}) N = 4	1.6[c] ±0.2	-	9.4 ±1.2	16.9 ±2.3	20.3 ±2.5	19.4 ±2.8	17.1 ±0.8
Kidney Activity (U/mg protein x 10^{-2}) N = 4	1.1 ±0.2	2.0 ±0.3	5.7 ±0.9	12.4 ±1.7	-	14.8 ±1.4	-

[a]From Sigma Kit No. 410-UV.

[b]International Units of activity at 25°C, pH 7.5.

[c]Values are mean ± S.E.M. for N number of fish.

Figure 4. Lineweaver-Burke plot of alanine aminotransferase (GPT) activity in liver and kidney tissue from rainbow trout at increasing assay concentrations of alanine. The Vmax for liver and kidney GPT are 20 U/mg x 10^{-2} and 12.5 U/mg x 10^{-2}, respectively, and the apparent Km values for alanine in the liver and kidney GPT catalyzed reactions are 5.6 mM and 5.0 mM, respectively.
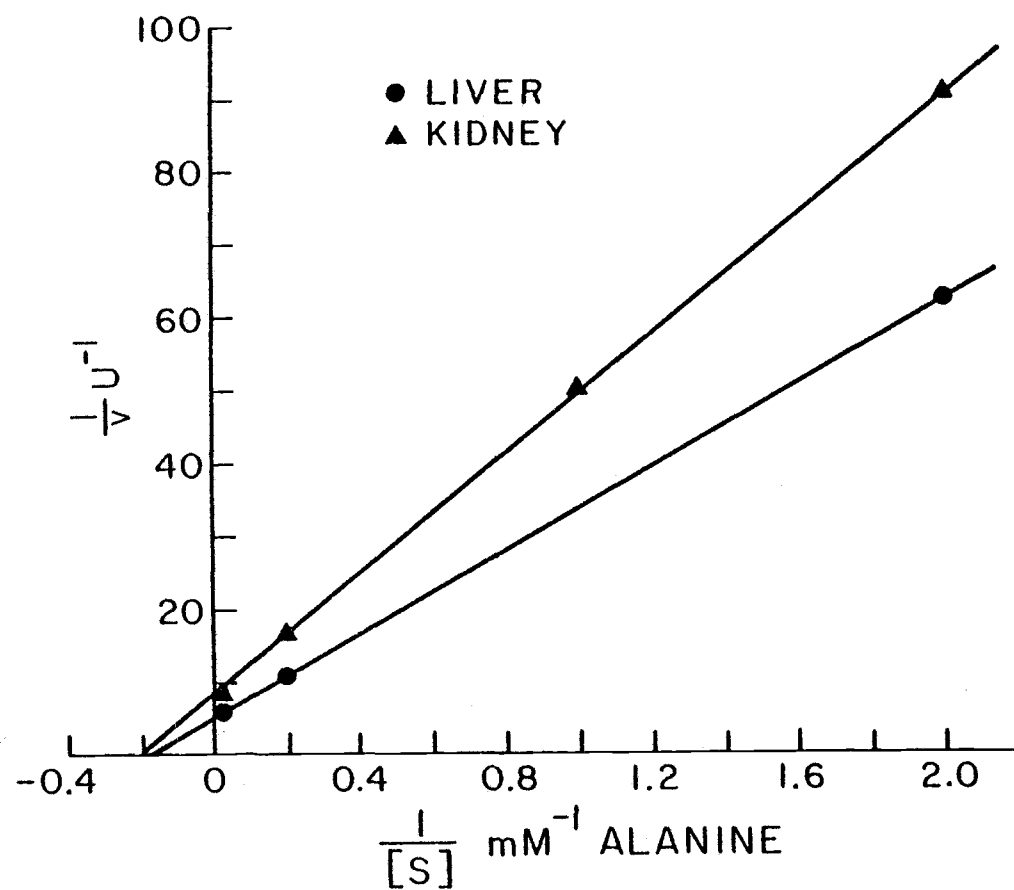
Figure 4.

$x\ 10^{-2}$ in the kidney. The apparent Km values for alanine in the

liver and kidney GPT catalyzed reactions were approximately 5.6

mM and 5.0 mM, respectively.

Comparison of Alanine Aminotransferase (GPT)
and Aspartate Aminotransferase (GOT) Activities
in Plasma and Tissue from Four Salmonids

All fishes in these experiments were fed the Donaldson diet

every day. The weight ranges for the fishes and acclimation

temperatures were: rainbow trout (58-138 g), $11.0^{\circ}C \pm 1.0^{\circ}$;

steelhead trout (24-58 g), $12.0^{\circ}C \pm 1.0^{\circ}$ (lab) and $11^{\circ}-14^{\circ}C$

(river); brook trout (140-182 g), $12.0^{\circ}C \pm 1.0^{\circ}$; kokanee salmon

(62-178 g), $12.0^{\circ}C \pm 1.0^{\circ}$. The season during which these values

were determined was: rainbow trout (summer), steelhead trout

(spring), brook trout (summer) and kokanee salmon (summer).

Plasma GOT activity was greater than plasma GPT activity

for all four salmonids (Table 7). Liver enzyme activities showed a

similar relationship, except for the rainbow trout in which liver

GPT activity was greater than GOT activity. The data agree with

the findings of Gaudet et al. (1975) that high tissue enzyme activities

are not necessarily reflected by high plasma enzyme activities, as

was suggested by Zimmerman et al. (1968). The highest GPT

activity in liver tissue was from the brook trout, the plasma from

which had the lowest GPT activity. Conversely, the plasma from

Table 7. Alanine aminotransferase (GPT), aspartate aminotransferase (GOT) activities in plasma and liver and protein concentration in liver from four members of the Family Salmonidae.

Parameter	Rainbow Trout (8)[a]	Steelhead Trout (lab) (14)	Steelhead Trout (river) (16)	Brook Trout (6)	Kokanee Salmon (15)
GPT Plasma Activity[b] (U/l)	9.7[c] $\pm$1.0	9.6 $\pm$0.9	11.0 $\pm$0.7	4.7 $\pm$1.4	12.6 $\pm$1.9
GOT Plasma Activity (U/l)	113.8 $\pm$10.2	188.9 $\pm$19.3	-	291.4 $\pm$17.5	130.9 $\pm$14.1
Liver Protein concen. (mg/100 mg tissue)	15.7 $\pm$0.5	13.5** $\pm$0.6	11.2 $\pm$0.4	13.9 $\pm$0.7	17.1 $\pm$0.6
GPT Specific Activity (U/mg protein x 10^{-2})	22.3 $\pm$2.6	23.4 $\pm$1.9	23.0 $\pm$2.1	30.3 $\pm$3.3	8.3 $\pm$0.8
GOT Specific Activity (U/mg protein x 10^{-2})	17.9 $\pm$1.5	34.6 $\pm$2.8	-	53.5 $\pm$5.6	14.9 $\pm$1.2

[a] Number of fish.

[b] International Units of activity at 25°C, pH 7.5.

[c] Values are mean $\pm$ S.E.M.

** Significantly different from Willamette River steelhead (P < 0.01).

the kokanee salmon had the highest GPT activity, while the liver

tissue had the lowest GPT activity. It appeared that all species had

the capability to synthesize liver protein, i.e. enzymes (Table 7).

Discussion

A comparison of plasma and liver GPT and GOT activities

found in my study with those activities in rainbow trout reported by

other workers demonstrates the variability of these enzyme

activities in normal fish (Table 8). Blood and biochemical para-

meters for fishes are known to depend on the strain, diet, sex,

age, time of year, holding conditions and disease states (Barnhart,

1969; Blaxhall, 1972; Hickey, 1976). Plasma activities for both

enzymes in this study were considerably lower than values reported

by Gaudet et al. (1975), which were 54 U/l and 259 U/l for GPT and

GOT, respectively for rainbow trout at 15°C. On the other hand,

in a sequel study Racicot et al. (1975) found plasma activities for

GPT of 26.6 U/l and 196 U/l for GOT, in one group of control fish,

and 15.6 U/l and 141 U/l for plasma GPT and GOT, respectively,

in another group of control fish. In the studies of Gaudet and

Racicot all fish were fed Purina Trout Chow[R] and, presumably,

were genetically similar, disease-free and maintained under identi-

cal conditions; nevertheless, considerable variation existed in their

values for mean plasma GPT and GOT activities in their control

Table 8. A comparison of alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) activities in plasma and liver from selected species of fishes.

Enzyme	Common Name and Water Temperature (°C)	Plasma Activity[a] (U/l)		Liver Activity (U/g)	Liver Activity (U/mg)	Reference
GPT	Rainbow trout (15)	54	(17)[b]	41 (2)	0.35 (2)	Gaudet et al. (1975)
GOT	Rainbow trout	259	(10)	33 (2)	0.30 (2)	Gaudet et al. (1975)
GPT	Rainbow trout (15)	26.6	(10)			Racicot et al. (1975)
		15.6	(7)			
GOT	Rainbow trout	196	(10)			Racicot et al. (1975)
		141	(7)			
GPT	Rainbow trout (15)	15	(20)			Sauer and Haider (1977)
GPT	Rainbow trout (12.5)	9	(12)			Sauer and Haider (1977)
GOT	Rainbow trout (15)	300	(21)			Sauer and Haider (1977)
GOT	Rainbow trout (12.5)	250	(14)			Sauer and Haider (1977)
GOT	Sockeye salmon (?)[c]	299	(7)			Bell (1968)
GPT	Native channel catfish (?)			31.4 (5)	0.27 (5)	Wilson (1973)
GOT	Native channel catfish (?)			45.8 (5)	0.35 (5)	Wilson (1973)
GPT	Cultured channel catfish (?)			42.6 (10)	0.31 (10)	Wilson (1973)
GOT	Cultured channel catsifh (?)			57.0 (10)	0.41 (10)	Wilson (1973)
GPT	Rainbow trout (10)			30.1 (20)		Smith et al. (1974)
GOT	Pink salmon (?)	309	(?) non-spawning			Marquez (1976)
GOT	Pacific herring (?)	1778	(?)			Marquez (1976)
GOT	Dogfish (?)	128	(?)			Marquez (1976)
GOT	Lingcod (?)	28	(?)			Marquez (1976)
GOT	Rainbow trout (15)			91.2 (5)		Freeman and Idler (1973)
GPT	Brook trout (15)			411.0 (10)		Freeman and Idler (1973)
GPT	Eel (20)			22.3 (7)		Inui (1969)
GOT	Eel (20)			196.7 (7)		Inui (1969)
GPT	Rainbow trout (12)	22.7	(49)			Statham et al. (1977)
GOT	Rainbow trout	169.0	(51)			Statham et al. (1977)

[a] International Units of activity under the assay conditions. [b] Values are the mean for the number of fish in parentheses.
[c] ? = Water temperature or number of fish not specified.

trout. It should be noted that different transaminase assay kits were used in their studies and may have accounted for the variability in the plasma GPT and GOT activities.

Statham et al. (1977) reported mean serum activities of 22.7 U/l for GPT and 169 U/l for GOT in control rainbow trout maintained at 12°C. The type of diet was not specified. In a recent study, Sauer and Haider (1977) measured GPT and GOT activities in plasma from rainbow trout, which had been acclimated to different water temperatures. At 12.5°C plasma GPT and GOT activities were 10 U/l and 250 U/l, respectively, and increased to 15 U/l and 300 U/l, respectively, at 15°C. The fish were fed Fukosalm[R], a commercial fish diet.

Liver activities (U/g tissue) for GPT and GOT were lower for fish in my study than liver activities reported by Gaudet et al. (1975), which were 41 U/g for GPT and 33 U/g for GOT. Their mean tissue activities were determined from two fish, and this sample size was not adequate to infer values representing normal, mean tissue enzyme activities. It is difficult to compare specific liver activities (U/mg protein) for GPT and GOT in my study with those listed by Gaudet. Their specific GPT activity, as presented, would be two to three orders of magnitude less than the values found in my study. Assuming that there was a misprint and the "iu/g protein" was actually "iu/mg protein," the specific liver GPT and GOT activities obtained in my study are less than those found by Gaudet.

In the determination of biochemical parameters, the analytical procedures are as important as the biological variables listed above. The lower GPT and GOT plasma and liver activities in my study might have been due to an inhibitory affect by one of the compounds in the assay mixture. Increasing concentrations of α-ketoglutarate and NADH are known to inhibit mammalian GPT and GOT activities (Bergmeyer and Bernt, 1974); however, the concentrations of α-ketoglutarate used by Gaudet et al. (1975) in Sigma Kit No. 155-UV and in my study were identical. Furthermore, the concentration of NADH in Kit No. 155-UV is higher than in Kit No. 410-UV. It appears that reactant concentrations were not the source of variation in enzyme activities between my study and that of Gaudet.

In the measurement of GPT and GOT activities, ammonia (NH_4^+) containing reagents, e.g. the LDH solution in Sigma Kits for GPT and GOT, can give higher apparent activities due to the concurrent measurement of GDH (see Appendix). In control fish, plasma GDH activity should be negligible, but liver homogenates and pathologic plasma may have considerable GDH activity.

The primary purpose of this experiment was to develop a reproducible analytical assay for GPT and GOT in plasma and liver from rainbow trout. As long as the precision of the measurements was relatively consistent, i.e. within ± 2 S.D., the concentrations of the assay reactants were not changed. These enzyme assay kits

and reagents are designed primarily for measuring enzyme activity in human plasma, and it is doubtful that the concentrations in the assay mixture are optimum for rainbow trout plasma or liver enzymes. Bergmeyer and Bernt (1974) determined the optimum conditions and concentrations for GPT and GOT activities in human serum but emphasized that these conditions and concentrations do not necessarily apply to sera or organ tissues from other species.

The differences in plasma and liver GPT and GOT activities between my study and those mentioned above may be due to some inherent variable in the rainbow trout or due to some unknown or ignored factor in the analytical procedures.

Although not frequently mentioned in mammalian toxicity studies, the hepatotoxic compound, CCl_4, can cause in vitro and in vivo hemolysis (Von Oettingen, 1955). Mammalian erythrocytes contain relatively greater levels of transaminase activities than an equivalent volume of plasma (Caraway, 1962). Gingerich et al. (1978) reported that CCl_4 caused considerable hemoglobinemia in rainbow trout treated by i. p. injection. Gaudet et al. (1975) determined that GOT activity was eight times greater in rainbow trout erythrocytes than in an equivalent volume of plasma; GPT activity in the red blood cells was negligible. They concluded that slightly hemolyzed blood remained suitable for plasma GPT and GOT measurements.

My results indicate that plasma GOT activity was increased by CCl_4-induced hemolysis but not by physical disruption of erythrocytes. Plasma GPT activity, on the other hand, was not consistently elevated by either treatment. The results of the CCl_4 treatment appear to support the findings of Gaudet et al. (1975).

The physical disruption of red blood cells produced plasma hemoglobin concentrations that were sometimes greater than those achieved by CCl_4 treatment. The reason for the lower hemoglobin ratios with physical hemolysis was because the non-hemolyzed (control) samples had slightly elevated hemoglobin concentrations.

The results suggest that the hemolytic effect of CCl_4, and the resultant release of intracellular enzymes, was different from physically produced hemolysis. CCl_4 has a high affinity for lipids, as does its biological metabolite, chloroform. The chemical structure and properties of membranes can vary from one tissue to another; however, all cells, including erythrocytes, have membranes with certain common constituents. The membranes consist primarily of protein and lipids, e.g. phospholipids such as phosphatidyl choline. In vitro studies with mammalian red blood cells have shown that CCl_4 is 10 times more active than chloroform in its hemolytic effect (Von Oettingen, 1955). The reactive compound can bind covalently and selectively to unsaturated fatty acid double bonds, displaying a great affinity for microsomal lipid, particularly

cholesterol esters and phosphatidylcholine (Reynolds, 1967). Presumably, the direct contact by CCl_4 on the outer and intracellular erythrocyte membranes results in the disruption of membrane structural and functional integrity and the "leakage" of intracellular components, including enzymes, into the plasma.

In vitro studies can only approximate the physiological conditions in the intact organism. It is not known, for example, what concentration of CCl_4 is present in the blood of rainbow trout after i. p. injection or how long the CCl_4 remains in contact with the erythrocytes. My in vitro results indicate that CCl_4 has the potential to cause in vivo hemolysis and can influence plasma enzyme measurements in the rainbow trout.

The first two experiments indicated: 1) that plasma GOT activity is greater and more variable than plasma GPT activity; 2) that in vitro, CCl_4-induced hemoglobinemia appears to alter plasma GOT activity more than plasma GPT activity. In addition, preliminary experiments with rainbow trout given CCl_4 by i. p. injection confirmed the findings of Gingerich et al. (1978) that CCl_4 produces considerable in vivo hemolysis; however, no additional, relevant information about the pathological response was obtained by the measurement of both plasma enzyme activities. The extra effort and expense required to measure GOT activity in future experiments was not justified, and the emphasis in the optimum

assay and CCl_4 toxicity experiments was placed on the measurement of plasma GPT activity.

The enzyme assay temperature may have little similarity to the usual environmental temperature of the organism; however, this in vitro temperature is important in comparative analysis of enzyme activities (see Appendix). Bell (1968) found that purified GOT from the liver of an adult coho salmon, Oncorhynchus kisutch, had activity which increased linearly from $10^{\circ}C$ to $30^{\circ}C$.

In Fig. 2 the deflection of the experimental curve from the hypothetical straight line at $>17^{\circ}C$ may be significant in the relationship between the environmental temperature of the organism and the optimum enzyme activity, i.e. the enzyme may have experienced conformational changes that resulted in decreased catalytic efficiency. Under laboratory conditions rainbow trout were capable of existing in water at $24^{\circ}C$ (Cherry et al., 1975). After acclimation to temperature of $15\text{-}18^{\circ}C$, these fish selected a similar temperature; however, when acclimated to temperatures above $20^{\circ}C$ or below $15^{\circ}C$, they preferred lower or higher water temperatures, respectively.

Growth rate, as an indication of food conversion efficiency, can be used to evaluate optimum temperature requirements. Atherton and Aitken (1970) demonstrated that water temperature and diet can influence growth rate in rainbow trout. Fish on a low fat (1 mg/g) diet showed optimum growth at $12^{\circ}C$, whereas the high fat

(95 mg/g) diet fish displayed optimum growth at 16°C. Wurtsbaugh and Davis (1977) established that ration level was important in the evaluation of optimum water temperature and growth of rainbow trout. They concluded that growth was enhanced by temperature increases up to 16.5°C when ration level was not restricted. The above studies demonstrated that the optimum water temperature for maximum food conversion efficiency, i.e. maximum metabolic homeostasis, in rainbow trout was between 12°C and 17°C.

The International Union of Biochemistry and the International Federation of Clinical Chemistry recommend a standard enzyme assay temperature of 30°C. Bergmeyer (1978) suggested that 25°C would be more practical for most situations. He contended that adequate reaction rates can still be achieved, and there would be less reagent and cuvette temperature fluctuation. In the measurement of enzyme activities in fishes, the assay temperature should approximate the environmental temperature of the animal; however, this is not always practical. The results of my study indicate that 25°C was the maximum assay temperature to practically measure liver GPT activity in these fish, and this temperature was used in future experiments. When enzyme activity is measured at a "non-physiological" temperature, it should be understood that this is an artificial situation and may lead to erroneous assumptions about the scope of enzyme activity in the natural environment.

My results indicate that optimum GPT activity in liver from rainbow trout can be measured at an assay pH of 7.5. Working with purified GOT from the liver of an adult coho salmon, Oncorhynchus kisutch, Bell (1968) determined the effect of assay pH on the initial velocity (activity) of the enzyme reaction at $25^{\circ}C$. His results suggested that there was no distinct optimum pH for GOT activity in the liver from this fish; however, the initial velocity was positively correlated to pH in the range of pH 5 to pH 10. In addition, Bell noted that GOT in liver (crude homogenate) from rainbow trout was most active in the alkaline pH range, i.e. >pH 8.0. This type of response to changes in pH has not been reported for this enzyme in mammalian plasma or tissues. The optimum pH range for GOT and GPT in plasma from humans is 7.3 to 8.0 and 7.3 to 7.8, respectively (Bergmeyer and Bernt, 1974).

The reason for the slight increase in GPT activity from pH 8.0 to 8.5 is not readily apparent. It may have been due to the small sample size and high variability of these fish; on the other hand, the enzyme may have been showing a pattern of increasing activity in the alkaline pH range as Bell (1968) reported for GOT in liver from rainbow trout.

CCl_4 has been shown to be hepatotoxic to many vertebrate species (Diaz-Gomez et al., 1975), including fishes (Bell, 1968; Gingerich et al., 1978; Inui, 1969; Racicot et al., 1975; Statham

et al., 1977). Moon (1950) and Stricker et al. (1968) reported that CCl_4 is also nephrotoxic to humans and laboratory mammals. GPT activity has been found in the liver, kidney and heart tissues from rainbow trout (Gaudet et al., 1975). No information is currently available regarding the nephrotoxic effect of CCl_4 in fishes; however, it is conceivable that measurable GPT activity could occur in the plasma from kidney damage in CCl_4-treated fish.

The kidney GPT activity obtained in this experiment was considerably less than the activity reported for rainbow trout by Gaudet et al. (1975); however, they also found that specific liver GPT activity was >30 %, i.e. approximately 40%, of the specific kidney GPT activity. Some reasons for the apparent differences in enzyme activities between different populations of rainbow trout were discussed previously.

The results indicate that optimum liver GPT activity can be measured with an alanine concentration between 50 and 100 mM in the assay mixture, and that alanine concentrations >100 mM appeared to inhibit enzyme activity.

The Michaelis constant (Km) for an enzyme is an important and useful characteristic and is fundamental to the mathematical description of enzyme kinetics and also to the quantitative assay of enzyme activity in different tissues. Isoenzymes catalyze the same reaction in different tissues but can differ significantly in their Km

requirements. Although there have been no isoenzymes reported for GPT in mammals or fish, the original intent of the Km studies with my trout was to characterize liver and kidney GPT by their apparent Km-alanine values for future application in CCl_4 toxicity experiments. If the Km values were different for the liver and kidney GPT, any GPT present in the plasma from tissue damage could similarly be characterized, and the tissue of origin identified. The apparent Km-alanine for liver and kidney GPT was 5.6 mM and 5.0 mM, respectively (Fig. 4) and, therefore, too similar to be applicable to the problem of differential organ damage by CCl_4. No attempt was made to determine the apparent Km values for α-ketoglutarate, the other substrate for GPT, in liver and kidney tissues from my fish. This was primarily because the GPT was not in a purified form, and the presence of any glutamate dehydrogenase and ammonia in the tissue homogenate could appreciably interfere with the measurement of GPT activity (see Appendix).

Bergmeyer (1978) determined that the apparent Km-alanine for GPT in human plasma was 21.9 mM. A higher Km is indicative of a lower substrate affinity for an active site on the enzyme molecule; however, the Km is not a static value but can vary depending on the experimental assay conditions, e.g. pH and temperature. We might deduce that the affinity of alanine for an active site on the GPT molecule, i.e. the formation of the

enzyme-substrate complex which determines the reaction velocity, is

four to five times greater in liver and kidney tissue of the rainbow

trout than in human plasma. This deduction, however, is per-

missible only for the specific experimental conditions. The results

may prove beneficial in the field of comparative biochemistry, but

we must remember that in vitro conditions limit the application of

of the data to conditions occurring in the natural environment where

the relationship between substrate concentrations and enzyme

activity remains undefined.

