Clinical methods to diagnose specific organ dysfunction have not been evaluated in fishes. To determine if an accepted clinical test of mammalian liver function could be applied to fishes, the hepatic disposition of the organic anion sulfobromophthalein (BSP) was studied in rainbow trout (Salmo gairdneri) treated with a model hepatotoxicant. Carbon tetrachloride (CCl₄) was used to model liver damage in the trout because it is widely used in this capacity in mammalian studies.

The initial objective was to evaluate the importance of the liver of the trout in the elimination of BSP. As in mammals, hepatic BSP accumulation in the rainbow trout appeared to be the most important factor contributing to plasma clearance of the dye while biliary excretion was the rate limiting step in the overall transfer of BSP from plasma to bile. Differences between trout and other
species with respect to rates of plasma clearance, hepatic accumulation, and biliary excretion of BSP could be explained when anatomical and physiological differences were considered.

The next objective was to determine the value of CCl$_4$ as a potential hepatotoxic agent in rainbow trout. Plasma half-lifes of BSP increased in proportion to the dose of CCl$_4$ administered and histological examination of liver sections indicated that morphological damage, including necrosis of hepatocytes surrounding central veins, also occurred following intoxication. In addition, hemoglobinemia was observed in fish as early as 12 h after treatment. To determine if high concentrations of hemoglobin had influenced the rate of BSP clearance, plasma clearance studies were conducted in two groups of fish following prolonged infusion of either hemoglobin or bilirubin. Results of these studies indicated that plasma BSP clearance was not affected by high plasma levels of either compound and it was therefore concluded that the CCl$_4$ induced plasma retention of BSP in the trout could not be explained by the intravascular hemolysis which attended the intoxication.

The final objective was to attempt to establish how CCl$_4$ intoxication was acting to induce plasma BSP retention. To determine if CCl$_4$ treatment had impaired hepatic processes associated with biliary BSP excretion, bile flow, bile BSP concentration and percent of metabolized BSP appearing in the bile were determined in
treated and control animals during prolonged, graded infusion of the dye. Results of these studies indicated that, unlike mammals, components of the hepatic excretory process were not impaired 24 h after CCl₄ treatment. Additional studies indicated that the rate of hepatic BSP accumulation following injection of a single dose of the dye was reduced in treated animals and suggested that processes of hepatic uptake and storage may have been impaired.

These studies indicate that the organic anion sulfobromophthalein is a useful compound with which to study liver function in trout. Furthermore, a test of liver dysfunction based on the rate of plasma BSP clearance may prove to be a useful method by which to diagnose liver damage in this fish.
Sulfobromophthalein Disposition in Rainbow Trout
(Salmo gairdneri) as Influenced by Carbon
Tetrachloride Intoxication

by

William Harold Gingerich

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Redacted for Privacy
Dean of Graduate School

Date thesis is presented November 2, 1976

Typed by Mary Jo Stratton for William Harold Gingerich
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SULFOBROMOPHTHALEIN DISPOSITION IN RAINBOW TROUT (Salmo gairdneri) AS INFLUENCED BY CARBON TETRACHLORIDE INTOXICATION

I. INTRODUCTION

Sensitive clinical methods are available to diagnose specific organ dysfunction in mammals; however, the literature indicates that few tests of this nature have been developed for fishes. The liver, one of the vital organs of fish, seems to be particularly sensitive to a variety of waterborne toxicants. In a review of the toxicity of chlorinated hydrocarbon pesticides to fishes, Johnson (1968) has stated that the liver is the most consistently damaged organ found in animals exposed to these compounds. Development of sensitive clinical methods to measure liver dysfunction in fish would therefore seem to provide a means whereby subtle physiological effects resulting from environmental toxicants and pollutants could be assessed. Successful use of liver function tests demands at least a basic understanding of the physiology of this organ in fish.

There has been increasing evidence that the liver of fish plays a major role in metabolism and biliary excretion of foreign compounds. In mammals, the hepatic microsomal mixed function oxidase system is thought to be primarily responsible for many of the biotransformation reactions. Components of this system have recently been demonstrated in the liver of several species of freshwater fish.
(Chen et al., 1967; Stanton and Khan, 1975) and studies by Buhler and Rasmussen (1968), Dewaide (1971), Ludke et al. (1972), Payne (1976), and Petersen (1976) have demonstrated that the livers of fishes are capable of a variety of biotransformation reactions. In addition, recent reports also have indicated that, as in mammals, biliary excretion may be an important route by which fish eliminate foreign compounds (Lech, 1973; Lech et al., 1973; Statham et al., 1976). Thus, a test of liver function based on the processes of hepatic excretion of a foreign compound may be possible in fish.

Because of its predilection for biliary excretion in mammals, the organic anion sulfobromophthalein (BSP) has been used extensively in clinical tests to evaluate liver function and as a model compound with which to study the general processes of biliary excretion. The transfer of BSP from plasma to bile is generally conceded to involve the processes of uptake of dye from the plasma and storage in hepatocytes, metabolism to more water soluble compounds, and active excretion into the bile (Goresky, 1965). Recent evidence indicates that biliary excretion is the major route by which BSP is eliminated in two species of cartilaginous fishes (Boyer et al., 1976c) and in a representative bony fish (Schmidt and Weber, 1973). The general processes of hepatic BSP excretion also appear to broadly conform to those which have been described in mammals (Boyer et al., 1976b).
To determine if BSP is a suitable compound with which to evaluate liver function in fish, several studies were conducted. The objective of the first study was to demonstrate the importance of the liver in the processes of plasma BSP clearance and biliary excretion in a representative fish. This problem was approached by estimating the rates of plasma clearance, hepatic accumulation, and biliary excretion of BSP in rainbow trout. To further demonstrate the dependence of plasma BSP clearance on normal liver function, the effects of surgical impairment of hepatic blood flow and bile flow on plasma clearance and hepatic accumulation were determined.

The objective of the second study was to evaluate the usefulness of plasma BSP clearance as a test to assess impaired liver function in the trout. Cutler (1974) and Hallesy and Benitz (1967) have established that plasma BSP clearance is a sensitive test by which to evaluate liver damage in mammals but this method has not previously been used for this purpose in fish. In addition, a suitable model hepatotoxicant has not been fully established for studies with fish.

One chemical that has frequently been used to produce liver damage in mammals is carbon tetrachloride (CCl₄). Intoxication by this compound is known to result in consistent pathological changes in the liver including triglyceride accumulation and centrilobular necrosis (Raisfeld, 1974). Carbon tetrachloride treatment also may result in some form of liver damage in fish. Bell (1968) found that
intraperitoneal administration of a mixture of CCl₄ and bromobenzene resulted in elevated plasma levels of glutamic oxaloacetic transaminase (GOT) in the serum of sockeye salmon (Oncorhynchus nerka) after 15 h; however, livers of these animals were not examined for evidence of morphological damage. Racicot et al. (1975) found that the hepatocytes of rainbow trout were intensely vacuolated following ip injection of CCl₄ and also reported that the plasma levels of several enzymes including GOT, glutamic pyruvic transaminase (GPT), and lactic acid dehydrogenase (LDH) were increased 6-10 times above control values 12 h after intoxication.

The problem of evaluating liver damage in fish was approached by determining the rate of plasma BSP clearance at intervals following CCl₄ treatment. Extrahepatic effects associated with CCl₄ intoxication also were evaluated as possible sources of error in this test.

The objective of the third study was to attempt to determine which of the processes of hepatic BSP disposition in trout were most affected by CCl₄ intoxication.

Carbon tetrachloride treatment of mammals is known to result in plasma BSP retention (Cutler, 1974). It is not clear from reports in the literature which of the hepatic processes associated with plasma BSP clearance are most affected by CCl₄ treatment. Brauer et al. (1955) infused BSP into dogs treated with CCl₄ and found that the ability of the liver to store BSP was not affected but that hepatic
extraction and biliary excretion of the dye were impaired. Klaassen and Plaa (1968) reported that bile secretion was reduced and hepatic metabolism and biliary excretion of BSP were impaired in rats treated with CCl₄. These investigators could not demonstrate differences in the capacities of the livers from treated and control animals to store BSP and therefore concluded that impairment of processes associated with biliary excretion contributed most to plasma BSP retention following CCl₄ treatment. Priestly and Plaa (1970) similarly found that metabolism and biliary excretion of BSP were most affected by CCl₄ intoxication. They showed that low rates of bile secretion were mainly responsible for impaired biliary BSP excretion early in the course of intoxication. After 24 h, however, they found that the hepatic BSP glutathione conjugating activity of the livers also was depressed. They concluded that while the BSP retention associated with CCl₄ hepatotoxicity was primarily the result of the impairment of transport processes at the onset of intoxication, impaired dye conjugation probably intensified this defect during the later stages. Contrary to these views Plaa and Hine (1960), using isolated and perfused rat livers, concluded that hepatic extraction of BSP from the perfusate, rather than decreased biliary excretion seemed to be the process most affected by CCl₄ induced liver injury. Maggio and Fujimoto (1966) injected a single dose of BSP into mice and measured its concentrations in plasma and liver. They found that
in animals treated with CCl$_4$, the hepatic concentrations of BSP were consistently lower and the plasma concentrations uniformly higher than those of controls. In addition they found that the relative amounts of BSP glutathione conjugates were similar in the plasma and liver of both treated and control animals. They concluded from these studies that the processes of hepatic uptake and storage were most affected by CCl$_4$ treatment. Thus, two conflicting hypotheses are advanced to explain CCl$_4$ induced plasma retention of BSP. One side indicates that plasma retention is the result of an impaired bile excretory process while the other side feels that dysfunction of the processes of hepatic uptake and storage most contribute to retention.

