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Assessment of Clinical Procedures to Evaluate Liver Intoxication in Fish

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ASSESSMENT OF CLINICAL PROCEDURES TO EVALUATE LIVER INTOXICATION IN FISH

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FOREWORD

The ability to generalize from the results of research on specific environmental toxicants and test organisms to classes of toxicants and organisms depends to a large extent on knowledge of the mechanism of toxicant action at the physiological level and the comparison of these mechanism access taxonomic lines. The research reported here evaluates the effect on liver function in the rainbow of several model liver toxicants used in mammalian tests, as measured by existing clinical diagnostic tests of liver dysfunction.

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ABSTRACT

Procedures were developed to clinically evaluate liver damage and liver function in rainbow trout following either acute intraperitoneal (i.p.) treatment or subacute bath exposure to selected mammalian hepatotoxic agents. Elevations in serum of liver specific enzymes such as aspartate aminotransferase (GOT), alanine aminotransferase (GPT) and alkaline phosphatase (AP) were investigated as potential indicators of hepatocellular damage. An exogenous test of liver function, plasma clearance of the organic anion sulfobromophthalein (BSP), also was investigated as a potentially useful test of overall liver function in the trout.

Histological damage was apparent in the livers of rainbow trout treated by i.p. injection with either carbon tetrachloride (CCl_4 or monochlorobenzene (MCB), however this damage could not be correlated consistently with indications of liver injury suggested by either the endogenous or exogenous tests of liver damage. Total plasma protein concentration decreased only in response to CCl_4 treatment but GPT activity in the plasma was significantly increased following treatment with both toxicants. Results also suggested that dietary factors may have influenced the response of fish to treatment by CCl_4. Plasma clearance of BSP was impaired following acute treatment with both toxicants.

Unlike acute i.p. treatment with MCB, treatment with CCl_4 resulted in significant weight gain in rainbow trout that was related to water retention. Anuria or severe oliguria with attendant proteinuria was evident as early as one hour after treatment and persisted for at least 24 h. Histological examination of the kidney revealed inconsistent damage to the proximal tubules of some treated fish. No detectable lesions were observed in the kidney of any fish earlier than 36 h after treatment indicating that direct effects of CCl_4 on the kidney probably were not responsible for the altered urine flow rates observed early in the course of the intoxication.

In a separate study trout were exposed continuously to two sublethal concentrations of monochlorbenzene (2.6 and 3.9 ppm) for 15 and 30 days. Fish treated with both concentrations of toxicant were anorexic during the first 15 days of exposure and a dose-related weight loss was observed in treated fish when compared to their paired controls. Total serum protein concentration was decreased and activity of serum GPT was increased in a dose-dependent manner at this time. Serum BSP concentrations in treated and non-fed control fish were similar but were significantly greater than BSP concentrations in the serum

of a separate group of fed control fish suggesting that food deprivation might greatly influence the disposition of this compound by the trout. Fish at both toxicant concentrations accepted food again between 15 and 30 days and appeared to tolerate better their exposure during this time period. After 30 days of exposure serum activities of GPT and AP from treated fish were elevated but were not significantly different from their paired controls. The impaired plasma clearance of BSP in treated fish observed at this time could not be related to MCB exposure.

The application of clinical tests to diagnose liver dysfunction in fishes following their exposure to environmental toxicants may be practical in controlled laboratory facilities Despite the considerable variation that exists between groups of fish, significant differences could be demonstrated between control and treated fish. Variation among groups of fish make intergroup comparison of the field populations increasingly difficult by these methods. Therefore, the use of such techniques should be employed to evaluate liver toxicity under precisely controlled laboratory studies. Their application to field studies does not seem advisable.

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SECTION I

INTRODUCTION

Biologists now realize the need for technical capabilities to measure the sublethal effects on aquatic organisms resulting from exposure to water-borne environmental toxicants. Short-term exposure tests designed to estimate the median tolerance limit (TLm) of chemicals to aquatic life yield little understanding of the effects of chronic exposure to low concentrations of these materials. On the other hand, while long-term exposure studies may be generally useful in establishing bioaccumulation rates and pharmacokinetic properties of certain toxicants in aquatic species, little is understood of how best to detect the subtle changes in physiological processes which result in the intoxication of the animal.

The application of existing diagnostic tests of organ function to the field of aquatic toxicology should shorten the search for fine indicators of pollutant-induced physiological dysfunction in fishes. Before this technology can be applied successfully to fishes however, a more fundamental understanding of specific physiological processes in these animals is essential. This understanding provides the investigator with a point of reference from which to compare and contrast similar physiological functions between divergent species and, in so doing, to identify appropriate tests that may find useful application in fisheries research.

The liver, one of the vital organs of a fish, has received limited study in this regard, even though morphological damage to this organ is most consistently reported in fish which have been exposed to a variety of halogenated hydrocarbons (Johnson, 1968). Because the liver is physiologically important to fishes for the maintenance of their metabolic homeostasis, this organ may be particularly sensitive to long-term sublethal exposure to a variety of toxicants. For this reason the development of techniques to evaluate liver function in fishes may be especially useful in detecting subtle pollutant-induced changes in physiological function.

Clinical diagnostic tests of liver dysfunction generally have been grouped into two broad categories, endogenous tests and exogenous tests. Endogenous tests usually require the measurement of specific enzymes in the plasma or serum or the estimation of serum concentrations of other biological chemicals such as bilirubin that are routinely handled by the liver. These biological chemicals generally increase or decrease in the serum when liver injury occurs. Exogenous tests usually involve the prior administration of an exogenous material that is specifically taken up or metabolized by the liver. Examples

of these tests are plasma clearance of liver specific dyes such as sulfobromophthalein (BSP) or the conjugation by the liver of exogenous benzoic acid with glycine and its subsequent urinary excretion as hippuric acid. The latter tests generally measure the true function of the organ in that the animal is given a quantity of material and the ability of the liver to process this load is evaluated.

In the present studies we have evaluated both endogenous and exogenous liver function tests in a representative cold water fish species, the rainbow trout (Salmo gairdneri). Endogenous tests used in these investigations included determination of changes in the serum activity of the enzymes alanine-aminotransferase (SGPT) and aspartate-aminotransferase (SGOT) and alkaline phosphatase (AP). Serum elevations in the activity of the two former enzymes are useful indicators of necrotic processes in the liver of mammals while elevations in the serum activity of the latter generally are associated with either an intra or extrahepatic pathological process (Plaa, 1968). The exogenous test evaluated in these studies was plasma clearance of the anionic dye sulfobromophthalein (BSP). Aspects of the functional capacity of the liver to transport this dye from the plasma to the bile also were studied.

In order to assess the usefulness of these tests in identifying liver dysfunction, trout were treated by intraperitoneal (i.p.) injection with two chemicals, carbon tetrachloride (CCl_4) and monochlorobenzene (MCB). Both of these agents produce consistent necrotic hepatotoxicity in mammals, including man, and in this respect they are commonly used to model liver toxicity in experimental animals (Raisfeld, 1974). In some studies a third model liver toxicant, alpha-napthylisothiocynate (ANIT), was used to produce liver dysfunction in the trout. This agent is used to model intrahepatic cholestasis in laboratory mammals (Plaa and Priestly, 1977). Finally, liver function tests were evaluated in a group of fish exposed to subacute concentrations of monochlorobenzene in the water for 15 and 30 days.

SECTION 2

RECOMMENDATIONS AND CONCLUSIONS

1) The tests used in these studies to clinically evaluate liver dysfunction in rainbow trout can be successfully applied to carefully controlled laboratory experiments. The variability in the values for these clinical tests between and among the different groups of test fish used in these studies suggest that their application to field studies would be impractical.

2) The evaluation of liver function through endogenous indicators such as change in the serum activity of liver specific enzymes was the most discriminating indicator of liver toxicity in trout used in acute and subacute exposure studies. Serum or plasma elevations in the activity of alanine aminotransferase were consistently observed following exposure to the model mammalian hepatotoxic agents carbon tetrachloride and monochlorobenzene. Furthermore, unlike serum aspartate amino transferase activity, alanine aminotransferase activity was not influenced by high concentrations of serum hemoglobin that resulted following acute exposure of trout to either carbon tetrachloride or monochlorobenzene.

3) Diagnosis of hepatic dysfunction in rainbow trout using serum alkaline phosphatase activity as a clinical indicator of bile stasis does not appear to be feasible. No differences in the serum activities of alkaline phosphatase were evident following either acute extrahepatic cholestasis created by ligation of the common bile duct and cystic duct or by treatment with the mammalian cholestatic agent alpha-napthylisothiocynate. Increased serum alkaline phosphatase activity was apparent in fish fed a high ration suggesting that diet may greatly influence the serum activity of this enzyme.

4) The plasma clearance rate of the organic anion sulfobromophthalein (BSP) can be used as a sensitive test of liver dysfunction in rainbow trout following their acute exposure to toxicants. This test appears to provide a direct method of detecting liver dysfunction in the trout, however it is relatively non-specific and may be influenced by factors not related to the liver. Our studies indicate that food deprivation can significantly influence the results of this clearance test in trout. Therefore it is recommended that this test not be used in long term studies where food consumption is reduced or lacking. Furthermore, the method requires a modest amount of technical expertise in the injection of the dye before it can be used successfully. The difficulties associated with this test may preclude its routine use as a diagnostic aid.

SECTION 3

MATERIALS AND METHODS

HOLDING FACILITIES AND CULTURE TECHNIQUES

Yearling rainbow trout (50-70 gm) were purchased from Roaring River fish hatchery, Scio, Oregon and transported to the Smith farm hatchery facilities of the Department of Fisheries and Wildlife where they were held until of a proper size for use in experiments (100-450 gm). Fish were held at densities of 25 kg per tank in 6000 liter circular tanks supplied with a continuous flow of well water (35 l/min). The well water temperature was constant at 13°C, the pH was 7.3 and the total alkalinity of the water as calcium carbonate was 80 mg/l. Other fish used in some experiments included Skamania River steelhead trout (Roaring River fish hatchery), kokanee salmon and brook trout. These fish were held at Smith farm under conditions similar to those described for rainbow trout.

Prior to use in experiments fish were transferred to laboratory holding facilities at either Nash Hall on the OSU campus or at Oak Creek Laboratory and acclimated to these facilities for at least one week. Fish taken to Nash Hall were held in a constant temperature room in 130 l aquaria supplied with continuously flowing (3 l/min) dechlorinated tap water (12.0°C ± 0.5°). A 12 h light:12 h dark photoperiod was maintained constantly. Fish were held at Oak Creek Laboratory in 500 l aquaria supplied with a continuous flow (10 l/min) of well water (11°C or 15°C). The photoperiod in the laboratory was adjusted every other week to conform to the natural photoperiod.

During the course of these studies diets from several sources were fed to the fish. In general, however, individual groups of fish were fed only one diet. Diets used in these studies included Purina Trout Chow (Ralston-Purina, St. Louis, Mo.), Donaldson Diet (Ore-Aqua, Inc., Newport, Oregon) and Siever Cup Diet (Murray Elevators, Murray, Utah). In all instances fish were fed a diet estimated to be slightly above their maintenance requirements.

DEVELOPMENT OF CLINICAL PROCEDURES TO EVALUATE LIVER TOXICITY IN RAINBOW TROUT

Endogenous Tests

Plasma and Serum Enzymes--

Alanine Aminotransferase (GPT) and Aspartate Amino Transferase (GOT) activities were determined in the liver and plasma from rainbow trout.

Fish were fed Purina Trout Chow daily but food was withheld the day before sampling. Blood was collected with tuberculin syringes, previously rinsed with sodium heparin, and deposited into small glass test tubes that had been rinsed with 10% potassium oxalate. Livers were removed and held on ice until homogenized.

The activity of GPT in kidney and liver tissue was compared in a separate group of fish acclimated to 15°C ± 1.0° and fed Purina Trout Chow daily. Liver and kidney tissues were removed and held on ice until homogenized.

The activities of GPT and GOT from the plasma and livers of rainbow trout, kokanee salmon and brook trout fed Donaldson Diet were compared. In another study a comparison of plasma and liver activities was made between steelhead trout held under laboratory conditions for 60 days or held in a fine meshed cage for 60 days in the Willamette River. Both groups of animals were fed Purina Trout Chow throughout the study. Liver and blood samples were collected as previously described.

A Gilford model 2400 recording spectrophotometer fitted with a Haake constant temperature recirculating water bath was used for all clinical assays. A constant temperature of 25°C was maintained in all cases except where otherwise indicated. Reagents were purchased from Sigma Chemical Co., St. Louis, Mo. and ammonia-free lactate dehydrogenase was obtained from Boehringer-Mannheim, San Diego, Calif. Whenever possible the procedures, concentrations and volumes as described in the appropriate Sigma Technical Bulletin were used for these tests.

Plasma or serum alanine aminotransferase (GPT) activities were measured by the method of Wroblewski and LaDue (1956), however tissue GPT assays were modified to accommodate the presence of glutamate dehydrogenase (GDH) as described by Bergmeyer and Bernt (1974). The final reaction mixture for GPT assays contained 2.0 ml NADH, 0.1 mg/ml; 0.1 ml lactate dehydrogenase, 28.8 µM/ml in 50% ammonia-free glycerol; 0.5 ml alanine, 0.4 M in 0.35 M phosphate buffer, pH 7.5; 0.2 ml alpha-ketoglutarate, 0.1 M in 0.1 M phosphate buffer, pH 7.5; and 0.2 ml of serum or diluted tissue homogenate. The enzyme reaction was initiated with alpha-ketoglutarate (AKG) after ensuring that the preliminary reaction had depleted extraneous substrates. The loss of absorbance at 340 nm was recorded for 5 min.

The GPT activities of blood and tissue were determined on the day that fish were sampled even though preliminary experiments indicated that serum GPT activity was stable for at least one week if the sample was stored under refrigeration (4°C). Tissue GPT activities were measured in the liver and middle or trunk portion of the kidneys. Whole organs were homogenized (10% u/v) in 0.25 M ice cold sucrose buffered at pH 7.4 with 0.05 M Tris-HCl, 0.025 M KCl, and 5mM $MgCl_2$ (TKM buffer, Cousins, et al., 1970). The homogenate was centrifuged for 15 min (600 xg; 0-4°C) to remove blood cells and cellular debris. The supernatant was used directly to determine the alanine-Km but was diluted five fold (1:4) with sucrose-TKM buffer for standard assays of GPT activity. The concentration of AKG used for the alanine-Km

experiments (7mM) was 10-20 times the known Km value for AKG in human plasma or pig heart GPT activities (Bergmeyer, 1978).

Plasma, serum and tissue (GOT) activities were determined by the method of Karmen (1955) and modified in a manner similar to that for GPT assays to account for the presence of endogenous GDH activity. Sample preparation and tissue handling techniques were the same as described for GPT analyses. The final reaction mixture for the GOT assay was 2.0 ml NADH, 0.1 mg/ml; 0.1 ml lactate dehydrogenase, 28.8 μ M/ml in 50% ammonia-free glycerol; 0.5 ml aspartate, 0.4 M in 0.35 M phosphate buffer, pH 7.5; AKG, 0.1 M in 0.1 M phosphate buffer; pH 7.5; and 0.2 ml of serum or diluted tissue homogenate.

Alkaline phosphatase (AP) activities were determined by modifying the method of Bessey et al., (1946) to accommodate the use of 2-amino-2-methyl-1-propanal buffer (1.5 M, pH 10.3). The final reaction mixture consisted of 1.0 ml buffer, 1.0 ml substrate solution (p-nitrophenyl-phosphate, disodium salt $6H_2O$, 0.4 mg/ml), and 50 μl of serum or diluted tissue homogenate. The buffer and substrate were mixed in a quartz cuvette, equilibrated to assay temperature, and the enzyme source added. Enzyme activity was determined by recording the change in absorbance at 410 nm and comparing this with a previously established calibration curve. Serum AP activity was stable at least 2 weeks when the samples were stored under refrigeration. Liver tissue was homogenized in chilled 0.15 M KCl (1:10, w/v) and centrifuged for 15 min. The resulting supernatant was used directly for protein determination and diluted (1:3, u/v) with 0.15 M KCl for determination of AP activity.

The influence of acute extrahepatic biliary obstruction on the serum activity of AP was determined in one group of five trout. Fish were prepared by ligation of the cystic duct and common bile duct and a blood sample was drawn from each animal after 3, 24, 48 and 72 h. In a second experiment the effect on a mammalian cholestatic agent (alpha-napthylisothiocynate, ANIT) was determined on the activity of AP in the serum in rainbow trout. A group of five fish was given ANIT 400 mg/kg i.p.) dissolved in salmon oil and after 3, 6 and 24 h a single blood sample (0.5 ml) was drawn from each fish by cardiac puncture. Control fish received an equivalent volume of salmon oil.

Other Endogenous Tests--

Plasma, serum and tissue protein concentration were determined by the biuret reaction (Gornall, et al., 1949) using Sigma kit no. 540. Volumes were adjusted to use 1.0 ml biuret reagent and 0.02 ml sample. A reagent blank was included with each protein determination to account for erroneously high protein concentrations resulting from the influence of turbidity in tissue samples or from increased absorbance due to high concentrations of bromosulfophthalein (BSP) in the sample. Serum albumin concentrations were measured by the bromocresol green method (Doumas and Biggs, 1972) as described in Sigma Technical Bulletin No. 630.

Serum proteins were separated by electorphoresis on cellulose acetate strips. Serum (3 μl) was applied to each strip, placed in an electrophoresis chamber containing 0.5 M tris-sodium barbital buffer (pH 8.8) and the separation was conducted for 20 min (4 milliamp per strip, 180 Vdc). Strips were stained with Ponceau S and then scanned, traced and the resulting peaks integrated using a scanning densitometer. A linear relationship was assumed for all protein bands between color intensity and protein concentration.

Serum hemoglobin concentrations were measured as cyanmethemoglobin using 0.5 ml of modified Drabkins solution (Richterich, 1969) and 50 μl of serum. Because intra-peritoneal administration of both carbon tetrachloride (CCl_4) and monochlorobenzene (MCB) resulted in increased intra-vascular hemolysis, serum hemoglobin values were used to correct for total serum or plasma protein concentrations as indicated in Sigma Technical bulletin 540. The concentration of bilirubin in the plasma was determined by the diazotization procedure of Malloy and Evelyn (1937).

Disposition of BSP by Rainbow Trout--

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 rates 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 fish (Schmidt and Weber, 1973). After immobilization, animals were weighed and placed into individual troughs of a plastic coated wire frame support within a plexiglas aquarium and allowed to recover at least 18 h.

Plasma clearance and hepatic accumulation of BSP were determined in two groups of five fish (220-290g). In experiments requiring timed serial sampling of blood from a single fish, a cannula 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 cannula consisted of PE tubing of known volume (50 μl). The shaft of a 23 gauge needle was attached to one end with the hub of the needle fitted to the other end. A suture in the caudal peduncle secured the cannula to the fish. A solution of BSP in physiological saline (either 5.0 or 10.0 mg/kg) was injected by the caudal vein cannula and 0.2 ml blood samples were taken 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 of BSP was estimated from the slope of a line visually fit to a plot of the points of the log of plasma BSP concentration vs time. The fractional turnover rate (f_t) of BSP was c = 0.693/T 1/2 where T 1/2 is the plasma half life of BSP in min and 0.693 is ln of 1/2.

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 puncture, 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.

The effects of impaired hepatic blood flow or bile flow on plasma clearance and hepatic accumulation of BSP were determined in three groups of five fish after preparation by the following surgical treatments. The cystic ducts and common bile ducts of the fish in the first group were ligated with 5-0 silk sutures. In the second group the cystic duct, common bile duct and hepatic portal vein were ligated. Sham surgery involving isolation of the ducts and vessels without ligation was performed in the fish of the third group. The incisions were closed with 4-0 surgical silk sutures and the trout were allowed a 10 h recovery period. Surgically prepared fish were used in experiments to determine either plasma clearance or hepatic accumulation of BSP as previously described.

To determine the rate of biliary excretion of a single dose of BSP the common bile duct was cannulated with PE 10 tubing of known volume (40 µl) and the cystic duct ligated (Schmidt and Weber, 1973). Bile duct cannulation was accomplished in anesthetized trout that had been fasted for at least 18 h. The fish was placed in dorsal recumbancy on a V-shaped trough positioned such that the head and gills were immersed in free flowing water. An incision (5 cm) was made along the linea alba and the abdomen retracted with butterfly forceps. The liver was identified at the anterior center of the incision and the gall bladder and common bile duct generally were found in the posterior right quadrant of the liver. Viscera were retracted to the left and the cystic duct was identified and ligated with a 5-0 silk suture. The common bile duct was isolated from the surrounding vasculature by careful blunt dissection with glass probes and exposed. Two ligatures of 4-0 silk were positioned along the bile duct, one proximal and one distal to the liver. A lateral incision was made in the common bile duct between the two ligatures with iris scissors and a length of polyethylene tubing (PE 10) was inserted through the incision toward the liver. The tubing was secured to the common bile duct by the two ligatures. The catheter was passed out through the posterior end of the incision and was fixed to the skin with 5-0 silk sutures. The incision was closed with 5-0 silk sutures and the animal allowed to recover for 12 h. With practice this procedure could be accomplished in 30-40 min.

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 cannula by a collar of PE 50 tubing. Bile flow rates were determined by recording the progress of the bile in the collecting cannula. 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. The biliary transport maximum (T_m) for BSP and metabolites was determined in spinal transected trout in a manner similar to that described by Schmidt and Weber (1973). These fish received 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 trout received a single dose of BSP (5.0 mg/kg) by the caudal vein to insure that the excretory capacity of the liver for BSP had been exceeded. Bile was collected into a length of PE 90 tubing as described earlier. Bile flow rates were determined every hour by recording the progress of the bile in the collecting cannula. Collecting cannulae were changed after 6, 12, and 15 h to prevent longitudinal mixing of the BSP in the tubing. No attempt was made to replace bile salts lost during these experiments.

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 nm on a Beckman DB spectrophotometer and converted to units of concentration by comparison with BSP reference standards. 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 ($\pm$ 0.02g) 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%.

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. 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 thin layer strips 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 (R_f) 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 either free or metabolized BSP fractions from the TLC strips into separate test tubes with alkaline buffer (Richterich, 1969) and the optical density read at 578 nm. 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.

ACUTE EXPOSURE STUDIES: CARBON TETRACHLORIDE

Determination of Median Lethal Dose

The median lethal dose of carbon tetrachloride to rainbow trout was estimated by the method of Brownlee et al. (1953). Basically this method involves treating the animal with a given dose of toxicant and if the animal dies the dose is decreased by a fraction of the original dose and the trial repeated. This procedure is replicated three successive times after obtaining one positive and one negative response with two successive doses. The dosage increment used in this study was a 25% increase in the first negative response obtained and the dosage interval ranged from 1.6 to 5.0 ml/kg (i.p.) of undiluted CCl_4. Control fish received a comparable dose of physiological saline (Wolf, 1963). Mortality was recorded every 24 h for 96 h. The LD50 value was estimated by dividing by five the sum of the last four consecutive doses of toxicant plus that dose of toxicant that would have been given as the sixth dose.

Dose and Time Response Studies: GPT

The effect of CCl_4 intoxication on the activity of GOT and GPT in plasma and liver was determined in fish fed either Purina Diet (Ralston Purina St. Louis, Mo.) or Donaldson Diet (ORE-AQUA, Newport, Ore.). Fish in all studies were weighed, marked with an identifying fin clip and allowed to recover in a 200 l aquarium supplied with continuously flowing well water (11.0°C or 15.0°C). After 24 h fish were given either undiluted CCl_4 (1.0 ml/kg or 2.0 ml/kg, i.p.) or an equivalent volume of Cortland's saline (Wolf, 1963). Blood and tissue samples were taken after 3, 6, 12, 18 or 24 h from fish fed the Purina diet or after 3, 6, 12, 18, 24, 36 or 48 h from fish fed the Donaldson diet.

Dose and Time Response Studies: BSP

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 rates 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 fish (Schmidt and Weber, 1973). After immobilization animals were weighed and placed into individual troughs of a plastic coated wire frame support

within a plexiglas aquarium and were allowed to recover at least 18 h.