Before enzymes or other biochemical parameters can be

utilized in the assessment of fish health or the response to toxi-

cants, it is necessary to have some knowledge of the normal

activities or concentrations in the particular species (Bell, 1968).

Some information is available regarding the activities of GPT and

GOT in the plasma and tissues, i.e. liver, from a few species of

fishes (Table 8).

In general, the results of the transaminase comparison

between the salmonid species in my study suggest that these enzymes

are more important in the liver metabolism of the brook trout than

the other species. Bell (1968) found a mean GOT activity of 299 U/l

in serum from sockeye salmon, the anadromous "cousin" of the land-

locked kokanee salmon, which had a mean plasma GOT activity of

130.9 U/l (Table 8). It is tempting to suggest that the greater

GOT activity in the plasma from the sockeye salmon reflects a
higher liver GOT activity and, therefore, that this enzyme plays a
more significant role in the liver metabolism of the anadromous
species. However, my results cannot support this deduction.
The specific enzyme activity in a tissue and the relationship between
tissue and plasma activities may be a species-dependent phenomenon
and controlled by mechanisms not yet determined.

The primary purpose of the steelhead-comparison experiment
was to determine the effect of different holding conditions, i.e.
water quality, on GPT activity in plasma and liver from a homogenous
population. The Skamania steelhead is well adapted to the water
conditions of the Willamette River and is stocked in this system to
provide a sport fishery. Whenever a comparison of enzyme activity
is made between different populations or species of fishes, the diet,
water temperature and assay conditions, i.e. assay temperature,
should always be considered as potential variables. Dietary protein
was shown to affect GPT activity in laboratory mammals (Szepesi and
Freeland, 1966, 1968; Waldorf et al., 1963), and channel catfish
(Wilson, 1973). Smith et al. (1974) found that GPT activity in
erythrocytes, liver and muscle from rainbow trout can be altered
by eliminating pyridoxal phosphate, the coenzyme for GPT and GOT,
from the diet. Acclimation temperature (Sauer and Haider, 1977)
and enzyme assay temperature (Pfeifer et al., 1977) have been shown

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to affect GPT activity in plasma and tissues from rainbow trout.

In this study, both groups of steelhead trout were maintained at

approximately the same temperature and fed the same diet. Enzyme

assay conditions were identical for both groups. The results indicate

that the GPT activity in the plasma and liver tissue from the labora-

tory and river fish were not significantly different ($P \leq 0.05$). The

concentration of protein in the liver was significantly higher

($P < 0.01$) in the laboratory fish. In conclusion, the results indicate

that variable environmental factors, i.e. photoperiod and water

quality, had no significant effect on the GPT activity in the plasma

and liver from these two groups of fish.

II. TOXICOLOGICAL EXPERIMENT #1: CARBON TETRACHLORIDE-INDUCED HEPATOTOXIC RESPONSE IN RAINBOW TROUT AS INFLUENCED BY TWO COMMERCIAL FISH DIETS

Introduction

The nutritional status of experimental animals has been shown to be an important factor in toxicological evaluation. The acute, toxic response of laboratory rats to organochlorine, organophosphate or carbamate compounds can be influenced by the quantity or quality of dietary protein (Baker and Street, 1970; Campbell and Hayes, 1976; DeFerreyra et al., 1974; Korsrud et al., 1976; Krijen and Boyd, 1971; McLean and McLean, 1966; Webb and Miranda, 1973). Mehrle et al. (1970) found that rainbow trout fed a 23% protein diet were more susceptible to chlordane toxicity than fish fed a 45% protein diet, and dietary methionine concentration altered DDT and dieldrin toxicities in rainbow trout (Mehrle et al., 1977). Many interactions between dietary constituents and exogenous chemical toxicity involve nutritional effects on the microsomal mixed function oxidase (MFO) enzymes in the endoplasmic reticulum of liver hepatocytes. Campbell and Hayes (1974) reviewed the importance of nutrition in drug biotransformation by the MFO enzyme complex.

In my study plasma alanine aminotransferase (GPT) activity was measured to assess the hepatotoxic response of rainbow trout to carbon tetrachloride (CCl_4). In the course of the investigation, the laboratory changed commercial brands of trout feed. The fish were fed the new diet for approximately 3 months before resumption of the toxicity studies. When they were treated with the same intraperitoneal (i. p.) dose of CCl_4 (1.0 ml/kg), plasma GPT activity was quantitatively and temporally different from the plasma enzyme response of fish on the previous diet.

The objectives of this study were to compare the effects of two commercial fish diets on alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) activities in plasma and liver from non-treated control rainbow trout and on the plasma GPT activity in CCl_4-treated fish. The plausible reasons for the variable hepatotoxic response are discussed.

Materials and Methods

Animals, Diet and Holding Conditions

Rainbow trout (100-250 g) were purchased from Roaring River Hatchery, Scio, Oregon. The fish were initially fed Purina Trout Chow [R] and held in 200 gallon aquaria supplied with continuously flowing well water at ambient temperature ($11.0\,^{\circ}C \pm 1.0^{\circ}$). They were transferred to another commercial fish diet, Donaldson-

Ore Aqua ^{R} , and the water temperature was maintained at $15.0^{\circ}C$ $\pm 1.0^{\circ}$. A 12L:12D photoperiod was used in all experiments. All fish had been acclimated to the holding conditions and diets for at least 3 months and were in apparently good health. They were fed daily, but food was withheld for 24 hours prior to an experiment. The Purina diet, CCl_4 study was performed in the winter, while CCl_4 experiments using Donaldson diet fish were carried out in the summer.

Experimental Procedures and Treatment with CCl_4

GPT and GOT activities in plasma and liver, and liver protein concentration were determined for non-treated control fish. These fish were taken directly from the "holding" aquaria and were not subjected to the experimental procedure discussed below.

Twenty-four hour "fasted" fish were weighed, fin marked and placed in 50 gallon aquaria supplied with continuously flowing well water ($11.0^{\circ}C$ or $15.0^{\circ}C$). After 24 hours, undiluted CCl_4 (1.0 ml/kg or 2.0 ml/kg) was given by i.p. injection, approximately equi-distant between the base of the pelvic fins and the distal edges of the pectoral fins. Control fish received an equivalent volume of Cortland saline (Wolfe, 1963). The injection procedure was done in a shallow, aerated water bath, which permitted opercular respiration but allowed for adequate restraint. Blood and tissue samples

were obtained at 3, 6, 12, 18, 24 hours post-treatment (Purina diet) and 3, 6, 12, 18, 24, 36 and 48 hours post-treatment (Donaldson diet).

Blood Collection, Tissue Homogenates and Analytical Procedures

These procedures were identical to those previously described in the Non-Toxicological Experiments. Liver enzyme activities were not determined for Cortland or CCl_4-treated fish.

Statistical Methods

Experimental data were converted to a quantal form and analyzed by the Fisher Exact Probability Test (Siegel, 1956). Values greater than two standard deviations ($P < 0.05$) from the mean control value were chosen to indicate a positive response in the treated fish.

Histological Studies

At 3 to 48 hours after treatment with CCl_4 or Cortland saline, the liver and a portion of the trunk kidney were removed from fish representing each dietary regimen. Within 3 minutes after the fish had been killed, sections of the liver and kidney were fixed in Bouin's Solution. Some control liver sections were also fixed in Carnoy #1 solution for glycogen identification. The tissues were

embedded in paraffin, and 6 μm sections were stained with hematoxylin and eosin (liver and kidney) or Best's carmine (liver) for glycogen resolution.

Results

Non-Treated Control Fish

GPT and GOT activities in plasma and liver, and liver protein concentrations were determined for non-treated control fish fed the Purina or Donaldson diets (Table 9). Plasma GPT and GOT activities were similar for fish on either diet. Liver GPT and GOT activities were 38% and 118% greater, respectively, for Donaldson diet than Purina diet fish. Liver protein concentration was 13% greater in the Purina diet fish.

CCl_4-Treated Fish

Plasma GPT activity for Cortland control fish fed the Purina diet was the same as non-treated Purina diet fish (Table 10). Cortland control fish fed the Donaldson diet showed a slight increase in plasma GPT activity over activity measured in the non-treated control fish.

Plasma GPT activity was maximum at 12 hours and 24 hours post-treatment with CCl_4 (1.0 ml/kg) for Purina and Donaldson diet fish, respectively (Table 10, Fig. 5). The GPT activity of 120 U/l

Table 9. A comparison of plasma and liver alanine aminotransfer-
ase (GPT), aspartate aminotransferase (GOT) activities
and liver protein concentration for rainbow trout fed two
commercial fish diets.

Parameter	Purina Diet	Donaldson Diet
GPT Plasma Activity[a] (U/l)	9.3 ± 1.3[b] (12)	9.7 ± 1.0 (8)
GOT Plasma Activity (U/l)	113.4 ± 27.4 (7)	113.8 ± 10.2 (8)
Liver Protein Concentration (mg/100 mg)	17.8 ± 0.9 (12)	15.7 ± 0.5 (8)
GPT Specific Activity (U/mg protein x 10^{-2})	16.1 ± 1.5 (12)	22.3 ± 2.6 (8)
GOT Specific Activity (U/mg protein x 10^{-2})	8.2 ± 0.5 (12)	17.9 ± 1.5 (8)

[a] International Units of activity at $25^{\circ}C$, pH 7.5.

[b] Values are mean $\pm$ S.E.M. for the number of fish in parentheses.

Table 10. Alanine aminotransferase (GPT) activity in plasma from rainbow trout post-treatment with CCl_4 (1.0 ml/kg, i.p.). Prior to treatment the fish had been fed different commercial fish diets.

Time[b]	GPT Plasma Activity (U/l)[a]	
	Purina Diet	Donaldson Diet
Mean Control (3-24)	9.3 ± 1.0[c] (15)	12.7 ± 1.4 (24)
3	40.7 ± 9.2 (10)	16.7 ± 1.5** (12)
6	101.4 ± 32.6 (7)	24.1 ± 2.2** (16)
12	120.5 ± 48.3 (3)	24.2 ± 2.2** (19)
18	93.0 ± 14.8 (7)	25.6 ± 3.0** (9)
24	22.9 ± 5.0 (4)	30.8 ± 4.1 (11)

[a] International Units of activity at $25^{\circ}C$, pH 7.5.

[b] Hours post-treatment.

[c] Values are mean $\pm$ S.E.M. for number of fish in parentheses.

** Significantly different from Purina Diet ($P < 0.01$).

Figure 5.   Alanine aminotransferase (GPT) activity in plasma from
rainbow trout fed two different commercial fish diets and
treated with CCl_4 (1.0 ml/kg, i.p.).   Experimental
control fish received Cortland saline.   Zero time mean
values are for non-treated control fish.   Values are
the mean $\pm$ SEM for the number of fish in parentheses.
Asterisks denote values for Donaldson diet fish that
are significantly different (**$P < 0.01$) from Purina
diet fish.
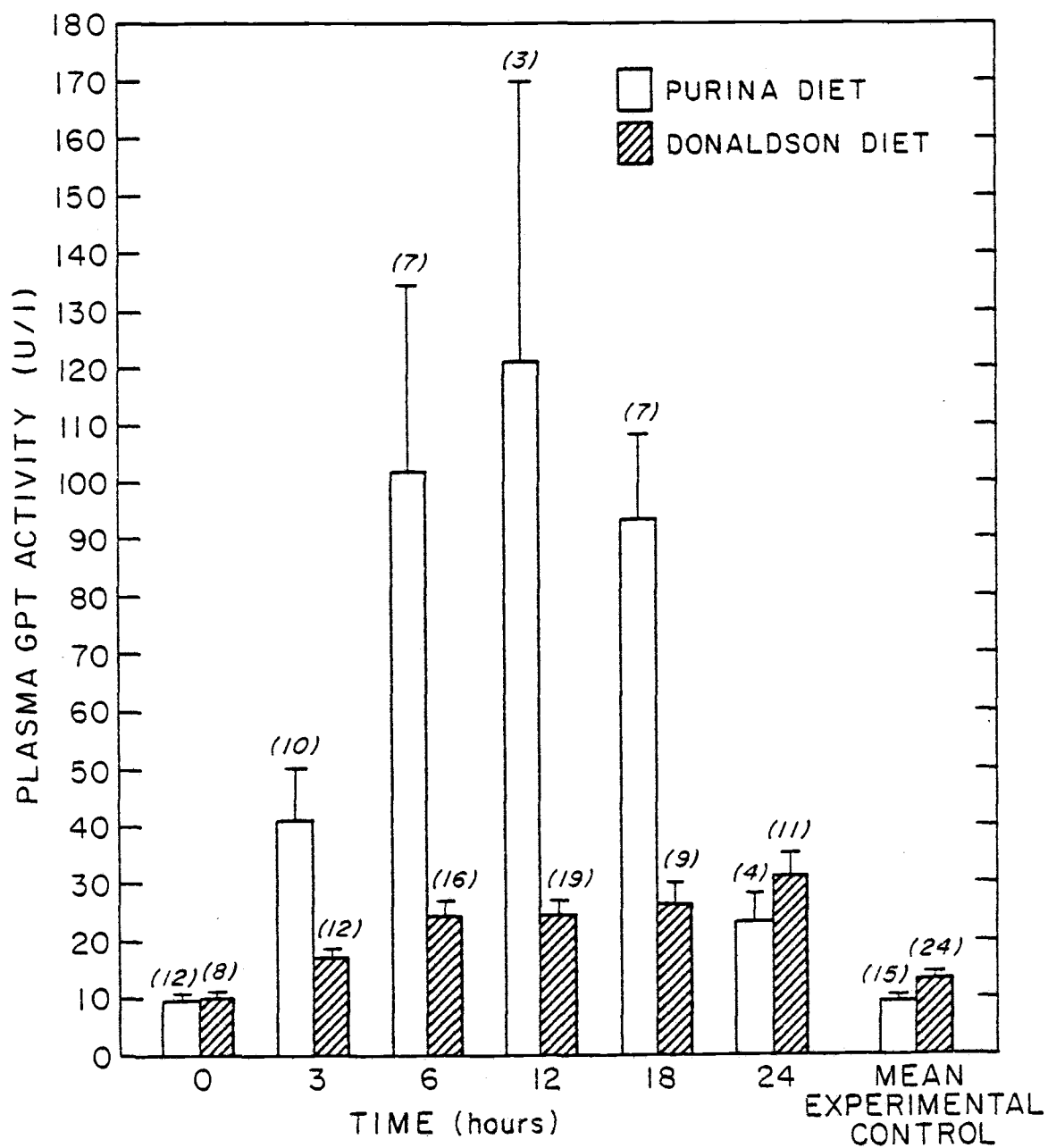
Figure 5.

at 12 hours for Purina diet fish was three times greater than the maximum enzyme activity at 24 hours for Donaldson diet fish. GPT activity showed the greatest variability in the plasma from Purina diet fish.

Plasma GPT activities for Donaldson diet fish after treatment with CCl_4 (1.0 ml or 2.0 ml/kg) are shown in Table 11 and Fig. 6. The maximum plasma enzyme activity for fish receiving CCl_4 (1.0 ml/kg) occurred at 36 hours post-treatment. The fish treated with CCl_4 (2.0 ml/kg) displayed a biphasic pattern of plasma GPT activity with maxima occurring at 3 hours and 36 hours post-treatment. The peak plasma enzyme activity of 87.9 U/l at 3 hours for Donaldson diet fish given CCl_4 (2.0 ml/kg) was less than the maximum activity of 120 U/l measured for Purina diet fish treated with one-half that does of CCl_4 (1.0 ml/kg).

The ratio of plasma GPT activity for treated fish at each sampling time over the mean control plasma activity was calculated for Purina and Donaldson diet fish given CCl_4 (1.0 ml/kg) and for Donaldson diet fish treated with CCl_4 (2.0 ml/kg) (Fig. 7). It is evident from this representation that the intensity of the hepatotoxic response, i.e. plasma GPT activity, was considerably greater for Purina diet fish than Donaldson diet fish treated with either dose of CCl_4.

Table 11. Alanine aminotransferase (GPT) activity in plasma from rainbow trout fed the Donaldson diet and treated with CCl_4 (1.0 ml or 2.0 ml/kg, i.p.).

Time	Dose:	GPT Activity $(U/l)^a$	
		CCl_4 (1.0 ml/kg)	CCl_4 (2.0 ml/kg)
Mean Control (3-48)		12.7 ± 1.4^c (24)	10.3 ± 1.2 (21)
3		16.7 ± 1.5 (12)	$87.9 \pm 24.5**$ (15)
6		24.1 ± 2.2 (16)	$72.2 \pm 23.4*$ (13)
12		24.2 ± 2.2 (19)	$50.1 \pm 8.3**$ (6)
18		25.6 ± 3.0 (9)	$57.2 \pm 14.1**$ (4)
24		30.8 ± 4.1 (11)	$44.7 \pm 2.8*$ (5)
36		48.3 ± 27.5 (5)	85.3 ± 31.9 (8)
48		29.5 ± 8.0 (6)	36.5 ± 4.2 (4)

[a] International Units of activity at 25^oC, pH 7.5.

[b] Hours post-treatment.

[c] Values are mean $\pm$ S.E.M. for number of fish in parentheses.

[*] Significantly different from trout treated with CCl_4 (1.0 ml/kg) (P < 0.05).

[**] Significantly different from trout treated with CCl_4 (1.0 ml/kg) (P < 0.01).

Figure 6.   Alanine aminotransferase (GPT) activity in plasma from rainbow trout fed the Donaldson Diet and treated with CCl_4 (1.0 ml or 2.0 ml/kg, i.p.). Experimental control fish received Cortland saline. Zero time mean values are for non-treated control fish. Values are the mean $\pm$ SEM for the number of fish in parentheses. Asterisks denote values for fish given 2.0 ml/kg that are significantly different (*$P < 0.05$, **$P < 0.01$) from fish given 1.0 ml/kg.
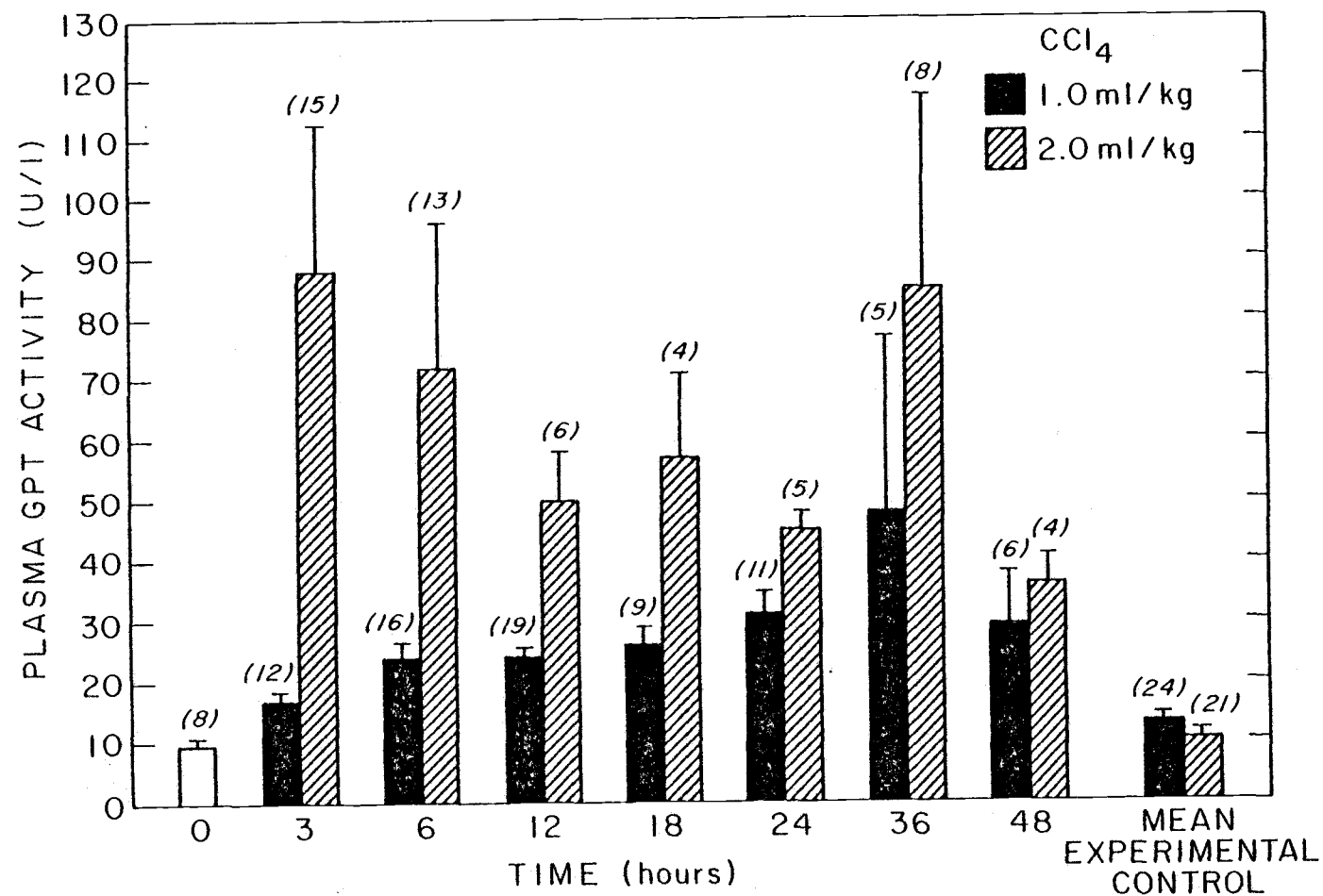
Figure 6.