The problem of evaluating which of the processes of hepatic BSP disposition were most affected by CCl$_4$ intoxication in trout was approached by comparing both hepatic accumulation and biliary excretion of this dye in treated and control fish.
II. ASPECTS OF HEPATIC DISPOSITION OF SULFOBROMOPHTHALEIN IN RAINBOW TROUT

Materials and Methods

Rainbow trout (300-500 g) were purchased from Roaring River fish hatchery, Scio, Oregon. Animals were held in a constant temperature room in 130 l plastic aquariums supplied with continuously flowing, dechlorinated city water (12.0°C ± 0.5). They were fed a commercial diet (Purina Trout Chow) every other day but food was withheld for 24 h prior to an experiment. A 12 h light:dark photoperiod was maintained throughout all experiments and new fish were allowed a one week acclimation period before use.

Animals used in all experiments were immobilized by transection of the spinal cord. This method of immobilization simplifies the technical difficulties associated with estimating biliary BSP excretion and does not appear to significantly alter either the rates of plasma clearance or biliary excretion of the dye relative to those of free swimming animals (Schmidt and Weber, 1973). After immobilization, animals were weighed and an identifying styrofoam float attached to the dorsal surface with a silk suture. Fish were then placed in individual troughs of a plastic coated wire frame support within a 40 l plexiglass aquarium having a continuous flow of chilled and dechlorinated city water (1.5 l/min) and allowed to recover at least 18 h.
Plasma Clearance, Biliary Excretion and Hepatic Accumulation of BSP

In experiments requiring timed serial sampling of blood from a single fish, a canula was inserted into the caudal vein at a point just ventral to the lateral line and immediately above the anterior insertion of the adipose fin. The canula consisted of PE 50 tubing of known volume (50 μl). The shaft of a 23 gauge needle was attached to one end of the tubing with the hub of the needle fitted to the other end. A suture in the caudal peduncle secured the canula to the fish. A solution of BSP in physiological saline (5.0 or 10.0 mg/kg) was injected as a single dose through the caudal vein canula and 0.2 ml blood samples were obtained every 15 min for one hour. Plasma volume was maintained by reinjecting an equivalent volume of heparinized (100 U.S.P. units/ml) saline following the withdrawal of each blood sample. The plasma half life (T₁/₂) of BSP was estimated from the slope of a line visually fit to a plot of the log of plasma BSP concentration vs time. The fractional turnover rate (Fₜ) of BSP was calculated from the formula $Fₜ = 0.693/T₁/₂$ where $T₁/₂$ is the plasma half life of BSP in min.

To determine the rate of biliary BSP excretion the common bile duct was canulated with PE 10 tubing of known volume (40 μl) and the cystic duct ligated (Schmidt and Weber, 1973). No attempt was made to replace bile salts lost during the experiment. After a 12 h
recovery period, a single dose of BSP (10.0 mg/kg) was injected into
the caudal vein and then bile flow was determined every half hour for
six hours. Bile was collected into PE 90 tubing which was volume
calibrated in 10 μl intervals and attached to the bile duct canula by a
collar of PE 50 tubing. Bile flow rates were determined by recording
the progress of the bile in the collecting canula. The bile produced in
each half hour period was obtained by cutting the tubing into segments
corresponding in length to the volume of bile produced during each
period.

To determine the concentration of BSP in the liver and plasma,
fish were sampled 15, 30, and 60 min after a single dose of BSP
(10.0 mg/kg) had been injected into the caudal vein. Each fish was
stunned by a blow to the head, a blood sample taken by cardiac punc-
ture, and the liver removed. Livers were perfused with 10 ml of
chilled physiological saline by the hepatic portal vein and then placed
on absorbent paper pads on ice.

Surgical Impairment of Blood
Flow and/or Bile Flow

Three groups of five animals each were prepared by the follow-
ing surgical treatments. The cystic ducts and common bile ducts of
the animals in the first group were ligated with 5-0 silk sutures. In
animals of the second group the cystic duct, common bile duct and
hepatic portal vein were ligated, while sham surgery involving isolation of the ducts and vessels without ligation was performed in animals of the third group. The incisions were closed with 4-0 surgical silk sutures and the animals were allowed an 18 h recovery period. Surgically prepared animals were used in experiments to determine either plasma clearance or hepatic accumulation of BSP as previously described.

Analytical Procedures

The concentration of BSP in the bile and plasma was estimated colorimetrically after appropriate dilution of each sample with alkaline buffer solution (Richterich, 1969). Absorbance was read at 578 mμ on a Beckman DB spectrophotometer and converted to units of concentration by comparison with reference standards of BSP. A blank for each sample was obtained by acidifying the sample with acid buffer solution (Richterich, 1969). The extinction coefficients of BSP and its metabolites in the bile and liver of trout were assumed to be equal (Combes, 1965; Whelan et al., 1970).

The concentration of BSP in the liver was determined by a modification of the method of Whelan et al. (1970). Livers were weighed, minced, and then homogenized on ice in Potter-Elvehjem tissue homogenizers. Approximately 0.5 g (± 20 mg) of the homogenate was weighed into a tared screw cap test tube and extracted twice
with 10 ml volumes of 75% methanol in water (v/v). After each addition of solvent the homogenates were shaken and then centrifuged for 10 min (1850 x g). The methanol supernates were combined and brought to a final volume of 25 ml with 75% methanol-water. Concentrations of BSP were determined from 100 μl samples of this final extract in a manner identical to that described for plasma and bile BSP. Recoveries of BSP using this method were greater than 97%.

Statistical Methods

Means of individual treatment groups were compared by Student's t-test for independent sample means (Steel and Torrie, 1961).

Results

Plasma Clearance, Biliary Excretion, and Liver Accumulation of BSP

Estimates of plasma half life and fractional turnover rates of BSP were the same for animals receiving either 5.0 or 10.0 mg/kg doses of the dye due to the similar logarithmic decline in the plasma BSP concentrations during the first hour after dye administration (Table 1). Assuming that the plasma volume of trout was 4.0% of the body weight (Houston and DeWilde, 1968) the estimated mean percentages of the initial dose of BSP remaining in the plasma
Table 1. Plasma BSP concentrations, plasma half lives and plasma fractional clearance rates in spinal transected rainbow trout receiving either 5.0 mg/kg or 10.0 mg/kg of BSP. Each value represents the mean ± SE of the number of animals in parentheses.

<table>
<thead>
<tr>
<th>Dose of BSP (mg/kg)</th>
<th>Plasma BSP Concentration (mg/100 ml)</th>
<th>Plasma half life (min)</th>
<th>Fractional clearance (%/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6)</td>
<td>6.25 ± 0.47</td>
<td>1.36 ± 0.17</td>
<td>0.54 ± 0.07</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7)</td>
<td>16.60 ± 0.35</td>
<td>6.91 ± 0.25</td>
<td>2.38 ± 0.19</td>
</tr>
</tbody>
</table>
Compartment after 60 min were 3.50% ± S.E. 0.31 and 2.43% ± S.E. 0.35 in groups receiving 10.0 mg/kg and 5.0 mg/kg respectively.

Small quantities of BSP were found in the bile as early as 30 min after animals had received the dye; however, maximal concentrations were present in the bile between 1.5 and 3 h following administration (Table 2). Bile flow rates were reduced in all fish between 1.5 and 2.5 h after dye injection and this reduction appeared to coincide with the time at which maximum transport of the dye would be expected across the canalicular membrane into the bile. After one hour the percent of the initial dose of BSP which had appeared in the bile ranged from 2.8% to 13.0% while after six hours these values ranged from 28.8% to 58.7%. This variability was due in part to differences in both bile flow rates and bile BSP concentrations between individual animals.

The hepatic content of BSP was highest 15 min after administration and thereafter both plasma and liver concentrations decreased (Table 3). Proportionately greater decreases in the dye concentrations were found in the plasma than in the liver between 15 and 60 min and this resulted in a steady increase in the liver to plasma concentration ratio of the dye. The absolute concentrations of BSP in the liver after one hour were from 38 to 49 times greater than those found in the plasma; however, it was not possible to determine the actual hepatocyte to plasma concentration gradient of the BSP since
Table 2. Bile flow, bile BSP concentration and percent of BSP dose\(^a\) appearing in bile of spinal transected rainbow trout with time after administration of the dye.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Bile Flow (µl/kg/min)</th>
<th>Bile BSP (mg/ml)</th>
<th>Accumulated BSP (percent)</th>
</tr>
</thead>
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<tr>
<td>0.5</td>
<td>1.76 ± 0.30(^b)</td>
<td>3.32 ± 0.38(^b)</td>
<td>2.5 ± 0.72(^b)</td>
</tr>
<tr>
<td>1.0</td>
<td>1.73 ± 0.31</td>
<td>6.03 ± 1.36</td>
<td>7.9 ± 2.94</td>
</tr>
<tr>
<td>1.5</td>
<td>1.27 ± 0.37</td>
<td>7.77 ± 1.76</td>
<td>14.8 ± 6.90</td>
</tr>
<tr>
<td>2.0</td>
<td>1.28 ± 0.37</td>
<td>8.39 ± 1.58</td>
<td>19.5 ± 8.61</td>
</tr>
<tr>
<td>2.5</td>
<td>1.68 ± 0.23</td>
<td>8.30 ± 1.10</td>
<td>23.8 ± 9.86</td>
</tr>
<tr>
<td>3.0</td>
<td>1.51 ± 0.26</td>
<td>6.57 ± 1.01</td>
<td>27.4 ± 10.1</td>
</tr>
<tr>
<td>3.5</td>
<td>1.63 ± 0.16</td>
<td>5.94 ± 1.42</td>
<td>30.8 ± 10.0</td>
</tr>
<tr>
<td>4.0</td>
<td>1.51 ± 0.13</td>
<td>5.58 ± 1.39</td>
<td>33.9 ± 9.73</td>
</tr>
<tr>
<td>4.5</td>
<td>1.46 ± 0.08</td>
<td>5.15 ± 1.41</td>
<td>36.6 ± 9.24</td>
</tr>
<tr>
<td>5.0</td>
<td>1.36 ± 0.11</td>
<td>4.68 ± 1.46</td>
<td>38.9 ± 8.86</td>
</tr>
<tr>
<td>5.5</td>
<td>1.39 ± 0.08</td>
<td>4.31 ± 1.31</td>
<td>41.3 ± 8.78</td>
</tr>
<tr>
<td>6.0</td>
<td>1.30 ± 0.15</td>
<td>4.05 ± 1.39</td>
<td>43.9 ± 8.63</td>
</tr>
</tbody>
</table>

\(^a\) 10.0 mg/kg BSP iv

\(^b\) Mean ± SE of three fish
Table 3. Liver and plasma BSP concentrations and liver:plasma ratio following administration of BSP (10.0 mg/kg) to spinal transected rainbow trout. Each value is the mean ± SE of five fish.