The effect of CCl_4 intoxication on plasma clearance of BSP was determined following administration of undiluted CCl_4 (0.2 or 2.0 ml/kg) or an equivalent volume of physiological saline. After 24 h a cannula was placed in the caudal vein and a single dose of BSP (5.0 mg/kg) in physiological saline was injected into the caudal vein. Blood samples (0.2 ml) were taken from the cannula 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 log plasma BSP concentration vs time.

Fish used in time-response studies received either undiluted CCl_4 (2.0 ml/kg i.p.) 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 vin for estimation of the plasma hemoglobin concentration.

The effect of high plasma concentrations of bilirubin or hemoglobin on plasma BSP clearance was determined in two groups of seven fish. Bilirubin was dissolved in a solution of 0.5 g Na_2CO_3 and 0.5 g NaCl per 100 ml water (Weinbren and Billing, 1956) and stabilized with 25 mg/100 ml of bovine serum albumin. Solutions, of appropriate concentration for each fish, were prepared in a darkened laboratory with the aid of a photographic dark room 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 cannula (PE 10 tubing). The wound was tightly closed with 4-0 surgical silk sutures and the fish were allowed a 30-60 min recovery period. A cannula was inserted into the caudal vein and a loading dose of bilirubin (7.0 mg/kg) was administered by this cannula 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 and 3 h after the infusion began BSP (5.0 mg/kg) was injected by the caudal vein cannula and serial blood samples were taken every 15 min for one hour. 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 brought to 300 milliosmol/kg 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 a single i.v.

injection of BSP (5.0 mg/kg) and blood samples were withdrawn by the caudal vein cannula every 15 min for one hour.

The influence of CCl_4 intoxication on the distribution of BSP between the liver and plasma was determined in four groups of five fish. Animals received either CCl_4 (2.0 ml/kg i.p.) or an equivalent amount of physiological saline. After 24 h BSP (10.0 mg/kg) was injected into the caudal vein and fish were sampled after 15, 30, 60, and 120 min. 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.

The influence of CCl_4 intoxication on the biliary excretion of BSP was determined in five control and three treated animals. Fish received either undiluted CCl_4 or an equivalent volume of physiological saline (2.0 ml/kg i.p.) and after 12 h the common bile duct was cannulated with PE 10 tubing of known volume (40 µl) and the cystic duct was ligated (Schmidt and Weber, 1973). An infusion cannula (PE 10 tubing) was inserted into the ventral intestinal vein and the incision closed with 4-0 surgical silk sutures. Free CCl_4 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 administered BSP by graded infusion over a 12 h period as indicated in a previous section of this report.

Effect of Acute CCl_4 Intoxication on Plasma Protein Concentration and Water Balance

In experiments to determine the effect of CCl_4 on plasma protein concentration and wet whole body weight change fish were weighed, given an identifying fin clip and held in 200 l aquaria supplied with continuously flowing well water. After 24 h undiluted CCl_4 (1.0 or 2.0 ml/kg, i.p.) or an equivalent volume of Cortland's saline (Wolf, 1963) was given. Blood samples were drawn and wet whole body weights were determined in individual groups of fish 0, 3, 6, 12, 18, 24, 36 and 48 h after treatment.

In a second group of experiments fish were weighed, fin clipped and held in 200 l aquaria and after 24 h individual groups of fish were treated with 0.25, 0.5, 1.0 or 2.0 ml/kg of undiluted CCl_4. Wet whole body weights were taken and blood samples drawn 24 h after treatment.

For experiments testing the influence of CCl_4 intoxication on urine flow rates fish were anesthetized in a solution of MS-222 (50 mg/l), weighed and a urinary catheter inserted into the urinary bladder and secured by sutures to the base of the anal fin. The fish was placed in a plexiglas restraining chamber within a larger aquarium supplied with continuously flowing well water (15°C ± 1.0°). Urine was collected into conical graduate centrifuge tubes positioned in a linear fraction collector. After 24 h fish

were removed from the aquaria, reanesthetized, reweighed and given a dose of undiluted CCl_4 (2.0 ml/kg, i.p.) or an equivalent volume of Cortland's saline (Wolf, 1963). Fish were repositioned in the restraining chamber and urine again was collected for hourly intervals over a 24 h period. A separate group of nine fish were catheterized and not disturbed for 48 h. Urine from this group was collected into separate tubes for hourly intervals for the entire 48 h period.

ACUTE EXPOSURE STUDIES: MONOCHLOROBENZENE

Determination of Median Lethal Dose

Four groups of 3 trout each were treated with MCB using a dose range of from 1.0-3.0 ml/kg. MCB was diluted with corn oil (1:1, vol:vol) and administered by i.p. injection. The number of dead animals per group was recorded daily for 3 days. The median lethal dose was calculated for 24, 48 and 72 h by the method of Weil (1952).

Time Response Studies: GPT

Experimental fish were weighed, marked with an identifying fin clip and treated with MCB (1.0 ml/kg, i.p.) diluted with an equal volume of corn oil. Control animals received a similar volume of corn oil. Fish from individual treatment groups were placed in separate 150 l aquaria supplied with continuously flowing (10 l/min) well water of constant temperature (15°C ± 1°).

Blood samples were drawn into heparinized tuberculin syringes from separate groups of fish 3, 8, 12, 24, 36, 48, 72 and 96 h after treatment and plasma was held on ice until enzyme analyses were performed.

Dose and Time Response Studies: BSP

Experimental fish used in dose and time response studies with BSP were handled in a manner similar to those used in studies of plasma GPT activity. Treated fish were weighed, marked with an identifying fin clip and were given either 0.5 or 1.0 ml/kg MCB, dissolved in corn oil, by i.p. injection. Fish treated with 0.5 ml/kg were sampled 24, 48 and 72 h after treatment and groups treated with 1.0 ml/kg were sampled after 3, 12, 24, 36, 48 and 72 h. Prior to sampling fish were administered a dose of BSP (5.0 mg/kg, i.v.) and after 45 min each fish was stunned by a blow on the head, a blood sample drawn from the caudal vein and the BSP concentration in the serum determined.

SUBACUTE EXPOSURE STUDIES: MONOCHLOROBENZENE

Preparation and Introduction of Toxicant

A saturated solution of monochlorobenzene was continuously produced by means of a self-regulating stock solution generator. The entire generator apparatus consists of a dilution water head box and a toxicant stock solution reservoir (Fig. 1). In principle of operation, the dilution water headbox functions to establish a constant head pressure which ensures a regulated

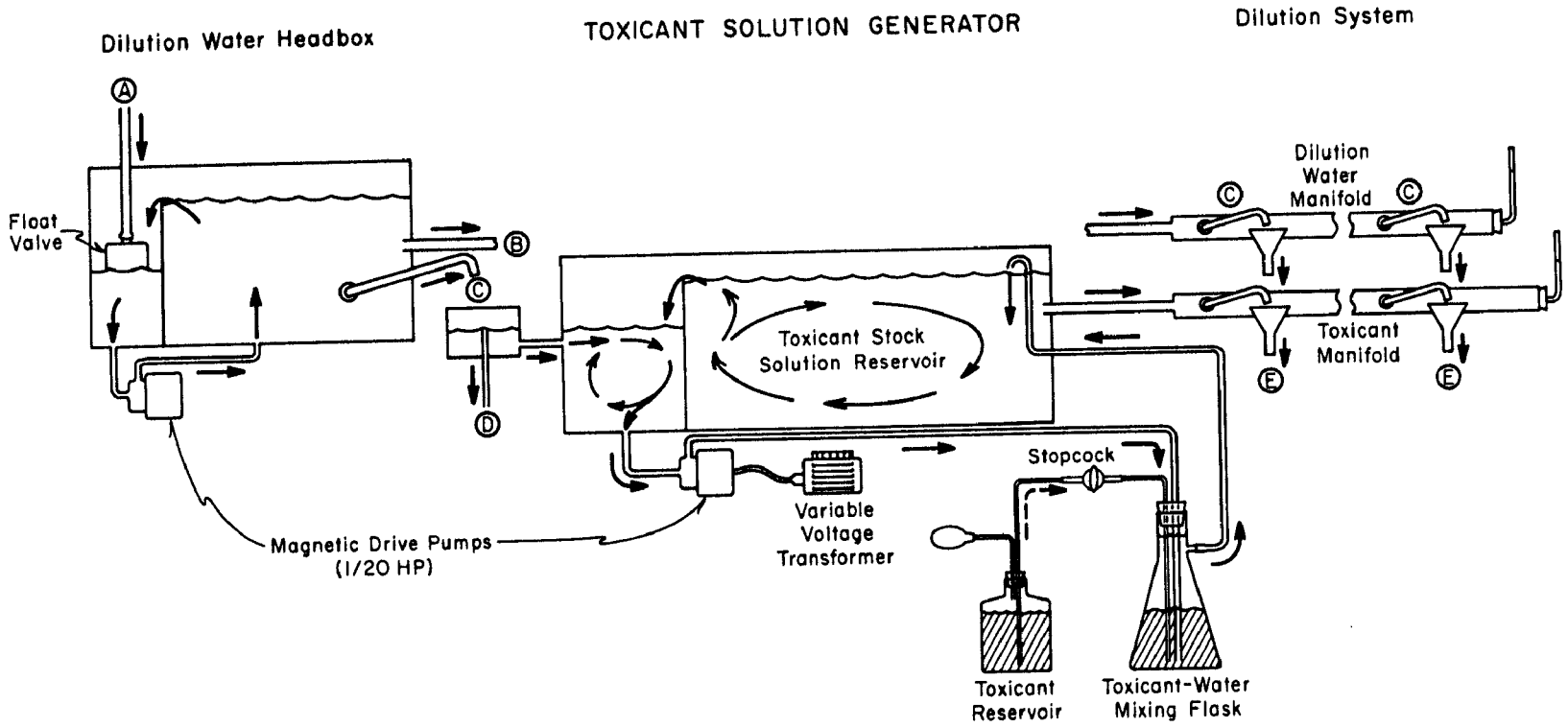

Figure 1. Toxicant solution generator

flow of water to both the main diluter apparatus and to the toxicant reservoir. The stock solution reservoir consists of a 200 liter glass aquarium separated into an affluent and effluent compartment by a glass partition. Water enters the affluent compartment by gravity flow and is pumped from this compartment by a small magnetic drive pump through a volume of monochlorobenzene and into the effluent compartment. The solution is pumped at a rate in excess of its rate of withdrawal from the reservoir and the excess solution flows over the glass partition and recirculates within the toxicant reservoir. The rate of recirculation of solution within the reservoir is adjusted by a variable voltage transformer connected to the recirculating pump. The pattern of water recirculation and inflow within the reservoir provides a constant and self-regulated renewal of stock toxicant solution. Diluent water and stock toxicant solution were delivered to test aquaria by a diluter system as described by Chadwick et al. (1972). Individual fish were held in separate glass aquaria that contained an exposure volume of 28 l and received a continuous flow (400 ml/min) of constant temperature (15°C ± 1.°) well water. Under these conditions a volume of diluted toxicant equal to the aquarium volume entered each test aquaria every 1.18 h and the 99 percent replacement time for toxicant solution in individual aquaria was estimated to be five hours.

Determination of Median Lethal Concentration

The 96 h median lethal dose of MCB was determined by bath exposure of five groups of 4 trout in a continuous flow bioassay. The 96 h median lethal concentration was determined by the logit linear regression method of Ashton (1972). Confidence interval estimates were made using a computer program designed by D. A. Pierce (Department of Statistics, Oregon State University). The concentration of MCB in the water was determined by direct injection of a water sample into a Waters Associates high performance liquid chromatograph fitted with a C18 microbond column and a model 444 UV absorbance detector.

Effects of Prolonged Subacute Exposure

Two groups of 8 trout were exposed continuously to two concentrations of MCB (2.6 or 3.9 ppm) for either 15 or 30 days and two separate groups of 8 fish served as individual controls for treated animals. All fish were held in separate aquaria and control fish were paired randomly with treated fish. Food was offered to all treated fish daily during the experiment and each control animal was fed a ration equivalent to the relative amount of food consumed by the exposed member of the pair. A second group of 8 control fish was held under conditions similar to those of the paired control animals but these fish were fed daily as much food as they would consume in a 15 min feeding period. Baseline control values were determined from two groups of 10 fish that were held in a community tank and were sampled either before or after the 30 day exposure study. Data for both baseline groups of fish were pooled after a test for homogeneity between variances of the two groups indicated that no differences existed between the two sample populations.

Half of the fish exposed to both concentrations of toxicant, their paired controls and half of the fish maintained on the unrestricted ration were sampled 15 days after the beginning of the exposure and the remaining fish were sampled after 30 days of exposure. Food was withheld and fish were weighed 24 h prior to sampling.

On the day of sampling, fish were injected with BSP (10 mg/kg, i.v.) and after 45 min they were stunned by a blow to the head, the wet whole body weight recorded and a blood sample was drawn from the caudal vein. A laparotomy was performed, observation of the gross pathology of the peritoneal cavity recorded and the liver and spleen removed, weighed and sections of each tissue fixed in Bouin's solution for histological examination.

GROSS PATHOLOGY AND HISTOLOGY

Observations of gross pathological changes in the peritoneal cavity were made and tissue samples were taken for histological studies in every experiment. Liver weight to body weight ratios were determined in spinal transected rainbow trout treated with CCl_4 (2.0 ml/kg, i.p.) or in free swimming trout treated with MCB (1.0 ml/kg, i.p.). Liver weight to body weight and spleen weight to body weight ratios were determined in trout exposed to both sublethal concentrations of MCB for 15 and 30 days.

Liver sections were taken from free swimming or spinal transected trout every six hours for 24 h following treatment with CCl_4 (2.0 ml/kg i.p.). Sections of trunk kidney were taken 24 h after treatment with CCl_4 (1.0 ml/kg) in conjunction with studies of water balance in trout. Liver slices were taken from trout 24, 48, 72 h after acute treatment with monochlorobenzene (1.0 ml/kg i.p.) and from fish both exposed to sublethal concentrations of MCB for either 15 or 30 days.

Tissue slices (generally 2-3 mm) were fixed in Bouin's fixative, embedded in paraffin and 6 µm sections were cut and stained with hemotoxylin and eosin. Some liver slices were fixed in Carnoy's #1 solution and 6 µm sections stained with Best's carmine stain for resolution of glycogen deposits.

STATISTICAL METHODS

Treatment group means of plasma BSP concentration, plasma osmolality, total plasma or serum protein concentration, serum hemoglobin concentration, whole wet body weight change, liver and spleen to body weight ratios and urine flow rates were compared by student's t test for independent sample means (Steel and Torrie, 1960). The variation among treatment groups was assessed for significance by Bartlett's test for homogeneity of variance (Sokal and Rohlf, 1969). Plasma or serum enzyme activity data were converted to a quantal form and analyzed by the Fischer Exact Probability Test (Seigel, 1954). Values greater than 2 standard deviations ($P < 0.05$) from the control values were chosen to indicate a positive response in treated fish.

SECTION 4

RESULTS

DEVELOPMENT OF CLINICAL PROCEDURES TO EVALUATE LIVER TOXICITY IN RAINBOW TROUT

Endogenous Tests

Plasma and Serum Enzymes--

The activity of alanine aminotransferase (GPT) and aspartate amino transferase (GOT) in plasma and liver were determined in the fall and winter from a group of fish held at 11.0°C ± 1.0°C and maintained on a ration of Purina Trout Chow. Plasma GOT activity was greater than that of GPT but liver GPT activity was greater than liver GOT activity (Table 1).

The effect of *in vitro* hemolysis on plasma GPT and GOT activity was determined in the spring on a group of fish (150-300 g) held at 15°C ± 1.0° and maintained on Donaldson Diet. Physically induced hemolysis did not tend to increase the activity of GPT or GOT in the plasma even though the plasma hemoglobin concentration was between 7 and 16 times greater than corresponding control plasma (Table 2). Carbon tetrachloride added to whole blood caused a dose-dependent increase in the hemoglobin concentration in the plasma and yet did not cause significant elevation in either GPT or GOT activity. At the highest dose of CCl_4 used, the plasma hemoglobin concentration was more than 430 times greater than that of the control and yet the activity of plasma GPT was the same as that of the control and plasma GOT activity was 1.4 times that of the control plasma.

A slight degree of correlation was evident between the plasma hemoglobin concentration and the plasma activity of both GPT and GOT. The correlation coefficient between plasma hemoglobin and GPT and GOT activities in physically induced hemolysis were 0.29 and 0.36 respectively. In the case of hemolysis induced by CCl_4 the correlation coefficients were 0.13 for GPT activity and 0.89 for GOT activity.

The effects of assay temperature and pH on GPT and GOT activity were determined in liver tissue from fish held at 11.0°C ± 1.0° and maintained on Purina Trout Chow. Liver GPT activity was measured at 7.5°C, 15°C, 25°C, 30°C and 37°C at pH 7.5 and at pH values of 6.5, 7.0, 7.5, 8.0 and 8.5 at 25°C. The mean liver GPT activity increased linearly from 7.5°C to 25°C and the GPT activity was four times greater at 37°C than at 7.5°C (Fig. 2). The change in reaction velocity with 10°C increase in temperature (Q_{10}) was estimated to be 2.4. The greatest variability in activity was found at assay temperatures above 25°C. An Arrhenius plot of this data suggested that structural and conformational changes in enzyme structure from thermal

TABLE 1. ALANINE AMINOTRANSFERASE (GPT) AND ASPARTATE AMINOTRANSFERASE (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°C, pH 7.5

[b]International Units of activity per g liver or per mg liver protein at 25°C, pH 7.5.

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

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)	6.9	1.0[c] (7)	16.6
CCl_4 Induced				
10µl[d]	1.2	261	1.2	261
20µl	1.5	328	1.3	328
30µl	0.8	386	1.3	386
40µl	0.8	233	1.1	233
50µl	1.0	430	1.4	430

[a]Ratio was determined as follows:

Physcially induced: $\frac{\text{Mean hemolyzed value}}{\text{Mean non-hemolyzed value (control}}$

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

There was an apparent sensitivity difference in the erythrocyte viability of the fish used for GPT versus those used for GOT determination, therefore Hb values are reported separately. The mean values for GPT for the hemolyzed group were 3.9 ± 1.6 and for non-hemolyzed 4.2 ± 1.5. The Hb for the GPT group was 173.6 ± 58.3 and 25.2 ± 2 for the hemolyzed and non-hemolyzed respectively. The mean values for GOT were 92.2 ± 12.2 and 89.7 ± 10.3, while Hb was 544.7 ± 174 and 32.8 ± 4.4 hemolyzed and non-hemolyzed respectively.

[b]Ratio is mean of 6 fish

[c]Ratio is mean of 7 fish.

[d]Volume of CCl_4 added to ml whole blood

[e]Control Values $\bar{x}$ ± SD used to calculate ratios

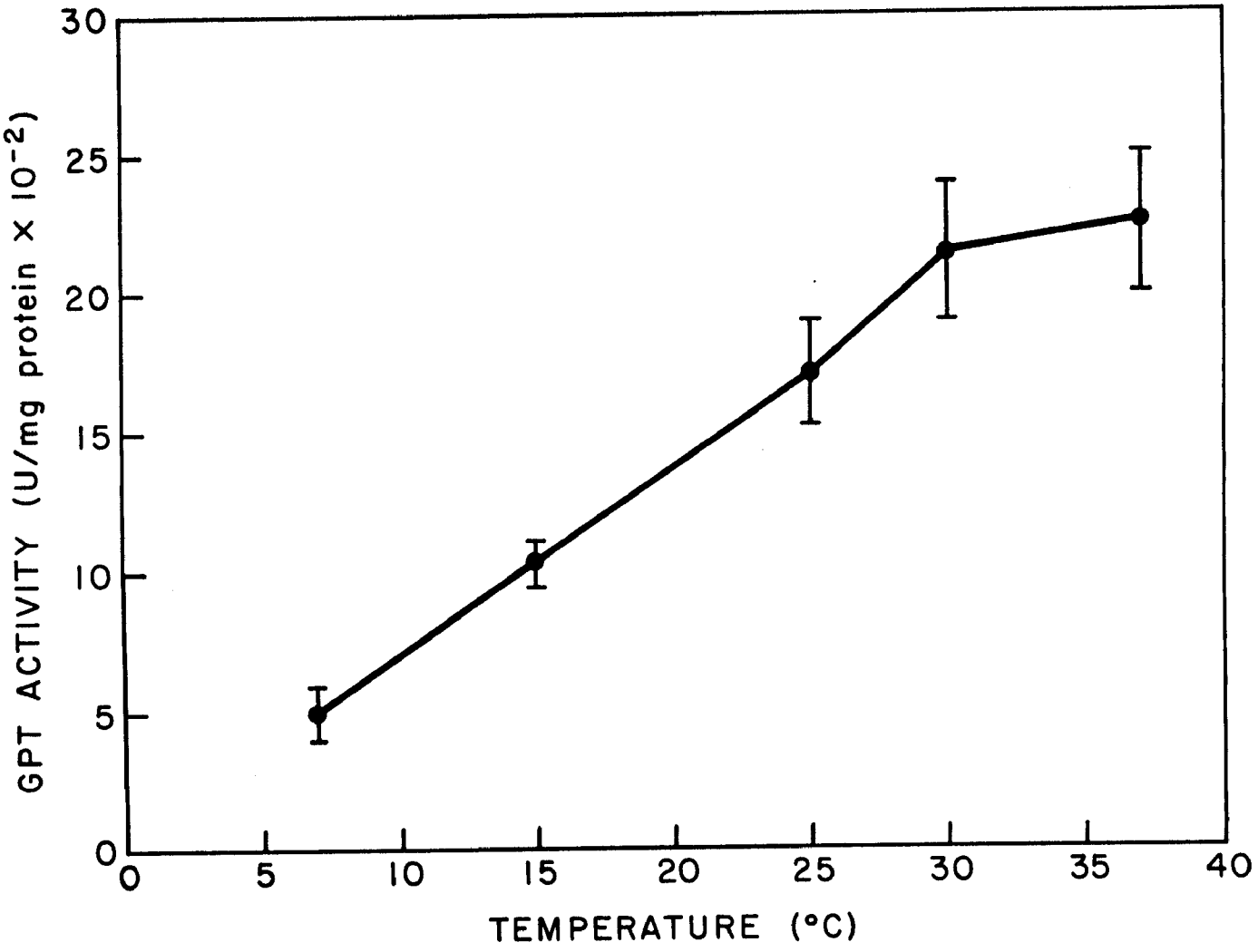

Figure 2. Alanine aminotransferase (GPT) specific activity in rainbow trout liver at increasing assay temperature. Values are the mean ±SEM for three fish. Assay pH was 7.5.

instability occurred at temperatures above 20°C (Fig. 3). The pH optimum for GPT activity was found to be 7.5 (Fig. 4).

The relative activities and enzyme characteristics of liver and kidney GPT were determined from a group of fish (175-300g) held at 15°C ± 1.0° and maintained on Purina Trout Chow. The specific activity of liver GPT was 30 percent greater than that in the kidney while the liver protein concentration was nearly 50% greater than that of the kidney (Table 3). The apparent Km and Vmax of liver and kidney GPT were determined from a Lineweaver-Burke plot of reciprocal reaction velocity vs reciprocal substrate concentration (Fig. 5). Optimum enzyme activity was measured with alanine concentrations between 50 mM and 100 mM and GPT activity was inhibited by alanine concentrations greater than 100 mM. The Vmax for GPT in liver and kidney were 20 mg x 10^{-2} and 12.5 mg x 10^{-2} respectively while the apparent Km values for alanine of the liver and kidney GPT catalyzed reactions were 5.6 mM and 5.0 mM respectively.

Plasma and liver activities were determined in four different salmonid species: rainbow trout (58-138g), steelhead trout (24-58g), brook trout (140-182g), and kokanee salmon (62-178g). Generally the plasma GOT activity was greater than the plasma GPT activity and, except for the rainbow trout, this relationship was similar in the liver tissue as well (Table 4). Differences ($P < 0.01$) in the mean liver protein concentration also were noted between steelhead reared in the laboratory (13.5 ± 0.6 mg/100 mg tissue) and those in holding cages in the Willamette River (11.2 ± 0.4 mg/100 mg tissue).