Figure 7. Alanine aminotransferase (GPT) activity ratio for rainbow trout fed Purina or Donaldson Diet and treated with CCl_4 (1.0 ml or 2.0 ml/kg, i.p.). Ratios were calculated as:

Treated Activity/Mean Experimental Control Activity
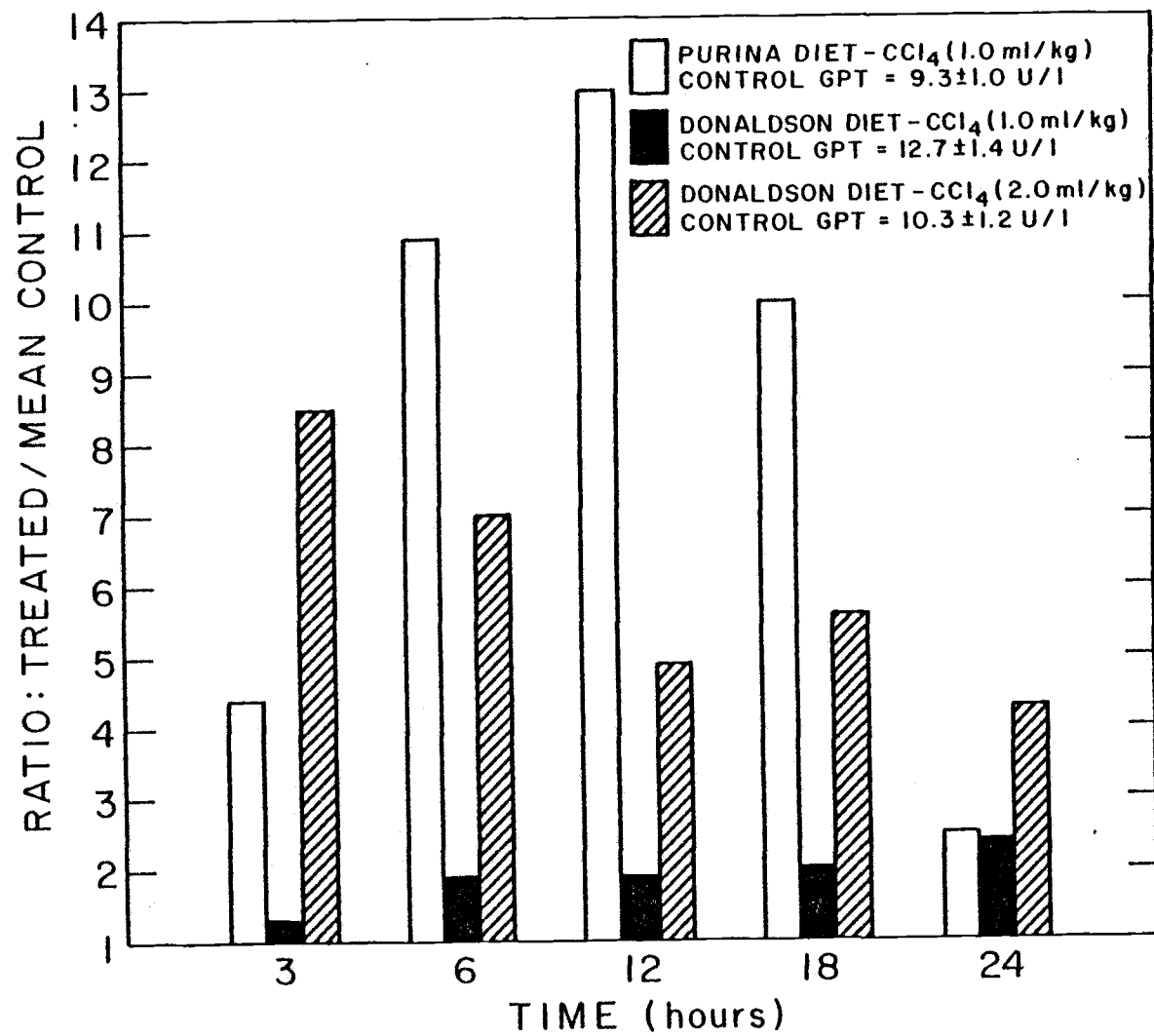
Figure 7.

Histopathology

The livers of many control and CCl_4-treated fish fed Purina Trout Chow ^{R} or the Donaldson diet showed considerable hepatocyte vacuolization from glycogen deposition. Histological sections of livers from fish fed Purina Trout Chow ^{R} were obtained only at 3, 6 and 12 hours post-treatment with CCl_4 (1.0 ml/kg), and none showed any abnormal morphological alterations, in spite of the fact that the highest plasma GPT activities occurred at 6 and 12 hours post-treatment. Donaldson diet fish, in general, displayed no correlation between the severity of hepatocyte damage and plasma GPT activity. Enzyme activity was greatest at 3 and 36 hours post-treatment with CCl_4 (2.0 ml/kg), and liver sections at these times showed significant glycogen deposition but no abnormal morphology. The liver of one Donaldson diet fish sampled at 6 hours post-treatment with CCl_4 (1.0 ml/kg) exhibited generalized areas of liquifactive necrosis. The plasma from this fish had the highest GPT activity (>500 U/l) of any CCl_4-treated fish, regardless of dose. Early hydropic changes appeared in the hepatocytes of other Donaldson diet fish at 6 hours and at 24 hours post-treatment with CCl_4 (2.0 ml/kg). The liver sections of some CCl_4-treated fish, irrespective of diet and sampling time, showed subcapsular damage characterized by coagulative necrosis and pyknosis.

Histological sections of kidney tissue from fish fed Purina Trout Chow ^{R} were negative as far as showing any structural pathology after treatment with CCl_4 (1.0 ml/kg). Hematopoietic cell necrosis, characterized by pyknosis or karyorrhexis, occurred in kidney tissue of Donaldson diet fish at 3 and 6 hours post-treatment with CCl_4 (1.0 ml/kg). Subtle changes, such as alterations in the brush border of the first and second proximal tubule segments, occurred in a Donaldson diet fish at 24 hours post-treatment (2.0 ml/kg). At 36 hours another Donaldson diet fish exhibited severe microdroplet degeneration of the first proximal tubule segment. No damage to glomeruli, distal tubule segments or collecting ducts was noted in any CCl_4-treated fish. Contrary to the liver histopathology, the severity of renal damage appeared better correlated with the level of plasma GPT activity.

In general, the histopathological examination failed to establish sufficient evidence to indicate a greater susceptibility of fish fed Purina Trout Chow ^{R} to the hepatotoxic and nephrotoxic effects of CCl_4.

Discussion

Non-Treated Control Fish

There is no adequate explanation for the difference between liver GPT and GOT activities in non-treated Purina and Donaldson

diet fish because of the variability introduced into the experiment. However, the results do suggest some experimental factors that might lead to an appropriate explanation. Schlisio and Nicolai (1978) showed that liver GOT activity in rainbow trout was more variable than GPT activity. Liver GOT activity in their fish appeared to follow a diurnal pattern which was not evident for GPT activity. In my study blood and tissue samples were obtained at approximately the same time each day; furthermore, it would appear unlikely that diurnal fluctuations could have accounted for the considerable variability in liver GOT activities. The greater liver GOT activity in the Donaldson diet fish may have been due to a higher concentration of a specific nutrient in the diet. The variation, on the other hand, may have been caused by the $4^{o}C$ difference in water temperature or by seasonal factors. Whiting and Wiggs (1977) reported that changes in tyrosine aminotransferase activity in liver tissue from brook trout fed Purina Trout Chow[R] may have been due to water temperature or a seasonal response; however, their fish were genetically dissimilar, which could have accounted for the variability in enzyme activity.

CCl_4-Treated Fish

The temporal pattern of plasma GPT activity in the Purina or Donaldson diet fish treated with CCl_4 (1.0 ml/kg) was different from

the response reported in previous studies. Racicot et al. (1975) found maximum GPT activity in plasma from rainbow trout fed Purina Trout Chow [R] at 6 hours and 18 hours post-treatment with CCl_4 (1.33 ml/kg, i.p.). The enzyme activity at these times was approximately five times greater than control GPT activity and similar to the maximum plasma GPT activity measured in my Donaldson diet fish treated with nearly twice the dose of CCl_4 (2.0 ml/kg). Statham et al. (1977), on the other hand, measured maximum plasma GPT activity in rainbow trout at 2 hours and 72 hours post-injection with CCl_4 (1.0 ml/kg, i.p.). Plasma enzyme activity at these times was nine times greater than control activity and greater than plasma GPT activities reported by Racicot or in my study.

In laboratory rats plasma GPT activity has been shown to reach maximum activity at 36 hours post-treatment with CCl_4 (1.0 ml/kg, i.p.) (Koeferl, 1972; Zimmerman et al., 1965). In addition, Koeferl found a biphasic temporal pattern for both GPT and GOT activities with peaks at 12 hours and 36 hours. The significance of this biphasic plasma enzyme pattern in rainbow trout and laboratory rats treated with CCl_4 is unknown. It may be related to the degree of liver damage, but histological evidence has been inconclusive.

Histopathology

The characteristic and reproducible central vein (central lobular) lesions seen in CCl_4-treated laboratory mammals (Recknagel, 1967) was not evident in my CCl_4-treated rainbow trout. Gingerich et al. (1978) noted that pericentral necrosis occurred in only 25% of their fish treated with CCl_4 (2.0 ml/kg, i.p.). The small number of histological sections, i.e. 2-3, at each sampling time in my study may have accounted for the low incidence of hepatocellular necrosis.

Racicot et al. (1975) found necrotic areas in the liver sections of 20% of fish examined at 18 hours post-treatment. The most prominent feature in the livers of their CCl_4-treated and control fish was the intense cellular vacuolization. They assumed that the vacuoles contained lipids because hepatic lipid accumulation is characteristic of CCl_4-treated laboratory mammals (Cornish, 1975); however, they did not confirm their hypothesis histochemically. In my study many of the control and CCl_4-treated fish had severe hepatocyte vacuolization, and, in general, Purina diet fish appeared to display this characteristic more than Donaldson diet fish. While appearing to result from a pathological condition, the vacuolization is common in the livers of healthy rainbow trout, and the vacuoles contain mainly glycogen.[10] Histochemical

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[10]Tissue sections were examined by Dr. Jerry Hendricks, Fish Pathologist, Department of Food Science, Oregon State University.

staining confirmed that the hepatocytes of my fish had significant quantities of glycogen. In support of these findings, Statham et al. (1977) found that CCl_4 had no effect on liver triglyceride levels in rainbow trout, and Sakaguchi and Hamaguchi (1975) reported no effect of CCl_4 on hepatopancreas lipids in the yellowtail. In addition, Statham noted vacuolization in control trout and severe vacuolization and focal and laminar necrosis in CCl_4-treated trout at 6 hours post-treatment (1.0 ml/kg). Vacuolization was present in 100% of the CCl_4-treated fish while laminar and focal necrosis occurred in 66% of the animals. In concurrence with the gross observations in this study, Statham noted consistent pathological lesions of the splenic capsule and parenchyma of CCl_4-treated fish.

The subcapsular hepatic necrosis was probably caused by direct contact of CCl_4 with the liver or due to bile spillage from the gall bladder when the livers were excised (Hendricks et al., 1976). In my study, the early pathological signs, such as cloudy cytoplasm (liver) and changes in the brush border (kidney) may have been indicative of subcellular alterations in hepatocyte or nephron organelle morphology that were not evident with the light microscope (X520).

Histopathological examination of the liver and kidney sections indicate that there was no definitive correlation between the degree

of hepatocyte or nephron damage and the dose of CCl_4, the diet or the time after treatment; furthermore, it was not possible to correlate plasma GPT activity with the degree of hepatocellular damage. The central vein necrosis, which is characteristic in CCl_4-treated mammals, was not a prominent histopathological feature of CCl_4 intoxication in my rainbow trout.

The results suggest that the diet of rainbow trout significantly altered the plasma GPT activity response to treatment with CCl_4. Two experimental conditions deserve mention and consideration as potential variables in this study: 1) the Purina diet fish were held at a lower temperature than the Donaldson diet fish, i.e., $11^{\circ}C$ vs $15^{\circ}C$; 2) CCl_4 was given to Purina diet fish in the winter and to Donaldson diet fish in the summer.

The hepatotoxicity associated with CCl_4 intoxication is, presumably, dependent on the biotransformation and activation of this compound by the MFO enzyme system of the hepatocytes (Butler, 1961; Rechnagel and Glende, 1963; Slater, 1966). Previous reports indicated that components of the MFO enzyme system occurred in various fishes, including rainbow trout (Chan et al., 1967; Chevion et al., 1977; Ludke et al., 1972; Stanton and Khan, 1975), and that rainbow trout were capable of hepatic biotransformation by the MFO system (Ahokas et al., 1977; Chambers and Yarbrough, 1976; Peterson et al., 1976). It has become apparent

that fish can perform many, and possibly all, of the reactions, i.e. oxidation, reduction and conjugation, utilized by mammals to detoxify or activate xenobiotics (Sierber and Adamson, 1977).

The biotransformation of CCl_4 to an active metabolite is a free radical reaction (Villarruel et al., 1975), which has higher activity at elevated temperatures. In laboratory rats Larson and Plaa (1965) demonstrated that CCl_4-induced liver necrosis can be delayed but not prevented when the body temperature of these mammals was lowered. If the biotransformation of CCl_4 to an active metabolite occurs in fish as it does in mammals, one might expect a greater hepatotoxic response and concurrent increase in plasma GPT activity at a higher temperature; however, the drug metabolizing enzymes of fish liver have temperature optimum generally lower than that of the corresponding mammalian system (Adamson et al., 1965; Buhler and Rasmusson, 1968a; Creaven et al., 1967; Lotlikar et al., 1976). Buhler and Rasmusson (1968a) determined that two biotransformation reactions, e.g. alanine hydroxylation and phenacetin dealkylation, of the MFO enzyme system of rainbow trout had optimum activities at incubation temperatures of $25^{\circ}C$ and $10^{\circ}C$, respectively. Previous studies with fishes have shown that liver intermediary metabolism (Dean, 1969; Evans et al., 1962), liver protein synthesis (Das and Prosser, 1967; Das, 1967; Dean and Berlin, 1969; Haschemeyer, 1968) and

enzyme activity associated with protein synthesis (Haschemeyer, 1969) were greater in fish that have been acclimated to lower temperature; however, the temperature ranges in the majority of these studies were greater than between my investigation and those of Racicot et al. (1975) and Statham et al. (1977).

The effect of water temperature on CCl_4 toxicity in fish is unknown. The results of Racicot et al. (1975) (rainbow trout at $15^{\circ}C$) and Statham et al. (1977) (rainbow trout at $12^{\circ}C$) suggested that plasma GPT activity was greater in the CCl_4-treated fish at the lower temperature ($12^{\circ}C$). Purina Trout Chow[R] was the diet used by Racicot, but Statham did not specify the diet. The variation in the plasma enzyme response in the studies of Racicot and Statham may have been dietary or genetically related. A comparison of my results for Donaldson diet fish at $15^{\circ}C$ with the Purina diet fish of Racicot at $15^{\circ}C$ demonstrated that their Purina fish had greater plasma GPT activity after treatment with CCl_4. A comparison of plasma enzyme activity for Purina diet fish at $11^{\circ}C$ and $15^{\circ}C$, i.e. between my study and Racicot, was inconclusive as far as showing an influence of temperature on the response to CCl_4.

Preliminary data obtained in the summer from a residual group of rainbow trout fed Purina Trout Chow[R] and held at $15^{\circ}C$ indicated that water temperature or the season did not alter the GPT enzyme activity in these fish at 3 or 6 hours post-treatment

with CCl_4 (1.0 ml/kg, i.p.). Six fish sampled at 3 hours had a mean plasma GPT activity of 56.3 U/l, and two fish at 6 hours post-treatment had a mean enzyme activity of 93.5 U/l. These plasma GPT activities were similar to the values obtained for Purina diet fish at $11^{\circ}C$ during the winter (Table 10).

Variable seasonal factors may have been responsible for the difference in the hepatotoxic response to CCl_4. Dewaide and Henderson (1970) found that activities of the MFO enzymes in the liver from the roach, Leuciscus rutilus, were dependent on seasonal and environmental factors. The highest enzyme activities occurred during the summer, the lowest during the winter. The seasonal variations were attributed to changes in endocrine and metabolic functions, food consumption or exposure to pollutants, but not to changes in temperature, stress, hepatic protein or DNA concentrations. Conversely, in a related study using hatchery rainbow trout, Dewaide and Henderson (1968) found no seasonal change in liver MFO enzyme activities. Presumably, the similar plasma GPT activities found in my winter and summer CCl_4-treated fish fed Purina Trout Chow [R] and held at $15^{\circ}C$ indicates that the season was not a factor in the hepatotoxic response.

Although inconclusive, the above results and discussion implicate the diet as a significant factor in the evaluation of CCl_4

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toxicity in my trout. Some of the dietary parameters that can

influence CCl_4 toxicity are discussed below.

Dietary Factors and the Hepato-toxic Response to CCl_4

Quantitative determination of the possible dietary factor(s)

responsible for the variation in hepatotoxic response in my study

would be a significant undertaking but was not the purpose of this

investigation; nevertheless, knowledge of the basic constituents in

the Donaldson diet and Purina Trout Chow [R], and the results of

previous studies, permit a hypothetical, yet tenable, explanation

for the variation in the hepatotoxic response in my rainbow trout.

Previous studies with mammals and fish demonstrated a

variation in the hepatotoxic response to organochlorine compounds

due to changes in dietary protein concentration (Korsrud et al.,

1976; McLean and McLean, 1966; Mehrle et al., 1974; Mehrle et

al., 1977; Seawright and McLean, 1967). The protein quality or

quantity in the Purina and Donaldson diets was probably not a

factor in the response of my trout to CCl_4. Purina Trout Chow [R]

(Large Fingerling Size #5105) contains not less than 40% total

protein, primarily from herring fish meal. The Donaldson diet

is approximately 40% total protein, which is obtained from herring

fish meal (30%) and other fish sources (10%) (Table 12). Forty

Table 12. Comparison of the basic ingredients in Purina Trout
Chow and Donaldson diets.

Ingredient	Purina[a]	Donaldson
Total Protein (%)	40[b]	40[c]
Amino Acids (%)[d]		
Total	16.3	none
Methionine	1.4	none
Grains and Cereals	yes	yes
Vitamin PreMix	yes	yes
Fats and Oils	yes	yes

[a] Purine Trout Chow: large fingerling size #5105.

[b] Chiefly herring fish meal and some soya protein.

[c] Approximately 31% herring fish meal (42% of total diet) with the
balance of protein from bottom fish (35% of total diet).

[d] Added to diet.

percent total protein is considered to be the minimum concentra-
tion for rainbow trout to insure normal metabolis homeostasis.[11]

Campbell and Hayes (1974) reviewed the effects of lipotropes
on biotransformation mechanisms. Lipotropes are compounds
which function as methyl donors or assist in methyl group transfer
during the synthesis of phospholipids, which are necessary for
normal MFO activity (Cooper and Feuer, 1973). The amino acid,
methionine, is the principal methyl donor in mammalian lipo-
tropic metabolism. Mehrle et al. (1977) reported that when dietary
methionine concentration was increased from 0.96 to 2.2%, the
toxicity of DDT and dieldrin to rainbow trout significantly increased
and decreased, respectively.

DDT is biotransformed primarily by a non-microsomal MFO
system to DDE (Sternburg et al., 1954). Addison and Willis (1978)
recently established that rainbow trout had a limited ability to
degrade and excrete DDT, presumably by mechanisms similar to
other organisms. The relationship of methionine to the "metabolism"
and toxicity of DDT in rainbow trout is unknown, but the toxicity is
probably not related to the MFO enzymes. In mammals, dieldrin
undergoes hydroxylation reactions catalyzed by liver microsomal
MFO enzymes. Chan et al. (1967) reported that microsomal
enzymes from rainbow trout liver could convert cyclodiene

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[11]Personal communication with Dr. W. Stott, Department of Food
Science, Oregon State University.

insecticides, e.g. aldrin, to the epoxides, and James et al. (1977) demonstrated the presence of an epoxide hydrase in the liver microsomes of three marine fishes. Macek et al. (1970) showed that the biological half-life of DDT was four times longer than dieldrin in rainbow trout. They concluded that the lower retention of dieldrin suggested "a greater capacity to metabolize and/or excrete dieldrin than DDT." An increase in MFO enzyme activities due to higher dietary methionine would increase the biotransformation of dieldrin to a more polar and less toxic metabolite. Although presumptive and speculative, this discussion affords an attempt to explain the decrease in dieldrin toxicity in the study of Mehrle et al. (1977).

If an increase in the concentration of dietary methionine can increase the activity of the microsomal MFO enzymes in rainbow trout, the biotransformation of CCl_4 to its active metabolite would similarly be enhanced. Purina Trout Chow[R] is fortified with 10 amino acids, and methionine is present in a concentration of 1.4%. The concentration of methionine in the Purina diet may have been sufficient to increase the hepatotoxic response, i.e. plasma GPT activity, of my rainbow trout to CCl_4.

An alternate explanation for the findings in my study involves the possible presence of trace contaminants in commercially formulated diets and dietary components. Schoettger and Mehrle

(1972) reported that the occurrence of organochlorine contaminants was widespread in commercial fish diets and dietary constituents. Although these workers did not find organochlorine contaminants in Purina Trout Chow[R], they indicated that chemical residues can vary considerably between feed lots.[12] Low level exposure to many compounds, including organochlorine derivatives, has been shown to induce MFO enzyme activities in laboratory mammals (Remmer, 1972). Induction of the MFO system in fish varies with the species of fish and the type of inducing agent; however, recent studies indicated the MFO system of trout liver is inducible by xenobiotics (Chambers and Yarbrough, 1976; Lidman et al., 1976; Payne and Penrose, 1975; Pedersen et al., 1974; Statham et al., 1978). If a chemical inducing agent was present as a contaminant in the Purina Trout Chow[R], the hepatotoxic response to treatment with CCl_4 would have been greater in my fish.

Another consideration to explain the variation in hepatotoxic response between the two groups of fish is the effect of dietary constituents on glutathione concentration in the liver. In mammalian systems glutathione is a nucleophile that acts to break down intracellular hydroperoxides in reactions catalyzed by glutathione peroxidase in the cytoplasmic fraction of the hepatocyte (O'Brien,

[12]Personal communication with Dr. P. Mehrle, Fish-Pesticide Research Laboratory, Fish and Wildlife Service, Columbia, Missouri.

1969). This mechanism protects the intracellular organelles, e.g. endoplasmic reticulum and mitochondrial membranes, from the peroxidative effects of free radicals, e.g. the active metabolite of CCl_4. Laboratory rats that were given glutathione prior to treatment with CCl_4 were protected against polysome disturbances and had improved amino acid incorporation into liver microsomal proteins (Gravela and Dianzani, 1970). Moreover, the prior administration of cysteine, which is required for glutathione synthesis, to laboratory mice decreased both the covalent binding of an active metabolite of acetaminophen to hepatocyte macromolecules and the severity of the resulting liver necrosis. DeFerreyra et al. (1974) showed that cysteine pretreatment in laboratory rats prevented the development of CCl_4-induced liver necrosis by an unknown process. At present no information is available regarding the effects of dietary cysteine or glutathione on the response of fish to hepatotoxic chemicals.

Visceral adipose tissue may have influenced the absorption and distribution of CCl_4 given by i.p. injection. Statham et al. (1977) found that adipose tissue of rainbow trout treated with $^{14}CCl_4$ (bath exposure: 1 mg/l for 2 hr) had the highest concentration of ^{14}C, which increased until 1.75 hours post-treatment. Peak liver concentration of ^{14}C occurred at 0.5 hours post-treatment followed by a slow elimination phase ($t_{1/2}$ = 39 hr). In my study, variable

quantities of visceral adipose tissue may have provided a storage depot for the CCl_4, reducing the availability to the liver and causing the variability in plasma GPT activity.

The results and discussion of this study have suggested that the type of diet fed to fish before or during a toxicity study is an important factor in the final evaluation of that experiment. The formulation and use of a standard research fish diet, as suggested by Mehrle et al. (1977), would be important steps toward the standardization of physiological and toxicological procedures in fisheries research. Diet standardization is necessary if physiological and biochemical parameters are to be used in the assessment of the toxicological response of the organism to chemical agents.