<table>
<thead>
<tr>
<th></th>
<th>Time (min)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Liver BSP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>concentration</td>
<td>(mg/g liver)</td>
<td>0.37 ± 0.02</td>
<td>0.40 ± 0.03</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>Hepatic BSP</td>
<td>(mg/100 g BW)</td>
<td>0.55 ± 0.02</td>
<td>0.53 ± 0.06</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>Plasma BSP</td>
<td>(mg/100 ml)</td>
<td>9.33 ± 0.31</td>
<td>3.40 ± 0.80</td>
<td>0.88 ± 0.03</td>
</tr>
<tr>
<td>Liver:plasma ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncorrecteda</td>
<td>4.0 ± 0.14</td>
<td>13.8 ± 2.54</td>
<td>42.1 ± 2.00</td>
<td></td>
</tr>
<tr>
<td>Correctedb</td>
<td>-</td>
<td>10.9 ± 1.93</td>
<td>32.0 ± 1.71</td>
<td></td>
</tr>
</tbody>
</table>

aLiver:plasma BSP concentration ratio not corrected for BSP in intrahepatic biliary space.

bLiver:plasma BSP concentration ratio corrected for BSP remaining in intrahepatic biliary tree. See text for details.
liver concentrations were contaminated with dye which previously had been excreted into the bile of the intrahepatic biliary space. Nevertheless, even when it was assumed that the volume of this space was 1% of the total wet liver mass and that the BSP concentration of bile in that space was 8.5 mg/ml, the corrected ratio of BSP in liver to plasma was not less than 20:1 in any of the fish sampled one hour after the dye had been given (Table 3).

Surgical Impairment of Hepatic Blood Flow and/or Bile Flow

To further establish the importance of normal liver function for BSP disposition in trout, plasma clearance and hepatic accumulation of BSP were estimated in animals having blood flow or bile flow artificially impaired by ligation of the hepatic portal vein or common bile duct. The influence of surgical impairment of hepatic blood flow and/or bile flow on the rate of plasma BSP clearance was dramatic (Figure 1). The level of BSP in the plasma of cystic-common bile duct ligated animals was more than four times that of sham treated control animals after 60 min while plasma levels of the dye in surgically treated animals were significantly higher (P < 0.01) after 30, 45, and 60 min. The effects of surgical impairment of hepatic blood flow as well as bile flow were even more striking since the plasma half life of BSP for this group (42 min) was nearly four times
Figure 1. Plasma disappearance curves of sham operated and surgically treated rainbow trout after receiving a single dose of BSP (10.0 mg/kg iv). (a) Sham operated controls, (b) cystic and common bile duct ligated, (c) cystic-common bile duct and hepatic portal ligation. Each point is the mean ± S.E. of five animals. Asterisk denotes values which are statistically significant (P < 0.05) from controls.
Plasma BSP Conc. (mg/100 ml) vs Time (min)
that of the sham treated animals and almost one and one-half times that of animals having only bile and cystic duct ligation. Comparison of the plasma concentrations of BSP indicated that fish having both restricted bile and hepatic blood flows retained significantly more (P < 0.05) dye in the plasma after 30, 45, and 60 min than did animals with cystic-common bile duct ligation and suggested that decreased rates of plasma clearance could be attributable to decreased hepatic blood flow.

The hepatic content of BSP and the plasma BSP concentrations were significantly altered (P < 0.05) by both surgical procedures (Figure 2). Liver concentrations of BSP in both treatment groups after 30 min were less than half of those in the livers of sham treated animals while plasma concentrations in surgically prepared animals were significantly elevated (P < 0.05). There was no apparent difference in the concentrations of BSP in the livers of control animals between 30 and 60 min; however, the liver concentration of dye increased in both groups of surgically treated animals during this interval. Differences in the hepatic content of the dye between these groups were reflected in the apparent rates of hepatic accumulation of the dye (Figure 3).
Figure 2. Hepatic content and plasma BSP concentrations in surgically treated rainbow trout 30 and 60 min after a single iv dose of BSP (10 mg/kg). (a) Sham operated fish, (b) cystic and common bile duct ligated, (c) cystic-common bile duct and hepatic portal ligated. Each value represents the mean ± S.E. of five animals. Asterisk denotes values which are different ($P < 0.05$) from controls.
Liver BSP Conc. (mg/100 g body wt.)

Plasma BSP Conc. (mg/100 ml)
Figure 3. Percent of a single dose of BSP (10.0 mg/kg) appearing in the liver 30 and 60 min after injection. (a) Sham operated fish, (b) cystic duct and common bile duct ligated fish, (c) cystic duct and common bile duct ligated and hepatic portal ligated fish. Values are the mean ± S.E. of five animals. Asterisks denote values which are significantly different (P < 0.05) from controls.
Discussion

Hepatic Uptake, Accumulation, and Excretion of BSP

These results indicate that BSP is selectively removed from the plasma and accumulated in the trout liver where it is then transported into the bile against a formidable concentration gradient. In mammals the transport of BSP from plasma to bile is generally conceded to involve several interdependent processes including active uptake by hepatocytes, binding of the dye to intracellular proteins, metabolism of the dye to more water soluble compounds and active excretion against a high concentration gradient into the bile (Goresky, 1965; Levi et al., 1969). The similarity of my results with those reported for mammals (Cantarow and Wirts, 1941; Klaassen and Plaa, 1967) suggests that many of the same processes by which mammals are able to excrete BSP may also be operating in trout.

The accumulation of more than half of the dose of BSP in the liver 15 min after injection of the dye indicates that egress of the dye from the plasma compartment of trout is primarily the result of uptake and accumulation of the dye by the liver, as occurs in mammals. That the process of BSP uptake is comparable in trout and rats is apparent by comparison of the respective fractional rates of BSP clearance. The fractional rate of clearance of a 50 mg/kg dose of
BSP in the rat is estimated to be 0.213 (Berthelot and Billings, 1966) which is less than three times the value found in trout (0.063) receiving 10 mg/kg. The apparent difference in the doses of the dye administered to the two species on the basis of body weight virtually disappeared when compared on the basis of liver weight. Rat liver is estimated to comprise 4.5% of the total wet body weight of the animal (Klaassen, 1973) while the mean liver weight of the trout used in these experiments was only 1.35% of the total body weight. Additionally, the sinusoidal surface area in rainbow trout is greatly reduced by the arrangement of hepatocytes in two cell thick cords (Weinbreb and Bilstad, 1955) and cardiac output in trout (60-100 ml/kg/min; Holeton and Randall, 1966) is less than one-third that reported for the rat (286 ml/kg/min; Prosser, 1975). Thus, taking these attributes into consideration, hepatic uptake of BSP in rainbow trout appears to be an efficient process.

Rapid uptake and accumulation of BSP by trout liver appears to be possible despite the fact that the organic anion binding protein ligandin, which is thought to facilitate these processes in mammals (Levi et al., 1969), has not been demonstrated in the liver cytosol from representatives of either cartilaginous (Boyer et al., 1976a) or bony fishes (Levine et al., 1971). Since recent evidence indicates that a close association exists between ligandin and the glutathione-S-transferase enzyme system (Habrig et al., 1974), it has been
suggested that this class of cytoplasmic enzymes also serves non-enzymatically in the cytosol of mammals as binding proteins for hepatic storage and transport of organic anions (Kaplowitz et al., 1975). The demonstration that ninhydrin reacting BSP conjugates are present in rainbow trout bile which are qualitatively similar in chromatographic characteristics to the glutathione conjugates of BSP in rat bile (Schmidt and Weber, 1973) suggests that trout also may have some hepatic glutathione-S-transferase activity. It would be of interest to determine whether some form of intracellular protein binding may be acting to facilitate hepatic BSP uptake and accumulation in trout in view of the apparent efficiency of these processes.

Comparison of the rates of hepatic uptake and accumulation with that of biliary excretion of BSP indicates that, as in mammals (Klaassen and Plaa, 1967), the latter process is probably the rate limiting step in the transfer of BSP from plasma to bile in trout. The dependence of biliary BSP excretion on the volume of bile produced per unit time has been established in rats (O'Maille et al., 1966). Comparison of the relative amount of a single dose of BSP excreted in the bile with the relative bile flow rate serves to further demonstrate this dependency in several unrelated species (Table 4). Since it has been established that biliary excretion is the major route by which BSP is eliminated in each species, this comparison is justified. Correlation coefficients of 0.9211 and 0.9996 were obtained when the
Table 4. Dependence of biliary excretion of a single intravenous dose of BSP on the bile flow rate in different species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose (mg/kg)</th>
<th>Bile Flow (µl/kg/min)</th>
<th>Dose excreted in bile after 6 h (µl/100 g liver/min)</th>
<th>Dose excreted in bile after 6 h (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogfish</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.12</td>
<td>10.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>10.0</td>
<td>1.5</td>
<td>11.1</td>
<td>43.4 ± 8.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rat</td>
<td>37.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>64.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>142.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>84.6&lt;sup&gt;d&lt;/sup&gt; ± 3.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>From Boyer (1976a)

<sup>b</sup>From Boyer (1976b)

<sup>c</sup>Mean ± SE

<sup>d</sup>From Klaassen (1975)

<sup>e</sup>From Klaassen and Plaa (1967)

<sup>f</sup>Based on estimate of bile flow in e and liver mass of 4.5% of body weight (Klaassen, 1973)
relative bile flow rate and the log relative bile flow rate, respectively, were correlated with the percent of the initial dose of BSP appearing in the bile after 6 h. These results suggest that there does seem to be a dependence of biliary BSP excretion on the rate of bile flow. Thus, the inherently low rates of bile secretion in trout, which were observed in this study and those which have been reported previously for both trout (Schmidt and Weber, 1973) and dogfish (Boyer et al., 1976a), may dictate the rate of biliary BSP excretion in these animals.