The effects of assay temperature and pH on alkaline phosphatase (AP) activity were determined from serum samples taken from sexually mature rainbow trout (170-250 g) maintained on Silver Cup Diet. The temperature optimum for serum AP was determined by comparing enzyme activity over a temperature range of from 8°C to 50°C at a pH of 10.3. The optimum pH range was determined by measuring enzyme activity in pH buffers ranging in value from 8.5 to 11.5 at 25°C. The mean serum AP activity increased linearly from 8°C to 25°C and enzyme activity at 35°C was three times greater than at 8°C (Fig 6a). The Q_{10} for serum AP activity was estimated to be 2.0 and increased variability in estimated enzyme activity was observed at temperatures above 25°C. The pH optimum for AP was found to be 10.3 (Fig 6b). An Arrhenius plot of enzyme activity and temperature indicated that the enzyme was stable in the range of temperatures between 8°C and 23°C (Fig. 7).

The mean activity of AP in the serum was estimated to be 30.56 ± 0.61 IU/l when calculated on a volume basis and the specific activity was 0.977 ± .019 IU/g serum protein. These values are considerably less than those calculated for the liver on either a per gram of liver basis (1.45 IU/g l ± 0.036) or per gram of liver protein (21.38 IU/g liver protein ± 0.89).

Serum activity of AP was not influenced by surgically created cholestasis in rainbow trout (Table 5) or by prior treatment with the mammalian cholestatic agent ANIT (Table 6).

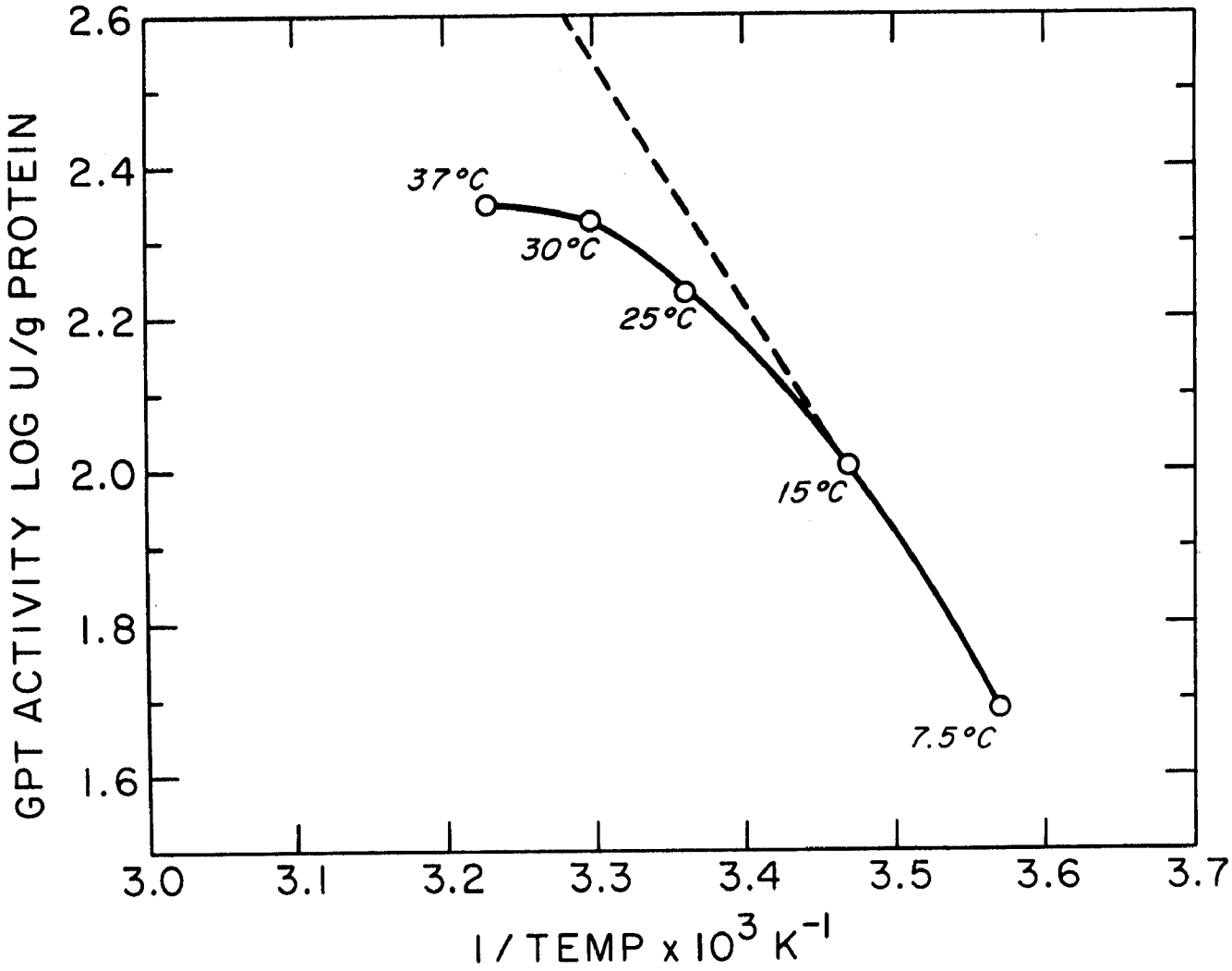

Figure 3. Arrhenius plot of alanine aminotransferase (GPT) activity in rainbow trout liver. Values are the mean of three fish. Assay pH was 7.5.

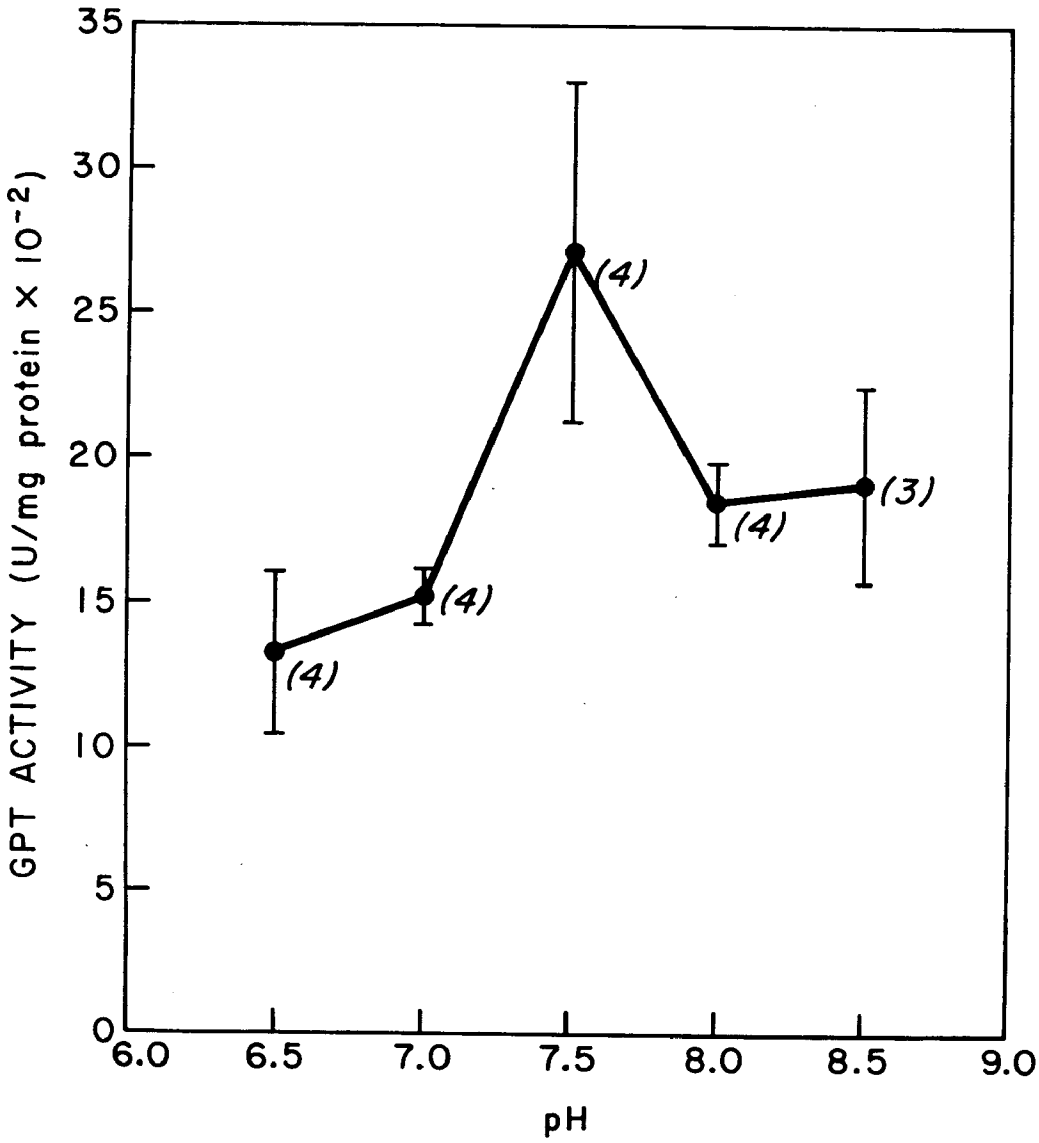

Figure 4. Alanine aminotransferase (GPT) specific activity in rainbow trout liver with increasing assay pH. Values are the mean ±SEM for fish at each pH. Assay temperature was 25°C.

TABLE 3. 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] ± 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°C, pH 7.5.

[b]Values are mean ±S.E.M. for 4 fish.

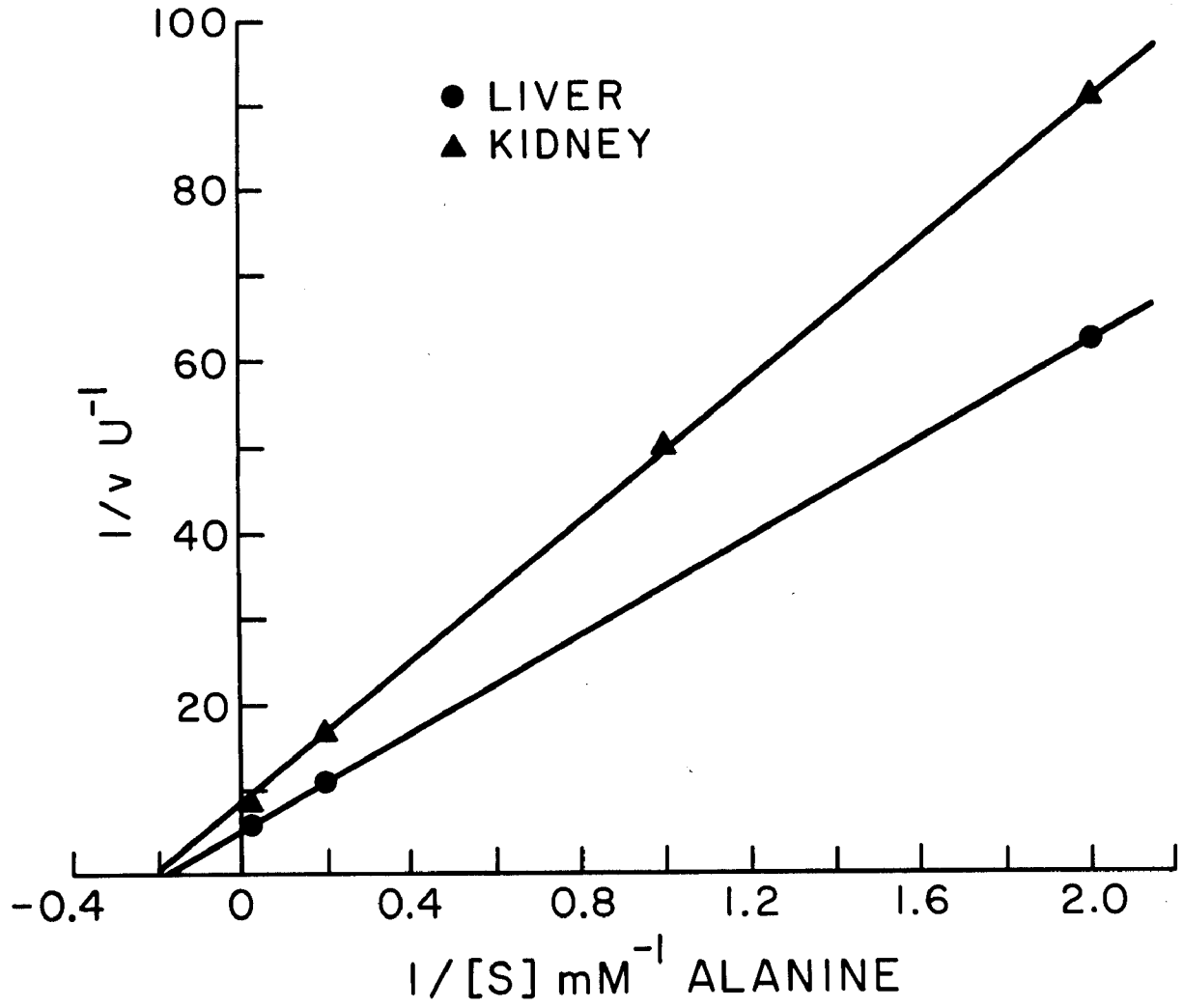

Figure 5. Lineweaver-Burke plot of alanine aminotransferase (GPT) activity in liver and kidney tissue from rainbow trout at increasing assay concentrations of alanine. The Vmax for liver and kidney GPT are 20 U/mg x 10^{-2} and 12.5 U/mg x 10^{-2}, respectively, and the apparent Km values for alanine in the liver and kidney GPT catalyzed reactions are 5.6 mM and 5.0 mM, respectively.

TABLE 4. 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] ±1.0	9.6 ±0.9	11.0 ±0.7	4.7 ±1.4	12.5 ±1.9
GOT Plasma Activity (U/l)	113.8 ±10.2	188.9 ±19.3	-	291.4 ±17.5	130.9 ±14.1
Liver Protein concen. (mg/100 mg tissue)	15.7 ±0.5	13.5** ±0.6	11.2 ±0.4	13.9 ±0.7	17.1 ±0.6
GPT Specific Activity (U/mg protein x 10^{-2})	22.3 ±2.6	23.4 ±1.9	23.0 ±2.1	30.3 ±3.3	8.3 ±0.8
GOT Specific Activity (U/mg protein x 10^{-2})	17.9 ±1.5	34.6 ±2.8	-	53.5 ±5.6	14.9 ±1.2

[a]Number of fish.

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

[c]Values are mean ±S.E.M.

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

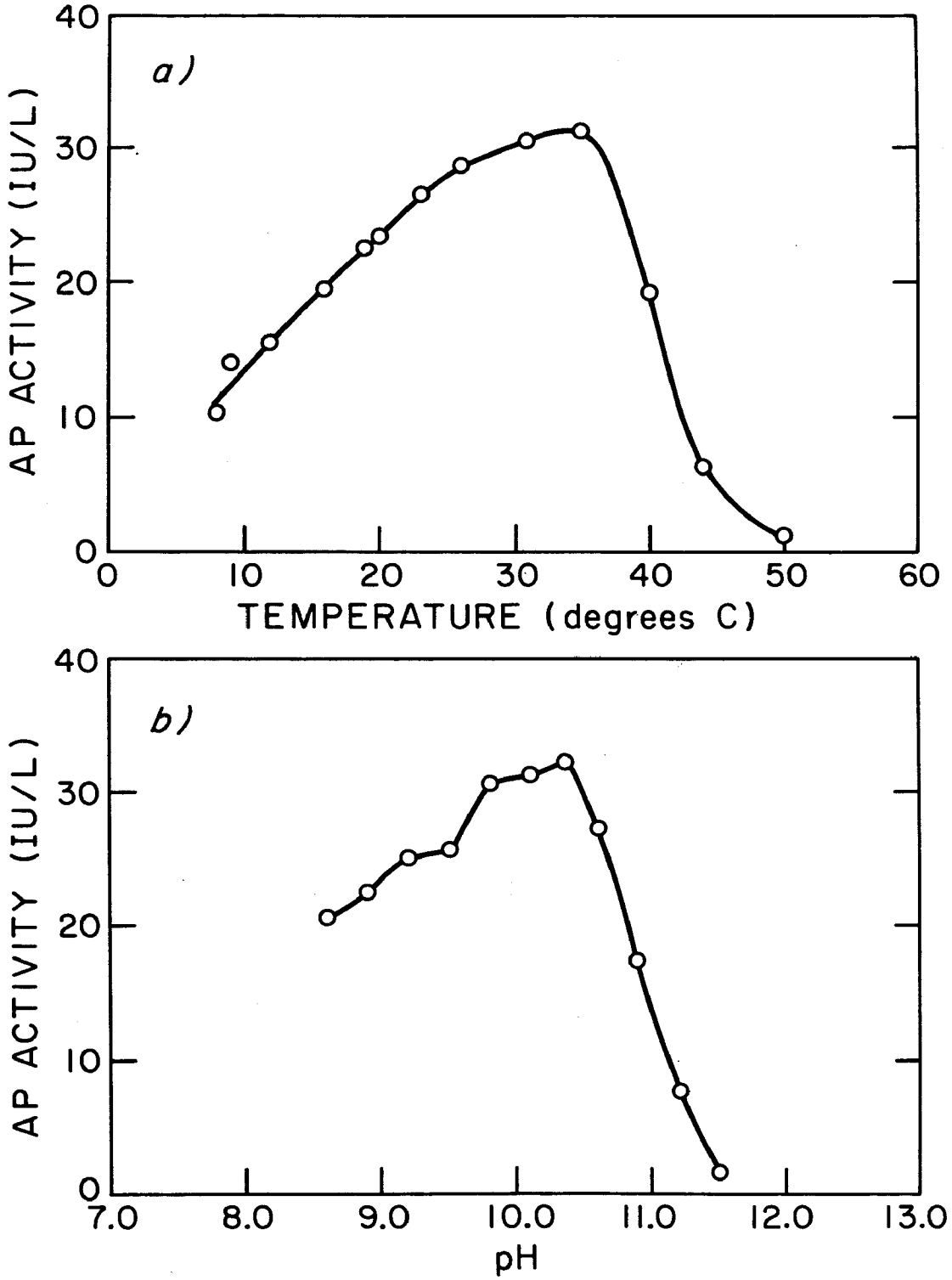

Figure 6a. Alkaline phosphatase (AP) activity with increasing assay temperature. Assay pH was 10.3. 6b. Alkaline phosphatase (AP) activity with increasing assay pH. Assay temperature was 25°C.

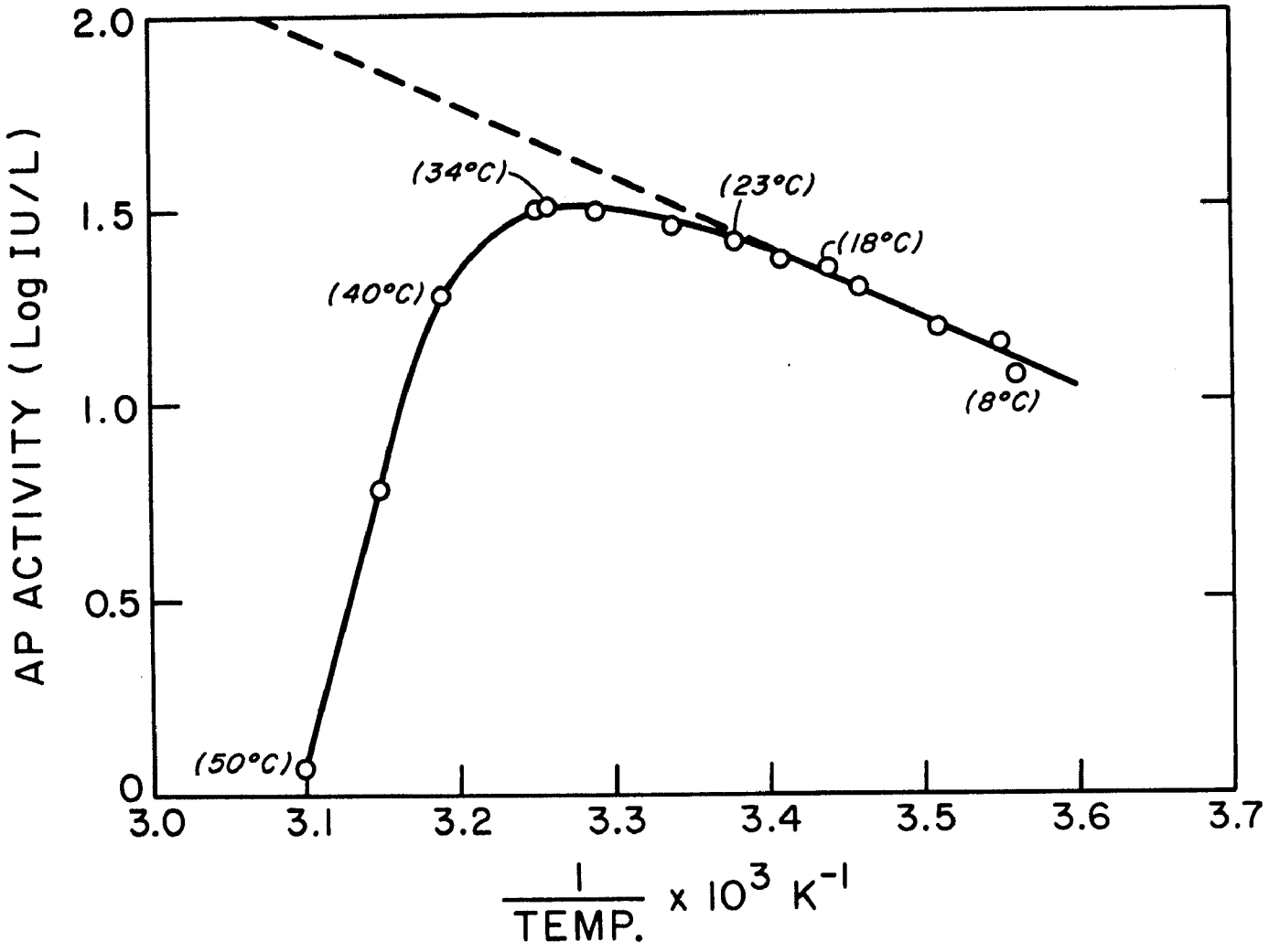

Figure 7. Arrehnius plot of alkaline phosphatase (AP) activity in rainbow trout liver. Assay pH was 10.3.

TABLE 5. SERUM ACTIVITY OF ALKALINE PHOSPHATASE (AP) IN RAINBOW TROUT FOLLOWING LIGATION OF THE CYSTIC DUCT AND COMMON BILE DUCT. EACH VALUE REPRESENTS THE MEAN ±SE OF FIVE FISH.

Time After Surgery (Hr)	Serum AP Activity (IU/gm serum protein)
3	0.339 ± 0.066
24	0.255 ± 0.057
48	0.270 ± 0.051
72	0.291 ± 0.031

TABLE 6. SERUM ACTIVITY OF ALKALINE PHOSPHATASE (AP) IN RAINBOW TROUT FOLLOWING TREATMENT WITH ANIT (400 MG/KG I.P.). VALUES ARE THE MEAN ±SE OF FIVE FISH.

Time After Treatment (Hr)	Serum AP Activity (IU/gm serum protein)
3	0.478 ± 0.0247
6	0.519 ± 0.035
24	0.487 ± 0.021
Control (24 h)	0.547 ± 0.082

Exogenous Tests

Disposition of BSP --

BSP disappeared from the plasma of trout which received doses of either 5.0 or 10.0 mg/kg at nearly equal rates. The half life and fractional turnover rate of BSP were estimated to be 11 min and 6.3%/min respectively in animals receiving either dose of BSP. Assuming that the plasma volume of trout was four percent of the wet body weight (Houston and DeWilde, 1969) the mean percentages of the initial dose of BSP remaining in the plasma compartment after 60 min were estimated to be 2.43% ± SE 0.35 (6 fish) and 3.5% ± SE 0.31 (5 fish) in groups of animals receiving 5.0 and 10.0 mg/kg of BSP respectively.

Following its injection BSP accumulated rapidly in the liver of trout. After 15 min the hepatic content of BSP was at its highest level (0.55 mg/100g body weight) and represented more than half of the injected dose of the dye (Table 7). Thereafter both hepatic content and plasma concentration of BSP declined. Proportionately greater decreases in the plasma concentration between 15 and 60 min resulted in a steady increase in the apparent 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. Because the liver homogenates included residual BSP within the intrahepatic biliary space it was not possible to determine the actual hepatocyte to plasma concentration gradient of the dye. However, even when it was assumed that volume of this space was one percent of the wet liver mass (Peterson et al., 1976) and that the BSP concentration 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 fish sampled 60 min after the dye had been administered.