III. TOXICOLOGICAL EXPERIMENT #2: THE EFFECT OF
CARBON TETRACHLORIDE ON THE TOTAL PLASMA
PROTEIN CONCENTRATION OF
RAINBOW TROUT

Introduction

Carbon tetrachloride (CCl_4) has become a model hepatotoxic

agent in mammalian experimental toxicology because of its ability to

cause reproducible liver damage (Raisfeld, 1974). Two characteris-

tic and apparently unrelated responses of CCl_4 intoxication are

hepatic lipid accumulation and necrosis. Previous studies with

mammals established that CCl_4 can depress hepatic protein syn-

thesis (Recknagel and Ghoshal, 1966; Smuckler et al., 1962;

Smuckler and Benditt, 1965); however, Recknagel (1967) concluded

that CCl_4-induced depression of protein synthesis was not the pri-

mary event leading to hepatic lipid accumulation or hepatic

necrosis. Smuckler et al. (1961) showed that CCl_4 reduced the

incorporation of amino acids into plasma proteins in laboratory

rats. Earlier reports indicated that CCl_4 decreased the total

plasma protein concentration in laboratory mammals (Berryman and

Bollman, 1943; Erickson et al., 1938).

Previous studies established that rainbow trout were sus-

ceptible to some of the hepatotoxic effects of CCl_4 reported in

higher vertebrates (Gingerich et al., 1978; Pfeifer et al., 1977;

Racicot et al., 1975; Statham et al., 1977). The plasma protein

concentration of teleosts can be affected by: season of the year (Denton and Yousef, 1975); stage of life cycle (Robertson et al., 1961a, 1961b; Triplett and Calaprice, 1974); diet (Koroleva, 1964); environmental temperature, photoperiod or oxygen concentration (Bouck and Ball, 1965; Guderly and Ulrickson, 1973; Meisner and Hickman, 1962; Poston, 1965; Umminger, 1970); and disease states (Cardwell and Smith, 1971; Hunn, 1964; Mulcahy, 1969). Zeitoun (1977) determined the effect of chlorine on blood parameters, including plasma protein concentration, of rainbow trout. Except for the recent study by Zeitoun, there is little information currently available regarding the effects of chemical agents on fish plasma proteins. The purpose of this study was to determine the effect of CCl_4 on the plasma protein concentration of rainbow trout.

Materials and Methods

Animals and Holding Conditions

Rainbow trout (100-250 g) were purchased from Roaring River Hatchery, Scio, Oregon. Fish were held in aquaria (200 gal) supplied with continuously flowing well water ($15.0^\circ C \pm 1.0^\circ$). A 12L:12D photoperiod was used throughout all experiments. Animals were fed a commercial fish diet, Donaldson-Ore Aqua[R], every day, but food was withheld for 24 hours prior to an experiment. All fish were in good health and allowed a one-week acclimation

period before they were used. The study was carried out during the months of September through November.

Experimental Procedure and Treatment with CCl_4

These procedures were identical to those described in Toxicological Experiment #1.

Blood Collection and Analytical Procedures

Blood collection was described in Toxicological Experiment #1.

Plasma protein concentration was measured by the biuret method (Gornall et al., 1949) and corrected for plasma hemoglobin concentration by the method outlined in Sigma Bulletin #540. Plasma hemoglobin concentration was determined by the cyan-methemoglobin method of Richterich (1969). Plasma osmolarity was measured with a Wescor[13] vapor pressure osmometer. Plasma albumin was determined by the method in Sigma Bulletin #630 which is based on the affinity of albumin for bromcresol green.

Statistical Methods and Histological Studies

These procedures were identical to those described in Toxicological Experiment #1.

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[13]Wescor Inc., Logan, Utah.

Results

Non-Treated Control Fish

Total plasma protein and albumin concentrations were 27.9 mg/ml and 13.7 mg/ml, respectively, in non-treated control trout (Table 13). Based on these measurements, albumin represented approximately 49% of the protein in the plasma.

CCl_4-Treated Fish

Plasma protein and albumin concentrations for Cortland-treated control fish were similar to values for non-treated control fish (Table 13). CCl_4 given at 0.25 ml or 0.5 ml/kg, i.p., did not significantly decrease ($P \leq 0.05$) the total plasma protein or albumin concentrations at 24 hours post-treatment (Table 13, Fig. 8). Trout treated with CCl_4 (1.0 ml/kg) showed a significant decline ($P < 0.05$) in total plasma protein but not in albumin concentration, and CCl_4 given at 2.0 ml/kg produced a highly significant reduction ($P < 0.01$) in both the total plasma protein and albumin concentrations. As early as 12 hours post-treatment, CCl_4 produced a significant decrease ($P < 0.05$) in plasma protein concentration (Table 14, Fig. 9). At 36 hours plasma protein concentration of CCl_4-treated fish was 55% less than Cortland controls.

Table 13. Plasma protein and albumin concentrations for rainbow trout at 24 hours post-treatment with CCl_4 (0.25 ml to 2.0 ml/kg, i.p.).

Dose of CCl_4	N	Plasma Protein (mg/ml)	Plasma Albumin (mg/ml)	Albumin[a]/ Plasma Protein
Control[b] (non-treated)	12	27.9 ± 0.8[d]	13.7 ± 0.4	49.1
Control[c] (treated)	6	28.3 ± 1.2	12.4 ± 0.6	43.8
CCl_4 (ml/kg)				
0.25	5	27.5 ± 1.3	12.3 ± 0.5	44.7
0.5	5	29.2 ± 2.6	12.5 ± 1.1	42.8
1.0	10	22.7 ± 2.2*	11.5 ± 1.0	50.7
2.0	9	16.7 ± 1.6**	8.0 ± 0.7**	47.3

[a] Value is ratio of albumin/total plasma protein x 100.

[b] Non-treated control fish were sampled from holding tank.

[c] Treated control received 0.25 ml to 2.0 ml/kg of Cortland saline, i.p.

[d] Values are mean $\pm$ S.E.M. for N fish.

*Significantly different from Cortland control ($P < 0.05$).

**Significantly different from Cortland control ($P < 0.01$).

Figure 8.   Total protein and albumin concentrations in plasma from rainbow trout treated with Cortland saline or CCl_4 (0.25 ml, 0.5 ml, 1.0 ml or 2.0 ml/kg, i.p.). Values are the mean $\pm$ SEM for the number of fish in parentheses. Asterisks denote values that are significantly different (*$P < 0.05$, **$P < 0.01$) from control fish.
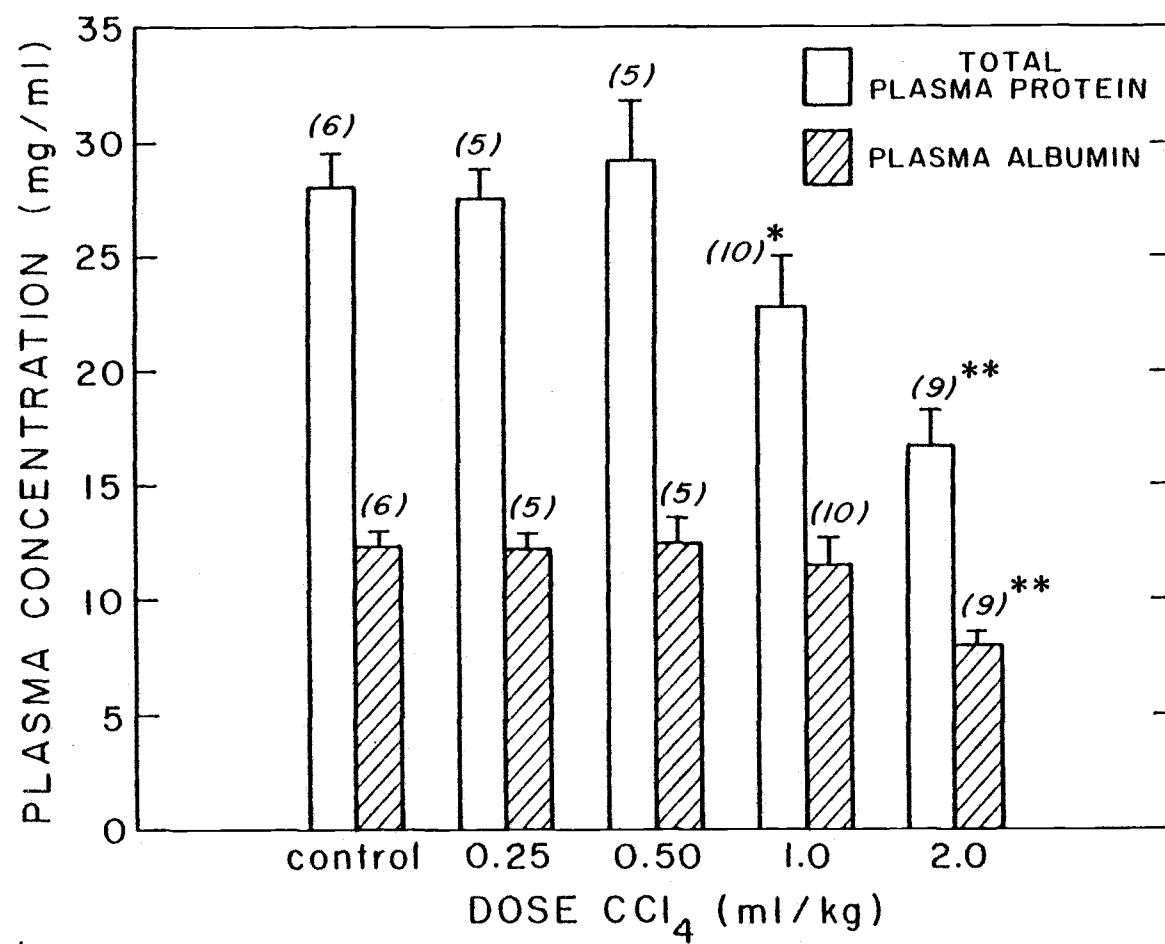
Figure 8.

Table 14. Plasma protein concentration, relative body weight change and plasma osmolarity for rainbow trout post-treatment with CCl_4 (2.0 ml/kg, i.p.).

Parameter	Time[a]						
	3	6	12	18	24	36	48
Plasma Protein (mg/ml)							
Control	28.0 $\pm$ 1.9[b]	28.6 $\pm$ 3.2	30.8 $\pm$ 3.2	30.9 $\pm$ 1.2	30.8 $\pm$ 1.2	30.8 $\pm$ 2.0	25.1 $\pm$ 3.6
	(3)	(3)	(3)	(3)	(3)	(3)	(3)
Treated	23.3 $\pm$ 2.2	20.8 $\pm$ 1.6	18.8 $\pm$ 1.2*	18.2 $\pm$ 0.8	14.4 $\pm$ 1.2*	13.7 $\pm$ 2.1	14.4 $\pm$ 1.2
	(9)	(7)	(5)	(4)	(5)	(8)	(4)
Weight Change (g/100 g BW)							
Control	-3.2 $\pm$ 1.3	-3.1 $\pm$ 0.6	-2.8 $\pm$ 1.3	-4.7 $\pm$ 0.3	-3.6 $\pm$ 0.8	-5.3 $\pm$ 1.0	-5.1 $\pm$ 0.6
	(3)	(3)	(3)	(3)	(3)	(3)	(3)
Treated	-1.2 $\pm$ 0.4	-1.7 $\pm$ 0.7	1.1 $\pm$ 0.7	4.6 $\pm$ 0.8*	4.5 $\pm$ 0.8*	6.4 $\pm$ 1.5**	1.6 $\pm$ 0.9*
	(9)	(7)	(6)	(4)	(5)	(8)	(4)
Plasma Osmolarity (m Os/kg)							
Control	306 $\pm$ 2	297 $\pm$ 4	285 $\pm$ 13	296 $\pm$ 7	290 $\pm$ 8	289 $\pm$ 3	295 $\pm$ 2
	(3)	(3)	(3)	(3)	(3)	(3)	(3)
Treated	293 $\pm$ 4*	283 $\pm$ 6	287 $\pm$ 4	280 $\pm$ 9	273 $\pm$ 2	268 $\pm$ 4**	270 $\pm$ 5*
	(13)	(12)	(6)	(4)	(5)	(8)	(4)

[a] Hours post-treatment.

[b] Values are mean $\pm$ SEM for number of fish in parentheses.

* Significantly different from Cortland control (P < 0.05).

** Significantly different from Cortland control (P < 0.01).

Figure 9. Plasma protein concentration for rainbow trout treated with Cortland saline or CCl_4 (2.0 ml/kg, i. p.). Values are the mean $\pm$ SEM for the number of fish in parentheses. Zero time mean value is for non-treated control fish. Asterisks denote values that are significantly different (*P < 0.05, **P < 0.01) from control fish.

Figure 9.

CCl_4-treated fish experienced an absolute, i.e. actual, and relative weight gain which started at 12 hours post-treatment and continued for the remaining sampling times, i.e. 18-48 hours (Table 14, Fig. 10). The maximum relative weight gain occurred at 36 hours post-treatment. Cortland-treated control fish, on the other hand, showed a decrease in relative body weight at each sampling time. In contrast to the weight change pattern exhibited by the fish given CCl_4 (2.0 ml/kg), trout treated with CCl_4 (1.0 mg/kg, i.p.) had an increase in relative body weight only at 3 and 48 hours (Fig. 11).

Plasma osmolarity of CCl_4-treated fish was less than control fish at all sampling times, except 12 hours (Table 14, Fig. 12). The minimum osmolarity in treated fish occurred at 36 hours and was 7% less than control fish.

Histopathology

The results of liver and kidney histopathology for Donaldson diet fish used in this study were presented in Toxicological Experiment #1.

Discussion

Non-Treated Control Fish

Previous studies that measured plasma protein and albumin concentrations in normal rainbow trout demonstrated the variability

Figure 10. Relative body weight changes for rainbow trout treated with Cortland saline or CCl_4 (2.0 ml/kg, i.p.). Values are the mean $\pm$ SEM for the number of fish in parentheses. Asterisks denote values that are significantly different (*$P < 0.05$, **$P < 0.01$) from control fish.
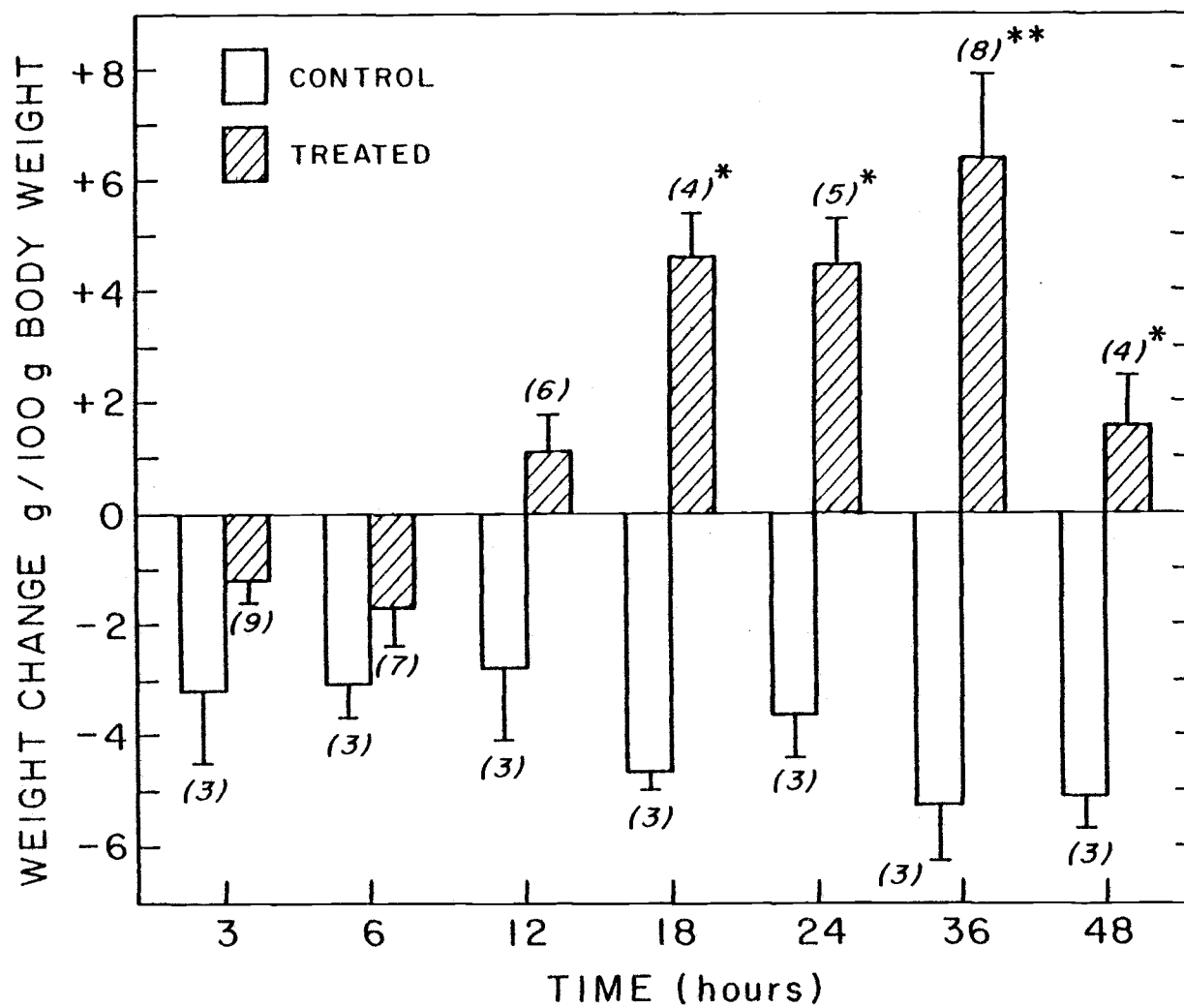
Figure 10.

Figure 11. Relative body weight changes for rainbow trout treated
with Cortland saline or CCl_4 (1.0 ml/kg, i.p.). Values
are the mean $\pm$ SEM for the number of fish in parenthe-
ses. Asterisks denote values that are significantly
different (*$P < 0.05$, **$P < 0.01$) from control fish.

Figure 11.

90
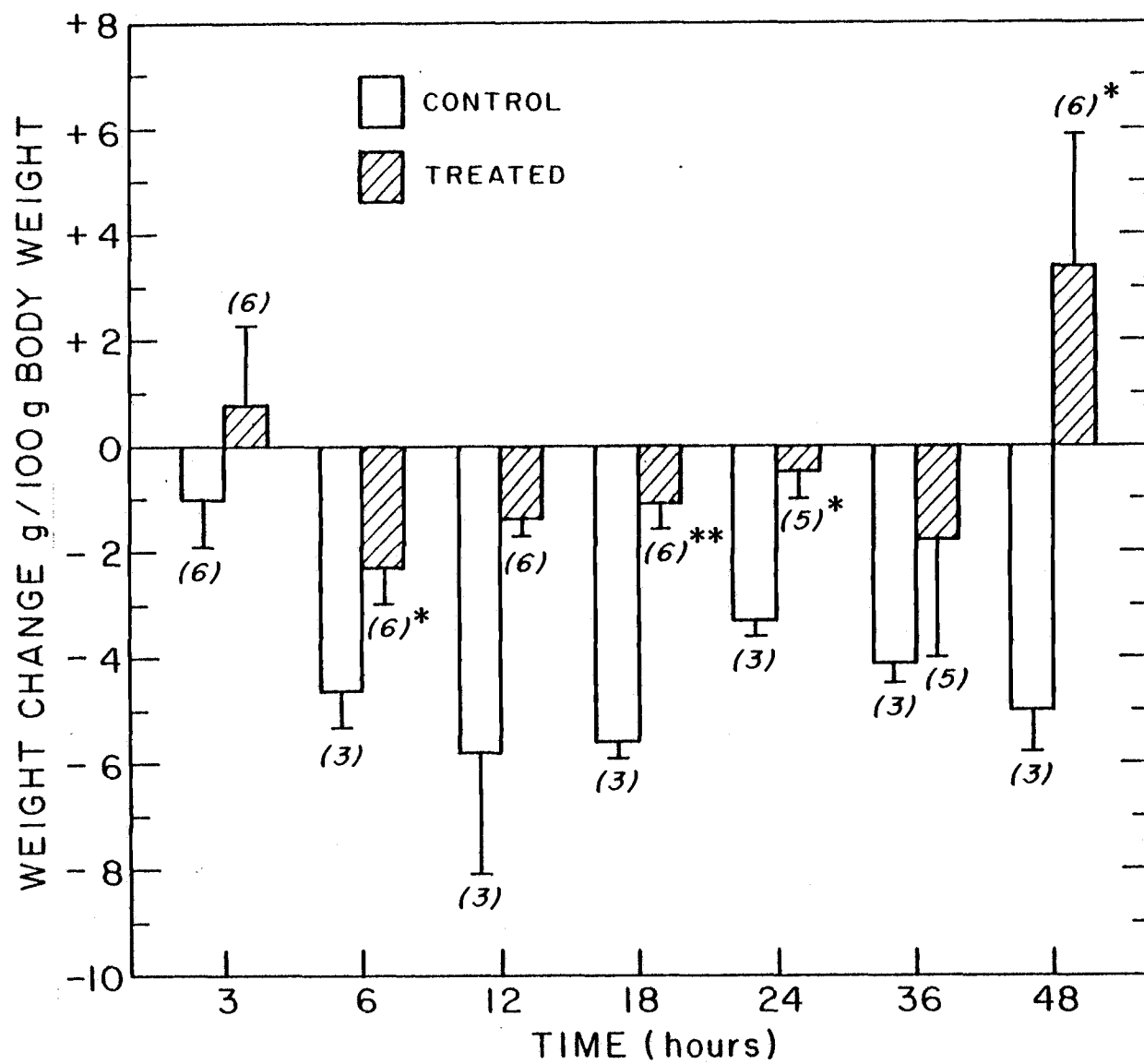
Figure 12. Plasma osmolarity for rainbow trout treated with
Cortland saline or CCl_4 (2.0 ml/kg, i.p.). Values
are the mean $\pm$ SEM for the number of fish in paren-
theses. Zero time mean value is for non-treated control
fish. Asterisks denote values that are significantly
different (*$P < 0.05$, **$P < 0.01$) from control fish.