Furthermore, if differences in the rate of biliary BSP excretion between the rat and the trout were due only to differences in the relative capacities of the canalicular membranes to excrete BSP into the bile, these differences should be apparent as differences in the concentrations of BSP in the bile after the maximum hepatic excretory rate ($T_m$) for the dye has been established. Under these experimental conditions the concentration of BSP in the bile of trout (11.8 ± 1.84; Chapter IV) is nearly the same as that reported for rat bile (15.6 ± 0.6; Klaassen and Plaa, 1968). Considering that conjugation may be an important process in the transfer of BSP into the bile (Whelan et al., 1970), the slight differences in bile BSP concentrations between rat and trout may be attributable to more efficient mechanisms for dye metabolism in the rat. Under conditions of maximum hepatic BSP excretion the proportion of metabolized BSP in rat bile (75%; Schulz and Czok, 1974) is nearly twice that found in
the bile of trout (40%; Chapter IV). Despite these quantitative differences in BSP metabolism, it appears that differences in the inherent rates of bile secretion rather than differences in active membrane transport capacities for BSP better explain differences in the rates of biliary BSP excretion between the two species.

Effects of Impairment of Bile Flow and Hepatic Blood Flow on Plasma Clearance and Liver Accumulation of BSP

The decrease in the rate of plasma BSP clearance which was observed in trout 24 h after experimental ligation of the cystic and common bile ducts confirms the results of similar studies by Schmidt and Weber (1975). In addition my results indicate that the rate of hepatic BSP accumulation was also reduced by this surgical procedure. Considering the efficiency of the processes of hepatic uptake and accumulation of BSP in the trout it is not immediately clear why these processes should be so severely reduced. If differences in the liver were due entirely to differences in the amount of dye that had been transferred into the intrahepatic biliary space it would be necessary for more than one-quarter of the injected dose of BSP to be actively transported into the bile of control fish within 30 min. Even if the rate of transport of the dye had equaled the maximum hepatic excretory rate (12.2 μg/kg/min; Chapter IV), less than 5% of the injected dose could have been transported into the bile
Berthelot and Billing (1966) have shown that experimental ligation of the common bile duct in rats 4 min after a single dose of BSP was given does not alter the fractional plasma turnover rate of the dye. Conversely, 24 h after ligation of the common bile ducts and cystic ducts in trout, the plasma half life of BSP was more than twice that of sham operated control fish. These differences suggest that prolonged bile stasis may produce cell wide biochemical or morphological changes in the hepatocytes which might reduce their functional capacity to take up and store BSP. Decreased activity of the membrane bound enzymes Mg$^{+2}$-ATPase and 5'-nucleotidase has been demonstrated in rat liver 24 h after experimental ligation of the common bile duct (Simon and Arias, 1973). Further, Vial et al. (1976) have recently demonstrated loss of microvilli on the bile canalicular surface and a variety of other ultrastructural alterations on the surfaces of rat hepatocytes after similar prolonged bile stasis. Thus, similar biochemical or morphological alterations of trout hepatocytes following experimental bile duct ligation may be partly responsible for the impaired plasma clearance and hepatic accumulation of the dye which were observed in this study.

These results suggest that, in the trout, the rates of hepatic uptake and accumulation of BSP as well as those of biliary excretion may well be dictated by physiological and anatomical factors which either act to: 1) delay transport of the dye to the liver, 2) reduce the
functional surface area available for plasma-hepatocyte contact, or 3) retard the transport of the dye away from this organ by low rates of bile secretion. Thus, the actual membrane mechanisms responsible for passage of BSP from plasma into the bile in trout are probably much more similar functionally to those of the rat than one would initially be led to believe on the basis of gross comparisons of these functions based on body weights.
III. CARBON TETRACHLORIDE-INDUCED PLASMA RETENTION OF SULFOBROMOPHTHALEIN IN RAINBOW TROUT

Materials and Methods

Rainbow trout (200-400 g) were purchased from Roaring River fish hatchery, Scio, Oregon and maintained under the conditions previously described (Chapter II). Unless stated otherwise, animals used in all experiments were immobilized by transection of the spinal cord (Schmidt and Weber, 1973). After transection, animals were weighed and an identifying styrofoam float was attached to the dorsal surface by a suture. They were then placed in individual troughs of a rubber-coated wire frame support within a 40 l plexiglass aquarium having a continuous flow of chilled, dechlorinated city water (1.5 l/min) and allowed to recover at least 18 h.

Histological Studies

Carbon tetrachloride (2.0 ml/kg ip) was given to one group of four spinal transected fish and a control fish was given a similar volume of physiological saline. The liver was taken from one fish every 6 h for 24 h and the control fish was sampled after 24 h. Sections of liver were fixed in Bouin's solution within 1 min after the fish had been killed. Tissue was embedded in paraffin and 8 μ sections were stained with hemotoxylin and eosin. A similar
experiment was performed using free swimming rainbow trout of the
Mt. Shasta strain in facilities provided by the Department of Food
Science and Technology, Oregon State University.

**Effect of CCl₄ Intoxication on**
**Plasma Clearance of BSP**

Animals were given undiluted CCl₄ (0.2 or 2.0 ml/kg) or an
equivalent volume of physiological saline. After 24 h a canula was
placed in the caudal vein as previously described (Chapter 1) and a
single dose of BSP (5.0 mg/kg) in physiological saline was injected
into the caudal vein through the canula. Blood samples (0.2 ml) were
taken from the canula every 15 min for one hour and plasma volume
was maintained by reinjecting an equivalent volume of heparinized
(100 U.S.P. units/ml) physiological saline following withdrawal of
each blood sample. The plasma half life of BSP was estimated from
the slope of a line visually fitted to a plot of the points of the log of
plasma BSP concentration vs time.

Fish used in time-response studies received either undiluted
CCl₄ (2.0 mg/kg ip) or an equivalent volume of physiological saline
12, 24, 48, 96, and 120 h prior to BSP administration. BSP (5.0 mg/
kg) was injected into the caudal vein and after 45 min a 0.2 ml blood
sample was taken by cardiac puncture. Immediately prior to
administration of the dye a blood sample was taken from the caudal
vein for estimation of the plasma hemoglobin concentration.
**Effect of Bilirubin or Hemoglobin Infusion on Plasma BSP Clearance**

Bilirubin (extinction coefficient 60.1 ± 0.2 mM; ICN Biochemicals, Cleveland, Ohio) was dissolved in a solution of 0.5 g Na$_2$CO$_3$ and 0.52 g NaCl per 100 ml water (Weinbren and Billing, 1956) and stabilized with 25 mg/100 ml of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). Solutions, of appropriate concentration for each fish, were prepared in a darkened laboratory with the aid of a photographic darkroom light and held overnight at 4°C in foil wrapped injection vials.

Animals were prepared for infusion experiments by exposing the ventral intestinal vein at a point between the pelvic fins and the anus and inserting an infusion canula (PE10). The wound was closed with 4-0 surgical suture silk and the fish were allowed a short recovery period. A canula was inserted into the caudal vein and a loading dose of bilirubin (7.0 mg/kg) was administered by this canula immediately prior to the start of infusions. Bilirubin was infused (40 μg/kg/min) for 4 h using a Sage model 341 variable speed syringe pump (Orion Research Inc., Cambridge, Mass.) and 3 h after the infusion began, BSP (5.0 mg/kg) was injected by the caudal vein canula and then serial blood samples were taken every 15 min for 1 h. Control fish received bilirubin vehicle in a similar manner over the same time period.
In experiments requiring the infusion of hemoglobin, a hemolysate was prepared from the blood of donor trout in a manner similar to that described by Ostrow et al. (1962). The tonicity of the hemolysate was restored to 300 milliosmolal with 5% (w/v) NaCl solution, the pH was adjusted to 7.3 with 0.15 M phosphate buffer and the hemoglobin content was adjusted with physiological saline to a concentration appropriate for each fish. A loading dose of hemoglobin (40 mg/kg) was administered and hemoglobin was infused (250 μg/kg/min) for 4 h. After 3 h animals received BSP (5.0 mg/kg iv) and blood samples were withdrawn by the caudal vein canula every 15 min for 1 h.

Analytical Procedures

Fifty microliter plasma samples were analyzed for BSP or hemoglobin content by the methods of Richterich (1969). The concentration of bilirubin in the plasma was determined by the diazotization procedure of Malloy and Evelyn (1937). In addition, plasma osmolality was determined in some experiments on 7 μl samples using a Wescor model 5100 vapor pressure osmometer (Wescor, Inc., Logan, Utah).

Statistical Methods

Means of individual treatment groups were compared by Student's t-test for independent sample means (Steel and Torrie, 1961).
Results

Gross Pathology and Histology

The pathological responses of rainbow trout to CCl₄ (2.0 mg/kg ip) were similar in transected and non-transected animals. Hemoglobinuria was evident after 12 h and continued for at least 48 h. Inspection of the peritoneal cavities of these animals revealed masses of dark red gelatinous material among the viscera and 7-10 ml of clear, dark red fluid could be aspirated from the abdominal cavity. Areas of hemorrhagic inflammation were evident in the linings of the peritoneal cavity and in sections of the large and small intestines while multiple, prominent thrombi were observed in the ventral intestinal vein. In addition, the surface of the liver and the spleen were mottled with blanched areas.