To further establish the importance of normal liver function in trout for the disposition of BSP, plasma clearance and hepatic accumulation of BSP were determined in fish having hepatic blood flow or bile flow occluded by experimental ligation. The influence of surgical impairment of hepatic blood flow and/or bile flow on the rate of plasma BSP clearance was dramatic. The concentration of BSP in the plasma of cystic-common bile duct ligated animals was more than four times that of sham operated animals after 60 min and plasma concentrations of the dye were significantly higher ($P < 0.01$) than controls after 30, 45 and 60 min. The added effects of impaired hepatic blood flow were even more striking since the estimated plasma half life of BSP from this group (42 min) was nearly four times that of sham treated animals (11 min) and almost one and one-half times that of animals having only ligated bile and cystic ducts (28 min). Ligation of the hepatic portal vein as well as the cystic-common bile duct resulted in ($P < 0.05$) in these animals after 30, 45 and 60 min than in animals having only ligated cystic and common bile ducts. These results suggested that decreased plasma clearance rates in the former group could be attributed to decreased hepatic blood flow.

Experimental ligation of the hepatic portal vein and/or the cystic and common bile ducts greatly influenced the distribution of BSP between plasma and liver (Figure 8). In fish of both surgically treated groups the

TABLE 7. LIVER AND PLASMA CONCENTRATIONS, PERCENT OF INJECTED DOSE AND LIVER:PLASMA CONCENTRATION RATIO OF BSP FOLLOWING A SINGLE IV INJECTION[a] TO SPINAL TRANSECTED RAINBOW TROUT.

BSP	Time (min)		
	15	30	60
Liver BSP concentration (mg/g liver)	0.37 ± 0.02[b]	0.40 ± 0.03[b]	0.35 ± 0.01[b]
Hepatic BSP content (mg/100g BW)	0.55 ± 0.02	0.53 ± 0.06	0.44 ± 0.02
Percent of injected dose of BSP in liver	54.7 ± 2.1	53.0 ± 5.6	44.0 ± 1.7
Plasma BSP concentration (mg/100 ml)	9.33 ± 0.31	3.40 ± 0.80	0.88 ± 0.03
Liver:plasma ratio			
uncorrected[c]	4.0 ± 0.14	13.8 ± 2.54	42.1 ± 2.00
corrected[d]	- -	10.9 ± 1.93	32.0 ± 1.71

[a] 10 mg BSP/kg.

[b] Mean ±SE of 5 fish.

[c] Mean liver:plasma BSP concentration ratio not corrected for BSP in intrahepatic biliary space.

[d] Mean liver:plasma BSP concentration ratio corrected for BSP remaining in biliary tree. See text for details.

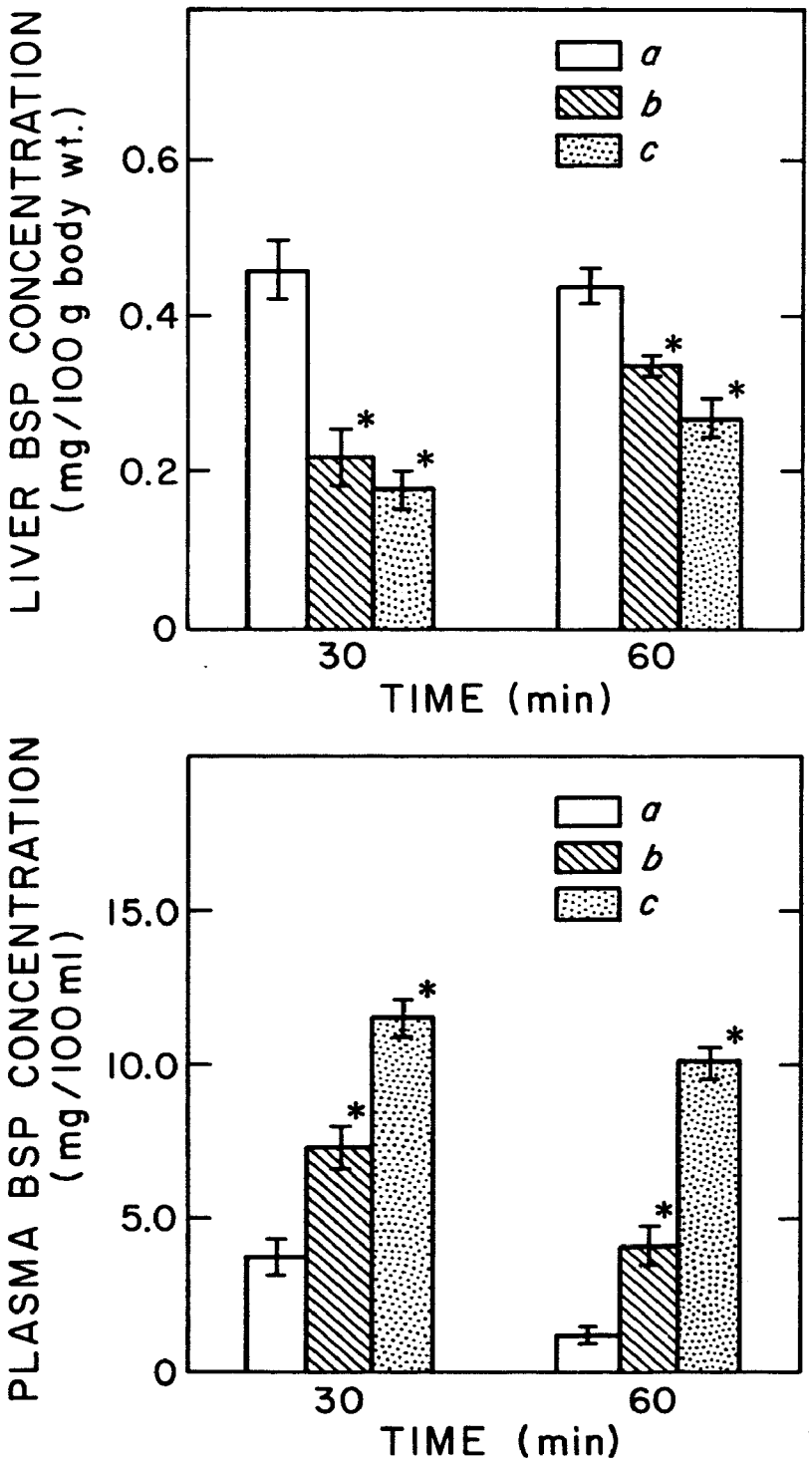

Figure 8. Liver BSP content and plasma BSP concentrations in sham treated control trout (a) and trout having either cystic duct-common bile duct ligation (b), or hepatic portal vein and cystic-common bile duct ligation (c) 30 and 60 min after a single i.v. dose of BSP (10 mg/kg). Values are the mean ±SE of five animals. Asterisks denote values which are significantly different ($P < 0.05$) from controls.

hepatic content of BSP was significantly lower ($P < 0.05$) than in fish on which sham surgery was performed. Plasma BSP concentrations were significantly higher ($P < 0.05$) in all surgically treated fish.

Two BSP fractions were separated from liver extracts of surgically treated rainbow trout by thin layer chromatography. The R_f value of the fastest migrating fraction was similar to that of BSP standards (0.7) and did not react with ninhydrin. The R_f value of the slower migrating fraction (0.38) was similar to that of fraction IV isolated from the bile and reacted with ninhydrin. Surgical impairment of bile flow or hepatic blood flow did not significantly influence the relative proportion of metabolized BSP in the liver homogenates. The mean percent of metabolized BSP in liver homogenates from five sham treated fish was 27.9% ± S.E. 2.3. In cystic-common bile duct ligated and cystic-common bile duct and hepatic portal ligated groups these values were 23.7% ± 1.5 and 24.6 ± 1.9 respectively.

When administered as a single intravenous injection (10.0 mg/kg), BSP was detected in the bile within 15 min even though maximum bile concentrations of the dye were not apparent until between 1.5 and 3 h after dosing. In general, the rate of bile flow was inversely proportional to the concentration of BSP in the bile. Maximum bile flow rates during the experiments ranged from 1.29 to 2.3 µl/kg/min and maximum BSP concentrations ranged from 7.28 to 11.50 mg/ml. Maximum rates of biliary BSP excretion occurred between 2 and 2.5 h after fish received the dye. By 6 h the accumulative excretion of BSP into the bile approached 50% of the injected dose of the dye. Maximum rates of biliary excretion ranged from 12.1 to 14.4 µg/kg/min.

The mean rates of biliary BSP excretion for five fish during prolonged, graded infusion were plotted with time (Figure 9). The rate of BSP excretion increased during the first nine hours of infusion and then remained relatively unchanged for the remainder of the infusion period. The maximum rate of biliary BSP excretion was considered to be the T_m for the dye and was estimated to be 12.1 ± 2.5 µg/kg/min (mean ± S.E.; 5 fish). Bile flow rates and bile BSP concentrations also remained relatively constant after 9 h and were found to be 1.24 ± 0.35 µl/kg/min and 11.8 ± 1.84 mg BSP/ml respectively.

The number of separable fractions of BSP in the bile of each fish increased with the time of dye infusion. Individual bands appeared in the order and at approximately the times indicated in Figure 10. The three BSP fractions having slowest mobility on the chromatograms reacted with ninhydrin indicating a probable association with amino acids. None of the BSP fractions reacted with aniline diphenylamine and therefore were probably not associated with carbohydrates as conjugates. Prolonged infusion of BSP also resulted in a steady increase in the proportion of metabolized dye which appeared in the bile. After a one-hour infusion period, metabolized BSP comprised only 24% of the total dye content of the bile but after 14 hours this value had increased to nearly 40%.

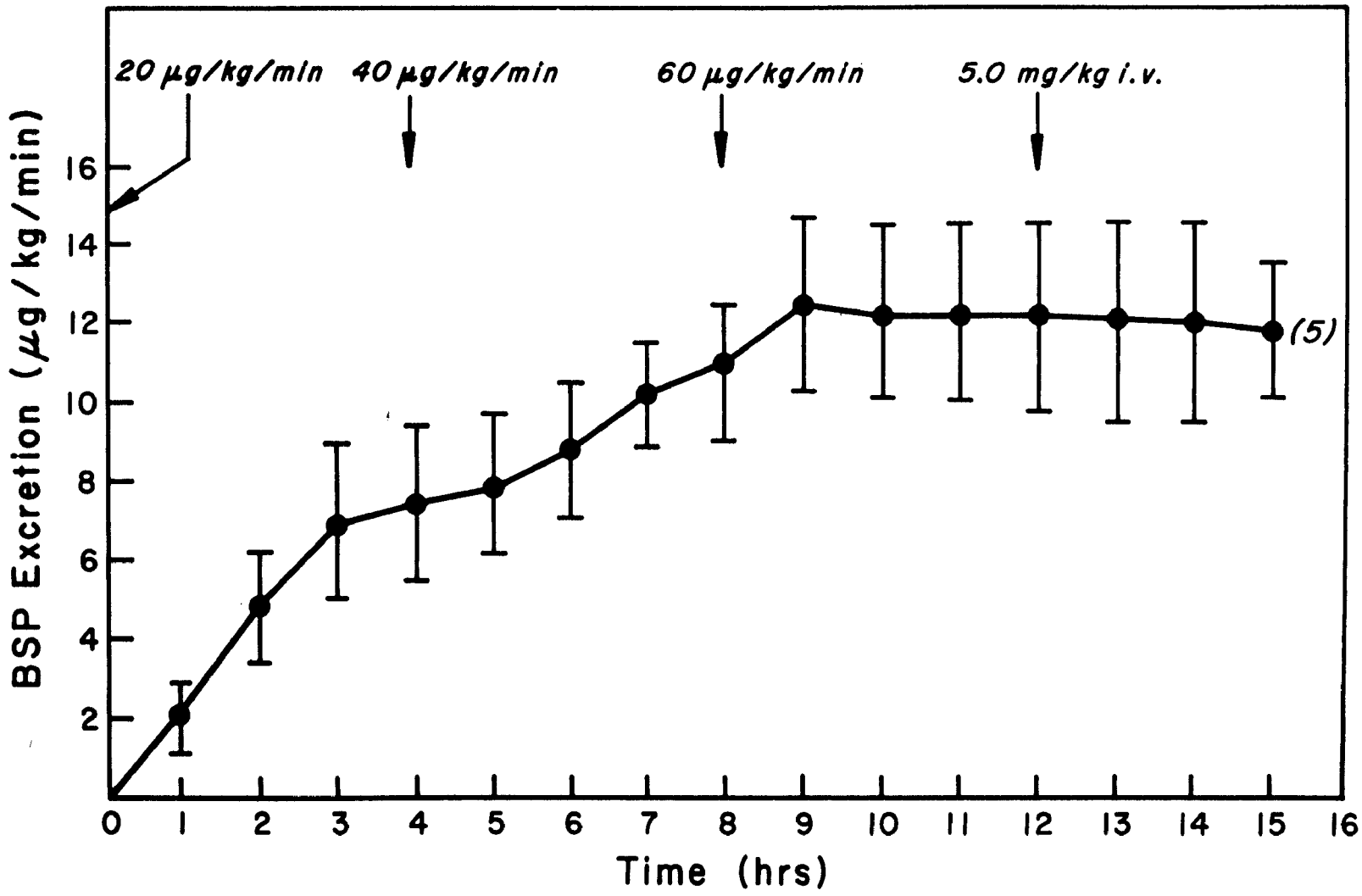

Figure 9. Biliary excretion of BSP during prolonged, graded infusion. Time on abscissa corresponds to time after the beginning of infusion. Each point is the mean ±SEM of samples from five fish.

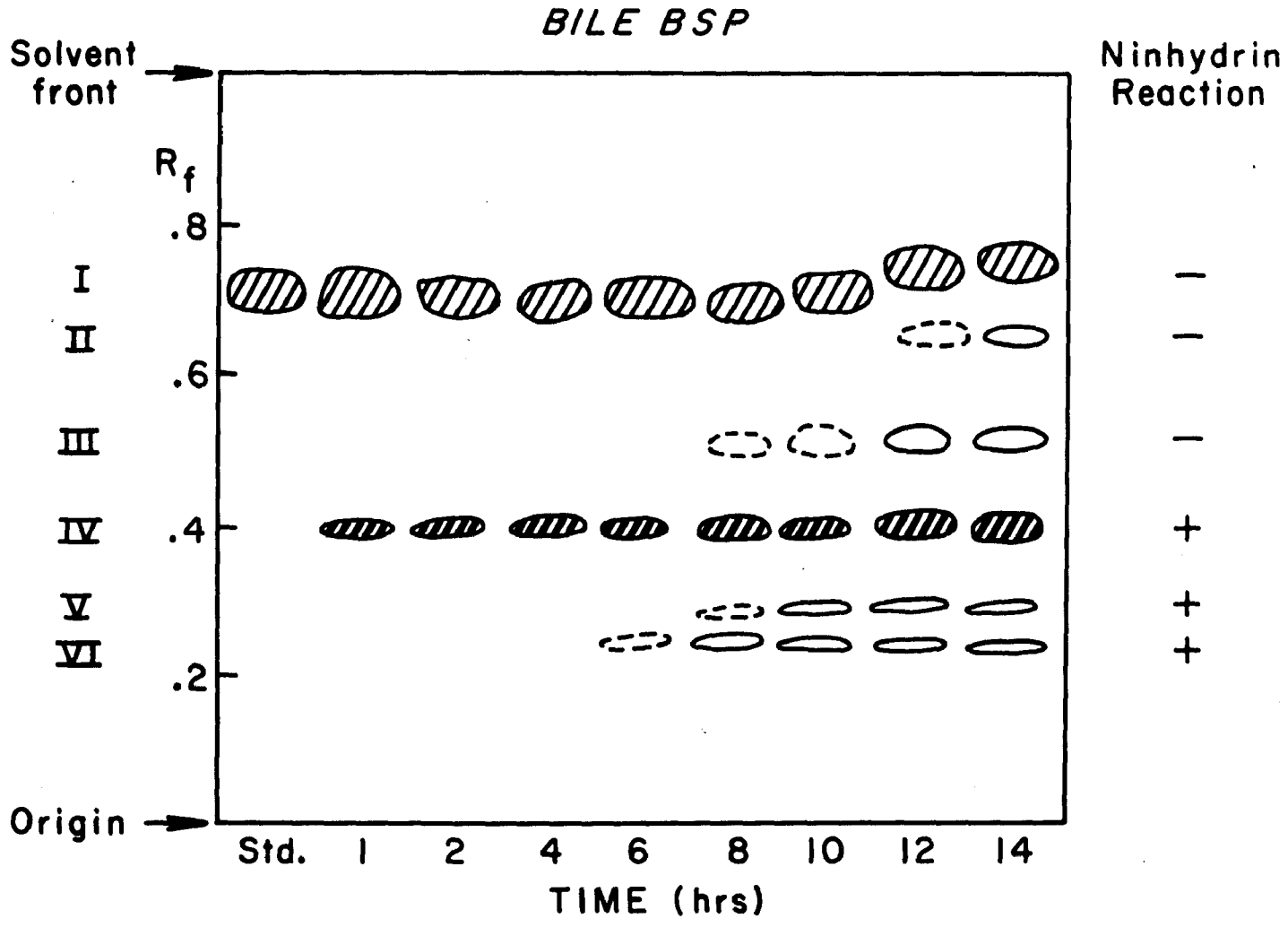

Figure 10. Representative chromatograms of BSP and BSP metabolites appearing in the bile of rainbow trout during prolonged infusion of the dye. Time on abscissa corresponds to the time after the beginning infusion. See text for details.

ACUTE EXPOSURE STUDIES: CARBON TETRACHLORIDE

Determination of LD50

The 24 h median lethal dose of undiluted CCl_4 administered intraperitoneally to rainbow trout was estimated to be 4.75 ml/kg. While observations of mortality were made at 24 h intervals for 72 h, all deaths occurred within the first 24 h.

Dose and Time Response Studies: GPT

Activities of GPT and GOT in the plasma were similar for fish maintained on either Donaldson or Purina Diets, however the specific activities of liver GPT and GOT, respectively were 38% and 118% greater in fish maintained on Donaldson Diet than those maintained on the Purina Diet (Table 8). Additionally, the mean liver protein concentration of fish mainted on the Purina Diet was 13% greater than that of fish maintained on the Donaldson Diet.

Plasma GPT activity was elevated in all fish treated with CCl_4 (1.0 ml/kg), however dramatic differences were apparent in the magnitude of this response between groups of fish maintained on different diets. The mean plasma GPT activities from trout fed the Purina Diet consistently were higher ($P < 0.01$) at all sampling times from 3 to 18 h after treatment than from trout maintained on the Donaldson diet (Fig. 11). Variability of response to treatment with CCl_4 was greater in fish fed the Purina Diet.

Plasma GPT activity increased in CCl_4 treated trout in a dose-related manner. Significant differences ($P < 0.05$) in mean plasma GPT activity were evident between fish treated with 1.0 ml/kg and 2.0 ml/kg at all periods after dosing from 3 h to 24 h (Fig. 12). A biphasic response pattern was noted in plasma GPT activity from fish treated with 2.0 ml/kg CCl_4 with activity maxima occurring at both 3 and 36 h post treatment. Plasma GPT activity in fish treated with 1.0 ml/kg CCl_4 increased steadily and was highest 36 h after treatment.

Dose and Time Response Studies: BSP

Significant ($P < 0.05$) retention of BSP was evident 30, 45, and 60 min after its administration of fish treated with 0.2 and 2.0 ml/kg of CCl_4 24 h earlier (Figure 13). The plasma half life of BSP was estimated to be 11 min in control animals and 15 and 32 min in animals receiving 0.2 and 2.0 ml/kg CCl_4 respectively, indicating some degree of dose dependence.

Significant ($P < 0.05$) retention of BSP was evident as early as 12 h after CCl_4 treatment and was still apparent after 120 h. Highest observable plasma retention of BSP was found after 48 h whereupon it slowly declined (Figure 14). Levels of BSP in the plasma of control animals were relatively constant.

The apparent hemolytic action of CCl_4 was reflected in sharply increased levels of hemoglobin in the plasma (Figure 15). Twelve hours after

TABLE 8. A COMPARISON OF PLASMA AND LIVER ALANINE AMINOTRANSFERASE (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°C, pH 7.5.

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

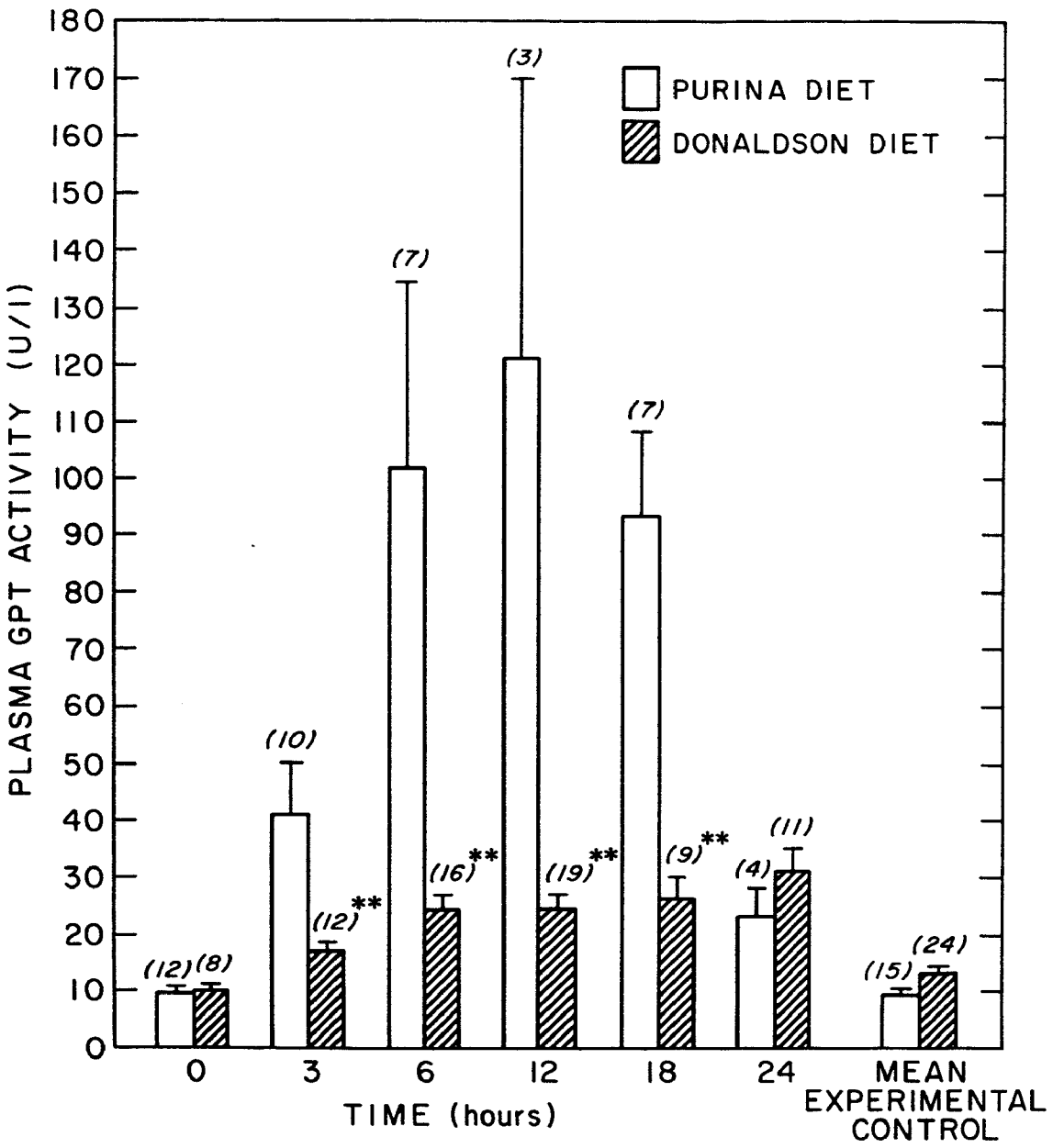

Figure 11. Alanine aminotransferase (GPT) activity in plasma from rainbow trout fed two different commercial fish diets and treated with CCl_4 (1.0 ml/kg, i.p.). Control fish received Cortland saline. Zero time mean values are for non-fed control fish. Values are the mean ±SEM for the number of fish in parentheses. Asterisks denote values for Donaldson diet fish that are significantly different ($P < 0.01$) from Purina diet fish.