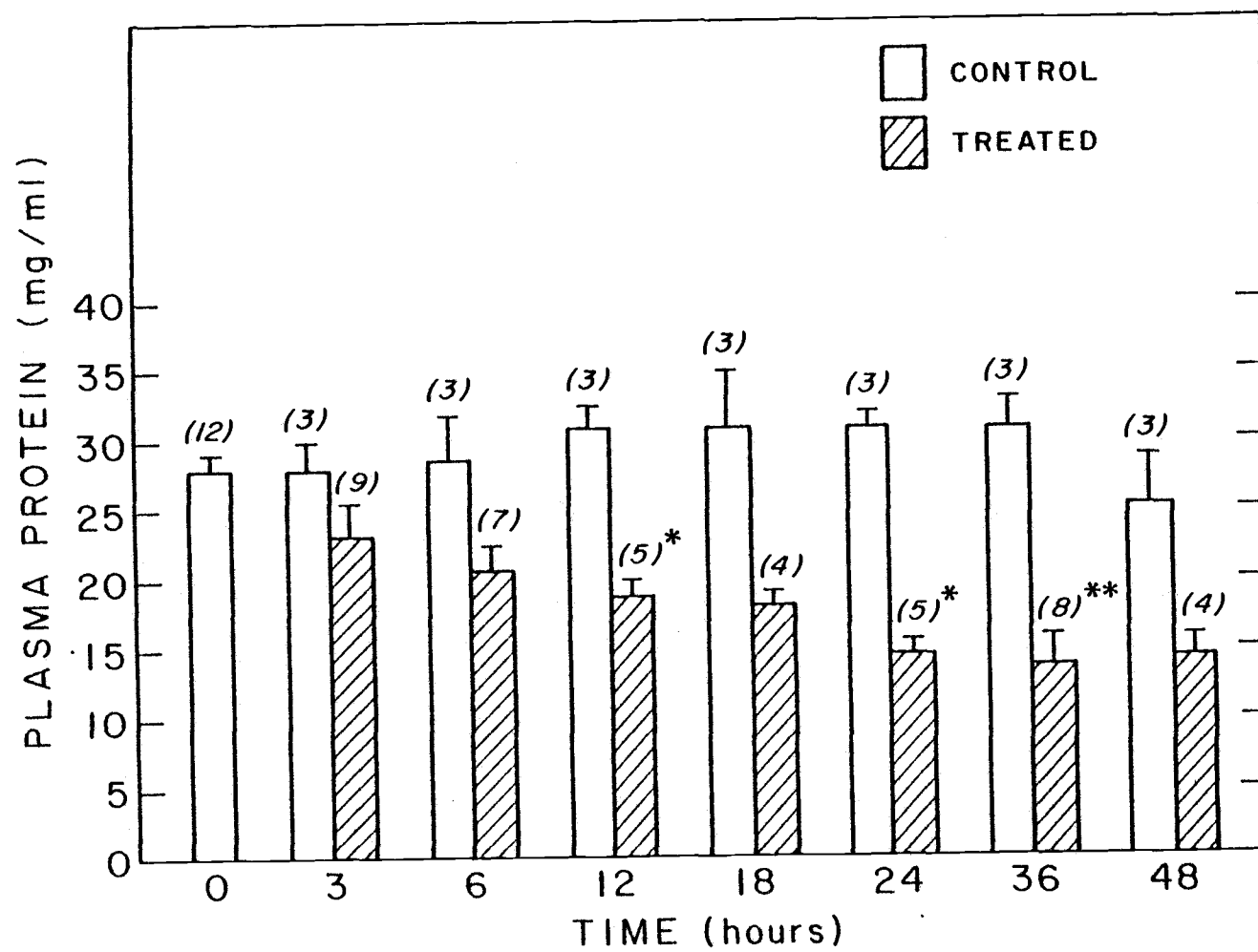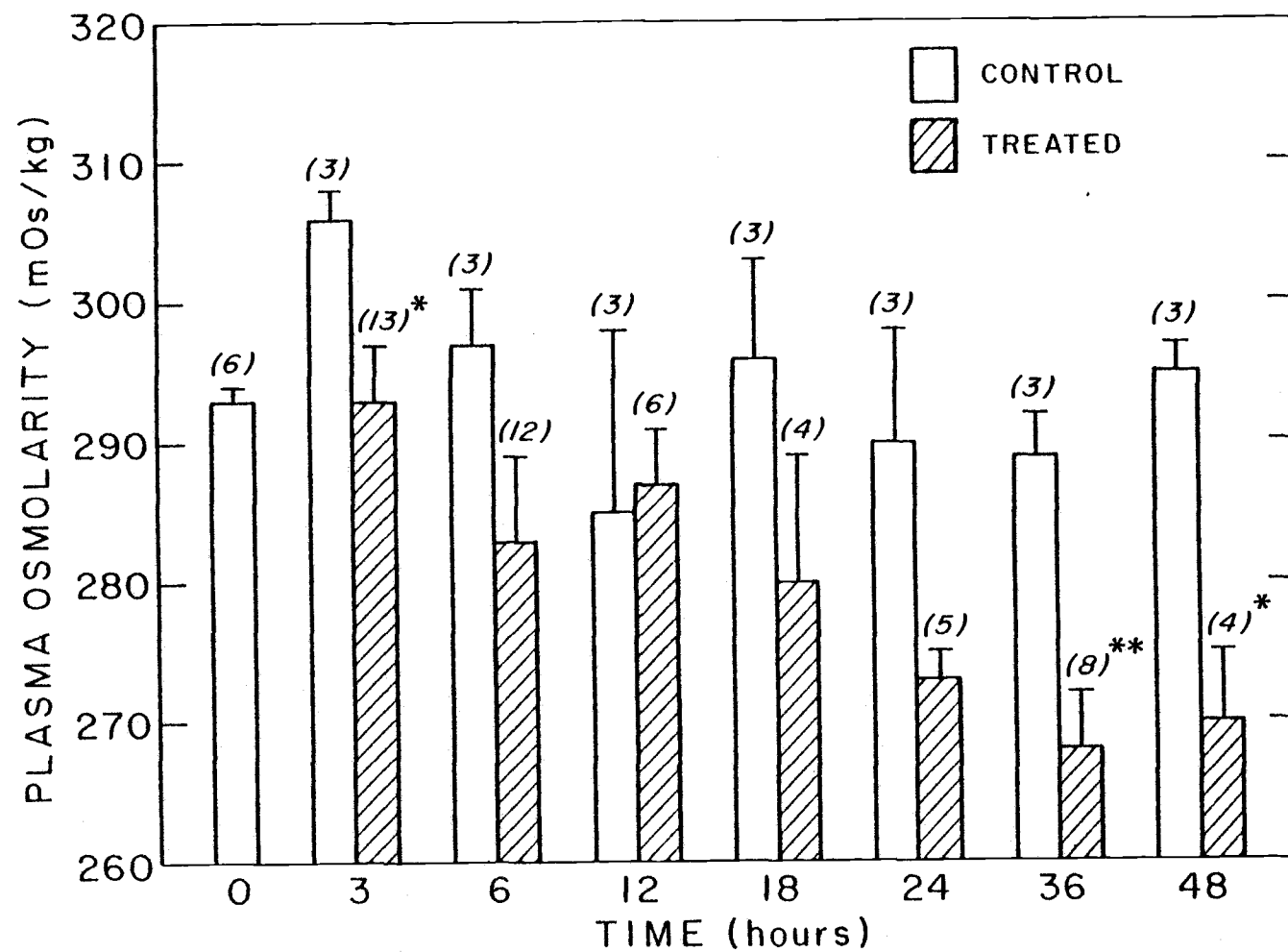Figure 12.

of these parameters. Sauer and Haider (1977) reported a plasma

protein concentration of approximately 34 mg/ml for rainbow trout

at 15°C. Denton and Yousef (1975) recorded a plasma protein con-

centration of 46 mg/ml for rainbow trout during November (water

temperature: 11-13°C). Meisner and Hickman (1962) determined

that the plasma albumin concentration for rainbow trout at 16°C was

44% of total plasma proteins, while Guderley and Ulrickson (1973)

found plasma albumin was 29% of total plasma protein. The appa-

rent discrepancy in plasma protein and albumin concentrations in

rainbow trout can be attributed to season of year, water tempera-

ture, strain of fish, diet and method of determination.

Plasma protein synthesis in higher vertebrates occurs pri-

marily in the liver. Investigations by Kenyon (1967) and Dean and

Berlin (1969) suggested that the liver of teleosts, e.g. rainbow

trout, had a similar capability. At present, the functional signi-

ficance of teleostean plasma proteins is unclear (Houston, 1973).

In order to maintain an effective plasma osmotic pressure, higher

vertebrates, e.g. humans, have a plasma protein concentration

approximately twice that found for trout in my study. The mean

arterial, systolic blood pressure in humans is 120 mm Hg; on the

other hand, the systolic pressure in the dorsal aorta of the rainbow

trout is approximately 35 mm Hg (Randall et al., 1965). The lower

blood pressure in the rainbow trout apparently indicates a reduced

plasma protein requirement to maintain blood flow and organ per-

fusion. Plasma proteins of teleosts have been implicated to function

in cardiovascular adjustments to temperature and in immune

response mechanisms (Houston, 1973).

CCl_4-Treated Fish

In my study the fish given CCl_4 (2.0 ml/kg) experienced a

50% decrease in total plasma protein concentration at 24 hours, but

they were alive and swimming. A comparable plasma protein

reduction in humans would have been followed by vascular collapse,

renal failure and death.

The increase in body weight and decrease in plasma osmolarity

in CCl_4-treated fish suggested that water was retained and that all

or part of the plasma protein reduction was due to an increase in

plasma volume. Blood or plasma volume changes were not

established for my fish. An in vitro dilution of trout plasma having

a known protein concentration and osmolarity established that

approximately 20% of the total protein decrease at 24 hours was

attributed to "simple" plasma dilution.

Fish given CCl_4 (1.0 or 2.0 ml/kg) showed a decrease in

plasma albumin concentration at 24 hours (Table 13), but the increase

in the albumin/total plasma protein ratio indicated that a part of the

reduction in plasma proteins was presumably from a loss of some

globulin fraction. Erickson et al. (1938) found that the plasma proteins in CCl_4-poisoned dogs were reduced mainly by a decrease in the albumin fraction. Bollman and Berryman (1943) reported a reduction of total plasma proteins, chiefly albumin, and a relative and absolute increase in the globulin fraction in CCl_4-treated laboratory rats. The implication that a specific protein fraction accounted for the plasma protein reduction in my fish is difficult because of the complex homeostatic mechanisms (in higher vertebrates) which control the level of plasma proteins (Zilva and Pannall, 1972). The loss of a particular fraction, e.g. albumin, is followed by an increased synthesis of that fraction, suggesting a feed-back stimulation of hepatic synthesis. The nature of this feedback control is unknown and apparently non-specific since there is a concurrent increase in hepatic synthesis of other plasma proteins.

Histopathology

The discussion of liver and kidney histopathology for Donaldson diet fish used in this study was presented in Toxicological Experiment #1.

Plasma Protein Decrease after Treatment
with CCl_4: Possible Mechanisms

The reduced plasma protein concentration in the CCl_4-treated fish can be attributable to several pathological processes. Some of

the mechanisms will be discussed, and a plausible hypothesis pre-sented to explain the decrease in plasma proteins.

Degradation within the Vascular System. Concurrent with the decrease in plasma protein concentration was the increase in plasma enzyme activities, e.g. alanine aminotransferase. Plasma GPT activity was maximum at 36 hours post-treatment with CCl_4 (1.0 ml or 2.0 ml/kg) (Tables 10 and 11); conversely, plasma pro-tein concentration was 55% less than control value at this time. CCl_4 had no effect on GPT activity in vitro (Curtis et al., 1972) and therefore would appear incapable of sufficient plasma protein dissolution in vivo to account for the decreased plasma protein con-centration in my fish. Furthermore, it seems unlikely, yet unproven, that there are proteolytic enzymes that can specifically degrade albumin and not an intracellular enzyme such as GPT.

Excretion. CCl_4 has been shown to be nephrotoxic to mammals (Moon, 1950; Stricker, 1958). The renal damage was confined mainly to tubule portions of the nephron, but albuminuria has been confirmed in some cases of CCl_4 toxicity (Von Oettingen, 1955). Glomerular and/or tubular capillary damage and subsequent "leakage" of albumin into the filtrate may have accounted for the albuminuria.

In my study histological examination of kidney tissues from CCl_4-treated trout showed no glomerular damage, but one fish had

proximal tubule necrosis at 36 hours post-treatment (CCl_4 2.0 ml/ kg). The Albustix [R] test was positive for proteinuria in 33 to 57% of the fish at the five test times (Table 19). An accurate, quantitative evaluation of the urinary protein was not possible with this method, but it did indicate that some plasma proteins may have passed through the glomerulus or entered the tubular lumen from damaged capillaries. Direct tubular damage by CCl_4 may have resulted in the loss of structural proteins into the urine and a positive Albustix [R] test for proteinuria.

Decrease in Plasma Protein Synthesis. The majority of plasma protein synthesis in teleosts is presumed to occur in the liver (Dean and Berlin, 1969; Kenyon, 1967). Smuckler and Benditt (1965) concluded that in laboratory rats given CCl_4 (p.o.) the decrease in liver protein and plasma protein synthesis was due to a change in ribosome structure. Previous studies showed no alteration in amino acid uptake or protein release from the liver (Smuckler et al., 1961, 1962). Labeled amino acid incorporation into fibrinogen and albumin was depressed as early as 3 and 4 hours, respectively, post-treatment, but plasma protein concentration and relative protein fractions were not altered during this period.

The half-life ($t_{1/2}$) for albumin in rainbow trout is not presently known; however, in the adult human, this time is

approximately 10 days (Sterling, 1951). The relative metabolic rate of poikilothermic animals is normally lower than homeotherms and presumably, the rates of protein catabolism would be lower for rainbow trout than mammals. However, in fish an organ may have a specific metabolic rate that is different from the "mean" metabolic rate of the entire organism. Evans et al. (1962) showed that the oxidative metabolism of trout liver was absolutely higher at $8°C$ than at $16°C$, despite the fact that whole animal, gill and muscle levels of oxygen consumption declined at the lower temperature. Das (1967), Das and Prosser (1967), Dean and Berlin (1969) and Haschemeyer (1968) demonstrated an increase in liver protein synthesis in various fishes, including rainbow trout, at lower water temperatures. Furthermore, no information is currently available regarding the effects of CCl_4 toxicity on specific organ or organismic metabolic rates.

The reduction in plasma proteins may have resulted from an impairment in hepatic blood flow. A decrease in amino acid availability and/or in hepatic protein secretory capability would reduce the total plasma protein concentration. Gingerich et al. (1978) suggested that a decrease in hepatic blood flow may have been responsible for impaired plasma clearance and hepatic accumulation of sulfobromophthalein in CCl_4-treated rainbow trout; however, data on hepatic blood flow in fishes and the changes

produced by CCl_4 are lacking. Based on available information, it appears unlikely that the > 50% reduction in plasma protein concentration in my CCl_4-treated trout at 24 hours can be attributed entirely to a decrease in liver protein synthesis.

Loss from the Vascular Compartment Due to Inflammation and Hemorrhage. Rainbow trout given CCl_4 by i.p. injection showed severe inflammation of the peritoneal cavity and general soft organ damage. Hemorrhagic fluid was characteristically present, and the spleen and intestines were consistently damaged. Previous work with laboratory mammals indicated that an i.p. injection of CCl_4 can cause an effusion of hemorrhagic fluid into the peritoneal cavity (Von Oettingen, 1955). Bollman et al. (1940) mentioned that hemorrhages were present in the gastro-intestinal tract of rats given CCl_4 (i.p.). They found that the blood of the rats had an increased prothrombin time and concluded that the decreased clotting ability and loss of blood were contributing factors in the death of the animals. CCl_4 can directly damage capillaries leading to diapedesis (Takasaka, 1925 from Von Oettingen, 1955). Rainbow trout treated with CCl_4 displayed a progressive inflammation of the urogenital opening and expelled small pieces of hemorrhagic tissue. The above examples from mammalian toxicological studies, and the observations of CCl_4-treated rainbow trout indicate that a probable loss of plasma proteins occurred via

hemorrhages into the peritoneal cavity or through the gastro-intestinal tract.

Presumably, a loss of plasma proteins via hemorrhage or capillary inflammation would have been accompanied by other lower molecular weight, plasma constituents, such as electrolytes. The decrease in plasma osmolarity of CCl_4-treated fish indicated a relative decline in electrolyte concentration, that was attributed to plasma dilution, i.e. increased blood volume, and/or capillary "leakage." The percent decrease in plasma electrolytes would, presumably, have been similar to the percent decline in plasma proteins. At 36 hours post-treatment plasma osmolarity had decreased by 7% of control value. Conceivably, there may have been a greater plasma electrolyte loss than indicated by the measured osmolarity, but electrolyte recruitment from the inter-stitial compartment could have maintained the plasma osmolarity. This transcapillary exchange of electrolytes and plasma proteins between the vascular and interstitial compartments has been impli-cated in temperature acclimation of freshwater fishes (Houston, 1973).

Several factors could have contributed to the decrease in plasma protein concentration in my CCl_4-treated rainbow trout. Plasma protein loss may have occurred in the urine due to renal damage. The hepatotoxic effects of CCl_4 may have resulted in

decreased plasma protein synthesis or impaired hepatic blood flow.

A decrease in prothrombin synthesis and direct capillary damage

would have permitted plasma protein loss via hemorrhages into the

peritoneal cavity or through the gastro-intestinal tract. Further

studies are needed to establish the relationship and relative

importance of these processes in CCl_4 intoxicated animals.

IV. TOXICOLOGICAL EXPERIMENT #3: THE EFFECT OF CARBON TETRACHLORIDE ON URINE FLOW RATE OF THE RAINBOW TROUT

Introduction

Carbon tetrachloride (CCl_4) is a model hepatotoxic agent that has been widely used in experimental mammalian toxicology (Dinman and Bernstein, 1968; Korsrud et al., 1972; Plaa and Larson, 1964; Recknagel, 1967). The majority of the current mammalian CCl_4 research has centered around the mechanisms of its hepatotoxic actions (Diaz-Gomez et al., 1975; Lindstrom and Anders, 1977; Suriyachan and Thithapandha, 1977). Previous studies with rainbow trout established that these lower vertebrates were susceptible to the hepatotoxic effects of CCl_4 (Gingerich et al., 1978; Pfeifer et al., 1977; Racicot et al., 1975; Statham et al., 1977).

In addition to its deleterious action on liver structure and function, CCl_4 has been reported to be nephrotoxic in humans and laboratory mammals (Moon, 1950; Plaa and Larson, 1965; Sirota, 1949; Stricker et al., 1968; Von Oettingen, 1955). Some workers have studied the effects of exogenous chemicals on salmonid renal function. Lloyd and Orr (1969) measured the urine flow rate of rainbow trout exposed to sub-lethal concentrations of ammonia. Hunn and Willford (1970) studied rainbow trout renal function after anesthetization with tricaine methane sulfonate (MS-222). Hunn and

Allen (1975) determined renal excretion characteristics for coho salmon, Oncorhynchus kisutch, after an acute, sublethal exposure to the lampricide, 3-trifluoromethyl-4-nitrophenol (TFM).

No information is currently available on the nephrotoxic response of fish to CCl_4. The purpose of this study was to determine the effect of an intraperitoneal injection of CCl_4 on the urine flow rate of the rainbow trout.

Materials and Methods

Animals and Holding Conditions

Rainbow trout (200-500 g) were purchased from Roaring River Hatchery, Scio, Oregon. Fish were held in aquaria (200 gal) supplied with continuously flowing well water ($15.0^{\circ}C \pm 1.0^{\circ}$). The room temperature was also kept at $15.0^{\circ}C$. Animals were fed a commercial fish diet, Donaldson-Ore Aqua^R, every day, but food was withheld for 24 hours prior to an experiment. A 12L:12D photoperiod was used throughout all experiments. All fish were allowed a one-week acclimation period before they were used in the experiments and were in good health. The study was done during the months of December through February.

Insertion of Urinary Catheter

A single fish was removed from the holding aquarium, anethetized in a solution of MS-222 (50 mg/l) for 3-5 minutes and

weighed. The fish was placed on a surgical table, and the desired level of anesthetization was maintained by irrigating the gills with either MS-222 (100 mg/l) or well water without anesthesia. Polyethylene tubing (P.E.-50; ID = 0.023", OD = 0.038") was inserted through the urogenital opening and into the urinary bladder to a depth of 1.5-2.0 cm. The catheter was sutured to the base of the anal fin. Urine usually entered the tubing after insertion into the bladder; however, positive pressure was applied to the tubing if urine had not appeared by 30-45 seconds. This procedure was normally completed in 10-15 minutes.

Urine Collection

The fish was placed in a Plexiglas ^{R} restraining chamber which prevented vertical and lateral body movements but permitted normal opercular respiration. The restraining chamber was located inside a 24" x 17" x 7" aquarium supplied with continuously flowing well water ($15.0^{\circ}C \pm 1.0^{\circ}$). Urine collection commenced when urine appeared at the tip of the tubing, approximately 15-20 minutes post-catheterization. The urinary catheter left the aquarium through a neoprene stopper directly under the fish and went to a fraction collector. Urine flowed by gravity and was collected at one hour intervals. The collection tubes were corked to prevent urine evaporation.

Treatment with CCl_4

After 24 hours of urine collection, the fish was removed from the restraining chamber, anesthetized in MS-222 (50 mg/l), weighed and injected with undiluted CCl_4 (2.0 ml/kg, i.p.), as previously described in Toxicological Experiment #1. Control fish received an equivalent volume of Cortland saline (Wolfe, 1963). The fish was returned to the chamber, and urine collection started within 5-10 minutes post-treatment. In an attempt to determine the effects of the Cortland-treatment procedure on urine flow rate, urine was collected from nine fish for 48 hours without interruption, i.e., non-treated control fish. All fish were weighed at the end of the 48 hour experimental period.

Analytical Procedures

Urine osmolarity was determined with a vapor-pressure osmometer. Approximate urine protein concentrations were obtained by a modified Albustix[14] dip-stick method. A standard protein solution was diluted to concentrations of 10, 20, 40 and 80 mg/100 ml. The color from the urine sample was compared to the color of the standard.

---

[14] Ames Co., Elkhart, Indiana.

Statistical Methods

Data were converted to a quantal form and analyzed by the Fisher Exact Probability Test (Siegel, 1965). Values greater than two standard deviations ($P < 0.05$) from the control values were chosen to indicate a positive response in the treated fish. Hourly mean urine flow rates (UFR) for non-treated control fish and Cortland-treated controls were compared by the Student's t-test for independent sample means (Simpson et al., 1960).

Histological Studies

Histological sections of the trunk kidney were obtained from fish that had not been catheterized but otherwise had been treated identically. The kidney section was removed 24 hours post-treatment with CCl_4 or Cortland saline (2.0 ml/kg). Sections were fixed, embedded and stained as previously described in Toxicological Experiment #1.

Results

Non-Treated Control Fish

The 48 hour collection period was divided into two 24-hour periods. The hourly mean UFR for the first and second 24-hour periods were 4.3 ml/kg/hr and 3.5 ml/kg/hr, respectively

Table 15. Mean urine flow rates and relative weight change for non-treated rainbow trout for 48 hours.

Parameter	Time[a]	
	1 - 24	25 - 48
Urine Flow Rate		
(ml/kg/hr)[b]	4.3 ± 0.4[d]	3.5 ± 0.5
(ml/kg/24 hrs)[c]	103.2 ± 11.0	82.7 ± 12.0
Weight Change	-2.8 ± 1.0[e]	
(g/100 g b.w.)		

[a]Hours post-catheterization.

[b]The hourly mean UFR for the first (hours 1-24) and second (hours 25-48) 24 hour periods.

[c]The 24 hour mean UFR for the first and second 24 hour period.

[d]Values are mean ± S.E.M. for 9 fish.

[e]Net weight change for 48 hours post-catheterization.

(Table 15). The decline in the UFR 24 hours post-anesthetization-catheterization was also reflected in the 24 hour mean UFR which was 103.2 ml/kg/24 hr for the first 24 hours and 82.7 ml/kg/24hr for the second 24 hours.

The mean weight loss per 100 g body weight during the 48 hour collection period was -2.8 g (Table 15).