The livers of fish receiving CCl₄ appeared to be slightly enlarged but the liver to body weight ratios of these animals were not significantly different from those of control animals. The mean liver weight of 15 fish treated with CCl₄ was 1.31% of body weight and values ranged from 0.98% to 1.81%. In control animals the mean liver weight of 15 animals was 1.37% of body weight and values ranged from 1.09% to 1.76%. These values were somewhat misleading, however, since animals receiving CCl₄ gained significantly (P < 0.05) more weight, presumably as water, after treatment and
maintained this weight for a longer time than did controls (Figure 4). Thus, even though livers of treated animals were enlarged, the concomitant increase in body weight negated demonstration of this effect.

After 24 h, fish receiving 0.2 ml/kg CCl₄ exhibited slight inflammation of the peritoneal cavity around the site of injection but thrombi were not observed in any of the major vessels of the splanchnic drainage. In addition, there was no evidence of hemoglobinuria during the first 24 h after intoxication. Livers of animals in this group were not taken for histological examination.

The livers from transected and non-transected control trout were similar histologically to those described by Weinbreb and Bilstad (1955). Slight vacuolization was evident in some hepatocytes; however, the majority of cells displayed a normally granular cytoplasm (Figure 5a). Morphological changes were evident in the liver taken from a non-transected fish 6 h after CCl₄ treatment. Necrosis was apparent both in the subcapsular region and in well defined areas surrounding the central veins (Figure 5b). Damage in the subcapsular region was characterized by coagulative necrosis and pyknosis (Figure 5c). Because of the apparent lack of well defined lobular structure in trout liver, the term pericentral is used to define areas surrounding central veins. Pathological changes in pericentral regions were characterized by liquifactive necrosis and karyolysis and necrotic areas were surrounded by a zone of swollen hepatocytes.
Figure 4. Relative weight gain in control animals and animals receiving CCl₄ 12, 24, 48, 96, and 120 h earlier. Values are the mean ± S.E. of the number of animals in parentheses. Asterisks indicate values that are significantly different (P < 0.05) from controls.
Figure 5. Liver sections from rainbow trout. Hemotoxylin and eosin stain.

(a) Control liver. 128 X.

(b) Peripheral and pericentral necrosis in liver 6 h after CCl₄ treatment. 20 X.

(c) Peripheral necrosis. 128 X.

(d) Pericentral necrosis. 128 X.
(Figure 5d). The essential aspects of the pericentral lesion were similar in one spinal transected animal 18 h after treatment.

It was not possible to assess the development of liver damage with time following treatment. Pericentral liver necrosis was evident in one free swimming fish after 6 h and in one spinal transected animal after 18 h. Eosinophilic degeneration and areas of slight hydropic degeneration were noted in sections of liver taken from non-transected fish after 12, 18, and 24 h while similar degenerative changes were noted after 6, 12, and 24 h in spinal transected fish. Cellular regeneration was not evident in livers from transected or non-transected individuals.

Effect of CCl₄ Intoxication on Plasma Clearance of BSP

Significantly higher (P < 0.05) levels of plasma BSP were found after 30, 45, and 60 min in fish treated with 0.2 and 2.0 ml/kg of CCl₄ 24 h earlier (Figure 6). The plasma half life of BSP was estimated to be 11 min in control animals and 15 and 32 min respectively in animals receiving 0.2 and 2.0 ml/kg CCl₄, indicating some degree of dose dependence.

Significant (P < 0.05) retention of BSP was evident as early as 12 h after CCl₄ treatment and was still apparent after 120 h. Retention of BSP was maximal after 48 h whereupon it slowly declined.
Figure 6. Plasma disappearance curves of BSP in control trout and trout treated 24 h earlier with CCl₄ (0.2 or 2.0 ml/kg ip). Each point represents the mean ± S.E. of the number of animals in parentheses. The asterisk indicates values which are significantly different (P < 0.05) from controls.
Plasma BSP Conc. (mg/100 ml)

Control (7)

2.0 ml/kg (7)

0.2 ml/kg (5)

Time (min)
(Figure 7). Levels of BSP in the plasma of control animals were relatively constant.

The apparent hemolytic action of CCl₄ was reflected in sharply increased levels of hemoglobin in the plasma (Figure 8). Twelve hours after receiving the toxicant the concentration of hemoglobin in the plasma was nearly 3.0 mg/ml but these levels slowly declined to those of controls by 120 h. Despite the apparent increase in total body water following CCl₄ intoxication, differences in plasma osmolality between treatment groups were not evident after 24, 48, 96, and 120 h.

**Effects of Bilirubin or Hemoglobin Infusion on Plasma BSP Clearance**

Previous studies have established that high plasma bilirubin concentrations can reduce the rate of plasma BSP clearance in rats (Hunton et al., 1961; Dragstedt and Mills, 1936), presumably by competing with BSP for processes associated with hepatic elimination (Clarenburg and Kao, 1973). Because preliminary studies indicated that bilirubin was the major bile pigment excreted by rainbow trout, it was possible that BSP retention was caused in part by competition for excretion with large quantities of bilirubin derived from hemolyzed red cells. To test this hypothesis fish received either bilirubin or an equivalent volume of bilirubin vehicle and then BSP was administered
Figure 7. Plasma BSP retention in rainbow trout following CCl₄ intoxication (2.0 ml/kg). Plasma dye concentrations were determined 45 min after a single dose (5.0 mg/kg iv) of BSP was administered. Values represent the mean ± S.E. of the number of animals in parentheses. Asterisks denote values which are significantly different (P < 0.05) from controls.
Plasma BSP Conc. (mg/100 ml)

- **CCl₄**
- **Control**

### Time (hrs)
- 12
- 24
- 48
- 96
- 120

### Data Points
- (5) * at 12 hrs
- (7) * at 24 hrs
- (7) * at 48 hrs
- (7) * at 96 hrs
- (6) * at 120 hrs
Figure 8. Plasma hemoglobin concentrations in control fish and fish receiving CCl₄ 12, 24, 48, 96, and 120 h earlier. Values represent the mean ± S.E. of the number of animals in parentheses. Asterisks denote values which are significantly different (P < 0.05) from controls.
and the rate of plasma BSP clearance determined. Animals receiving bilirubin tended to retain more BSP in their plasma than controls, but the difference was significant (P < 0.05) only 60 min after BSP administration (Figure 9). The plasma half life of BSP in control fish was 14 min while that of animals receiving bilirubin was 18 min. In a similar study it was found that high levels of hemoglobin in the plasma had no significant effect on the rate of plasma BSP clearance (Figure 10. The plasma half life of BSP was estimated to be 14 min in both groups.

Discussion

The development of pericentral liver necrosis in rainbow trout following CCl₄ intoxication is not unlike the centrilobular liver necrosis that routinely develops in mammals after treatment with this toxicant. In mammals, it is not clear whether these lesions result from the irreversible binding of active intermediates of CCl₄ metabolism to critical cellular elements (Klaassen and Plaa, 1969; Castro et al., 1972) or whether these active intermediates precipitate a peroxidative attack on lipid structural elements (Rechnagel, 1967). In either case, it is generally felt that the hepatotoxicity associated with CCl₄ intoxication is related to metabolism of the compound. In view of recent reports which indicate that components of the mixed function oxidase system are present in various fish, including rainbow
Figure 9. Plasma disappearance curves of BSP in control fish and fish loaded with bilirubin. Values represent the mean ± S.E. of the number of fish in parentheses. Asterisk denotes a value which is significantly different ($P < 0.05$) from controls.
Figure 10. Plasma disappearance curves of BSP in control fish and fish loaded with hemoglobin. Values are the mean ± S.E. of the number of animals in parentheses.
trout (Chan et al., 1967; Ludke et al., 1972; Stanton and Khan, 1975), and that rainbow trout are capable of hepatic biotransformations by this mixed function oxidase system (Peterson et al., 1976), it is conceivable that the pericentral liver necrosis that develops in trout following CCl₄ treatment is the result of metabolism to active intermediates.

In the present study only one animal in four from both the transected and non-transected groups developed necrotic lesions in pericentral regions of the liver, even though minor degenerative changes were found in the livers of all treated animals. The reason for this variability is not known. Differences in the nutritional status among fish used in this experiment may be responsible in part, since diet is known to greatly influence both metabolism of CCl₄ and the degree of its hepatotoxic response in rats (Seawright and McLean, 1967). In addition, unequal rates of uptake or differences in distribution of the toxicant may have contributed to this variability. Statham and Lech (1976) have reported that rainbow trout dosed with undiluted $^{14}$CCl₄ (1.0 ml/kg ip) accumulated highest levels in the mesenteric fat surrounding the gastrointestinal tract followed by intermediate concentrations in the heart, gills, and liver. In the present study, large amounts of visceral fat may have reduced the effective dose of CCl₄ by providing a storage depot for the toxicant.
Necrosis in the subcapsular region was probably caused by direct contact of CCl$_4$ with the liver. Conversely, it is not likely that the pericentral necrosis was caused by direct contact with high concentrations of the toxicant. If this were the case, one would expect periportal hepatocytes also to be damaged since cells in this region should be exposed to higher levels of CCl$_4$ sooner than those in the pericentral region. No evidence of periportal necrosis was found in livers of any animals.