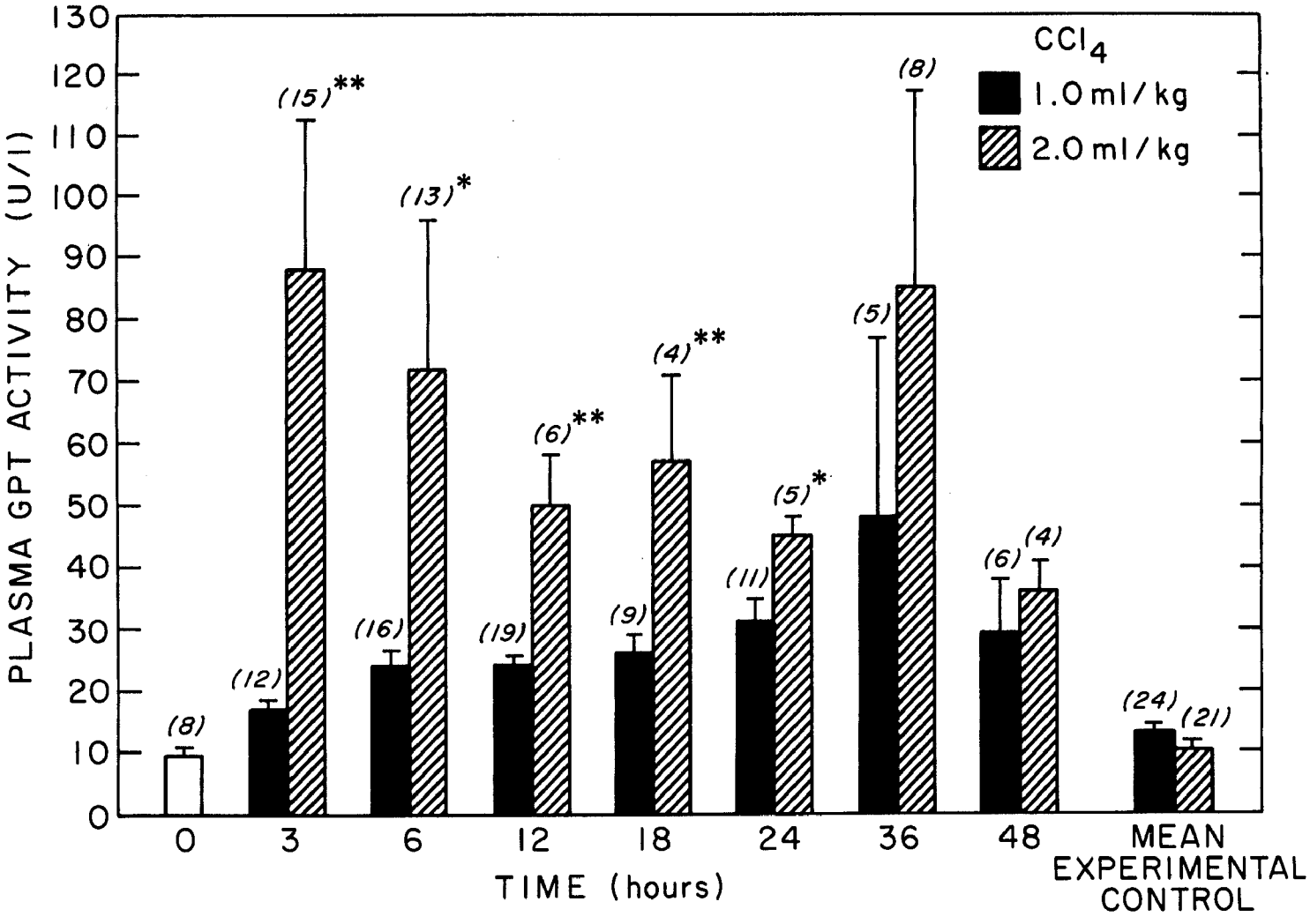

Figure 12. Alanine aminotransferase (GPT) activity in plasma from rainbow trout fed the Donaldson diet and treated with CCl_4(1.0 or 2.0 ml/kg, i.p.). Control fish received Cortland saline. Zero time mean values are for non-treated control fish. Values are the mean ±SEM of the number of fish in parentheses. Asterisks denote values for fish given 2.0 ml/kg i.p. that are significantly different (*P < 0.05, **P < 0.01) from fish given 1.0 ml/kg.

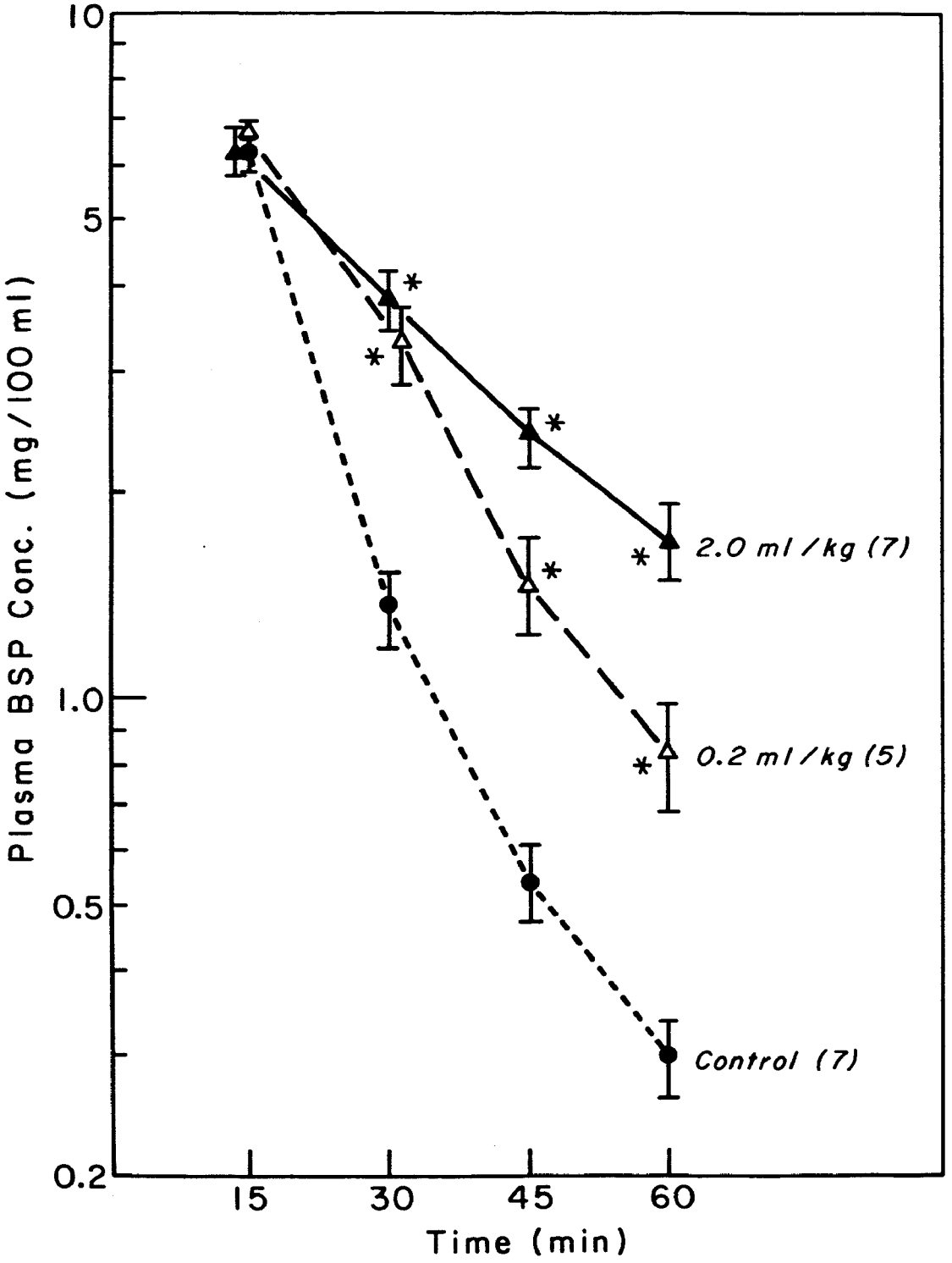

Figure 13. Plasma disappearance curve for BSP in control trout and trout treated 24 h earlier with CCl_4 (0.2 or 2.0 ml/kg, i.p.) Each point represents the mean ±SEM of the number of fish in parentheses. Asterisks indicate values which are significantly different ($P < 0.05$) from controls.

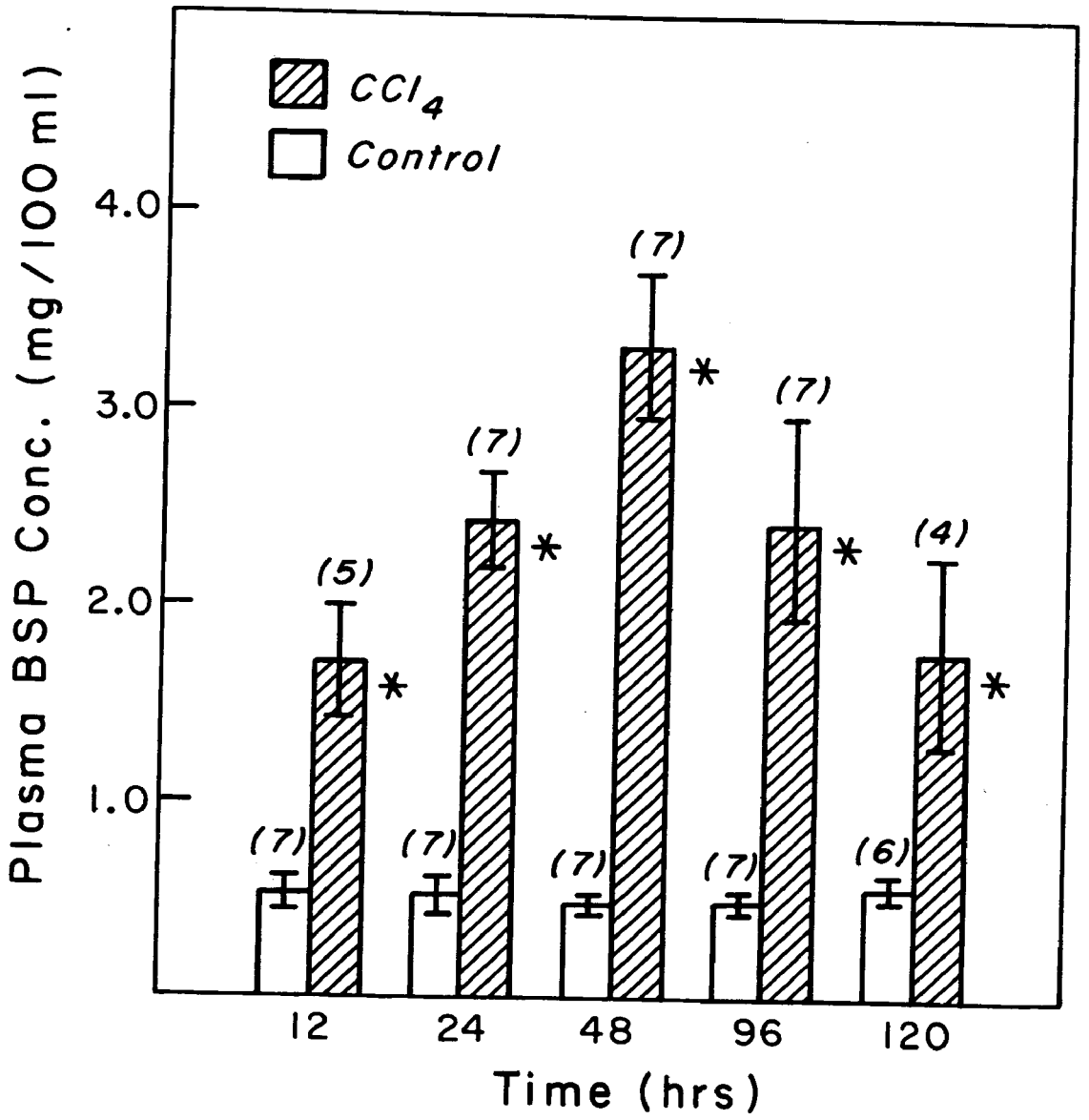

Figure 14. Plasma BSP retention in rainbow trout following CCl_4 treatment (2.0 ml/kg i.p.). Plasma dye concentrations were determined 45 min after a single dose of BSP (5.0 mg/kg, i.v.) was administered. Values represent the mean ±SEM of the animals in parentheses. Asterisks denote values which are significantly different ($P < 0.05$) from controls.

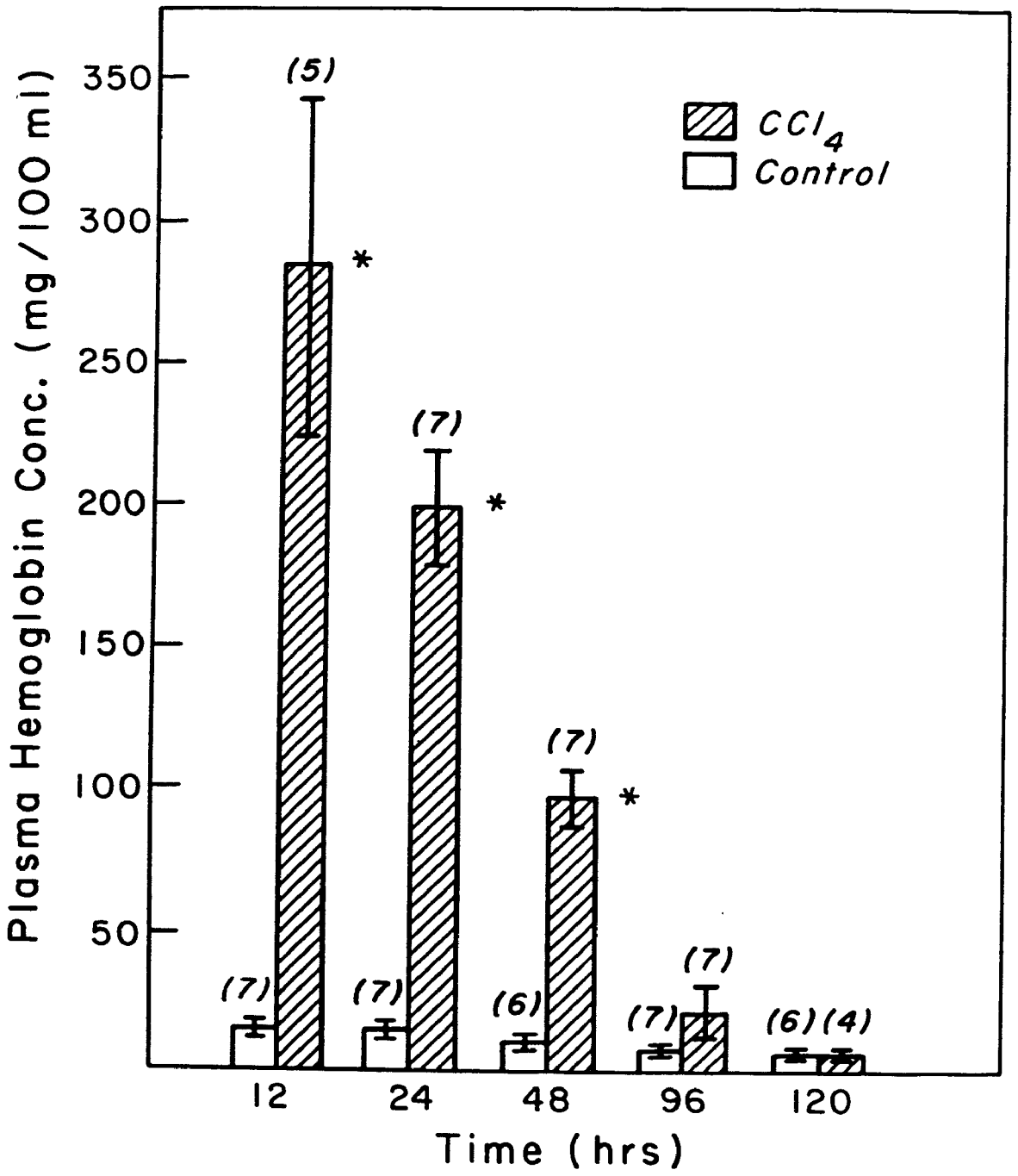

Figure 15. Plasma hemoglobin concentrations in control fish and fish receiving CCl_4 12, 24, 96 and 120 h earlier. Values represent the mean ±SEM of the number of fish in parentheses. Asterisks denote values which are significantly different ($P < 0.05$) from controls.

treatment the concentration of hemoglobin in the plasma was nearly 3.0 mg/ml but these levels slowly declined to those of the controls by 120 h. Despite the apparent increase in total body water following CCl_4 intoxication, differences in plasma osmolality between treatment groups were not evident after 24, 48, 96, and 120 h.

Previous studies have established that bilirubin can reduce the rate of plasma BSP clearance in rats (Hunton, et al., 1961; Dragstedt and Mills, 1936), presumably by competing for some process involved with its hepatic elimination (Clarenburg and Kao, 1973). Because preliminary studies indicated that bilirubin was the major bile pigment excreted by rainbow trout (unpublished observations), it is possible that BSP retention was caused in part by competition for excretion with large quantities of endogenous bilirubin derived from hemolyzed red cells. To test this hypothesis fish were administered either bilirubin or an equivalent volume of bilirubin vehicle prior to BSP administration and the rate of plasma clearance determined. Animals receiving bilirubin tended to retain more BSP in their plasma than controls, but the difference in plasma BSP concentrations was significant ($P < 0.05$) only 60 min after BSP administration. 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. The plasma half life of BSP was estimated to be 14 min in both groups.

The hepatic content of BSP (mg BSP/100 g body weight) in animals receiving CCl_4 was significantly different ($P < 0.05$) from those of controls 15, 60, and 120 min after the dye was given while plasma BSP concentrations in treated animals were significantly higher ($P < 0.01$) than those of controls at all times (Table 9). Concentrations of BSP in the plasma and liver of control animals declined uniformly throughout the experimental period. In treated animals the hepatic content of BSP appeared to increase until at least 60 min after the dye had been injected even though plasma BSP concentrations decreased during the entire period.

BSP accumulated in the livers of control animals at a faster rate than in animals receiving CCl_4 (Figure 16). 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 CCl_4, 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.

Bile flow rates, bile BSP concentrations and the rates of biliary BSP excretion were not significantly different between treated and control groups at any time during the experiment. 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 10. The concentration of BSP in the bile of both groups was highest at this time and remained at these levels for the duration of the experiment.

TABLE 9. LIVER AND PLASMA BSP CONCENTRATIONS FOLLOWING ITS ADMINISTRATION (10 MG/KG IV) TO CONTROL FISH AND FISH RECEIVING CCL_4 (2.0 ML/KG I.P.) 24 HOURS EARLIER. VALUES ARE THE MEAN ±SE OF 5 ANIMALS. ASTERISKS DENOTE VALUES WHICH ARE SIGNIFICANTLY DIFFERENT ($P < 0.05$).

	Time after BSP injection (min)			
	15	30	60	120
Control				
Liver				
(mg/gl)[a]	0.37 ± 0.02	0.40 ± 0.03	0.35 ± 0.01	0.14 ± 0.02
(mg/100 g bw)[b]	0.55 ± 0.02	0.53 ± 0.06	0.44 ± 0.02	0.21 ± 0.01
Plasma				
(mg/100 ml)	9.33 ± 0.31	3.40 ± 0.80	0.88 ± 0.33	0.52 ± 0.01
Carbon tetrachloride				
Liver				
(mg/gl)	0.19 ± 0.03*	0.40 ± 0.04	0.40 ± 0.03	0.27 ± 0.02*
(mg/100 g bw)	0.25 ± 0.04*	0.51 ± 0.09	0.56 ± 0.04*	0.36 ± 0.03*
Plasma				
(mg/100 ml)	17.14 ± 1.46*	8.40 ± 1.19*	1.65 ± 0.17*	1.45 ± 0.18*

[a] mg/g liver

[b] mg/g body weight

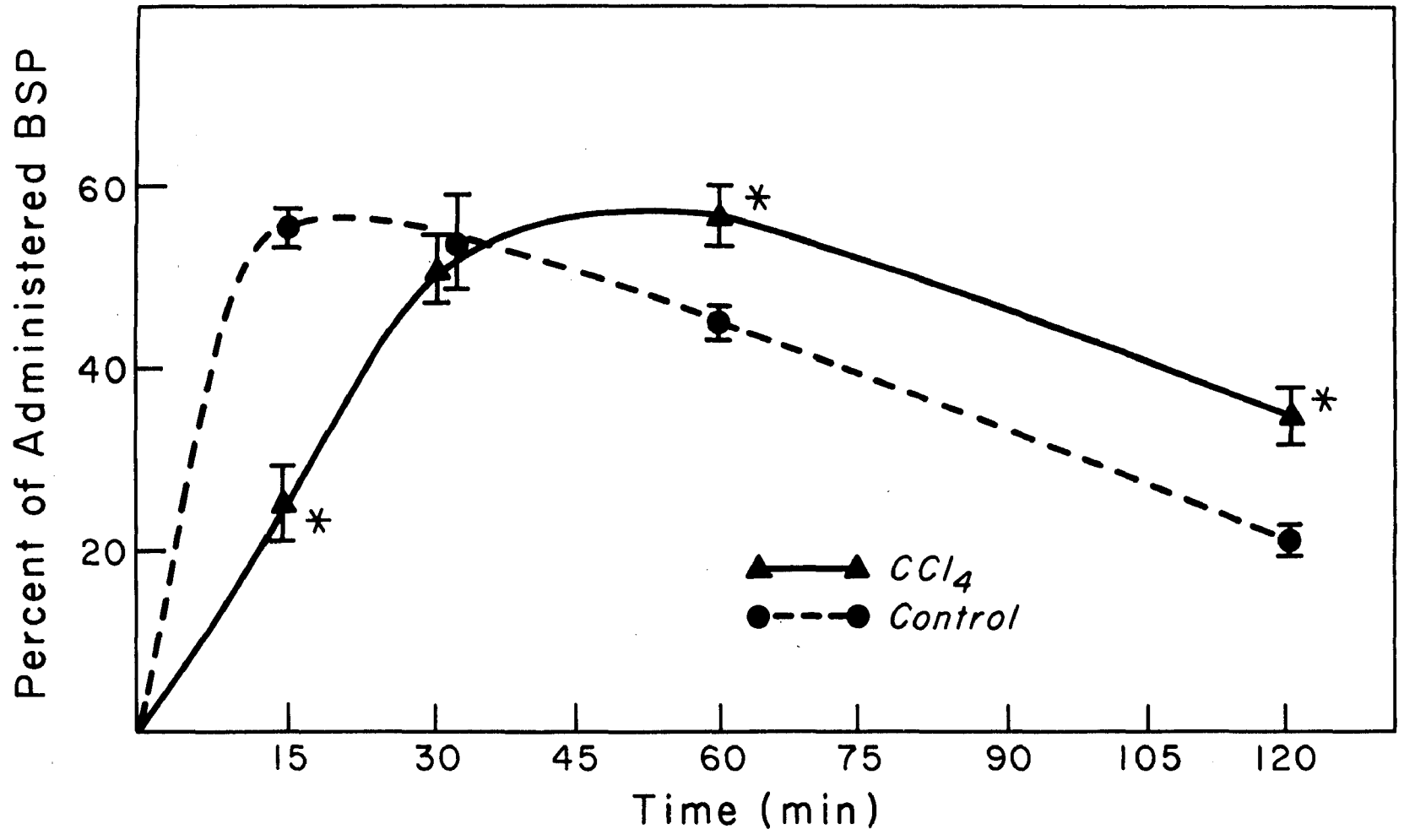

Figure 16. Percent of a single dose of BSP (10.0 mg/kg) appearing in the liver of trout 15, 30, 60 and 120 min after injection in control fish and fish treated with CCl_4 24 h earlier. Each point represents the mean ±SEM of five animals. Asterisks denote values which are significantly different ($P < 0.05$) from controls.