Mean urine osmolarity decreased during the 48 hour collection period from 76 mOs/kg (first hour) to 65 mOs/kg (48th hour) (Table 16).

Table 16. Urine osmolarity for non-treated rainbow trout determined every 12 hours for 48 hours.

Urine Osmolarity	Time[a]				
(m Os /kg)	1	12	24	36	48
Non-treated Fish	75 ± 9[b]	69 ± 3	67 ± 2	66 ± 7	65 ± 7

[a] Hours post-catheterization.
[b] Values are mean $\pm$ S.E.M for 9 fish.

Urine was tested for protein at 6-hour intervals during the 48-hour period. Based on the sensitivity of the Albustix[R] method, all urine samples were negative, i.e. <10 mg protein/100 ml urine.

In Fig. 13 the 48 hour collection period was arbitrarily divided into eight 6-hour periods. The hourly mean UFR (ml /kg/hr) for nine fish was determined for each 6 hour period. There

Figure 13. Urine flow rate for non-treated control rainbow trout
for 48 hours post-catheterization. Values are the
hourly mean $\pm$ SEM for nine fish during each 6-hour
time period.
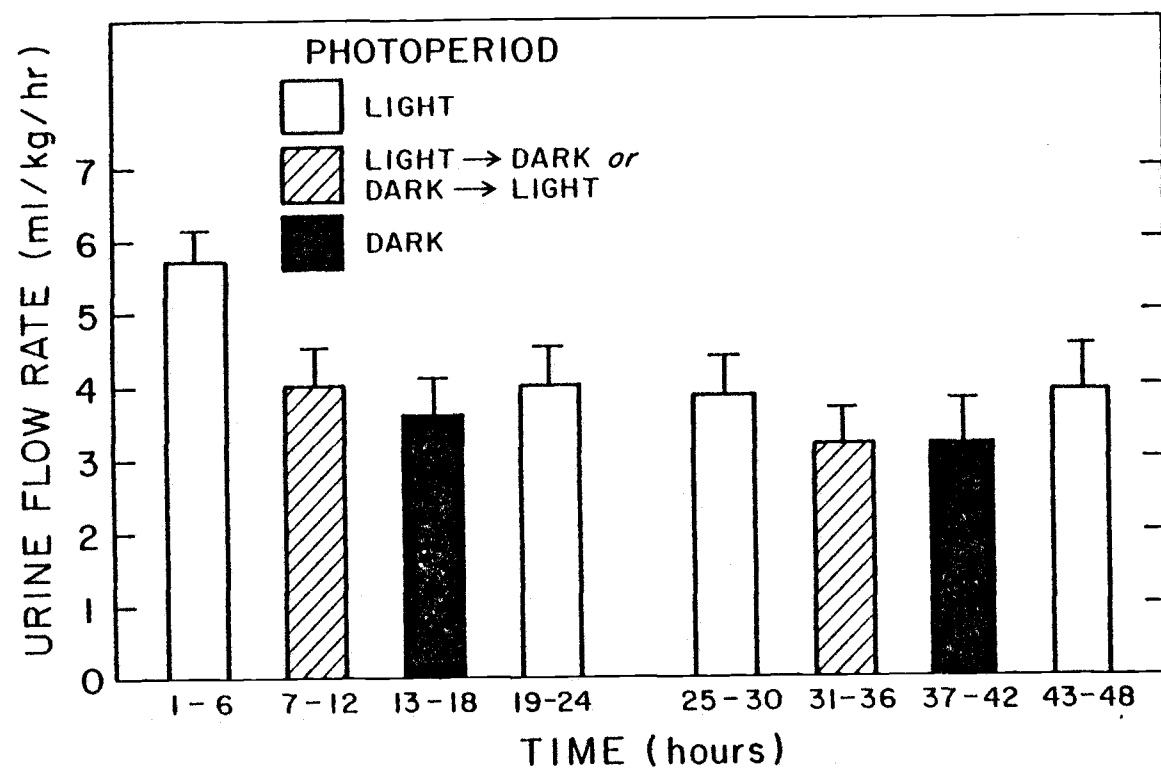
Figure 13.

appeared to be a biphasic, i.e. diurnal, pattern to the 24 hour UFR which may have been attributed to the photoperiod and the resultant activity levels. The lowest hourly mean UFR occurred during the all dark portion of the first 24 hour period and during the light/dark and all dark portions of the second 24 hour period. The high UFR during the initial 6 hour period probably reflected the effects of the stress imposed by the anesthetization and/or catheterization as mentioned by previous workers (Hunn and Willford, 1970).

A regression of body weight (g) and UFR (ml/kg/hr) for the 24 hours post-catheterization was plotted for 34 fish (Fig. 14). There was a slight, negative correlation (r = -0.16) between UFR and body weight. The reason for this phenomenon is apparently the higher gill surface area to body weight ratio in the smaller fish (Hickman and Trump, 1969).

Cortland Saline Treated Fish

After a 24 hour pre-treatment urine collection period, control fish received an i.p. injection of Cortland saline (2.0 ml/kg). Both the hourly and 24 hour mean UFR increased slightly after treatment (Table 17). The hourly mean UFR increased from 4.1 ml/kg/hr to 4.3 ml/kg/hr, and the 24 hour mean increased from 98.1 ml/kg/24hr to 104.1 ml/kg/24 hr. This increase was not significant (P ≤ 0.05). Cortland saline treated fish still retained

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Figure 14. Correlation between urine flow rate and body weight for
rainbow trout for 24 hours post-catheterization. Each
point represents one fish (N = 34). Correlation coeffi-
cient (r) = -0.16. Equation for the line which best fits
the data:

Urine flow rate = (-.003)(body weight) + 5.4 ml/kg/hr
  (ml/kg/hr)   (g)
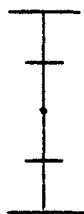
represents mean urine flow rate ± one SEM and ± one
SD at the mean body weight for the 34 fish.
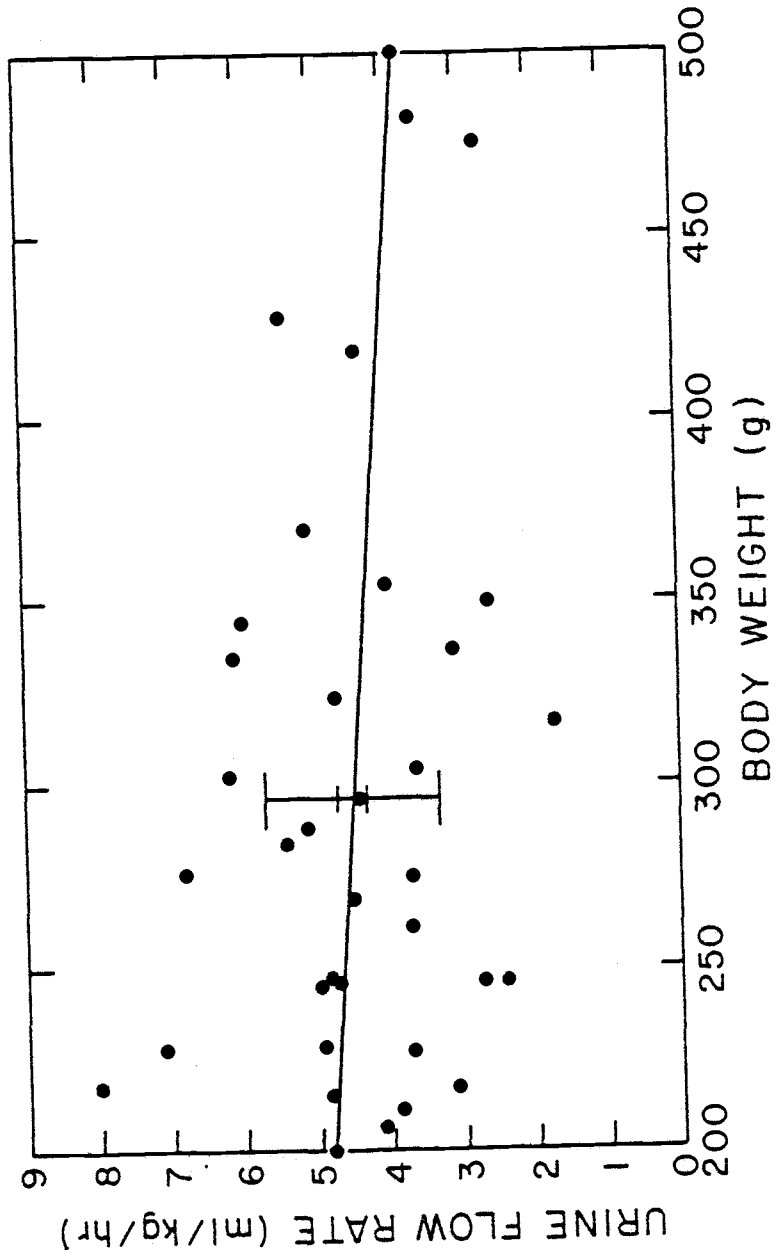
Figure 14.

Table 17. Mean urine flow rates and relative weight change for rainbow trout during 24 hours pre-treatment and 24 hours post-treatment with Cortland saline or CCl_4 (2.0 ml/kg, i.p.).

Parameter	Pre-treatment (24 hours)	Post-treatment (24 hours)
Urine Flow Rate (ml/kg/hr)[a]		
Control (8)	4.1 ± 0.3[b]	4.3 ± 0.3
CCl_4 (10)	4.1 ± 0.5	1.0 ± 0.4**
(ml/kg/24 hrs)[c]		
Control	98.6 ± 6.7	104.1 ± 7.5
CCl_4	98.7 ± 11.5	23.2 ± 10.1**
Weight Change (g/100 g b.w.)		
Control	-2.3 ± 0.4	-0.3 ± 0.3
CCl_4	-2.5 ± 0.4	$+3.7 \pm 0.7$**

[a] The hourly mean UFR for 24 hours pre-treatment and 24 hours post-treatment.

[b] Values are mean $\pm$ S.E.M. for 8 (control) and 10 (CCl_4) fish.

[c] The 24 hour mean for 24 hours pre-treatment and 24 hours post-treatment.

** Significantly different from Cortland control values (P < 0.01).

a biphasic urinary excretion pattern (Fig. 15), but the post-treatment rates for the four 6-hour periods were slightly higher than the UFR for those same periods in the non-treated 48 hour control fish (Fig. 13). The hourly mean UFR for the initial 6-hour period post-treatment was significantly different (P < 0.05) from the value for non-treated controls during the 25-30 hour period.

CCl_4-Treated Fish

CCl_4-treated fish, on the other hand, showed a dramatic decrease in the hourly mean and 24 hour mean UFR (Table 17). The hourly mean UFR decreased from 4.1 ml/kg/hr during the pre-treatment period to 1.0 ml/kg/hr post-treatment with CCl_4. The 24 hour mean UFR decreased from 98.7 ml/kg/24 hr pre-treatment to 23.2 ml/kg/24 hr during the post-treatment period. These changes in UFR were highly significant (P < 0.01); in addition, the hourly mean UFR and 24 hour mean UFR for Cortland-treated control fish and CCl_4-treated fish were significantly different (P < 0.01) (Table 17).

The diurnal pattern of urine flow was completely abolished in the CCl_4-treated fish (Fig. 16). There appeared to be an attempt by these fish to restore the UFR as indicated by the slight rate increases in the 13-18 hr and 19-24 hr periods.

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Figure 15. Urine flow rate for Cortland-treated (2.0 ml/kg, i.p.) control rainbow trout for 24 hours pre-treatment and 24 hours post-treatment. Values are the mean $\pm$ SEM for eight fish during each 6-hour time period. Asterisks denote value that is significantly different (*P < 0.05) from non-treated control fish during a similar time period post-catheterization.

Figure 15.

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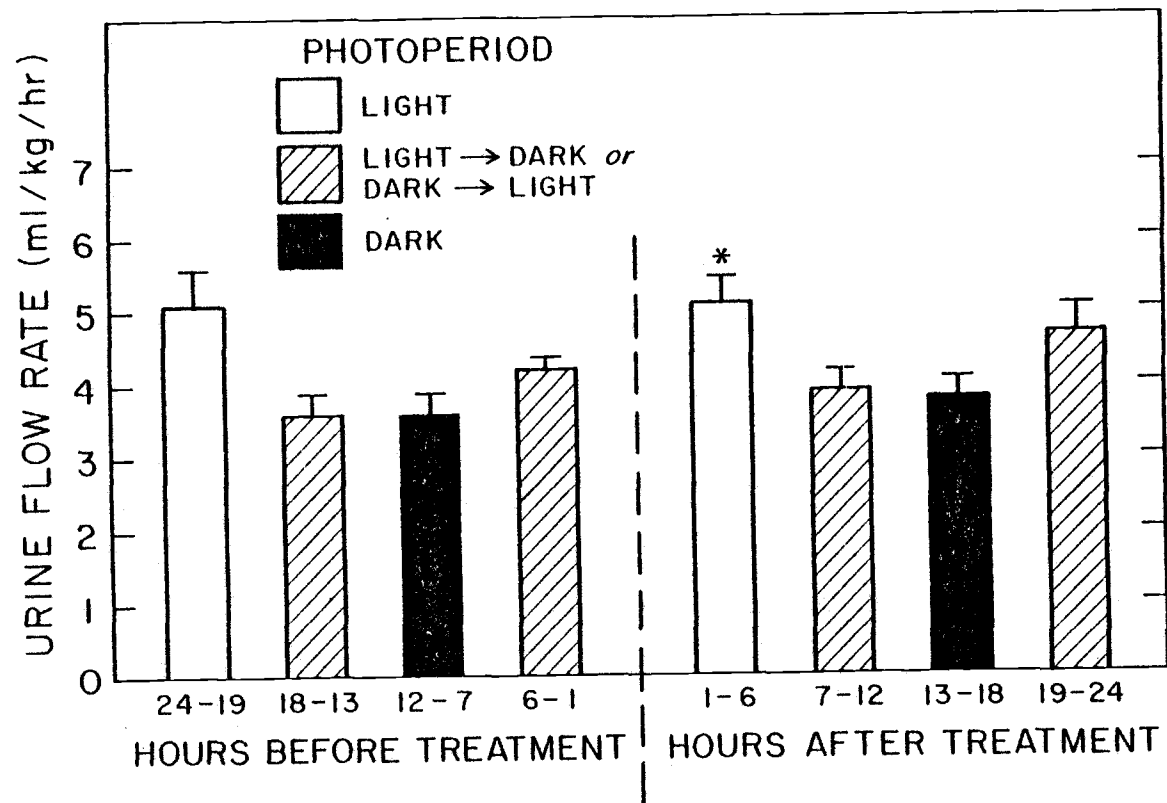
Figure 16. Urine flow rate for CCl_4-treated (2.0 ml/kg, i.p.)
rainbow trout for 24 hours pre-treatment and 24 hours
post-treatment. Values are the hourly mean $\pm$ SEM
for 10 fish during each 6-hour time period. Asterisks
denote values that are significantly different (**$P < 0.01$)
from Cortland-treated controls during the same time
periods post-treatment.
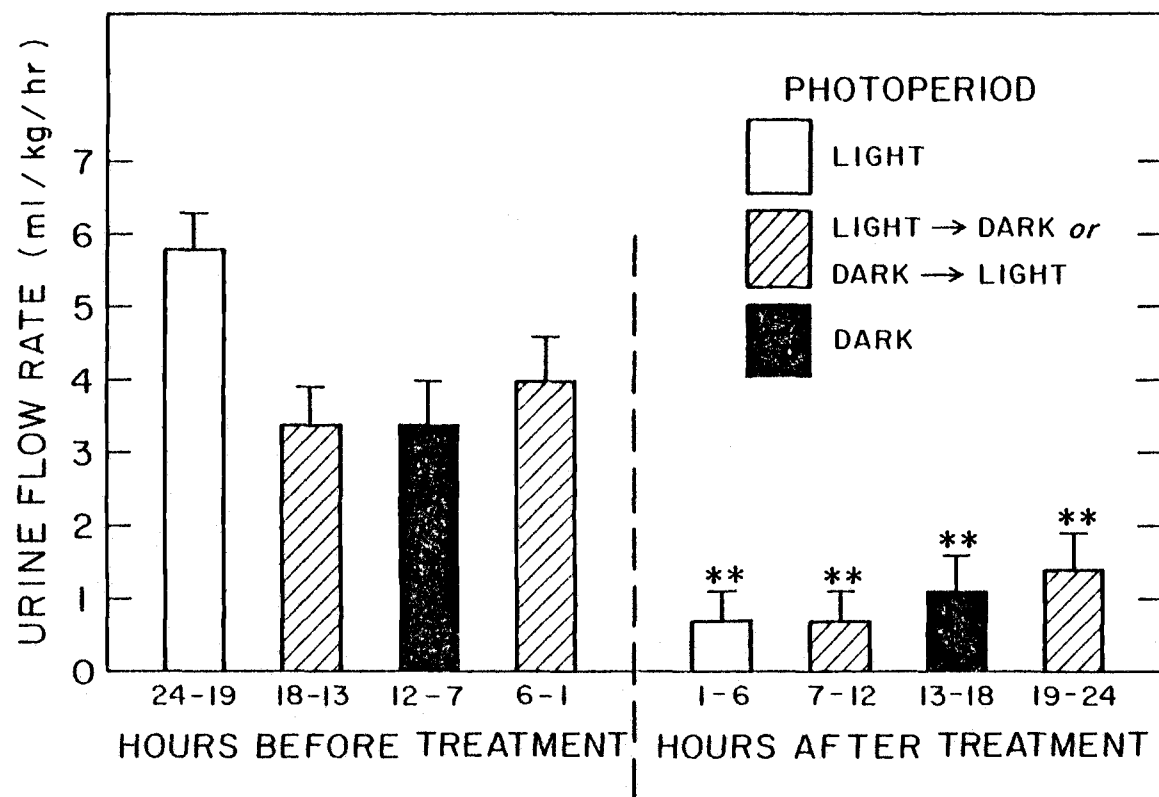
Figure 16.

CCl_4-treated fish showed a significant (P < 0.01) increase
in relative body weight when compared to control fish (Table 17).
A negative correlation of 0.80 was found between UFR (ml/kg/24
hr) and the relative change in body weight (g/100 g B.W.) for the
CCl_4-treated fish, 24 hours pre-treatment and 24 hours post-
treatment (Fig. 17).

During the 24 hours post-treatment, the urine osmolarity of
Cortland-injected control fish remained stable at the times tested
(Table 18). On the other hand, the urine osmolarity of the CCl_4-
treated fish increased significantly (P < 0.01) when compared to
the control values.

Urine was tested for protein at 6 hour intervals during the
24 hours post-treatment (Table 19). All urine samples from
Cortland-treated fish were negative, i.e. < 10 mg protein/100 ml
urine/hr. Some of the CCl_4-treated fish had proteinuria in the
range of 10-80 mg/100 ml/hr.

Histopathology

The results of kidney histopathology for Donaldson diet fish
used in this study were presented in Toxicological Experiment #1.

Figure 17. Correlation between urine flow rate and body weight change for CCl_4-treated (2.0 ml/kg, i.p.) rainbow trout during 24 hours pre-treatment and 24 hours post-treatment. Correlation coefficient (r) = -0.80. N = 10 fish.
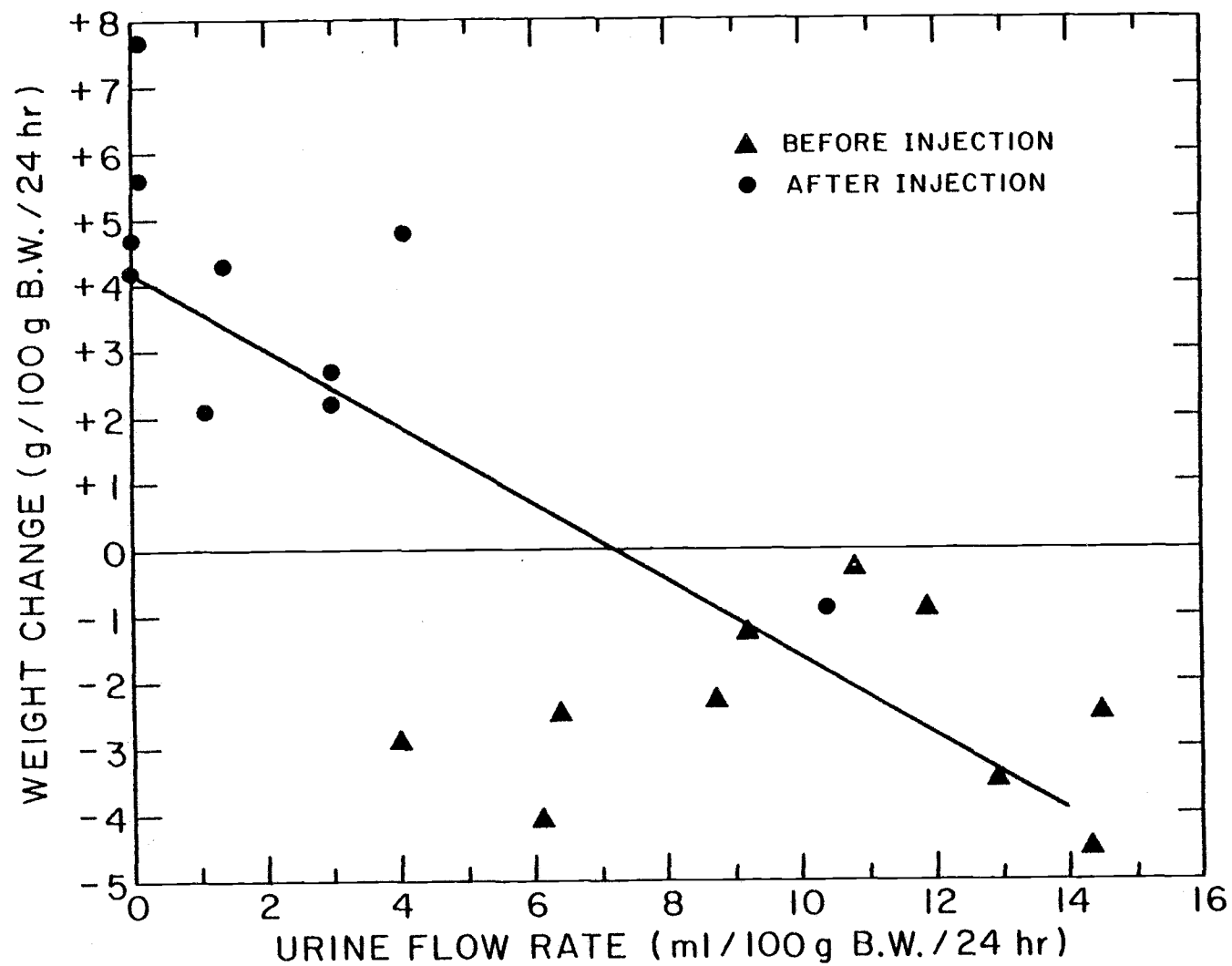
Figure 17.

Table 18. Urine osmolarity for rainbow trout treated with Cortland saline or CCl_4 (2.0 ml/kg, i.p.) determined every 12 hours for 24 hours pre-treatment and 24 hours post-treatment.