The intense hemolytic response that occurred in animals receiving CCl$_4$ has not been reported in similar studies with fish or mammals. The level of CCl$_4$ used in the present studies was higher (2.0 ml/kg) than doses used previously in trout (1.33 ml/kg; Racicot et al., 1975) and rats (1.0 mg/kg; Klaassen and Plaa, 1968). Moreover, administration of undiluted CCl$_4$ could facilitate its rapid uptake into a variety of tissues and result in the localization of high concentrations of this compound in relatively confined areas. Thus, direct contact may contribute greatly to the hemolytic effect of CCl$_4$. It is not likely that hypotonic shock was responsible for the hemolysis since the osmolality of random plasma samples from treated and control fish was similar. Also, plasma hemoglobin concentrations in treated animals were decreasing even though fish continued to gain weight (i.e. take up water).
It was not unexpected that intoxication by CCl$_4$ should result in weight gain in these fish. Carbon tetrachloride is known to produce renal lesions in laboratory animals which result in oliguria and anuria (Stricker et al., 1968). In addition, Smith et al. (1970) have reported severe renal congestion in chinook salmon (Oncorhynchus tshawtscha) which had been rendered anemic by the hemolytic agent phenylhydrazine. Similar processes leading to renal impairment could result in excessive water retention in animals treated with CCl$_4$. The slight and transient weight gain in control animals was probably due to effects related to handling and surgical stress of transection (Stevens, 1973).

Results of these studies indicate that decreased plasma clearance or increased plasma retention of BSP may be a useful criteria by which to evaluate liver dysfunction in fish. A significant decrease in plasma clearance was detected in animals receiving as little as 0.2 mg/kg ip of CCl$_4$. In addition, when plasma retention of BSP was used to estimate the time course of functional liver damage, plasma concentrations of BSP were found to be significantly higher than controls for as long as 120 h after treatment. It is also apparent that plasma BSP clearance is not greatly influenced by high levels of bilirubin or hemoglobin in the plasma which might be expected to develop after prolonged exposure to certain classes of toxicants. Studies by Hallesy and Benitz (1963) and Cutler (1974) have
established the usefulness of BSP plasma clearance as a test to predict liver dysfunction in laboratory animals. Yet it was pointed out in both of these investigations that morphological changes are more discriminating of liver damage in long term studies than are functional changes. This is probably true in fish. In my limited histological studies some form of degenerative change was evident in the livers of all animals receiving CCl₄ in acute doses. This is not to imply that in chronic exposure studies a similar relation between functional impairment and morphological alteration would be as readily apparent. Further consideration should be given to the potential use of this method in chronic exposure experiments.

Ligation of the hepatic portal vein in rainbow trout can impair the rate of plasma BSP clearance, presumably by reducing its transport to the liver (Chapter II). While it was not possible to evaluate the effects of CCl₄ on hepatic blood flow in these animals, alteration and redistribution of hepatic portal and arterial blood supplies must be recognized as a possible source of plasma BSP retention. Lautt and Plaa (1974) could find no evidence of decreased total hepatic blood flow in the intact cat 24 h after oral administration of CCl₄ (1.0 ml/kg). Nevertheless, it is possible that the general inflammation in the peritoneal cavity and the presence of thrombi in the ventral intestinal vein may have caused changes in blood flow patterns within the splancnic drainage of sufficient magnitude to influence blood flow to the liver.
The method of BSP plasma clearance is a relatively fast and sensitive test to indicate liver dysfunction in rainbow trout. This test also might be applicable to other fishes since Boyer (1976b) has demonstrated that two species of cartilaginous fishes also excrete BSP primarily by the bile. Provided that the limitations imposed by the non-specific nature of the test are recognized, this could prove to be a useful method of evaluating liver function in fish.
IV. THE EFFECT OF CARBON TETRACHLORIDE ON HEPATIC ACCUMULATION, METABOLISM, AND BILIARY EXCRETION OF SULFOBROMOPHTHALEIN IN RAINBOW TROUT

Materials and Methods

Rainbow trout (300-500 g) were obtained from Roaring River fish hatchery, Scio, Oregon and were maintained under the conditions described in Chapter II). Animals used in all experiments were immobilized by transection of the spinal cord (Schmidt and Weber, 1973). Identifying styrofoam floats were attached to the dorsal surface of each animal with a piece of silk suture. Then the animals were weighed and placed in the individual troughs of a rubber-coated wire frame support within a 40 l plexiglass aquarium having a continuous flow of chilled and dechlorinated city water (1.5 l/min) and allowed to recover at least 18 h.

Distribution of BSP in Liver and Plasma

Animals received either CCl₄ (2.0 ml/kg ip) or an equivalent amount of physiological saline. BSP (10.0 mg/kg) was injected into the caudal vein 24 h following treatment and fish were sampled 15, 30, 60 and 120 min later. Each fish was stunned by a blow to the head, a blood sample taken by cardiac puncture, and the liver removed. Livers were perfused with 10 ml of chilled physiological
saline by the hepatic portal vein and placed on absorbent paper pads over ice.

**Biliary BSP Excretion**

Fish received either undiluted CCl₄ or an equivalent volume of physiological saline (2.0 ml/kg ip) and after 12 h the common bile duct was canulated with PE 10 tubing of known volume (40 μl) and the cystic duct was ligated (Schmidt and Weber, 1973). An infusion canula (PE 10) was inserted into the ventral intestinal vein and the wound closed with 4-0 surgical silk sutures. Free CCl₄ was not apparent in the peritoneal cavities of animals treated 12 h earlier. No attempt was made to replace bile salts lost during the experiment.

Animals were loaded with BSP by graded infusion over a 12 h period. The initial infusion rate (20 μg/kg/min) was maintained for 4 h and then the rate was increased to 40 and then 60 μg/kg/min in two ensuing 4 h periods. After 12 h the infusion was discontinued and the animals received a single dose of BSP (5.0 mg/kg) by the caudal vein to insure that the biliary BSP excretory capacity had been exceeded. Bile was collected into lengths of PE 90 tubing which were attached to the bile duct canula by a collar of PE 50 tubing and volume calibrated in 10 μl intervals. Bile flow rates were determined every half hour by recording the progress of the bile in the collecting canula. These canulae were changed after 6, 12, and 15 h to prevent
longitudinal mixing of the BSP in the tubing. The bile produced in each hour period was obtained by cutting the tubing into segments corresponding in length to the volume of bile produced during each individual period.

**Analytical Procedures**

The concentrations of BSP in the bile and plasma were estimated colorimetrically by the procedure of Richterich (1969) as described in Chapter II. The concentrations of BSP in the liver were determined by a modification of the method of Whelan et al. (1970) as outlined in Chapter II. The extinction coefficients of free BSP and its metabolites in the bile and liver were assumed to be equal (Combes, 1965; Whelan et al., 1970).

Liver extracts were prepared for chromatographic separation of free and metabolized BSP by the method of Whelan and Combes (1971). The residue containing the dye was reconstituted with equal volumes (20 μl) of distilled water and 75% methanol in water (v/v). A portion (10 μl) of the reconstituted extract was applied to TLC strips and chromatographed. BSP was extracted from the plasma by adding 5 vol of 1:1 acetone/methanol mixture to 1 vol of plasma with constant agitation on a vortex mixer. The mixture was allowed to stand 15 min and then centrifuged (15 min, 1850 x g) to separate the precipitated protein. A portion of the supernate (4 ml) was transferred
to a test tube and evaporated to dryness under a slight stream of air on a 40°C water bath, the residue was reconstituted as previously described for liver extracts and a sample (10 μl) applied to a TLC strip and chromatographed. Samples of bile (2.5 or 5.0 μl), collected 1, 2, 4, 6, 8, 10, 12 and 14 h after the start of BSP infusion, were applied directly to TLC strips and chromatographed.

Free BSP and its metabolites were separated by thin layer chromatography on precoated microcrystalline cellulose TLC strips (Baker-flex, J.T. Baker Chemical Co., New Jersey) following the procedure of Whelan and Plaa (1963). Ninhydrin reagent (Nutritional Biochemicals Co., Cleveland, Ohio) was sprayed on chromatographs to detect amino acid conjugates of BSP while aniline diphenylamine reagent (Sigma Chemical Co., St. Louis, Mo.) was used to detect carbohydrate conjugates. Standards of BSP were prepared by adding a solution of BSP in physiological saline to freshly collected plasma, bile or to liver homogenates. BSP fractions not having relative mobility (Rf) values similar to those of the BSP standards were considered to be metabolites of the dye.

The proportion of metabolized dye that appeared in liver extracts or bile was determined by eluting from the TLC strips either free or metabolized BSP fractions into separate test tubes with alkaline buffer (Richterich, 1969) and recording the optical density at 578 μm. The relative contribution of metabolized BSP was determined as the
ratio of the optical density of the metabolized BSP to the sum of the optical densities of both free and metabolized BSP. The optical densities of all samples were within the linear portion of the calibration curve prepared for BSP.

**Statistical Methods**

Means of individual treatment groups were compared by Student's t-test for independent sample means (Steel and Torrie, 1961). Differences between the metabolite ratios were tested using a one-way analysis of variance after data had been transformed into the arcsin square root percentage to conform to the assumptions of normality imposed by the test (Sokal and Rohlf, 1969).

**Results**

**Distribution of BSP in Liver and Plasma**

The hepatic content of BSP in animals receiving $\text{CCl}_4$ was significantly different ($P < 0.05$) from those of controls 15, 60, and 120 min after the dye was given while plasma concentrations of treated animals were significantly higher ($P < 0.01$) than those of controls at all times (Table 5). Concentrations of BSP in the plasma and liver of control animals declined uniformly throughout the experimental period, but in intoxicated animals the hepatic content of BSP continued
Table 5. Liver and plasma BSP concentrations following its administration (10 mg/kg iv) to control fish and fish receiving CCl₄ (2.0 ml/kg ip) 24 hours earlier. Values are the mean ± SE of five animals. Asterisks denote values which are significantly different (P < 0.05) from controls.