TABLE 10. BILE FLOW, BILE BSP CONCENTRATION AND RATE OF BILIARY BSP EXCRETION 12 HOURS AFTER BEGINNING INFUSION OF BSP IN CONTROL FISH AND FISH RECEIVING CCL_4 (2.0 ML/KG I.P.) 36 HOURS EARLIER. VALUES ARE THE ±SE OF THE NUMBER OF ANIMALS IN PARENTHESES.

	Bile Flow	Bile BSP	Bile BSP Excretion
	(µl/kg/min)	(mg/ml)	(µg/kg/min)
Control (5)	1.24 ± 0.35	11.8 ± 1.84	12.1 ± 2.49
CCl_4 (3)	0.67 ± 0.19	11.0 ± 1.76	6.8 ± 2.49

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 17). 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 a decrease in the concentration 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 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 12.1 µg/kg/min in control fish, however it was not possible to demonstrate a sustained rate of biliary BSP excretion in animals receiving CCl_4 due to the variable rates of biliary BSP excretion (Figure 17).

Chromatography of plasma and liver extracts and bile indicated that the separable fractions of BSP were qualitatively similar in control and treated fish. A single BSP fraction was found on chromatograms of plasma extracts that did not react with ninhydrin and this fraction migrated with mobility similar to that of the plasma BSP standard. Two BSP fractions were present on chromatograms of liver extracts; the fastest of which had an Rf value similar to that of the liver BSP standard. The slowest migrating fraction reacted with ninhydrin and was assumed to be an amino acid conjugate of the dye. The mean percent of metabolized BSP present in liver extracts of control animals represented 19.6% of the total amount of BSP in the liver and ranged from 18.5% to 20.5%. In treated animals the mean value was 18.9% and ranged from 17.6% to 21.0%. The number of separable fractions of BSP in the bile of fish from both groups increased with the time of infusion. No qualitative differences in these fractions were evident between treated and control fish and the pattern of metabolites that appeared in the bile was similar to that previously described (Gingerich et al. 1977). The BSP fraction which demonstrated the greatest mobility had an Rf value similar to that of the bile BSP standard and did not react with ninhydrin. The three fractions exhibiting lowest mobility reacted with ninhydrin while the two fractions of intermediate mobility 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 total metabolized dye which appeared in the bile of both treated and control fish (Figure 18). The relative amount of total metabolized BSP increased by 32% over initial levels in the bile of CCl_4 treated fish and by 39% in the bile of control animals between 1 and 14 h after infusion of the dye began. The bile of CCl_4 treated trout contained a slightly higher proportion of metabolized dye throughout the infusion period than did that of control animals; however, these differences were not significant. Some

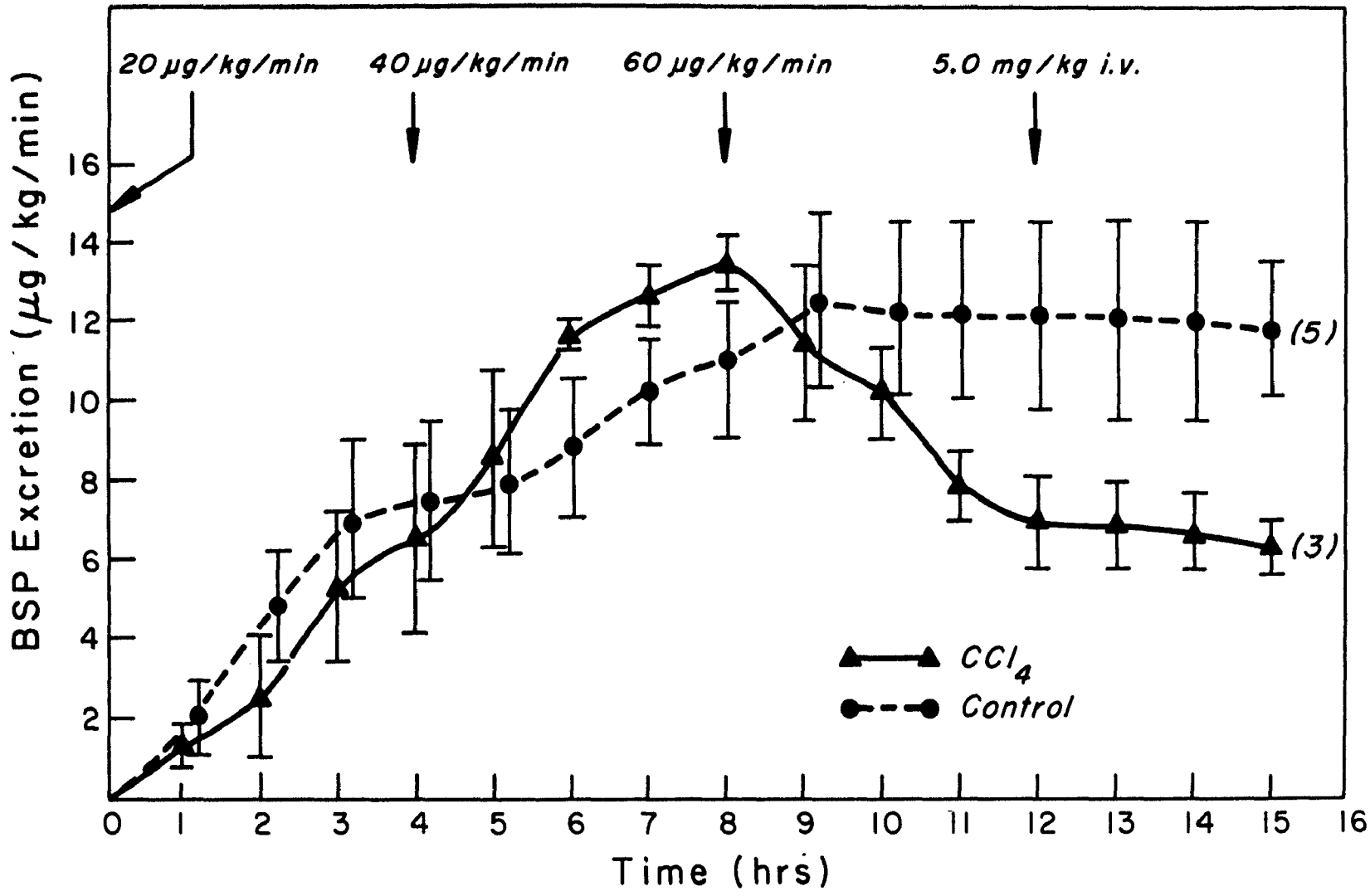

Figure 17. Biliary excretion of BSP by control trout and trout treated with CCl_4 24 h prior to the start of BSP infusion. Time on the abscissa corresponds to time after the beginning of infusion. Each point is the mean ±SEM of the number of animals in parentheses.

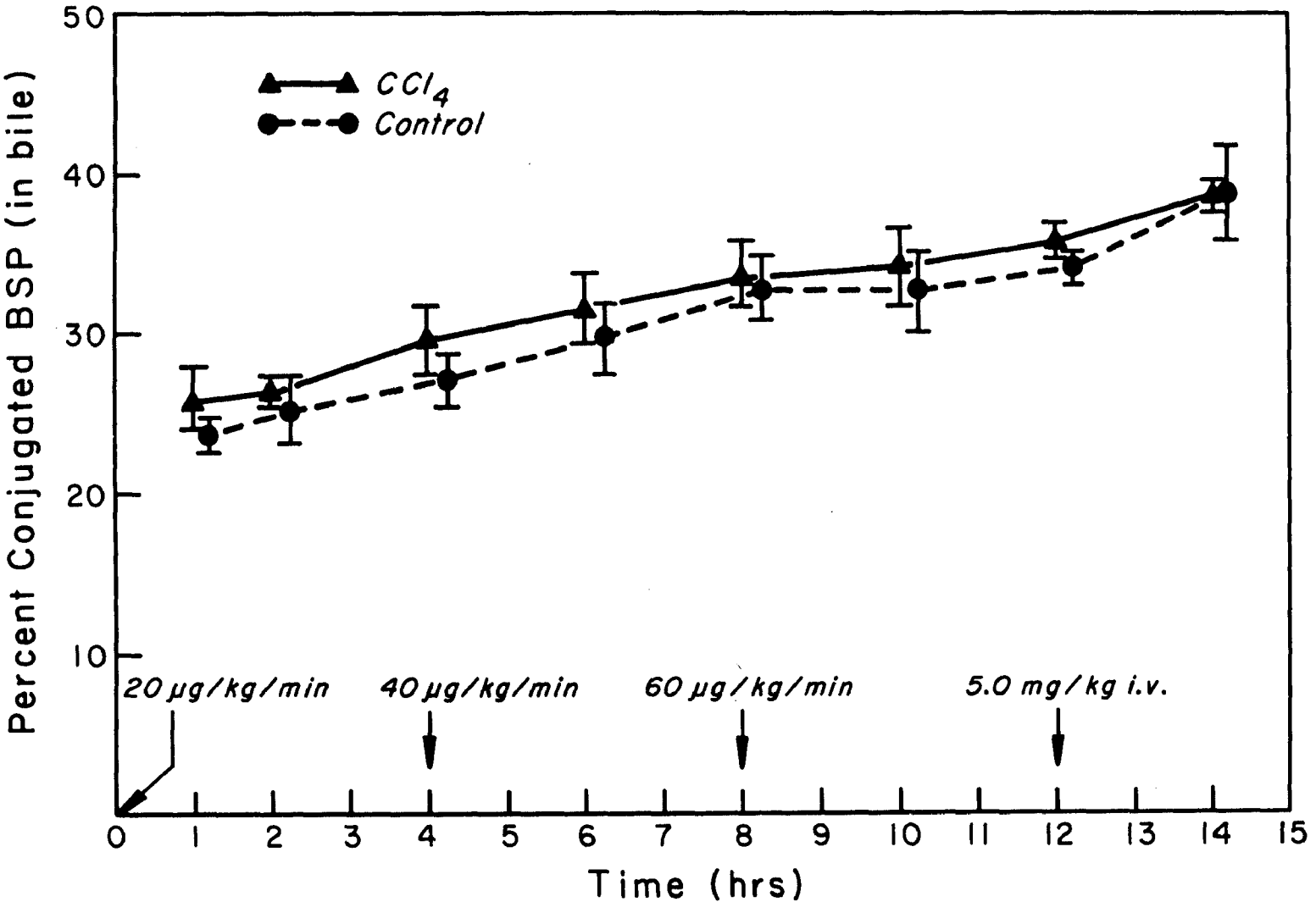

Figure 18. Total metabolized BSP appearing in the bile of control fish or fish treated with CCl_4 during continuous, graded infusion of BSP. Time on abscissa represents time after the start of infusion. Each value represents the mean ± SEM of at least three fish.

error may be associated with these percentage estimates however, because it was assumed that the extinction coefficients of metabolized BSP in the trout bile were similar to that of the unconjugated dye. While there is 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.

Effect of CCl_4 Intoxication on Plasma Protein Concentration and Water Balance

A dose-dependent decrease in the plasma total protein concentration was observed in rainbow trout treated with CCl_4 24 h earlier. The plasma total protein concentrations of trout treated with 1.0 and 2.0 ml/kg CCl_4 (i.p.) were 80 percent and 59 percent respectively of control fish treated with Cortland's saline (Table 11). No differences in the mean plasma total protein concentration were evident in groups of fish receiving either 0.25 or 0.5 ml/kg CCl_4. A significant decrease ($P < 0.01$) in plasma total protein concentration was apparent as early as 12 h after treatment with CCl_4 (2.0 ml/kg i.p.) and protein concentrations remained depressed for at least 36 h (Table 11).

The pattern of whole wet body weight change in fish treated with CCl_4 was considerably different from those treated with Cortland's saline. Fish treated with CCl_4 (2.0 ml/kg) either lost less weight than control fish or tended to gain weight. Differences ($P < 0.05$) in this pattern of weight change were evident as early as 12 h after treatment and continued for at least 36 h (Table 12). Plasma osmolality of treated fish also was less ($P < 0.05$) than that of control fish as early as 3 h after treatment and remained depressed for at least 48 h (Table 12).

Urine flow rates (UFR) in control trout treated with Cortland's saline had a tendency to be elevated however these increases were never significant (Fig. 19). The hourly mean UFR increased from 4.1 ml/kg/h to 4.3 ml/kg/h and the mean urine output for 24 h increased from 98.1 ml/kg to 104 ml/kg. Additionally, a diurnal pattern in the mean UFR remained evident following treatment with Cortland's saline. Conversely, urine flow rates in trout treated with CCl_4 (2.0 ml/kg i.p.) were decreased dramatically within 1 h after treatment (Fig. 20). The mean hourly UFR decreased from the pre-treatment rate of 4.1 ml/kg/h to 1.0 ml/kg/h within one hour post-treatment and the mean pre- and post-treatment urine outputs for 24 h were 98.7 ml/kg and 23.2 ml/kg, respectively. The diurnal pattern of urine flow evident in control fish was abolished in fish receiving CCl_4. When compared to control fish the urine osmolality of trout treated with CCl_4 was increased (Table 13), in part by the resultant proteinuria (Table 14).

TABLE 11. 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 ±S.E.M. for N fish.

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

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

TABLE 12. PLASMA PROTEIN CONCENTRATION, RELATIVE BODY WEIGHT CHANGE AND PLASMA OSMOLALITY 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[b] ± 1.9 (3)	28.6 ± 3.2 (3)	30.8 ± 3.2 (3)	30.9 ± 1.2 (3)	30.8 ± 1.2 (3)	30.8 ± 2.0 (3)	25.1 ± 3.6 (3)
Treated	23.3 ± 2.2 (9)	20.8 ± 1.6 (7)	18.8 ± 1.2* (5)	18.2 ± 0.8 (4)	14.4 ± 1.2* (5)	1 3.7± 2.1 (8)	14.4 ± 1.2 (4)
Weight Change (g/100 g BW)							
Control	-3.2 ± 1.3 (3)	-3.1 ± 0.6 (3)	-2.8 ± 1.3 (3)	-4.7 ± 0.3 (3)	-3.6 ± 0.8 (3)	-5.3 ± 1.0 (3)	-5.1 ± 0.6 (3)
Treated	-1.2 ± 0.4 (9)	-1.7 ± 0.7 (7)	1.1 ± 0.7 (6)	4.6 ± 0.8* (4)	4.5 ± 0.8* (5)	6.4 ± 1.5** (8)	1.6 ± 0.9* (4)
Plasma Osmolality (m Os/kg)							
Control	306 ± 2 (3)	297 ± 4 (3)	285 ± 13 (3)	296 ± 7 (3)	290 ± 8 (3)	289 ± 3 (3)	295 ± 2 (3)
Treated	293 ± 4* (13)	283 ± 6 (12)	287 ± 4 (6)	280 ± 9 (4)	273 ± 2 (5)	268 ± 4** (8)	270 ± 5* (4)

[a] Hours post-treatment.

[b] Values are mean ±SEM for number of fish in parentheses.

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

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

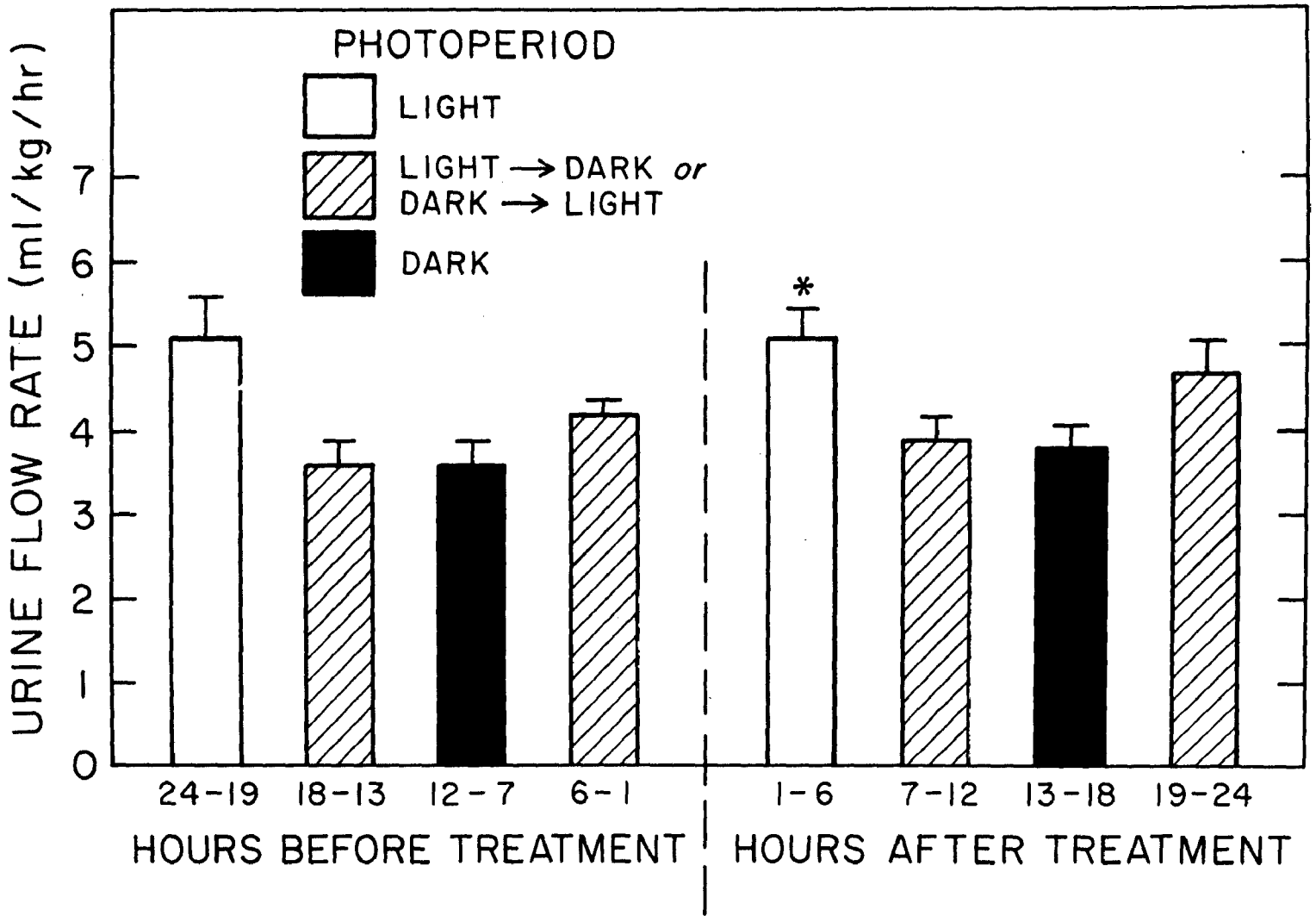

Figure 19. Urine flow rate of Cortland-treated (2.0 ml/kg, i.p.) control rainbow trout for 24 h pre-treatment and 24 h post-treatment. Values are the mean ±SEM for eight fish during each 6 h time period. Asterisks denote value that is significantly different ($P < 0.05$) from non-treated control fish during a similar time period post catheterization.

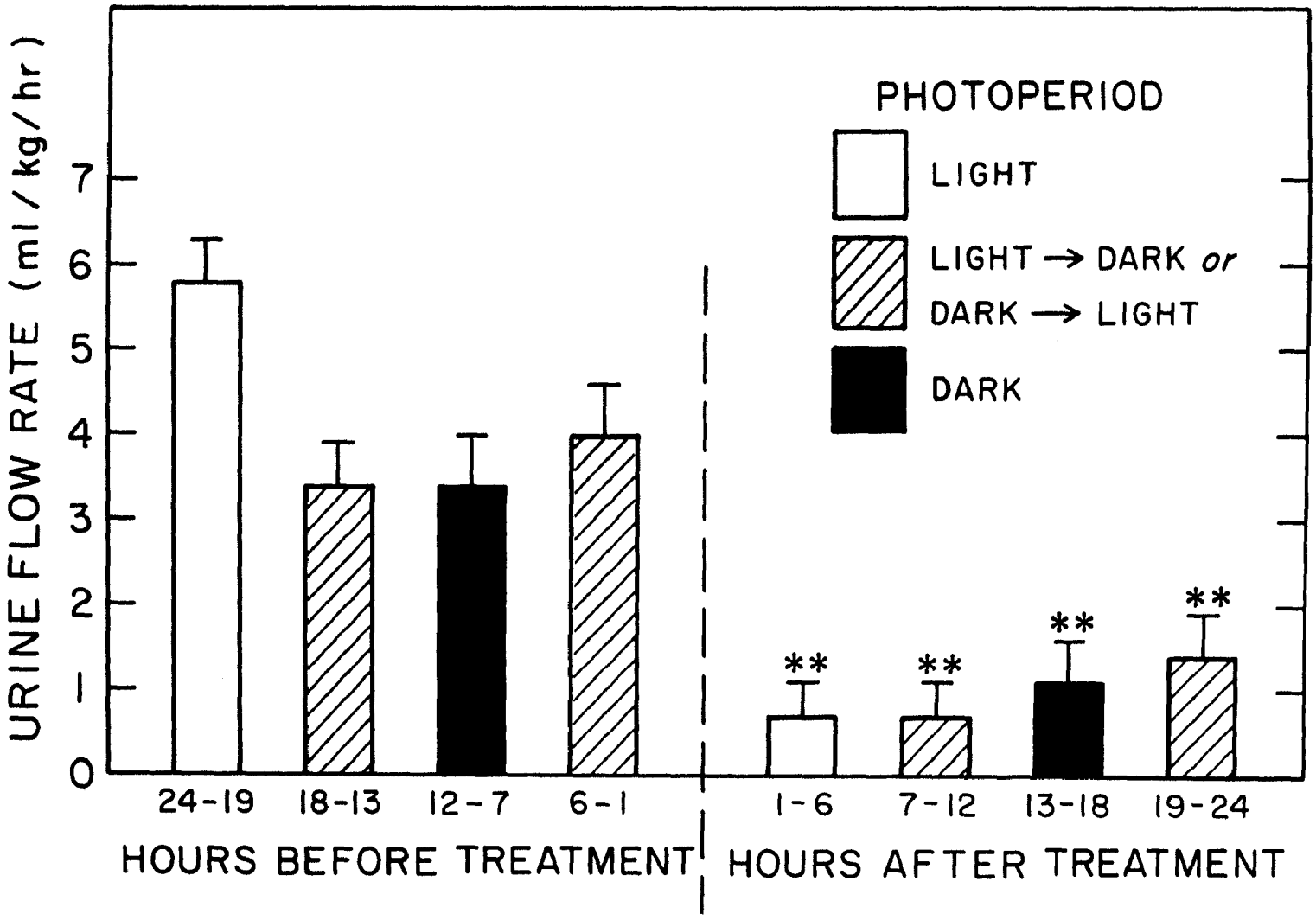

Figure 20. Urine flow rate for CCl_4 treated rainbow trout (2.0 ml/kg, i.p.) for 24 h pre-treatment and 24 h post-treatment. Values are the hourly mean ±SEM for 10 fish during each 6 h time period. Asterisks denote values that are significantly different ($P < 0.01$) from Cortland-treated controls during the same time periods post-treatment.