Urine Osmolarity (mOs /kg)	Hours Pre-treatment			Hours Post-treatment		
	24	12	1	1	12	24
Control (8)	73 ± 4[a]	66 ± 1	61 ± 2	60 ± 3	$61 + 2$	60 ± 2
CCl_4 (10)	75 ± 6	58 ± 4	56 ± 4	76 ± 3**	104 ± 16**	88 ± 9

[a]Values are mean $\pm$ S.E.M. for 8 (control) and 10 (CCl_4) fish during a one hour period.

**Significantly different from Cortland control value ($P < 0.01$).

Table 19. Protein concentration[a] in urine from rainbow trout treated with Cortland saline or CCl_4 (2.0 ml/kg, i.p.).

	Time[b]				
	1	6	12	18	24
Number of Fish Showing Protein in Urine					
Control (8)[c]	0/8	0/8	0/8	0/8	0/8
CCl_4 (10)	3/9[d]	4/7	4/7	3/7	4/7
Range of Urinary Protein (mg/100 ml/hr)					
Control	neg[e]	neg	neg	neg	neg
CCl_4	10-40	10-40	10-80	10-80	10-80

[a] Approximate concentrations determined by Albustix[R].

[b] Hours post-treatment with Cortland saline or CCl_4 (2.0 ml/kg, i.p.).

[c] No control fish had any urinary protein, at the level of sensitivity of this method (10 mg/100 ml).

[d] Some CCl_4 treated fish did not pass sufficient urine to measure the protein concentration.

[e] Neg = <10 mg/100 ml.

Discussion

Non-Treated Control Fish

The rainbow trout is a hyperosmotic regulator. It is continually faced with an influx of water from the environment, and the kidney functions primarily as a water excretory organ. This is accomplished by renal glomeruli which filter the blood and by renal tubules which have a low water permeability but can efficiently absorb monovalent ions, e.g. sodium, from the glomerular filtrate (Hickman, 1965). The urine of these fish is characteristically hypoosmotic to the plasma (Fromm, 1963; Hunn and Willford, 1970). The rainbow trout under resting conditions does not drink in fresh water (Shehadeh and Gordon, 1969); therefore, the net volume of urine excreted should balance the quantity of water entering via the gills.

The results of my study indicate that these rainbow trout exhibited a diurnal UFR, probably the result of increased activity during daylight hours. This was not surprising since heightened metabolic activity would cause greater branchial blood flow and increased water influx via the gills (Mackay and Beatty, 1968; Wood and Randall, 1972).

The 24 hour mean UFR found in this study was in the range for control fish in previous studies using rainbow trout (Hunn,

1969; Hunn and Willford, 1970). The hourly mean UFR of 3.5 ml/kg/hr for the second 24 hour period was higher than the control UFR of 2.5 ml/kg/hr at 15°C found by Lloyd and Orr (1969) but similar to the pretest value of 3.3 ml/kg/hr (16°C) reported by Swift and Lloyd (1974). In both of these earlier studies, the control UFR was determined 24 hours post-anesthetization-catheterization, as suggested by Hunn and Willford (1970).

The decrease of 14% in urine osmolarity during the 48 hour collection period was less than the 54% decrease during an equivalent collection period reported by Hunn and Willford (1970).

Cortland Saline-Treated Fish

My results show a slight, but significant ($P < 0.05$) increase in the hourly mean UFR for Cortland-treated controls during the initial 6 hour period post-treatment. This increase may have been caused by handling, re-anesthetization and/or the injection of saline. Holmes and Stainer (1966) reported an insignificant increase in the UFR of MS-222 anesthetized rainbow trout during the 20 hours post-treatment with a single i.p. dose of 0.5 ml NaCl (0.8%).

CCl_4-Treated Fish

The results of my study show that CCl_4 (2.0 ml/kg, i.p.) produced an oliguria or anuria in rainbow trout as early as one

hour after treatment. This decrease in UFR was still apparent 24 hours after treatment. CCl_4 poisoning in humans, whether by inhalation or ingestion, has been reported to produce oliguria or anuria within 1 to 3 days following exposure (Guild et al., 1958; Moon, 1950; Sirota, 1949). Cornish and Ryan (1964), on the other hand, found a two-fold increase in urine volume for the first 24 hours after exposing rats to CCl_4 vapors. Also using rats, Stricker et al. (1968) showed an increase in urine volume for the first 24 hours after an oral dose of CCl_4 (2.5 ml/kg).

Previous studies with salmonid fishes demonstrated that the stress of handling, exposure to sub-lethal concentrations of chemicals or hypoxic conditions, increased UFR (Hunn, 1969; Hunn and Allen, 1975; Lloyd and Orr, 1969; Swift and Lloyd, 1974). Lloyd and Orr (1969) attributed the diuretic response in rainbow trout exposed sub-lethal levels of ammonia to an increase in gill permeability to water, rather than a direct action on the kidney.

Gingerich et al. (1978) reported a significant weight gain in rainbow trout 24 hours post-treatment with CCl_4 (2.0 ml/kg, i.p.). My results indicate that the weight gain experienced by CCl_4-treated trout was related to impaired water clearance reflected by a decrease in UFR.

Urine osmolarity increased in the trout treated with CCl_4. A decrease in the ability of the proximal or distal tubules to

absorb electrolytes from the glomerular filtrate would have been reflected by an increase in urine osmolarity and may have been due to subtle changes in the integrity of the tubule epithelial cells.

In mammalian toxicology, proteinuria is frequently indicative of renal pathology. It is usually associated with glomerular damage but can be due to renal tubular damage and even occur when there is no pathological condition (Foulkes and Hammond, 1975). The proteins in the urine that are associated with glomerular damage are of relatively high molecular weight, e.g. albumin, while those associated with tubular damage tend to be of lower molecular weight. Toxicological Experiment #2 reported that CCl_4-treated trout experienced a reduction in total plasma protein concentration after 24 hours. This suggested that the plasma proteins may have been lost through damaged glomeruli. The urine of Cortland-treated control and CCl_4-treated fish was tested for protein using a simple dipstick method (Albustix ^{R}) which can detect protein at a concentration of 10 mg/100 ml. This method appeared adequate since the reduction in plasma protein concentration was one to two orders of magnitude higher than this minimum level of sensitivity. The highest hourly urine protein concentration recorded in the CCl_4-treated fish was approximately 80 mg/100 ml/hr. This concentration was higher than control values (all were negative, i.e. < 10 mg/100 ml), but was

not consistent and did not adequately account for the reduction in

plasma proteins. The interpretation of proteinuria is difficult

since these tests do not differentiate between structural and

plasma proteins. For example, CCl_4 could directly damage the

kidney tubules and release structural proteins into the urine.

Quantitative and qualitative measurements of urinary protein

should be considered in toxicity studies before much significance

is placed on proteinuria. These tests should be interpreted with

adequate knowledge about specific organ toxicity of the compound

and should be used in conjunction with histopathological evaluations.

Histopathology

The oliguria and anuria produced by CCl_4 in my trout cannot

be explained solely on the basis of kidney damage. The histologi-

cal sections of the kidney tissue were inconclusive as far as

showing any widespread tissue damage. No glomerular damage

was observed, and only one kidney showed proximal tubular

damage. Hematopoietic cell necrosis and early pathological signs,

such as changes in the brush border of the proximal tubules, may

have been indicative of more subtle changes in nephron micro-

structure that led to functional impairment. Stricker et al. (1968)

noted that kidney changes related to CCl_4 intoxication in the rat

were limited to the proximal tubules. As early as 2 hours after

treatment they found alterations in mitochondrial morphology. At 24 hours there was epithelial cellular swelling which was characterized by swollen microvelli and the loss of basilar interdigitations. They concluded that these cellular and mitochondrial alterations affected energy utilization for the active uptake of sodium ions from the glomerular filtrate. This hypothesis was supported by their finding of an increased urinary sodium concentration up to 48 hours after treatment; however, renal ATP levels remained unchanged (Smuckler et al., 1968).

Oliguria/Anuria after Treatment with CCl_4: Possible Mechanisms

The present data are not sufficient to explain the pathological process by which CCl_4 decreased UFR in the rainbow trout. Some of the possible mechanisms will be presented and discussed below.

Gill permeability to water may have been altered in the CCl_4-treated fish, but I have no evidence to support this hypothesis. I did find a negative correlation between UFR and change in body weight. The relative increase in body weight post-treatment with CCl_4 indicated that the decrease in UFR was not due solely to decreased branchial water influx.

UFR in fresh water teleosts is directly proportional to glomerular filtration rate (GFR) (Hickman, 1965). A nearly constant proportion of the water and salts filtered by the glomeruli is reabsorbed by the tubules; therefore, variation in UFR is achieved by changes in GFR. Hammond (1969) showed that fluctuations in dorsal aortic blood pressure in the lake trout were positively correlated with changes in GFR. In higher vertebrates there are numerous factors that can affect the GFR, but the two most important are probably changes in renal blood flow and arterial blood pressure.

Sirota (1949) concluded that in humans the reduction in renal blood flow and abnormal tubular back diffusion were important factors contributing to the resultant oliguria after CCl_4 exposure. In acute tubular necrosis, the decrease in GFR could be partly due to an increase in intraluminal pressure resulting from edema of the surrounding tissues. The increase in intraluminal pressure would reduce the hydrostatic pressure gradient essential for normal plasma filtration. Hemoglobinemia frequently occurred in my CCl_4-treated trout. Gingerich et al. (1978) reported a similar response in their rainbow trout injected with CCl_4 (2.0 ml/kg). Hemoglobin aggregation in the renal vascular system may have impaired blood flow and caused a reduction in GFR and, subsequently, in UFR.

Other studies with laboratory mammals indicated that CCl_4

can produce a fall in systemic blood pressure (Von Oettingen,

1955). It appears plausible that the early oliguria and anuria in

my CCl_4-treated fish may have been due to a decrease in arterial

blood pressure and/or a decrease in renal blood flow rather than

a direct effect on the kidney. Gingerich et al. (1978) suggested

that CCl_4 produced a decrease in hepatic blood flow in rainbow

trout; however, earlier studies with laboratory rats treated with

CCl_4 failed to resolve this question (Plaa, 1975).

Research concerned with endocrine control of renal mecha-

nisms in teleosts is far from complete, but much evidence has

accumulated implicating various hormonal-substances in the

overall "hydromineral" balance of these fishes (Johnson, 1973).

The pituitary hormone, prolactin, appears to be the most

important hormone for regulation of hydromineral balance in

fresh water fishes. Prolactin is thought to increase GFR, reduce

renal tubular water reabsorption and reduce urinary sodium (Lam,

1972). Cortisol, from the interrenal tissue of the head kidney,

is thought to be the most important hydromineral hormone in salt

water teleosts; however, GFR, which had been reduced by inter-

renalectomy in the fresh water eel, was restored to the normal

rate by cortisol (Chan et al., 1969). Other hormonal substances,

including adrenalin, angiotensin urophysial, Stannius corpuscle

extracts or pituitary arginine vasotocin, may also regulate (increase) urine flow in fresh water, possibly through a blood pressure effect (Chester Jones et al., 1969a).

Assuming similar hormones and mechanisms of control in rainbow trout as in other fresh water teleosts, it is possible that CCl_4 can exert a direct effect on one or more endocrine glands and disrupt the homeostatic renal mechanisms controlled by these hormones. Previous studies in mammalian toxicology indicated that CCl_4 may alter endocrine gland(s) function to varying degrees (Von Oettingen, 1955). CCl_4, like chloroform, has anesthetic properties leading to central nervous system (CNS) toxicity, e.g. confusion, incoordination and coma (Cornish, 1975). In acute exposure studies with mammals, the decrease in respiratory function was due to depression of the respiratory center, and the fall in blood pressure was primarily the result of vasodilation and central vascular paralysis. Based on the above information, the possibility exists that CCl_4 may reduce GFR, and therefore UFR, through non-specific actions on the CNS.

V. SUMMARY AND CONCLUSIONS

Non-Toxicological Experiments

The non-toxicological experiments with Roaring River Hatchery rainbow trout established the following:

1. Plasma aspartate aminotransferase (GOT) activity was greater and more available than plasma alanine aminotransferase (GPT) activity.

2. Liver GPT activity, conversely, was greater and more variable than liver GOT activity.

3. GPT activity was greater in liver tissue than kidney tissue.

4. In vitro, CCl_4-induced hemolysis increased plasma GOT activity but not plasma GPT activity, and in vitro, physically-induced hemolysis did not increase the plasma activity of either transaminase enzyme.

5. Reproducible plasma and tissue GPT activities were measured at assay conditions of $25^{\circ}C$ and pH 7.5, with an alanine concentration of 67 mM (Sigma Bulletin No. 410-UV). An alanine concentration of > 100 mM appeared to inhibit liver GPT activity.

6. The apparent Km values for alanine in the GPT catalyzed reaction were similar for liver and kidney tissue; there-fore, plasma Km determinations could not be used to

differentiate between liver and kidney damage from CCl_4.

7. Transaminase activities in plasma and liver from four salmonids confirmed that high liver activities are not always reflected by high plasma enzyme activities.

8. The rainbow trout was the only salmonid in which liver GPT activity was greater than liver GOT activity.

9. Photoperiod and water quality had no significant effect on plasma and liver GPT activity in steelhead trout held in the Willamette River when compared to laboratory fish.

Toxicological Experiments

The toxicological experiments with carbon tetrachloride (CCl_4) demonstrated that plasma GPT activity appeared to be a sensitive indicator of hepatocellular damage in the rainbow trout. Up to 24 hours post-treatment, fish fed Purina Trout Chow ^{R} and treated with CCl_4 (1.0 ml/kg) had greater plasma GPT activity than Donaldson diet fish treated with CCl_4 (1.0 ml or 2.0 ml/kg). In general, liver and kidney histopathology failed to show any correlation between the degree of tissue damage and the dose of CCl_4, the diet, the time of sampling post-treatment or plasma GPT activity. Some of the dietary factors that can affect CCl_4 toxicity in laboratory mammals and fishes were examined in an attempt to explain the variable hepatotoxic response in these

fish. Methionine added to Purina Trout Chow may have been responsible for the greater plasma GPT activity in these fish, but a detailed dietary analysis is necessary before the influence of diet on the toxicological response can be adequately evaluated.

Fish treated with CCl_4 (2.0 ml/kg) experienced a significant reduction in total plasma protein concentration as early as 12 hours post-treatment. The concomitant increase in body weight and decrease in plasma osmolarity suggested that the decrease in plasma protein was the result of plasma dilution; however, it was established that dilution did not account for the entire reduction. The peritoneal inflammation and hemorrhages produced by CCl_4 were implicated as significant pathological conditions contributing to the plasma protein loss.

Urine flow rate was significantly decreased in CCl_4-treated fish. It was determined that the inability of the kidney to remove body water was responsible for the weight gain experienced by these fish. Kidney histopathology did not disclose consistent morphological damage which could account for nephron dysfunction. Previous studies with laboratory mammals demonstrated that CCl_4 can decrease blood pressure and renal blood flow, and the early anuria or oliguria found in this study suggested that similar mechanisms were operating in the rainbow trout.

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APPENDIX

PART A: THE USE OF SERUM ENZYMES IN CLINICAL MEDICINE AND EXPERIMENTAL TOXICOLOGY

I. Historical Background

The use of serum (plasma) enzyme measurements as an indication of organ dysfunction had its origin in clinical medicine in the 1930's. Brodansky (1933) pioneered the use of serum alkaline phosphatase activity in the diagnosis of bone and liver diseases. King and Armstrong (1934) and Armstrong et al. (1934) measured serum alkaline phosphatase activity in hepato-biliary disease, and Gutman et al. (1936) used serum acid phos-phatase activity in the diagnosis of prostatic carcinoma. Warburg and Christian (1943) observed that intracellular enzymes involved in carbohydrate metabolism also occurred in the serum of diseased laboratory animals. LaDue et al. (1954) found that serum aspartate aminotransferase activity was transiently elevated after myocardial infarction. Since the "discovery" of LaDue and its application in human cardiology, serum enzyme analysis in clinical medicine has progressed to include screening for diseases of the liver, skeletal muscle and bone, as well as in the diagnosis of certain congenital and acquired anemias, and malignancies. These techniques have become important tools in the laboratory evaluation of specific organ toxicity from drugs or other

chemicals and in the process have permitted some insight into

cellular mechanisms and the events leading to cellular death.

II. Plasma Enzymes in the Diagnosis of Organ Damage: Factors to Consider

A. Organ Specificity

A few serum enzymes are specific for damage to an individual organ and selection of a relatively organ specific enzyme enhances the capability to interpret the effects of elevated serum activity due to a pathological condition. Specific metabolic reactions are catalyzed by enzymes more restricted in their tissue distribution, and an increase in the serum activity of these "organ specific" enzymes infers some degree of limited organ dysfunction or damage. Examples of "organ specific" enzymes are ornithine carbamyltransferase (OCT) and sorbitol dehydrogenase (SDH), which are found in the liver, and acid phosphatase in the prostate gland.

Major advances in clinical diagnosis have originated from the studies of enzymes involved in metabolic pathways (Schmidt and Schmidt, 1974). These "metabolic" enzymes are present in most tissues and include lactate dehydrogenase (LDH), alanine aminotransferase (GPT), aspartate aminotransferase (GOT) and

isocitrate dehydrogenase (ICDH). GPT is relatively specific for liver tissue and subsequent elevation in serum activity usually suggests hepatic dysfunction or damage. GOT and ICDH, which occur predominantly in the liver and heart, show moderate tissue specificity. LDH is found in all tissues and has a low tissue specificity.

In a study correlating serum and tissue activities of GPT and GOT in six vertebrates, Zimmerman et al. (1968) determined that the liver of all six species had the highest tissue GPT activity, but that GOT activity showed no specific tissue distribution. Serum GPT activity was positively correlated with liver GPT activity in all species. They also demonstrated that the enzyme assay method, i.e. colorimetric or kinetic, can influence the measurement of enzyme activities and apparent tissue distribution. The relative enzyme activities obtained by the two methods varied for a particular organ and species.

The serum activity of enzymes which occur in multiple molecular forms, i.e. isoenzymes, can be useful indicators of specific organ damage. Isoenzymes catalyze the same biochemical reaction but have characteristic substrate requirements, i.e. K_m concentrations, in each organ. Some of the enzymes that show such heterogeneous properties are LDH, ICDH and GOT. The techniques of differential isoenzyme analysis have the potential

to become the most sensitive and specific indices of organ damage
(Cornish, 1971).

B. Plasma Enzyme Activity

The total enzyme activity in serum of healthy animals is
normally low. Plasma enzymes, such as pseudocholinesterases,
lipoprotein lipases and the enzymes involved in blood coagulation,
are secreted by the liver into the circulation where they serve a
physiological function. The activities of these enzymes are
normally higher in serum than in liver tissue. Intracellular or
"metabolic" enzymes lack serum substrates and cofactors and
have low activity levels in the serum.

The use of serum activities of "metabolic" enzymes as an
assessment of organ dysfunction or damage assumes a priori
that cellular injury results in the release of intracellular enzymes
into the extracellular fluids and eventually into the blood.
Cornish (1971) mentioned the difficulty in determining whether
enzymes were lost from the cell due to a toxic effect on cellular
function, or whether effects on permeability and loss of cellular
enzymes lead to biochemical imbalance and cellular death.
Wilkinson (1970b) discussed the empirical value of most serum
enzyme tests and indicated they were beneficial "despite our lack

of understanding of the mechanisms involved in the entry and removal of intracellular serum enzymes."

It is apparent that serum enzyme activity reflects the result of two opposing factors, the increase in serum activity caused by enzyme synthesis and release into the blood, and the decrease in serum activity caused by inactivation or serum clearance. Young (1974) suggested four theories that attempted to explain the mechanism(s) of enzyme passage from cells into serum: 1) normal cell turnover; 2) changes in cell membrane permeability; 3) alterations in cellular energy; and 4) intracellular structural protein binding and enzyme complexation due to alterations in tissue integrity. He indicated that cell turnover, especially of erythrocytes, can provide a significant contribution to usual serum enzyme activity levels, but in pathological conditions, it was possible to equate increased serum enzyme activities with cellular damage in various organs.

There have been conflicting reports concerning the degree of cellular damage necessary to produce measurable changes in serum enzyme activity. An earlier hypothesis emphasized that the increase in serum enzyme activity was the result of equalization of the enzyme concentration between the cell and the extracellular compartment upon the death of the cell. Schmidt and Schmidt (1974) stated that this hypothesis required revision. The

lack of significant serum enzyme activity in starvation (White, 1958) and neuromuscular atrophies (Dreyfuss et al., 1958) indicated that "normal" destruction of cells was not necessarily correlated with the release of cellular components. The elevation in serum enzyme activities has been associated with the non-morphologic alterations characteristic of hypoxia, hypothermia and shock (Henley et al., 1966). In vitro studies with rat skeletal muscle or diaphragm tissue suggested a direct correlation between the release of aldolase from cells and increased membrane permeability (Zierler, 1958). Factors causing the increased permeability included glucose deficiency, anoxia, altered electrolyte concentrations, and interference with cellular metabolism by iodoacetate, cyanide or dinitrophenol. Schmidt and Schmidt (1974) maintained that the release of intracellular enzymes resulted from a decrease in cellular integrity caused by an imbalance between energy production and expenditure.

The intracellular location of enzymes may influence their release into the blood stream. Cytoplasmic enzymes, e.g. GPT, are more likely to be released into the circulation in reversible inflammatory processes characterized by an increase in membrane permeability (Wilkinson, 1970b); however, widespread destruction of cells (necrosis) is followed by increased serum activities of mitochondrial enzymes, e.g. glutamic dehydrogenase (GDH). The

serum activities of organelle specific enzymes have been measured

by several workers in their evaluation of the hepatotoxic response

in carbon tetrachloride (CCl_4)-treated laboratory mammals

(Cornish and Adefuin, 1966; Curtis et al., 1972; Rees and Sinha,

1960; Zimmerman et al., 1965). In general, serum activities of

predominantly cytoplasmic enzymes increased earlier than activi-

ties of enzymes that were found in both the cytoplasm and mito-

chondria. Enzymes found only in the mitochondria showed the

latest peak in plasma activity; however, Zimmerman et al. (1965)

determined that the peak serum activity of cytoplasmic GPT was

similar to mitochondrial GDH. They suggested that "factors other

than intracellular location can determine the serum (enzyme)

pattern of (hepatotoxic) response to CCl_4 poisoning." Curtis et al.

(1972) concluded that there were numerous variables which can

affect plasma enzyme activities, e.g. synthesis, degradation,

membrane permeability, intracellular binding, plasma clearance,

and that "the degree of elevation in plasma enzyme activity cannot

be expected to indicate the severity of injury to a sub-cellular

organelle."

Schmidt et al. (1967) devised a chromatographic method for

separating the cytoplasmic and mitochondrial isoenzymes of GOT

in serum. In a case of viral hepatitis they found a temporal

correlation between serum activities of mitochondrial GOT and

GDH and indicated this procedure may be valuable as an estimation of relative cellular damage.