<table>
<thead>
<tr>
<th></th>
<th>Time after BSP Injection (min)</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>30</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/g L)</td>
<td></td>
<td>0.37 ± 0.02</td>
<td>0.40 ± 0.03</td>
<td>0.35 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>(mg/100 g bw)</td>
<td></td>
<td>0.55 ± 0.02</td>
<td>0.53 ± 0.06</td>
<td>0.44 ± 0.02</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td>9.33 ± 0.31</td>
<td>3.40 ± 0.80</td>
<td>0.88 ± 0.03</td>
<td>0.52 ± 0.01</td>
</tr>
<tr>
<td><strong>Carbon Tetrachloride</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/g L)</td>
<td></td>
<td>0.19 ± 0.03*</td>
<td>0.40 ± 0.04</td>
<td>0.40 ± 0.03</td>
<td>0.27 ± 0.02*</td>
</tr>
<tr>
<td>(mg/100 g bw)</td>
<td></td>
<td>0.25 ± 0.04*</td>
<td>0.51 ± 0.09</td>
<td>0.56 ± 0.04*</td>
<td>0.36 ± 0.03*</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td>17.14 ± 1.46*</td>
<td>8.40 ± 1.19*</td>
<td>1.65 ± 0.17*</td>
<td>1.45 ± 0.18*</td>
</tr>
</tbody>
</table>

*a mg/g Liver

*b mg/g body weight
to increase until 60 min after the dye had been injected even though plasma BSP concentrations decreased during the entire period. While the maximum BSP content was not significantly different in the livers of either group, the time at which these levels were achieved was 45 min earlier in control fish.

BSP accumulated in the livers of control animals at a faster rate than in animals receiving CCl₄ (Figure 11). After 15 min the amount of BSP found in the livers of control animals was more than twice that found in the livers of treated animals and represented approximately 55% of the injected dose of the dye. Even though as much as 57% of the injected dye eventually was found in the livers of animals treated with CCl₄, this level was not attained until 60 min after animals had received the dye. The levels of BSP in both groups of animals decreased uniformly between 60 and 120 min.

**Biliary BSP Excretion**

Bile flow rates, bile BSP concentrations and biliary excretion of BSP were not significantly different between treated and control groups at any time during the experiments. Twelve hours after the infusion began bile flow rates, bile BSP concentrations, and biliary BSP excretory rates were stable in both groups and are presented for comparison in Table 6. The concentrations of BSP in the bile of both groups reached their highest levels at this time and remained at
Figure 11. Percent of a single dose of BSP (10.0 mg/kg) appearing in the liver of trout 15, 30, 60, and 120 min after injection in control fish or fish treated with CCl₄ 24 h earlier. Each point represents the mean ± S.E. of five animals. Asterisks denote values which are significantly different (P < 0.05) from controls.
The graph shows the percent of administered BSP (Bilirubin Succinyl Sulfonate) over time (in minutes) for two different groups: CCl₄ and Control. The CCl₄ group is indicated by the filled triangles, and the Control group by the filled circles. The time points are marked at 15, 30, 45, 60, 75, 90, 105, and 120 minutes. The data points are connected by lines, and error bars represent the standard deviation. Asterisks indicate statistically significant differences between the groups.
these levels for the duration of the experiment.

Table 6. Bile flow, bile BSP concentration and rate of biliary BSP excretion 12 h after beginning infusion of BSP in control fish and fish receiving CCl₄ (2.0 ml/kg ip) 36 h earlier. Values are the mean ± SE of the number of animals in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Bile Flow (µl/kg/min)</th>
<th>Bile BSP (mg/ml)</th>
<th>Biliary BSP Excretion (µg/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (5)</td>
<td>1.24 ± 0.35</td>
<td>11.8 ± 1.84</td>
<td>12.1 ± 2.49</td>
</tr>
<tr>
<td>CCl₄ (3)</td>
<td>0.67 ± 0.19</td>
<td>11.0 ± 1.76</td>
<td>6.8 ± 2.49</td>
</tr>
</tbody>
</table>

The mean rates of biliary BSP excretion in control and intoxicated fish were not significantly different at any time during the course of the infusion (Figure 12). However, when these rates were integrated over the 15 h infusion period, the total amount of BSP excreted in the bile was estimated to be 8.36 mg/kg and 6.88 mg/kg in control and treated animals respectively. The apparent decrease in the rate of biliary BSP excretion in treated animals after 11 h was due to a decrease in the rate of bile flow rather than to decreases in the concentrations of BSP in the bile. The bile flow rates in both groups of animals declined during the infusion period. Bile flow in control animals dropped approximately 35% from 1.92 µl/kg/min to 1.24 µl/kg/min over a 7 h period and this lower rate was maintained for the remainder of the experiment. Over a similar 7 h period bile flow
Figure 12. Biliary excretion of BSP in control trout and trout treated with CCl₄ (2.0 ml/kg) 24 h prior to the start of BSP infusion. Time on the abscissa corresponds to time after the beginning of infusion. Each point is the mean ± S.E. of the number of animals in parentheses.
BSP Excretion (µg/kg/min) vs Time (hrs)

- 20 µg/kg/min
- 40 µg/kg/min
- 60 µg/kg/min
- 5.0 mg/kg i.v.

**CCl₄**

**Control**

(3) 20 pg/kg/min
(4) 40 pg/kg/min
(5) 60 pg/kg/min

Time (hrs): 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
in treated fish dropped 64% from 1.97 μl/kg/min after 5 h to 0.7 μl/kg/min at 12 h. This bile flow was maintained for the remainder of the experiment. The peak sustained rate of biliary BSP excretion was considered to be the biliary transport maximum (Tm) for the dye. This value was estimated to be 12.2 μg/kg/min in control fish; however, it was not possible to demonstrate a sustained rate of biliary BSP excretion in animals receiving CCl₄ due to the variable rates of bile BSP excretion (Figure 12).

**BSP and Metabolites in Plasma, Liver, and Bile**

Chromatography of plasma and liver extracts and bile indicated that the separable fractions of BSP were qualitatively similar in treated and control fish. A representative chromatogram is illustrated in Appendix A-I. A single band was found on chromatograms of plasma extracts that did not react with ninhydrin and migrated with mobility similar to that of the plasma standard. Two BSP fractions were present on chromatograms of liver extracts; the fastest (I) of which did not react with ninhydrin and had Rf values similar to those of the liver BSP standard. The slowest migrating fraction (II) did not appear on chromatograms of the liver BSP standard, reacted with ninhydrin and was assumed to be an amino acid conjugate of the dye. The relative amount of the dye in this fraction did not vary
significantly between experimental groups when samples were taken at the same time or among the same group when sampled at different times. The mean percent of metabolized BSP which was present in the liver extracts of control animals represented 19.6% of the total amount of BSP and ranged from 18.5% to 20.5%, while in treated animals the mean value was 18.9% of the total BSP and ranged from 17.6% to 21.0%.

The number of separable fractions of BSP in the bile of all fish increased with the time of infusion and qualitative differences were not evident in BSP metabolite patterns between treatment groups. A representative chromatogram of biliary BSP metabolites is illustrated in Appendix A-II. Individual bands appeared in the bile in the order I, IV, VI, V, III, and II. The Rf values of individual fractions were relatively consistent when the same bile sample was separated on different TLC strips or when bile samples from different fish were chromatographed on the same strip. The BSP fraction which demonstrated the greatest mobility (I) had an Rf value similar to that of the bile standard, did not react with ninhydrin and therefore was assumed to be unconjugated BSP. The three fractions that had the lowest mobility (IV, V, VI) reacted with ninhydrin while the two fractions of intermediate mobility (II, III) did not. None of the BSP fractions reacted with aniline diphenylamine and therefore were probably not associated with carbohydrates.
Prolonged infusion of BSP resulted in a steady increase in the proportion of metabolized dye which appeared in the bile of both treated and control fish (Figure 13). The relative amount of metabolized BSP increased by 32% in the bile of CCl$_4$ treated fish and by 39% in the bile of control animals between 1 and 14 h after dye infusion began. The bile of CCl$_4$ treated animals contained a slightly higher proportion of metabolized dye throughout the infusion period than did that of control animals; however, these were not significant differences. Some error may be associated with these percentage estimates because it has been assumed that the extinction coefficients of metabolized BSP in trout bile were similar to that of the unconjugated dye. While there appears to be no experimental evidence to support this assumption for BSP metabolites in trout bile, Combes (1965) and Whelan et al. (1970) have reported that the extinction coefficients of the major BSP metabolites in rat bile are similar to free BSP.

Discussion

As in mammals, significant plasma retention of BSP is evident in rainbow trout following CCl$_4$ intoxication (Chapter III). While it appears that some form of functional liver damage has occurred in the trout, it is not clear which of the processes responsible for hepatic disposition of the dye have been affected. In the present study after a
Figure 13. Percent of total biliary BSP appearing as metabolites in bile of control fish or fish treated with CCl₄ (2.0 mg/kg) during continuous graded infusion of BSP. Time on abscissa represents time after the start of infusion. Each value represents the mean ± S.E. of the number of animals in parentheses.
Percent Conjugated BSP (in bile)

- CCl₄
- Control

Time (hrs)

20 µg/kg/min  40 µg/kg/min  60 µg/kg/min  5.0 mg/kg i.v.
single dose of BSP the rate of its appearance in the liver was much slower in animals receiving CCl₄ than in control animals and yet maximum hepatic concentrations of the dye were similar in both groups of animals. These results may be explained by assuming that CCl₄ has affected processes associated with either 1) hepatic uptake and storage of the dye, 2) biliary excretion of the dye, or 3) transport of the dye to the liver.