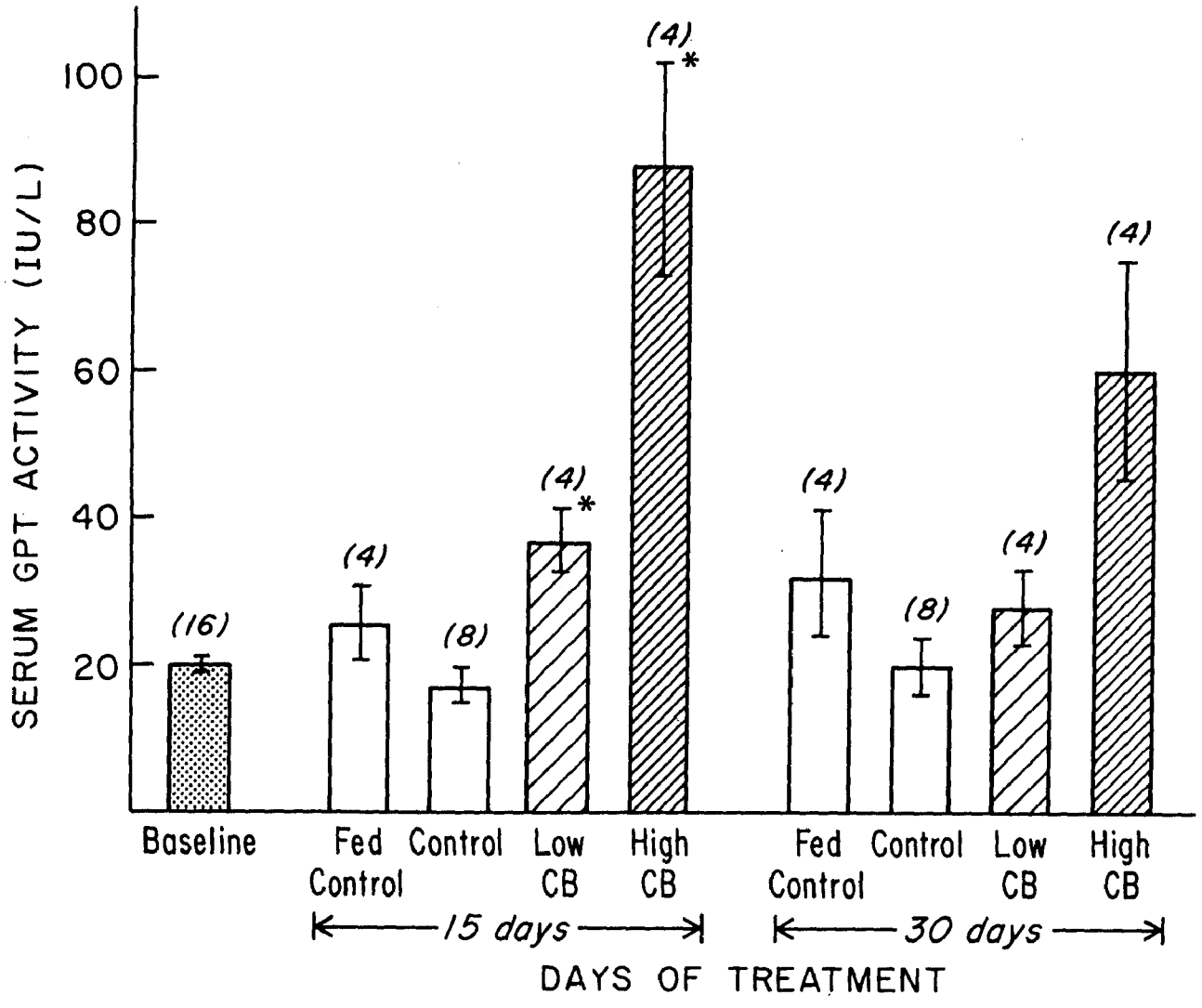

Figure 25. Mean plasma GPT activities from baseline, fed and paired control trout and trout exposed to two subacute concentrations of MCB (2.6 ppm and 3.9 ppm) for 15 and 30 days. Values are the mean ±SEM of the number of animals in parentheses.

TABLE 17. ELECTROPHORETIC DISTRIBUTIONS OF SERUM PROTEINS (GM/100 ML: MEAN ± SE) FROM TROUT AFTER 15 DAYS OF EXPOSURE TO CONCENTRATIONS OF MONOCHLOROBENZENE.

	Fraction	Fed Control (2)[a]	Control (2)	Low CB (1)	High CB (3)
	I	.406 ± 1.55[b]	0.209 ± .020	.524	.241 ± .142
Zone I	II	.763 ± 0.343	0.521 ± .008	.579	.273 ± .079
	III	.907 ± .123	0.513 ± .127	.841	.507 ± .166
	IV	.681 ± .149	0.472 ± .154	.607	0.559 ± .154
	V	0.200 ± 0.051	0.201 ± .063	.342	.204 ± .044
Zone II	VI	0.432 ± 0.059	.185 ± .034	.593	.390 ± .103
	VII	0.203 ± 0.010	.225 ± .122	.324	.233 ± .086
	VIII	0.025 ± 0.012	.095 ± .050	.131	.064 ± .029
	Ratio[c]	1.3420	1.1515	0.9497	0.6810

[a] Number of fish sampled

[b] Mean ± SE

[c] Mean ratio of the sum of proteins in Zone I to sum of proteins in Zone II.

weight of 15 fish treated with CCl_4 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 CCl_4 gained significantly ($P < 0.05$) more weight 24 h after treatment, presumably as water, and maintained this weight for a longer time than did controls (Figure 26). 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 CCl_4 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 27a). Morphological changes were evident in the liver taken from non-transected fish 6 h after CCl_4 treatment. Necrosis was apparent both in the subcapsular region and in well defined areas surrounding the central veins (pericentral regions) (Figure 27b). Damage in the subcapsular region was characterized by coagulative necrosis and pyknosis (Figure 27c). Pathological changes in pericentral regions were characterized by liquifactive necrosis and karyolysis and necrotic areas were surrounded by a zone of swollen hepatocytes (Figure 27d). The essential aspects of the pericentral lesion were similar in only 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.

Marked tissue damage was not evident in the kidneys of trout receiving CCl_4. In the sections of kidney examined no glomerular damage was observed at any of the times following treatment and evidence of proximal tubule damage was found in one one fish treated 36 h earlier with CCl_4.

Gross Pathology and Histology: MCB

Unlike trout receiving CCl_4, no significant wet whole body weight changes were observed in trout treated with monochlorobenzene (Fig 28). Slight inflammation of the peritoneal cavity and in sections of both the

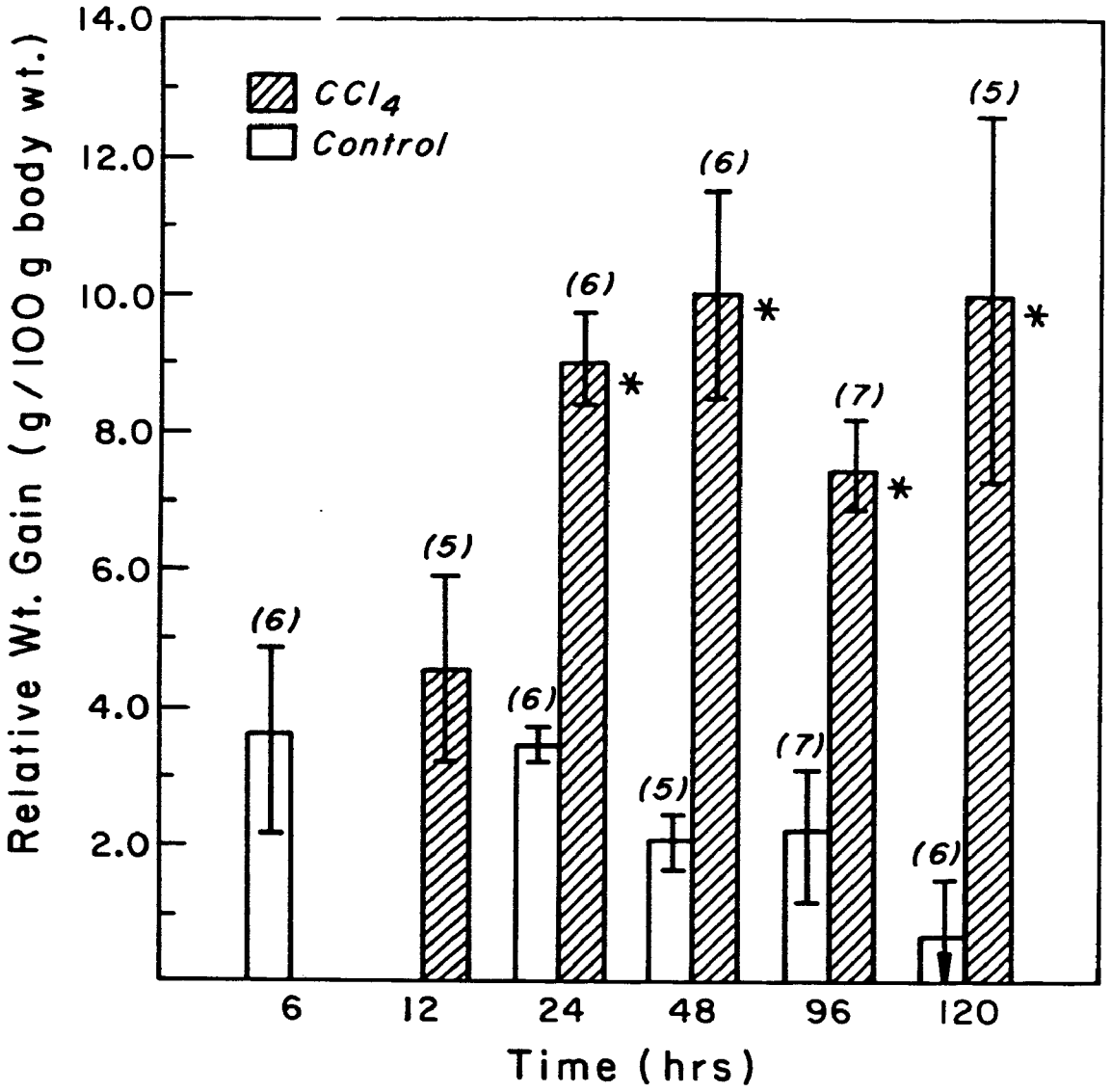

Figure 26. Relative weight gain in spinal transected control trout and spinal transected trout receiving CCl_4 (2.0 ml/kg, i.p.) 12, 24, 48, 96 and 120 h earlier. Values are the mean ±SEM of the number of fish in parentheses. Asterisks denote values that are significantly different ($P < 0.05$) from controls.

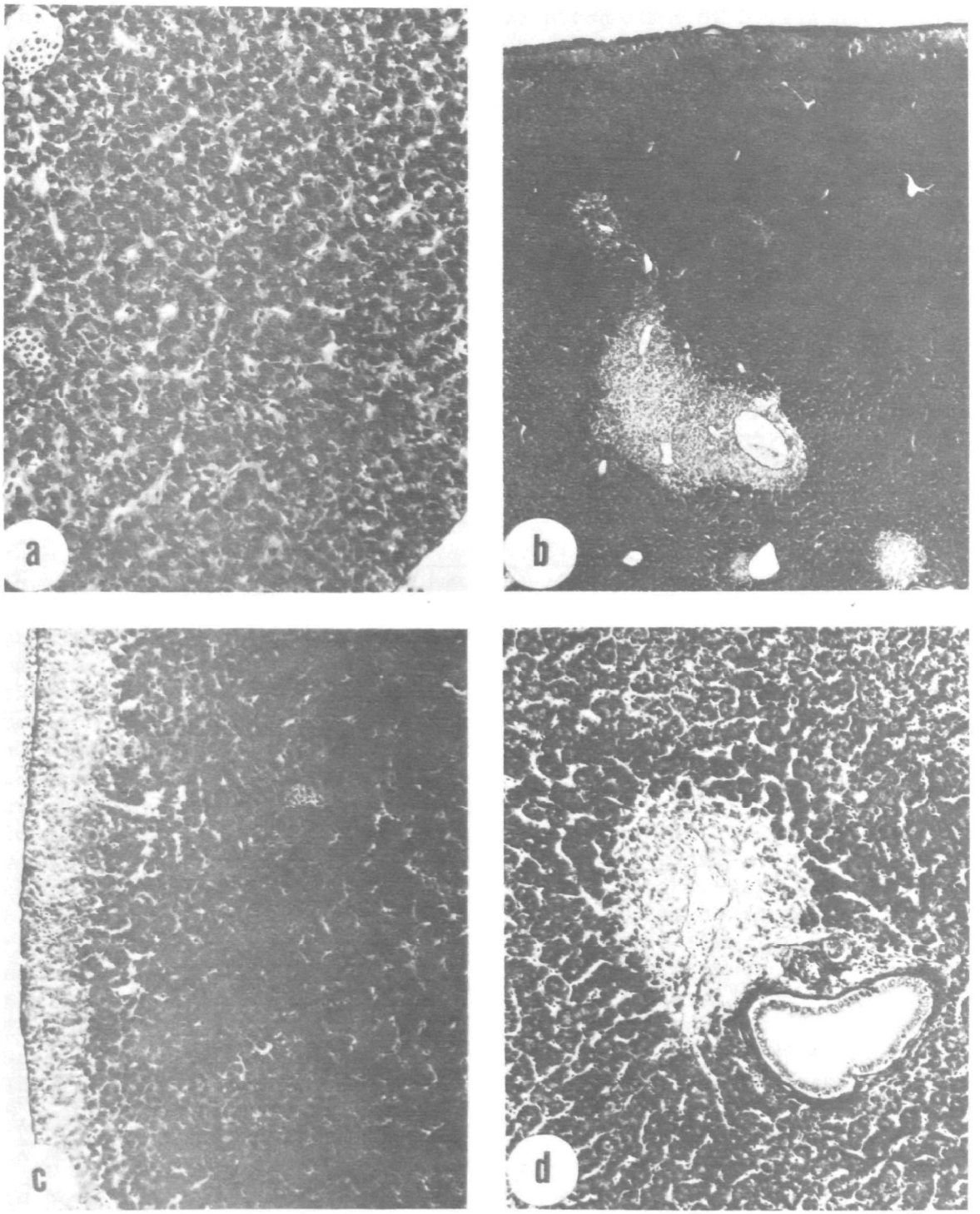

Figure 27. Liver sections from rainbow trout. Hemotoxylin and eosin stain. (a). Control liver 128x. (b) Peripheral and pericentral necrosis in trout liver 6 h after CCl_4 treatment 20x. (c) Peripheral necrosis 128 x. (d) Pericentral necrosis 128 x.

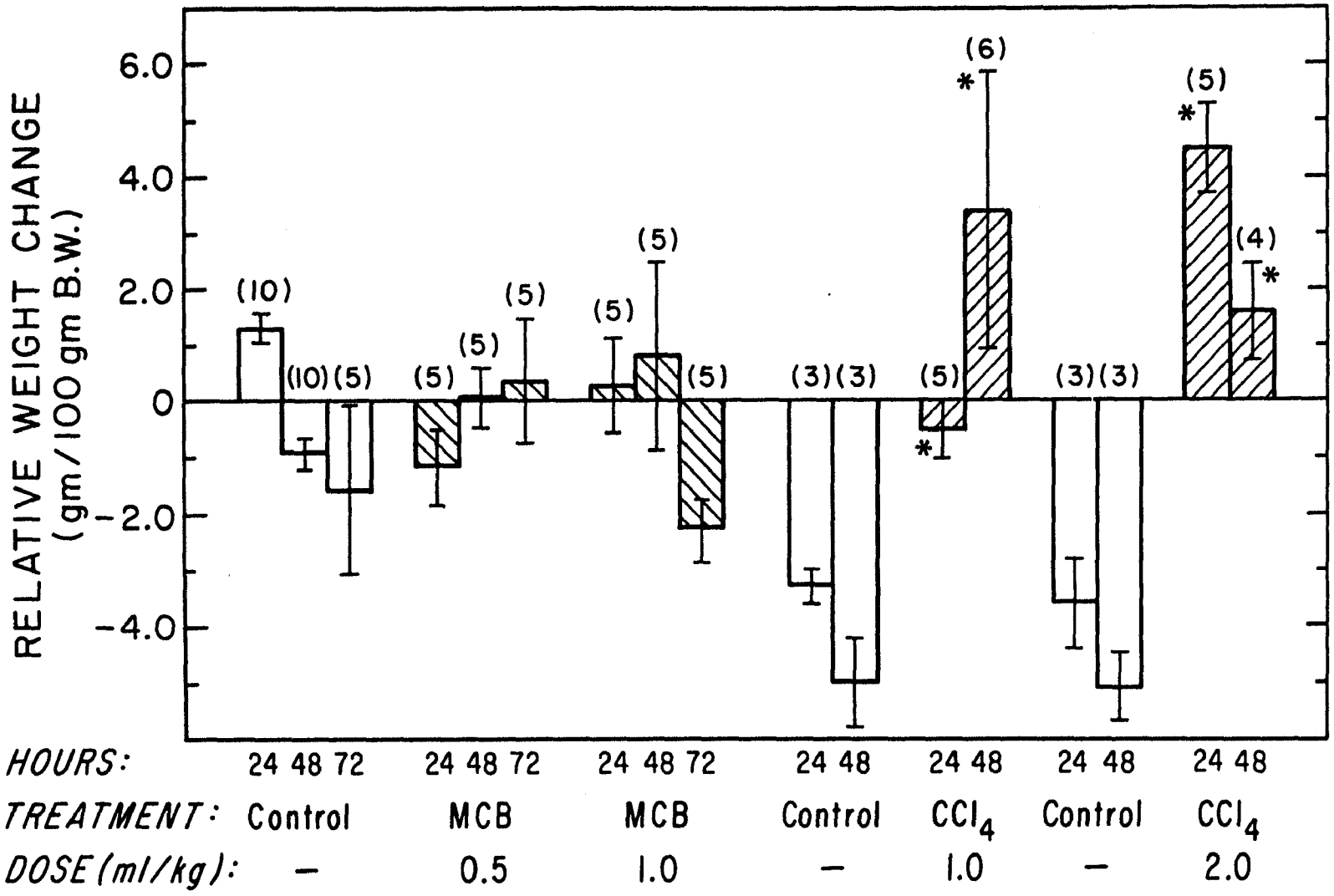

Figure 28. Relative weight change in rainbow trout following treatment with either MCB (0.5 of 1.0 ml/kg, i.p.) or CCl4 (1.0 or 2.0 ml/kg i.p.). Values represent the mean ±SEM of the number of animals in parentheses. Asterisks denote values that are significantly different ($P < 0.01$) from controls.

large and small intestine were observed in all fish from all treatment groups. Enlargement of the spleen was noted in 3 of 5 fish after 24 h and 48 h and in 2 of 5 fish after 72 h. Even though the liver weight to body weight ratios were higher in all treatment groups than in the respective controlgroups, these differences were never significant. The mean liver weight in 7 control fish was 0.76 percent of body weight while in four groups of five treated fish the mean liver weight comprised 0.86, 1.07, 0.99, and 0.94 percent of the final body weight after 24, 36, 48, and 72 h respectively. Mild hydropic degeneration of hepatocytes surrounding central veins was noted in 1 of 2 fish taken from histological examination at 8, 24, and 48 h and moderate pericentral necrosis was observed in one fish 8 h after treatment. The glycogen content of the livers of treated fish generally was reduced relative to that of the controls.

Fish exposed to subacute concentrations of MCB were irritable and non-excitable throughout the experimental period. Respiratory rates, as opercular beats per minute, of resting fish were increased in response to the dose of MCB. The mean number of opercular beats per minute in both the high concentrations (11.4 BPM ± SE 4.0) and low concentration (91 BPM ± SE 2.8) of MCB was nearly twice that of the paired control fish (67 BPM ± SE 2.1). Oxygen consumption rates for fish exposed to MCB were variable. The mean oxygen consumption of trout exposed to the low concentration of MCB (0.60 $mgO_2/kg/min$ ± 0.15) was actually less than that calculated for the pooled control group (0.72 $mgO_2/kg/min$ ± .05) while the mean O_2 consumption of fish held in the high MCB concentration was nearly 4 times that of the control (2.86 $mgO_2/kg/min$ ± SE 0.30). In addition, fish exposed to MCB were anorexic for at least the first 23 days of the experiment and consequently negative whole body weight changes were recorded for experimental fish after 15 and 30 days of exposure (Fig. 29). Fish sampled after 15 days had lost weight in a dose-dependent manner and mean weight loss in both treatment groups was less ($P < 0.05$) than that of the pooled mean of the non-fed control group. A similar trend in weight change was observed in fish sampled after 30 days but these results were complicated by the fact that fish in both treatment groups began to accept food during the second 15 days of exposure. One half of the fish (2/4) in the low MCB exposure group and 1/4 in the high MCB exposure group accepted food before the experiment was terminated on day 30 of exposure. The mean relative food consumption rates of fish held in the high and low MCB concentrations were 1.0 gm/100 gm body weight/day and 1.5 gm/100 gm body wt/day, respectively. In contrast, fish offered an unrestricted ration consumed an average of 3.5 gm/100 gm body weight/ day.

At the times of sampling, fish in both treatment groups appeared to be tetanic and emaciated relative to their controls and a dramatic decrease in the white muscle mass of treated fish was particularly evident. Inspection of the peritoneal cavities of exposed fish revealed few remarkable differences when compared to their paired controls. The livers and spleens of fish in all groups except the fed control fish were reduced in size but otherwise were unremarkable. The liver weight to body weight ratios of non-fed fish were less than those fed an unrestricted ration and relative spleen weights of treated fish were smaller and less variable than were

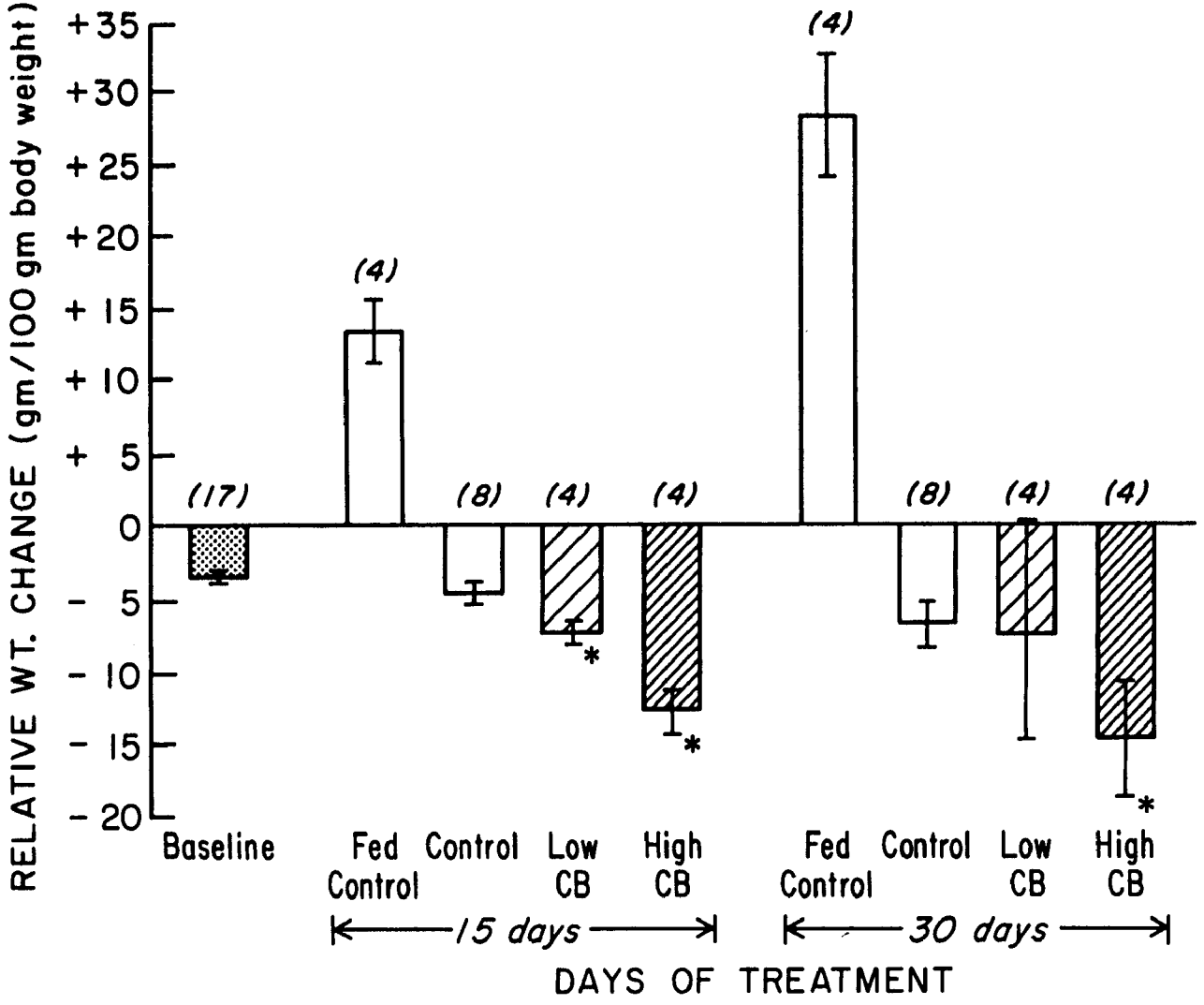

Figure 29. Relative weight change in rainbow trout following treatment with either MCB (0.5 of 1.0 ml/kg, i.p.) or CCl_4(1.0 or 2.0 ml/kg i.p.). Values represent the mean ±SEM of the number of animals in parentheses. Asterisks denote vlaues that are significantly different ($P < 0.01$) from controls.

those of unexposed control animals (Table 18). No histopathological alterations were observed in livers or spleens taken from treated or control fish.