The rate of enzyme removal from the serum is characteristic for each enzyme and can be different in each species. This temporal variability in serum activity can be a significant consideration in the use of these procedures in clinical diagnosis and the interpretation of experimental results. At present, the mechanisms of serum protein elimination are poorly understood. According to Young (1974), plasma enzyme activity can be reduced by: 1) an active uptake into intact cells; 2)inactivation or degradation in the intra- or extravascular space; and 3) excretion. He stated that there was probably no active uptake of serum enzymes into cells because of the high energy expenditure required to transport a high molecular weight protein across cell membranes, and because there was no difference in enzyme activity in the arterial and venous circulation of organs. Posen (1970) suggested that irreversible intravascular denaturation of enzymes by exo- or endopeptidases or by non-enzymatic mechanisms may be important in the elimination process. Alternately, Schmidt and Schmidt (1974) proposed that intravascular degradation was unlikely and supported the theory of cellular uptake. Posen (1970) showed that the removal of either the liver or kidneys from experimental animals had no effect on the rates of enzyme

elimination from the blood. He indicated that serum enzymes were not significantly excreted in the urine, bile, saliva or perspiration. In common with other proteins, serum enzymes may be digested in the small intestine, and the amino acids excreted or reincorporated into "new" protein.

In rats and mice denatured bovine serum albumin is cleared mainly from the blood by the liver macrophages (Kupffer cells), but a considerable fraction of the protein is also removed by the kidneys (Mego, 1973). Mego (1971) observed that the liver and kidneys of three species of frogs participated equally in the uptake of denatured ^{125}I-labeled bovine albumin from the blood. Dannevig and Berg (1978) indicated that the kidney was the major organ in the char, Salmo alpinus L., for the removal of foreign serum albumin from the blood. The kidney of fishes may play a major role in the removal of exogenous and endogenous blood-borne proteins.

Blood sampling techniques and some chemicals, e.g. CCl_4, can cause considerable hemolysis. Some enzymes have higher activities in erythrocytes than in serum, and their release from damaged red blood cells can give erroneously elevated serum activities. For example, LDH, GOT and GPT activities were 67, 40 and 7 times, respectively, greater in human red blood cells than in plasma (Caraway, 1962). When using serum

transaminase activities in the assessment of liver damage,

samples with appreciable hemolysis should be avoided.

PART B: THE LABORATORY ASSESSMENT OF CHEMICALLY-INDUCED ORGAN DAMAGE IN FISHES: FACTORS TO CONSIDER

I. Animal Selection

In experimental fish toxicology, animal availability and genetic variability are of paramount importance. Rainbow trout, Salmo gairdneri, of consistent size and sufficient numbers are readily obtainable from state fish hatcheries. The acquisition of these "domesticated" trout from one hatchery within a particular geographic area usually insures fish of the same strain and similar genetic background. They can be easily maintained under laboratory conditions provided there is a continual supply of fresh, cool water. The hatchery rainbow trout has become an excellent cool water species for work in comparative toxicology and in the evaluation of chemical effects on an aquatic organism.

II. Organ Selection

The mammalian liver is the most complex and important organ involved in metabolic homeostasis and can be used to evaluate specific organ dysfunction or damage due to toxicant exposure. The interactions in the liver between carbohydrate, lipid and protein intermediary metabolism are of fundamental importance to the entire organism. The liver of fishes appears to be equally important in their intermediary metabolism

(Baldwin and Reed, 1976; Cowey and Sargent, 1977; Gumbmann et al., 1958; Hochachka, 1969; Hochachka and Somero, 1973; Johnston, 1977; Nicholls et al., 1976; Phillips and Hird, 1977a, 1977b; Tarr, 1968). Interrelated to the above metabolic processes in fishes are other important hepatic functions including: bile synthesis and secretion into the intestine as a means of removing foreign compounds (Lech, 1973; Lech et al., 1973; Statham et al., 1976), biotransformation of endogenous and exogenous compounds (Ahokas et al., 1977; Buhler and Rasmussen, 1968a; Chambers and Yarbrough, 1965; Ludke et al., 1972; Peterson et al., 1976), plasma protein synthesis (Kenyon, 1967) and detoxification of metabolic ammonia to glutamine (Campbell, 1972).

The liver has a prominent role in lipid metabolism and tends to concentrate lipid soluble compounds. In general, chlorinated hydrocarbons, e.g. DDT and CCl_4, and organic phosphate pesticides, e.g. parathion, are relatively insoluble in water but are readily absorbed by non-polar media, such as fatty acids and oils. Earlier work established that organ residues of chlorinated hydrocarbons in fish were proportional to the concentration of lipids in the organ (Holden, 1962, 1965, 1966). Recently, Statham et al. (1977) showed that $^{14}CCl_4$ concentration in the liver of rainbow trout was maximum at 2 hours post-injection. Johnson's (1968) review of fish exposure to pesticides noted that the liver was the

most consistently damaged organ in fishes exposed to halogenated hydrocarbon and organic phosphate pesticides.

The liver of rainbow trout was chosen as the organ to evaluate in my study because: 1) its importance in intermediary metabolism, xenobiotic biotransformation and possible ammonia detoxification; 2) its ability to accumulate potentially harmful, exogenous chemicals; and 3) the availability of suitable methodology to evaluate liver function and/or damage.

III. Toxicant Selection

CCl_4 is a model hepatotoxic agent that has been widely used in experimental animal toxicology (Cameron and Karunaratne, 1936; Fowler, 1970; Plaa and Larson, 1964; Rechnagel, 1967). The consistency with which CCl_4 produces hepatic necrosis and lipid accumulation in laboratory animals has led to its widespread use as a tool in biochemical studies of liver function and damage (Cornish, 1975; Raisfeld, 1974). Recent studies have demonstrated that salmonids treated with CCl_4 experienced liver pathology similar to that in mammals (Bell, 1968; Gingerich et al., 1978; Racicot et al., 1975; Statham et al., 1977).

CCl_4 was chosen as the toxicant in this study because of its proven ability to produce consistent and reproducible hepatic dysfunction and structural damage.

IV. Methods to Evaluate Liver Damage

In his review on serum enzymes as diagnostic aids, Cornish (1971) contended that one goal of the toxicologist should be "to detect early physiological or biochemical changes in response to chemical insult." In the field of clinical medicine numerous liver function tests have been developed for the detection of hepatic dysfunction or damage. These tests include: 1) measurement of serum bilirubin as an indication of secretory function; 2) excretory capability by the measurement of sulfobromophthalein (BSP) removal from the blood; 3) conjugation of benzoic acid with glycine to form hippuric acid, as an estimation of biotransformation; 4) sugar tolerance tests as an indication of carbohydrate metabolism; 5) plasma albumin:globulin ratios as an evaluation of protein synthesis; and 6) serum cholesterol:cholesterol ester ratios as an indication of lipid metabolism (Harper, 1973). However, many liver function tests differ widely in their sensitivity during pathological processes. Cornish (1971) stated that due to the reserve functional capacity of the liver, most of these tests in mammals were not particularly sensitive. Cutler (1974), on the other hand, concluded that the hippuric acid test, plasma albumin concentration and the BSP test were sensitive functional procedures in the rat for the detection of liver injury produced by long-term administration of CCl_4. In addition, Cutler found that plasma

GPT activity was a sensitive index of liver cell necrosis. In the assessment of hepatic dysfunction or damage due to chemical insult, altered serum enzyme activities appear to be a relatively sensitive and specific index and can provide a significant contribution toward achievement of the above goal proposed by Cornish.

Such biochemical data should be correlated with histopathological evaluations. Grice et al. (1971) and Korsrud et al. (1972) concluded that serum enzyme activities and isoenzyme patterns were important supplements to histopathological examination of tissues during toxicological investigations.

V. Selection of an Enzyme(s) to Assess Liver Damage by CCl$_4$

Wilkinson (1970b) stated that the transaminases (aminotransferases), GPT and GOT, and alkaline phosphatase appeared to be the most useful serum enzymes in the assessment of liver damage. The activity of serum alkaline phosphatase, unlike that of the transaminases, was enhanced to a greater extent in obstructive hepatobiliary disease than in hepatitis or chemical intoxication. The measurement of serum ICDH activity along with serum GPT and GOT would help differentiate hepatic diseases from other damaged organs rich in GOT, e.g. cardiac tissue. The so-called "liver specific" enzymes, OCT, GDH and SDH, were relatively

insensitive and generally did not help in differential diagnosis (Schmidt and Schmidt, 1974; Wilkinson, 1970b); however, some investigators have found OCT useful in the diagnosis of biliary obstruction.

Alanine aminotransferase (GPT) and aspartate aminotrans-ferase (GOT) were selected as the enzymes to evaluate the hepato-toxic response of the rainbow trout to CCl_4. Previous studies with mammals (Cornish, 1971; Wilkinson, 1970b) and fishes (Bell, 1968; Inui, 1968, 1969; Racicot et al., 1975; Statham et al., 1977) have established these enzymes as relatively sensitive and specific indicators of CCl_4-induced hepatic damage.

The activities of GPT and GOT can be determined by various methods, but the most popular have been: 1) the kinetic assay using the procedures of Wroblewski and LaDue (1956) and Karmen (1955) for GPT and GOT, respectively; 2) the colorimetric assay using 2,4-dinitrophenylhydrazine (Reitman and Frankel, 1957). Wilkinson (1970b) indicated that the kinetic methods were more precise, sensitive and reliable, since the course of the reaction can be followed; however, kinetic methods required more elaborate instrumentation and more expensive substrates. Guisti et al. (1969) compared GPT and GOT serum activities using the kinetic methods of Wroblewski and LaDue (GPT) and Karmen (GOT) to the colorimetric methods of Reitman and Frankel (GPT and GOT).

These workers concluded that the colorimetric methods were less reliable than the kinetic procedures. Kinetic assays would be more applicable to studies with poikilothermic animals, since the reaction temperature can be varied; on the other hand, the colorimetric assays for GPT and GOT require an incubation at 37°C (Sigma Bulletin #505).

Enzyme assay conditions should be optimal to insure that maximum activity is measured; however, pursuant to this goal, the procedure must also remain practicable. Bergmeyer (1978) suggested that the term "optimized methods" be used when both aspects are considered.

The amount of enzyme present in the serum is determined by its activity, an indirect approximation of its actual concentration, and for this reason catalytic activity measurements can be standardized and compared only on the basis of the assay conditions. Some of the factors that affect enzyme activity are discussed below.

Like all chemical reactions, enzyme-catalyzed reactions are sensitive to temperature. Each enzyme has an optimum temperature which can be influenced by other assay conditions, e.g. substrate concentration. This optimum temperature can vary for the same enzyme in different species, particularly poikilo-thermic organisms. When enzyme activities for the same or

different species are compared, the activity at a particular assay temperature should always be evaluated with respect to the temperature at which the enzyme normally or usually functions in nature.

Maximum sensitivity in enzyme activity can be achieved at the optimum pH, because this is the point at which the pH curve has a minimum slope (Bermes and Forman, 1970). The optimum pH not only depends on the nature and ionic strength of the buffer, but also varies with temperature and substrate concentration. Maximum buffer capacity is achieved when the optimum pH of the reaction is as close as possible to the pKa of the buffer.

Substrate concentrations should be high enough that the velocity of the enzyme catalyzed reaction is dependent on the amount of enzyme, i.e. zero order kinetics must be maintained. Theoretically, substrate concentrations should be 50 to 100 times the Km value, i.e. the Km equals the substrate concentration that will produce a reaction velocity (activity) of one-half the naximal velocity. There are occasions when optimum substrate concentrations cannot be used. Limited substrate solubility or substrate inhibition of enzyme activity, e.g. in a two-substrate reaction as GPT or GOT, are problems that must be considered.

For two-substrate reactions Bergmeyer (1978) listed three general principles for the selection of substrate concentrations:

1. The pair which, for a small change in concentration of one substrate, produces the smallest change in the concentration

of the other substrate (smallest variation).

2. The pair with the lowest concentration of both substrates that will produce the maximal activity (the most economical pair).

3. The pair that offers the least difficulty in practical performance, e.g. solubility of substrates, inhibition by substrates or products.

Practical performance is particularly important in the GPT and GOT reactions. At increasing concentrations, α-ketoglutarate, the second substrate, competitively inhibits both GPT and GOT. The use of the optimum concentration of alanine, the specific substrate for GPT, is precluded by its insolubility at higher concentrations. In the GPT reaction a high concentration of the product, pyruvate, can inhibit enzyme activity. It is not always possible to use the optimum substrate concentrations, but the most economical pair of concentrations should be selected to give reproducible results.
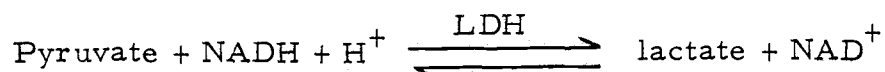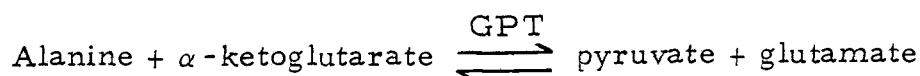
Coenzymes are "cosubstrates" in an enzymatic reaction, since they chemically participate in the reaction. Pyridoxal phosphate, vitamin B-6, is a coenzyme for most transaminases, including GPT and GOT. It is tightly bound to GPT and does not have to be added to the reaction mixture (Green et al., 1945). In the case of GOT, pyridoxal phosphate is not tightly bound to the

enzyme but is usually present in sufficient concentrations in serum and tissues that it does not have to be added to the reaction mixture (Green et al., 1945).
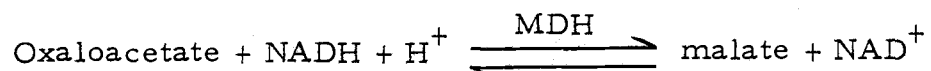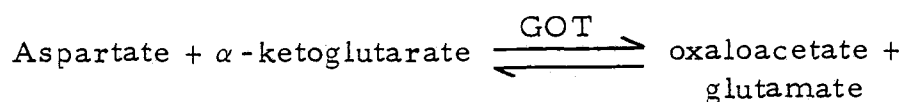
In the determination of GPT and GOT activities in animal tissues, e.g. especially liver and kidney, the presence of ammonia can give higher apparent activities. These tissues are good sources of GDH, which requires α-ketoglutarate and ammonia as substrates. In both transaminase reactions and the GDH reaction, the oxidation of NADH is directly proportional to the reaction of substrates with enzymes and serves as a measure of the enzyme activities. To avoid the concurrent measurement of GDH activity, Bergmeyer and Bernt (1974) suggested that the addition of the substrates to the reaction mixture be reversed. In the case of GPT, a preliminary reaction with α-ketoglutarate measures the GDH activity (and some pyruvate), and a second reaction started with alanine (specific substrate for GPT) measures GPT activity and any remaining GDH activity. The difference between the ΔE/min values for the two reactions is used to calculate the GPT activity (see reactions below).
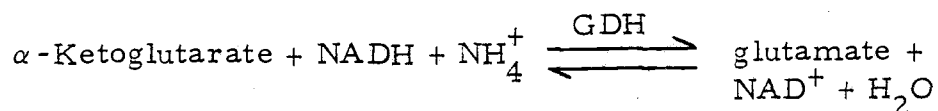
Enzyme Assay Reactions

Alanine Aminotransferase (GPT)[1]

$$\text{Alanine} + \alpha\text{-ketoglutarate} \underset{\longleftarrow}{\overset{\text{GPT}}{\longrightarrow}} \text{pyruvate} + \text{glutamate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \underset{\longleftarrow}{\overset{\text{LDH}}{\longrightarrow}} \text{lactate} + \text{NAD}^+$$

Aspartate Aminotransferase (GOT)

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \underset{\longleftarrow}{\overset{\text{GOT}}{\longrightarrow}} \text{oxaloacetate} + \text{glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \underset{\longleftarrow}{\overset{\text{MDH}}{\longrightarrow}} \text{malate} + \text{NAD}^+$$

Ammonia Reaction

$$\alpha\text{-Ketoglutarate} + \text{NADH} + \text{NH}_4^+ \underset{\longleftarrow}{\overset{\text{GDH}}{\longrightarrow}} \text{glutamate} + \text{NAD}^+ + \text{H}_2\text{O}$$

[1]GPT: alanine aminotransferase EC 2.6.1.2

  GOT: aspartate aminotransferase EC 2.6.1.1

  GDH: glutamate dehydrogenase EC 1.4.1.3

  LDH: lactate dehydrogenase EC 1.1.1.27

  MDH: malate dehydrogenase EC 1.1.1.37

NADH: nicotinamide-adenine dinucleotide (reduced)

NAD^+: nicotinamide-adenine dinucleotide (oxidized)

NH_4^+: ammonia

PART C: ENZYME ANALYSIS IN THE RAINBOW TROUT,
<u>SALMO GAIRDNERI</u>: THE USE OF PLASMA
TRANSAMINASE ACTIVITIES AS AN INDEX
OF LIVER DAMAGE DUE TO CCl_4
INTOXICATION

I. <u>Historical Background</u>

The use of plasma enzyme analysis as an assessment of organ

damage due to biological or chemical insult has been widely used in

clinical medicine and experimental toxicology. Until recently,

application of these methods to fish had been minimal. The

increased interest in enzyme analysis in fish has been due to:

1) the search for biochemical tests which can evaluate the health of

cultured fish and their response to environmental parameters;

2) the need for suitable aquatic organisms which can monitor the

physiological and biochemical effects of the increasing numbers of

environmental chemicals.

II. <u>Factors Affecting Transaminase Activities in Fishes</u>

<u>Diet</u>

Wilson (1973) measured GPT, GOT and GDH activities in

tissues from native and cultured channel catfish. He concluded

that the higher protein diet of the cultured fish was responsible

for the generally higher enzyme activities, particularly GPT, in

the liver and kidney tissues. Smith et al. (1974) found significant alterations in GPT activities of erythrocytes, muscle and liver from rainbow trout fed a diet deficient in pyridoxine, the coenzyme for GPT.

Endogenous Compounds (Hormones)

Freeman and Idler (1973) showed that injection of gluco-corticosteroids increased liver GPT and GOT activities in both rainbow and brook trout and suggested that these hormones played an important role in salmonid gluconeogenesis. Elevated plasma glucocortiosteroid concentrations have been recorded in spawning Pacific salmon (Robertson, 1958), and Marquez (1976) found higher serum GOT activity in spawning pink salmon, O. gorbuscha, than in the non-spawning or pre-spawning fish. It has been established that spawning salmon do not feed on their upstream journey, and gluconeogenesis may be a significant energy producing mechanism for swimming and spawning. Grant and Mehrle (1973) showed that rainbow trout, which were exposed to the pesticide, endrin, and forced to swim, had reduced serum glucocortico-steroid, i.e. cortisol, concentrations compared to those fish that were only forced to swim. Presumably, this decrease in plasma cortisol concentration could result in a reduction in liver trans-aminase activities and in gluconeogenesis. The results of Freeman and Idler (1973) agreed with those of Storer (1967), who found that

injections of cortisol in goldfish, Carassius auratus, resulted in a

significant increase in liver GPT activity; however, their results

were contrary to the conclusions of Chan and Cohen (1964) that

glucocorticosteroids did not stimulate liver transaminase activities

in animals phylogenetically below the reptiles. The results of

these studies indicate that transaminase activities in fishes may

be regulated by glucocorticosteroid concentrations. Young (1974)

suggested that in mammals various hormones, including gluco-

corticosteroids, aided in the regulation of normal tissue and

plasma enzyme activities.

Exogenous Compounds (Xenobiotics)

Freed et al. (1965) utilized in vitro and in vivo enzyme

activity measurements in their assessment of the effects of hydro-

cyanic acid and pentachlorophenol on coho salmon, O. kisutch,

and the cichlid, Cichlasoma bimaculatum. In a preliminary toxi-

cological study, Bell (1968) realized the diagnostic value of serum

enzymes in the evaluation of chemical organ damage and fish

disease. Serum GOT activity increased in juvenile sockeye salmon,

O. nerka, after intraperitoneal (i.p.) injection with equivalent

volumes of bromobenzene and CCl_4 or an infection of bacterial

kidney disease. In conclusion, Bell stressed the need to establish

"normal" or "standard" physiological values for cultured fishes

before serum enzyme measurements would be clinically useful. Inui (1968, 1969) showed that the increase in plasma GPT and GOT activities in CCl_4 treated eels was the result of hepatocellular damage and the release of these enzymes from the liver. Lane and Scura (1970) demonstrated a significant increase in serum GOT activity in the sailfin mollie, Poecillia latipinna, exposed to dieldrin for 1 to 3 days; however, there was no significant correlation between plasma enzyme activity and the time of exposure at the higher pesticide concentrations, i.e. 12 ppb. Mehrle and Bloomfield (1974) measured various enzyme activities in the liver and brain tissues from rainbow trout exposed to sublethal concentrations of dieldrin and suggested that chronic exposure to this pesticide may alter ammonia detoxification. Liver GOT activity increased only at the highest dieldrin concentration, i.e. 430 µg/kg, but liver GDH activity increased at all dieldrin concentrations. Gaudet et al. (1975) determined the activities for eight enzymes in plasma and tissue from rainbow trout, and Racicot et al. (1975) used these enzymes in their assessment of the toxicological response of these fish to i.p. injection of CCl_4. They concluded that plasma GPT and GR (glutathione reductase) activities were sensitive indicators of CCl_4 induced liver damage. Sakaguchi and Hamaguchi (1975) and Sastry and Agrawal (1975) measured various serum enzyme activities in the yellowtail,

Seriola dorsalis, and the catfish, Heteropneustes fossils, respectively, after i.p. injection of CCl_4. For the yellowtail, maximum serum GPT and GOT activities occurred at 24 hours post-treatment (CCl_4, 1.0 ml/kg). Working with rainbow trout, Statham et al. (1977) showed a significant elevation in plasma GPT and GOT activities as early as 4 hours and 2 hours, respectively, after treatment with CCl_4 (1.0 ml/kg i.p.). In another group of fish these workers found that the plasma activities of these enzymes were not elevated after a "bath-type" exposure to CCl_4 under static water conditions. Other studies have utilized plasma transaminase activities in the evaluation of heavy metal toxicity in fish. McKim et al. (1970) found a significant elevation in serum GOT activity in the brook trout, Salvelinus fontinalis, after exposure to sublethal concentrations of cupric chloride. In a similar study, Christensen et al. (1972) demonstrated an increase in serum GOT activity in the brown bullhead, Ictalurus nebulosus, following 6 days of exposure to sublethal concentrations of cuprous sulfate. Wekell and Brown (1973) measured ornithine aminotransferase activity in the tissues of numerous fresh water and marine fishes. They demonstrated that mercuric chloride had a significant inhibitory effect on ornithine aminotransferase activity in the liver from rainbow trout.

Other Factors

Whiting and Wiggs (1977) reported that changes in tyrosine aminotransferase activity in liver tissue from brook trout may have been due to water temperature or season; however, the fish were genetically dissimilar, which could have accounted for the variability in enzyme activity.

Sauer and Haider (1977) measured GPT and GOT activities in plasma from rainbow trout which had been acclimated to water temperatures between 3.5° and $21.5^{\circ}C$. In general, plasma GPT and GOT activities were greater at the higher water temperatures.

Schlisio and Nicolai (1978) showed that liver GOT activity in rainbow trout was more variable than liver GPT activity and appeared to follow a diurnal activity pattern.