The accumulation of more than half of the dose of BSP in the livers of control animals 15 min after injection indicates that the initial rapid loss of the BSP from the plasma compartment is primarily the result of its hepatic uptake and accumulation. In contrast, the hepatic BSP content of animals receiving CCl₄ was less than half of that of control animals after 15 min. Since it can be assumed that only minimal amounts of dye have been excreted into the bile at this time in both groups of animals, it appears that differences in the hepatic BSP content are not due to differences in rates of biliary excretion. Furthermore, the capacity of the livers of treated animals to take up and store the dye seem to have been exceeded by the dose of the dye that was given (10.0 mg/kg) since liver BSP concentrations continued to increase until at least 60 min even though plasma concentrations of the dye declined throughout the experimental period. In contrast, highest BSP content was found in the livers of control animals 15 min after injection of the dye and thereafter both plasma
and liver concentrations declined. Thus, in spite of consistently higher plasma BSP concentrations, the rate of hepatic dye accumulation in treated animals was much slower. These results suggest that CCl₄ intoxication caused impairment of the processes of hepatic dye uptake and accumulation. Maggio and Fujimoto (1966) reached a similar conclusion in their studies with mice. They showed that 24 h after receiving CCl₄ the hepatic concentrations of BSP were consistently lower than those of control animals even though plasma concentrations of the dye were higher. It has been suggested by others (Klaassen and Plaa, 1968) that the decrease in hepatic BSP concentrations reported by Maggio and Fujimoto was due to decreased biliary excretion of the dye, which is known to occur in rats following treatment with CCl₄ (Klaassen and Plaa, 1968; Priestley and Plaa, 1970). Decreasing biliary excretion of BSP does not appear to be a factor in the present studies since the hepatic excretory function in trout receiving CCl₄ was similar to that of control animals for at least 34 h following CCl₄ treatment.

That similar maximum hepatic concentrations of BSP were found in treated and control animals is not inconsistent with the view that some aspect of uptake or storage has been affected by CCl₄ intoxication. Liver BSP concentrations represent not only dye that has been taken up and stored by the hepatocytes, but also includes dye that has been excreted into the intrahepatic biliary space. After
60 min a considerable portion of the dye that was found in liver extracts of treated animals may have been contributed from dye within the canaliculi and ductules. The apparently unimpaired biliary excretory mechanism in treated animals would tend to support this view.

Surgical impairment of hepatic blood flow by hepatic portal ligation decreases both the rates of plasma clearance and the liver accumulation of BSP in rainbow trout (Chapter 1). Studies by Eckhardt and Plaa (1963) also have shown that administration of vasoactive compounds which increase hepatic resistance can reduce plasma clearance of BSP by reducing hepatic blood flow. It is possible that a decrease in hepatic blood flow in the trout following CCl₄ intoxication may be responsible for differences in the distribution of BSP between liver and plasma by impairing transport of dye to the liver. Lautt and Plaa (1974) have shown that there is no change in the total hepatic blood flow in intact cats 24 h after oral administration of CCl₄, even though a slight increase in hepatic arterial blood flow was observed. While the effects of CCl₄ intoxication on hepatic blood flow were not evaluated in trout used in this study, the possibility that this factor may have influenced these results should be recognized.

Even though the biliary excretory maximum for BSP was not established in animals receiving CCl₄, defects in the hepatic
excretory process should be apparent with doses of BSP that do not exceed the excretory maximum if such defects are having a major affect on plasma dye clearance. My results indicate that bile flow rates, percent of total metabolized BSP appearing in the bile and total bile BSP concentrations were similar in treated and control fish until at least 34 h after CCl₄ treatment. These results are not consistent with those reported for rats treated with CCl₄. Several studies have established that decreases in bile flow, bile BSP concentration, and hepatic BSP-glutathione conjugating activity are primarily responsible for plasma dye retention in these animals following CCl₄ induced liver injury (Klaassen and Plaa, 1968; Priestly and Plaa, 1970). Even though the hepatic excretory function in trout was not impaired early in the course of CCl₄ intoxication, these results do not exclude the possibility that components of this system are not affected at a later time. The decrease in biliary excretion in trout 12 h after the start of BSP infusion was apparently due to decreased bile flow. If bile secretion in rainbow trout was impaired by latent effects of CCl₄ intoxication one would expect to find evidence of a progressive increase in plasma dye retention. This does occur since maximum plasma retention of BSP is not apparent in trout receiving CCl₄ for at least 48 h after the toxicant has been administered (Chapter III). It is therefore possible that defects in the processes of uptake, accumulation, and biliary excretion may simultaneously contribute to
plasma retention of the dye late in the course of intoxication. Evaluation of components of hepatic excretory function 48 h after CCl₄ treatment would help to resolve this point.

The general decrease in the rate of bile flow in both treated and control animals following prolonged infusion of BSP above the excretory maximum has been reported in similar experiments in both rainbow trout (Schmidt and Weber, 1973) and rats (Schulz and Czok, 1974). While it is likely that this decrease is due to surgical stress or bile salt depletion, recent studies by Schulz and Czok (1974) indicate that elevated levels of unconjugated BSP may partially contribute to the decrease in bile flow in rats. It has been suggested that high levels of unconjugated BSP in the liver may act to reduce bile flow by reducing the ATP content of the liver since BSP can competitively inhibit the translocation of phosphate and adenosine diphosphate into the liver mitochondria (Laperch and Odea, 1976). The relatively high levels of unconjugated BSP which were found in liver extracts from trout (approximately 75%) would not exclude this as a possible explanation of decreased bile flow in these animals after prolonged infusion of BSP.

The relative importance of the conjugating mechanism to the overall process of biliary BSP excretion in fishes has not been established. If dye conjugation was the important prerequisite for this process in fishes that it appears to be in mammals (Whelan et al.,
1970; Priestly and Plaa, 1970b), a much higher proportion of metabolized dye would be expected in their bile. The relative proportion of metabolized BSP initially present in the bile of control and treated fish was approximately 25% of the total dye concentration of the bile. While this value is nearly twice those which have been reported in the bile of two species of cartilaginous fishes (Boyer et al., 1976a), it represents only one-third of the amount of conjugated BSP which appears in the bile of rats (Whelan et al., 1970; Schultz and Czok, 1974). Furthermore, it appears that the rate of bile secretion may be most responsible for the overall process of biliary BSP excretion in fish (Chapter II). Thus, even if CCl₄ intoxication had decreased the BSP metabolizing activity of the liver it is unlikely that this would influence biliary excretion sufficiently to cause plasma retention.

The apparent increase in the percent of metabolized BSP which was found in the bile of control and treated fish following prolonged infusion of the dye was not expected. Infusion of BSP above the biliary Tm in rats results in a decrease in the relative amount of glutathione conjugate and an increase in the relative amount of free BSP appearing in the bile (Schulz and Czok, 1974). The increased proportion of metabolized BSP in trout bile may be due to anomalies of hepatic blood flow which do not permit immediate and uniform distribution of the dye to all sinusoidal surfaces or it may be due to incorporation of minor pathways of BSP metabolism after major
pathways have become saturated. Identification of the separable fractions of BSP in trout bile would prove useful in understanding more fully the nature of the processes responsible for biliary excretion of this dye by trout.

The use of BSP tests as a tool in the clinical diagnosis of liver dysfunction in trout may be useful providing that the limitations of the techniques are recognized. Thus, the measurement of hepatic BSP concentrations must be interpreted not only in terms of the processes of uptake and accumulation of the dye, which are not differentiated by this method of analysis, but also in terms of hepatic excretory function. Further, the techniques which have been developed to measure hepatic excretory function and hepatic storage capacity in small mammals (Klaassen and Plaa, 1967) do not seem practical in trout because of the toxic affects of high BSP plasma concentrations (Schmidt and Weber, 1973) and the length of time necessary to establish a maximal rate of biliary BSP excretion.
V. SUMMARY AND CONCLUSIONS

These studies have attempted to determine whether liver function in fish can be adequately evaluated by methods based on the plasma clearance of the organic anion sulfobromophthalein (BSP). In the first study the dependence of plasma BSP clearance on hepatic elimination was established. The data indicate that, as in mammals, hepatic uptake, accumulation, and biliary excretion are primarily responsible for plasma BSP clearance in the trout. Apparent differences in these processes between the trout and mammals based solely on relative body weight, are greatly reduced when anatomical and physiological differences are recognized.

In the second study, plasma BSP clearance was evaluated as a method by which to estimate functional liver impairment. Carbon tetrachloride ($\text{CCl}_4$) intoxication resulted in hepatic lesions in areas surrounding central veins in several fish which were not unlike those that occur in the mammalian liver following treatment with this toxicant. Rates of plasma BSP clearance in treated animals could be correlated with the dose of toxicant and were not greatly affected by high plasma concentrations of endogenous substances such as hemoglobin or bilirubin.

In the last study the effect of $\text{CCl}_4$ intoxication on specific hepatic processes associated with plasma BSP clearance was evaluated.
To determine which of these processes were most affected by CCl₄ treatment, the rates of hepatic accumulation and biliary excretion of BSP were estimated in treated and control animals. In addition, the relative amounts of metabolized BSP also were determined in liver and bile samples from both groups of animals. The data indicate that processes of biliary excretion, including bile secretion and BSP metabolism are not reduced 24 h after CCl₄ treatment. Impaired rates of hepatic BSP accumulation suggest that the processes of uptake and storage are probably most affected by the intoxication. The impairment of these processes in trout probably contributes most to the plasma BSP retention following CCl₄ treatment.

The results of these studies indicate that tests of liver function based on plasma BSP clearance are practical in fish under controlled laboratory conditions. While the test appears to be a sensitive indicator of liver dysfunction, plasma clearance may be altered by factors other than liver impairment. Increased plasma BSP retention may be the result of competition for some aspect of hepatic disposition with another compound or by reduction in hepatic blood flow. Provided that the general limitations of this test are recognized, this method should prove useful in assessing functional liver damage in fish.


APPENDIX
APPENDIX A-I

Figure 1. Representative chromatogram of BSP from extracts of plasma and liver of standards, control fish or fish receiving CCl$_4$ 24 h earlier.
APPENDIX A-II

Figure 2. Representative chromatograms of BSP appearing in the bile of rainbow trout during prolonged infusion of BSP. Time on abscissa corresponds to time after start of BSP infusion.
Bile BSP

Solvent front

Time (hrs)

Ninhydrin Reaction

+ +

Std. 1 2 4 6 8 10 12 14

R_f

.8

.6

.4

.2

Origin

I II III IV V VI