TABLE 18. LIVER WEIGHT TO BODY WEIGHT AND SPLEEN WEIGHT TO BODY WEIGHT RATIOS IN FISH EXPOSED TO SUBACUTE CONCENTRATIONS OF MONOCHLOROBENZENE FOR 15 AND 30 DAYS.

		15 days				30 days			
	Baseline (19)	Fed Control (4)	Control (8)	Low CB (4)	High CB (4)	Fed Control (4)	Control (8)	Low CB (4)	High CB (4)
Liver wt/ Body wt ratio (gm/100 gm bw)	1.153[a,b] ±0.025	1.675[b] ±0.138	0.6961 ±0.0135	0.670 ±0.037	0.931* ±0.059	1.358[b] ±0.121	0.755 ±0.052	0.865 ±0.149	0.873 ±0.066
Spleen wt/ Body wt ratio (gm/100 gm bw)	0.101[b] ± .008	0.120[b] ± 0.042	0.222 ± .050	0.119* ±0.019	0.137* ±0.011	0.065[b] ±0.004	0.130 ±0.029	0.077* ±0.007	0.105 ±0.024

* Denotes values significantly different ($P < 0.05$) from paired control

[a] Mean ± SE

[b] Denotes values significantly different from non-fed control

SECTION 5

DISCUSSION

Endogenous Tests: Plasma, Serum and Tissue Enzymes

A comparison of plasma and liver GPT and GOT activities found in these studies with those activities in rainbow trout reported by other investigators demonstrates the variability of these enzyme activities in normal fish (Table 19). Blood and biochemical parameters 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 IU/l and 259 IU/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 IU/l and 196 IU/l for GOT, in one group of control fish, and 15.6 IU/l and 141 IU/l for plasma GPT and GOT, respectively, in another group of control fish. In both of these studies all fish were fed Purina Diet and, presumably, were genetically similar, disease-free and maintained under identical conditions. Nevertheless, considerable variation exists in values for mean plasma GPT and GOT activities of rainbow trout. It should be noted that different transaminase assay kits were used in their studies and may have accounted in part for some of the variability in the plasma GPT and GOT activities.

Statham et al. (1978a) reported mean serum activities of 22.7 IU/l for GPT and 169 IU/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 IU/l and 250 IU/l, respectively, and increased to 15 IU/l and 300 IU/l, respectively, at 15°C. The fish were fed Fukosalm, a commercial fish diet.

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 activity. In control fish, plasma GDH activity should be negligible, but liver homogenates and pathologic plasma may have considerable GDH activity.

Our primary purpose in these investigations 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

TABLE 19. A COMPARISON OF ALANINE AMINOTRANSFERASE (GPT) AND ASPARTATE AMINOTRANSFERASE (GOT) ACTIVITIES IN PLASMA AND LIVER FROM SELECTED SPECIES OF FISHES.

Enzyme	Common Name 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(?)	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 catfish (?)			57.0 (10)	0.41 (10)	Wilson (1973)
GPT	Rainbow trout (10)			30.1 (20)		Smith et al. (1974)
GOT	Pink salmon (?)	309	(?)			Marquez (1976)
		non-spawning				
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. (1978)
GOT	Rainbow trout	169.0	(51)			Statham et al. (1978)

[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.

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.

Carbon tetrachloride can cause in vitro and in vivo hemolysis of mammalian erythrocytes (Von Oettingen, 1955) and this hemolysis does add significantly to the transaminase activity in the plasma (Caraway, 1962). The results of this study indicate that plasma GOT activity was increased by CCl_4-induced hemolysis but not by physical disruption of erythrocytes while plasma GPT activity was not consistently elevated by either treatment. These results appear to support the findings of Gaudet et al. (1975).

The hemolytic effect of CCl_4 and the resultant release of intracellular enzymes appears to be different from the physically induced hemolysis. CCl_4 has a high affinity for lipids, as does its metabolite, chloroform. The chemical structure and properties of membranes can vary from one tissue to another, and yet 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 surface and intracellular erythrocyte membranes results in the disruption of membrane structural and functional integrity and thereby causes loss of the 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. Our in vitro experiments indicate that CCl_4 has the potential to cause hemolysis in vivo that may 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 to a greater extent than plasma GPT activity. Since the simultaneous measurement of both plasma transaminases provided little relevant information concerning the nature of the pathological response of trout to CCl_4 intoxication only plasma GPT activity was measured.

The enzyme assay temperature may have little similarity to the environmental temperature of the organism; however, this *in vitro* temperature is important in comparative analysis of enzyme activities. Bell (1968) found that purified GOT from the liver of an adult coho salmon, *Oncorhynchus kisutch*, had activity which increased linearly from 10°C to 30°C. The International Union of Biochemistry and the International Federation of Clinical Chemistry has recommended a standard enzyme assay temperature of 30°C, but Bergmeyer (1978) suggests that 25°C would be more practical for most situations. He contends 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. Our studies indicate that 25°C was the maximum assay temperature to measure practically the liver GPT activity in these fish, and this temperature therefore was used in subsequent 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.

Carbon tetrachloride has been shown to be hepatotoxic to many vertebrate species (Diaz Gomez et al., 1975), including fishes (Bell, 1968; Gingerich et al., 1978a; Inui, 1969; Racicot et al., 1975; Statham et al., 1978a). Moon (1950) and Stricker et al. (1968) reported that CCl_4 also is 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), however no information is currently available regarding the nephrotoxic effect of CCl_4 in fishes. It is conceivable that measurable GPT activity could occur in the plasma from kidney damage in fish following CCl_4 intoxication. 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 approximately 40% of the specific kidney GPT activity.

The Michaelis constant (Km) is an important and useful characteristic of the enzyme 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 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 damaged tissue identified. The apparent Km-alanine for liver and kidney GPT was 5.6 mM and 5.0 mM, respectively and, therefore too similar to be applicable to the problem of identifying specific 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 tissue. 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.

Diseases of the liver, bone, small intestine, kidney and placenta all may contribute to increased activity of serum AP (Zimmerman and Henry, 1969). Serum alkaline phosphatase activity particularly is increased during both intra and extrahepatic cholestasis (Steiner et al. 1965), and for this reason it has been a useful diagnostic index of liver function in mammalian toxicology. In the present series of investigations serum alkaline phosphatase activity was not found to be useful as a diagnostic aid in assessing liver dysfunction in rainbow trout. Unlike mammals, (Baker et al., 1978) serum alkaline phosphatase activity in experimental trout was not increased following either acute extrahepatic cholestasis (ligation of cystic and common bile ducts) or by treatment with a mammalian cholestatic agent (alpha-napthyisothiocynate) even though the time course for these experiments was at least 48 h.

Exogenous Tests: Disposition of BSP by Rainbow Trout

Comparison of hepatic uptake and accumulation of BSP with its biliary excretion indicates that, as in mammals (Klaassen and Plaa, 1967) the latter is probably the rate limiting step in the transfer of this compound from plasma to bile in the trout. Biliary excretion appears to be dependent both on the rate of bile secretion and the capacity of the membrane systems to actively transport the compound into the bile. Thus, the rate of biliary excretion may be limited either by a reduced rate of bile secretion or a reduced capacity for active transport of the compound. Differences in rates of biliary excretion between trout and rats are not well explained by assuming the latter possibility since the concentration of BSP in trout bile at the transport maximum (11.8 mg BSP/ml bile) is similar to that of rat bile under similar experimental conditions (15.6 mg BSP/ml; Klaassen and Plaa, 1968). Therefore, differences in the inherent rates of bile flow between individual species are more likely to explain differences in the rates of biliary BSP excretion. The dependence of the rate of canalicular bile secretion on biliary excretory rate of BSP has been established in rats (O'Maille et al., 1966). Comparison of bile flow rates with the percent of a single dose of BSP excreted in the bile by several species suggests that inherent rates of bile secretion are most responsible for interspecific differences in biliary BSP excretory capacity (Table 20). The percent of a single dose of BSP secreted by each species after six hours was well correlated with the log of relative bile flow based on wet liver weights among three species ($r = 0.996$). Thus, differences in the biliary excretory capacity of BSP between these species may be explained as differences in the inherent rates of bile secretion rather than as differences in canalicular transport processes.

The relative importance of conjugation to the overall process of biliary BSP excretion has not been established in fishes. If dye conjugation was the important prerequisite for this process in the trout that it appears to be in the rat (Whelan et al., 1970; Priestly and Plaa, 1970b), a much higher proportion of metabolized dye should be expected in fish

TABLE 20. DEPENDENCE OF BILIARY EXCRETION OF A SINGLE INTRAVENOUS INJECTION OF BSP ON THE BILE FLOW RATE IN DIFFERENT SPECIES.

Species	Dose (mg/kg)	Bile Flow (µl/kg/min)	Bile Flow (µl/100 g liver/min)	Percent Dose Excreted in Bile after 6 h	
Dogfish	1.0[1]	1.23[2]	1.12[2]	10.0[1]	
Rainbow Trout	10.0	1.5	11.1	43.4	8.6[3]
Rat	37.5	64.0[5]	142.0[6]	84.6	3.2[4]

[1] From Boyer et al (1976a)

[2] Calculated from Boyer et al. (1976 b)

[3] Mean ±SE

[4] From Klassen (1975)

[5] From Klassen and Plaa (1967)

[6] Based on estimate of bile flow in ref. 5 and liver mass of 4.5% of body weight (Klaassen, 1973).

bile. Metabolized BSP initially represented approximately 25 percent of the total dye concentration of the bile in rainbow trout. Even though this value is nearly twice that which has been reported previously in the bile of several cartilagenous fishes (Boyer et al., 1976b,c), it represents only about one third of the amount of conjugated BSP which appears in the bile of rats (Whelan et al., 1970; Shultz and Czok, 1974). Studies comparing the relative rates of biliary excretion for free and conjugated BSP would be useful in determining the relative importance of conjugation for biliary excretion of this particular compound in the trout.

The apparent increase in the percent of metabolized BSP which was observed in trout bile during prolonged dye infusion was not expected. Infusion of BSP above the biliary T_m 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 number of BSP fractions, as well as the increased proportion of metabolized BSP in trout bile, may be the result of anomalies in hepatic blood flow which do not permit immediate and uniform distribution of the dye to all sinusoidal surfaces or the result of 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 the trout.

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, the present studies indicate that the rate of hepatic BSP accumulation also is severely reduced by this surgical procedure. Considering the relative efficiency of hepatic uptake and accumulation of BSP in the trout it is not immediately clear why surgically created cholestasis should impede these processes. Differences in the hepatic BSP content of the livers of sham and cystic-common bile duct ligated fish do not seem to be the result of differences in the amount of dye that was transferred into the canalicular and ductular biliary space. If this were the case more than one quarter of the injected dose of BSP would need to have been actively transported into the bile of sham treated fish within the initial 15 min period. Even if the net rate of transport of BSP had equaled the maximum biliary excretory rate (12.1 µg/kg/min) less than five percent of the injected dose of BSP could have been transported into the bile during this time. Thus, the differences in hepatic BSP content are more likely the result of altered uptake or storage capacities of the livers following experimental ligation. Such impairment may be due to cell wide biochemical and/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 shown recently that prolonged bile stasis results in a loss of microvilli on the bile canalicular surface and other ultrastructural alterations on the surfaces of rat hepatocytes. Similar biochemical and morphological alterations of trout hepatocytes following experimental bile duct ligation may

be responsible in part for the impaired plasma clearance and hepatic accumulation of BSP observed in this study.

These observations indicate that efficient mechanisms for hepatic uptake and biliary excretion of the organic anion BSP are present in the rainbow trout. Furthermore, the processes associated with the transfer of this compound from the plasma to bile in the trout appear to be relatively similar to those described for mammals. The results suggest that, as in mammals, hepatic excretory function in this fish may be an equally important route of elimination for certain classes of foreign compounds.

Acute Exposure Studies: Carbon Tetrachloride

The temporal pattern of plasma GPT activity in the Purina or Donaldson Diet fed fish treated with CCl_4 (1.0 ml/kg) was different from responses reported in previous studies. Racicot et al. (1975) found maximum GPT activity in plasma from rainbow trout fed Purina Trout Chow 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 our Donaldson Diet fish treated with nearly twice the dose of CCl_4 (2.0 ml/kg). Statham et al. (1978a) 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 those plasma activities reported by Racicot et al. (1975) or those found in our studies. 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 in rats with peak activities 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.

The results suggest that the diet of rainbow trout may have significantly altered the plasma GPT activity response to treatment with CCl_4. Previous studies with mammals and fish have demonstrated a variation in the hepatotoxic response to organochlorine compounds due to changes in dietary protein concentration (Korsrud et al., 1976; McLean and McLean, 1967). The protein quality or quantity in the Purina and Donaldson Diets was probably not a factor in the response of our trout to CCl_4. Purina Trout Chow (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%). Forty percent total dietary protein is considered to be the minimum concentration required by rainbow trout to insure normal metabolic homeostasis (Personal Communication, Dr. W. Stott; Department of Food Science, Oregon State University).

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 synthesis of the phospholipids necessary for normal mixed function oxidase (mfo) activity (Cooper and Feuer, 1973). The amino acid methionine is the principal methyl donor in mammalian lipotropic 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.

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 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 the trout to CCl_4.

An alternate explanation for these findings 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, they indicated that chemical residues can vary considerably between feed lots (Personal communication, Dr. P. Mehrle, Fish-Pesticide Research Laboratory, Fish and Wildlife Service, Columbia, Missouri). 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 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., 1976). If a chemical inducing agent was present as a contaminant in the Purina diet, the hepatotoxic response to treatment with CCl_4 would have been greater in our fish.

Another hypothesis to explain the variation in hepatotoxic response between the two groups of fish is the effect of dietary constituents on the 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, 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. (1978a) found that adipose tissue of rainbow trout treated with $^{14}CCl_4$ (bath exposure; 1 mg/l for 2 h) 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 h). In our studies, 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.

Results of our experiments with BSP suggest that plasma clearance of this dye may be a useful criterion by which to evaluate liver dysfunction in fish following acute exposure to toxicants. A significant decrease in plasma clearance was detected in fish receiving as little as 0.2 ml/kg i.p. of CCl_4. When plasma retention of BSP was used as an index of liver dysfunction, elevated levels of BSP were found in the plasma as long as 120 h after treatment. It is also apparent that plasma BSP clearance is not influenced by abnormally high levels of plasma hemoglobin which might 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 may also be true in fish. In the present histological studies some form of degenerative change was evident in the livers of all trout receiving CCl_4 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.

As in mammals, intoxication of rainbow trout with CCl_4 results in demonstrable morphological damage to the liver and plasma retention of BSP (Gingerich et al., 1978a). Because the processes of hepatic accumulation, metabolism and biliary excretion of this organic anion in the trout appear to conform to those of mammals (Schmidt and Weber, 1973; Gingerich et al., 1977, 1978b), it was of interest to investigate which of these processes in trout were most affected by CCl_4 treatment.

The accumulation of more than half of the dose of BSP in the livers of control animals 15 min after its injection indicates that egress of the dye from the plasma compartment was primarily the result of its uptake and accumulation by the liver. In contrast, the hepatic BSP content of treated fish was less than half that of the controls after this time. Furthermore, the apparent net rate of hepatic BSP accumulation was slower in treated fish despite plasma BSP concentrations that should have favored its hepatic uptake.

Maggio and Fujimoto (1966) similarly found that the concentration of BSP in the livers of mice treated with CCl_4 was less than that of controls following a single injection of BSP and concluded that impairment of uptake or storage was most responsible for the decrease in plasma BSP clearance. From the present study it appears that BSP plasma clearance in treated fish may be retarded in part by impaired uptake or storage; processes which were not differentiated by the methods used in this study.

While extracts of whole livers would be expected to be contaminated with residual BSP in the canalicular and ductular spaces, it does not seem likely that differences in hepatic BSP concentrations could result from differences in the amounts of BSP within these spaces. Bile BSP concentrations and rates of bile secretion in treated and control trout were similar during the first nine hours of BSP infusion. Even if the rate of biliary BSP transport in these animals had equalled the maximum rate of biliary BSP excretion as determined during the infusion experiments (12.1 µg BSP/kg/min), less than five percent of the injected dose of BSP would have been transported into the bile after 15 min. The difference in the total amount of hepatic BSP found in livers of treated and control fish 15 min after injection of the dye was more than 25 percent of the injected dose. Therefore, it appears that differences in the amount of BSP in the intrahepatic biliary space cannot adequately account for differences in the amount of BSP found in liver extracts of treated and control fish.

Results of the infusion experiments suggest that the excretory capacity of the liver was not greatly reduced 24 h after CCl_4 treatment. Bile flow rates, bile BSP concentration and total metabolized BSP appearing in the bile of treated fish were similar to those of control animals for at least 9 h after the start of BSP infusion and for 33 h after CCl_4 treatment. These results are in contrast to similar studies which have demonstrated that impaired biliary excretory function is the factor which most contributes to plasma BSP retention in rats following CCl_4 treatment (Klaassen and Plaa, 1968; Priestly and Plaa, 1970a). The possibility that impairment of biliary excretory function in treated fish occurs at a time later in the course of the intoxication cannot be excluded. The decrease in the rate of BSP excretion nine hours after the beginning of the infusion was the result of decreased bile flow. Furthermore, the greatest plasma BSP retention observed in treated animals during a time-course study was found 48 h after CCl_4 was administered (Gingerich et al., 1978a). Therefore, it may be possible that a decrease in bile flow may have contributed in part, to plasma BSP retention at some time beyond the temporal limits which were chosen for the biliary excretion studies.

The relative importance of BSP conjugation to the overall process of BSP excretion in fish has not been established. If metabolism of this compound was the important prerequisite for its biliary excretion in fishes that it appears to be in mammals (Whelan et al., 1970; Priestly and Plaa, 1970b), a higher proportion of metabolized BSP would be expected in their bile. The proportion of metabolized BSP in the bile of both treated and control trout was approximately 25 percent during the first hour of the infusion. This value is only one-third that reported for rat

bile (Schulz and Czok, 1974). However, despite these quantitative differences, the biliary excretory capacity of the trout liver does not appear to be overtly deficient. The concentration of BSP in the bile of trout during maximum rates of biliary dye excretion (11.8 mg/ BSP/ml) is not greatly different from that found in rat bile under similar experimental conditions (15.6 mg BSP/ml, Klaassen and Plaa, 1968). Therefore, it appears that even if CCl_4 intoxication had resulted in decreased hepatic BSP metabolism, it is unlikely that this would have affected biliary excretion of this compound sufficiently to cause its plasma retention.

It is possible that a decrease in hepatic blood flow following exposure to CCl_4 may be responsible for both impaired plasma clearance and hepatic accumulation of BSP by limiting its transport to the liver. Impairment of hepatic blood flow by ligation of the hepatic portal vein has been shown to decrease plasma clearance and hepatic accumulation of BSP in rainbow trout (Gingerich et al., 1977). Intraperitoneal administration of undiluted CCl_4 is known to result in general inflammation of the peritoneal cavity and the formation of thrombii in the ventral intestinal vein (Gingerich et al., 1978a). It is possible that these changes may have altered blood flow in the splanchnic drainage sufficiently to influence blood flow to the liver.

The use of function tests incorporating the organic anion BSP to evaluate liver dysfunction in trout may be useful providing that limitations of the technique are recognized. Thus, measurement of hepatic BSP concentrations must be interpreted not only in terms of the processes of uptake and accumulation; but also in terms of hepatic excretory function. Further, the techniques which have been successfully applied to study hepatic excretory function and storage capacity in small mammals (Klaassen and Plaa, 1967) do not seem practical in the trout because of the toxic effects of high plasma BSP concentrations (Schmidt and Weber, 1973) and the length of time necessary to establish a maximal rate of biliary excretion.

The increase in body weight and decrease in plasma osmolality in CCl_4-treated fish suggested that body water was retained in these animals and that the reduction in plasma protein concentration was due in part to an increase in the plasma volume. An *in vitro* dilution of trout plasma, of known protein concentration and osmolality, established that approximately 20% of the total protein decrease at 24 hours could be attributed to dilution of the plasma proteins with water retained in the fish.

Plasma albumin concentration in fish treated with CCl_4 was lower than that of controls at 24 hours posttreatment, but the increase in the albumin/total plasma protein ratio indicated that a part of the reduction in plasma proteins presumably was due to a loss of some globular 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. Berryman and Bollman (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 the treated 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 feed-back control is unknown and apparently non-specific since there is a concurrent increase in hepatic synthesis of other plasma proteins.

Intoxication with CCl_4 (2.0 ml/kg, i.p.) also produced an oliguria or anuria in rainbow trout as early as one hour after treatment which 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 during 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 to sub-lethal levels of ammonia to an increase in gill permeability to water, rather than a direct action on the kidney. Gingerich et al. (1978a) reported a significant weight gain in rainbow trout 24 hours post-treatment with CCl_4 (2.0 ml/kg, i.p.). The results of these studies indicate that the weight gain experienced by CCl_4-treated trout was related to impaired water clearance as reflected by a decrease in UFR and 24 h urine output.

Urine osmolality 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 as 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 dysfunction and is associated with glomerular renal tubular damage. This condition may occur even in the absence of demonstrable histopathology (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. We have found that CCl_4-treated trout experienced a reduction in total plasma protein concentration after 24 hours, in part due to dilution by retained body water. The presence of protein in the urine suggests that some proteins also may have been lost through damaged glomeruli or renal tubules.

The significance of this proteinuria is difficult to establish since this test does 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 of specific organ toxicity of the compound and should be used in conjunction with histopathological evaluations.

Acute Exposure Studies: Monochlorobenzene

Elevated plasma GPT activity (Pfeifer et al.,1977; Racicot et al., 1975; Statham et al., 1978a) and attenuated clearance of plasma BSP (Gingerich et al., 1978a) both have been used as diagnostic criteria by which to assess liver dysfunction in rainbow trout. In the present study MCB intoxication produced elevations in plasma enzyme activity at 8 and 72 h post-treatment. While a significant increase in plasma enzyme activity occurred only at 72 h, it is interesting to note the secondary increase in enzyme activity at 8 h. Biphasic responses in plasma GPT activity have been reported previously in both rats (Koeferl, 1972) and in rainbow trout treated with CCl_4 (Pfeifer et al., 1977; Racicot et al., 1975; Statham et al., 1978a). A similar biphasic pattern of plasma enzyme activity may be associated with MCB intoxication in the trout and may be indicative of the pathological processes occurring during the intoxication.

Significant retention of BSP was evident in the plasma of fish at 3, 12, and 24 h after MCB treatment. While it is possible that anesthetic effects associated with the MCB intoxication may have influenced plasma clearance of BSP by altering total hepatic blood flow, it is not likely that this effect was solely responsible for the significant plasma retention of the BSP observed in this study. Histological studies confirmed the presence of some degenerative changes in the hepatocytes as early as 8 h after treatment and evidence of minor degenerative changes were observed in livers of fish sampled at 24 and 48 h post treatment.

A comparison of the relative liver toxicities of MCB and CCl_4 following their i.p. administration of trout suggest that CCl_4 may be more hepatotoxic than is MCB. Significant changes in both the plasma GPT activity and concentration of BSP from plasma were apparent following treatment of trout with CCl_4 at all doses of this toxicant employed and at all sample periods used in these studies. In addition, CCl_4 did produce histological alterations in the livers of exposed trout including necrosis of hepatocytes surrounding central veins. In contrast, the results of studies with MCB were variable and inconclusive. Significant alterations of the clinical indicators of liver dysfunction were evident only at the highest doses of MCB used and only at specific times either early or late in the course of the intoxication. These results suggest that the liver of the rainbow trout may be more sensitive to intoxication by CCl_4 than by MCB.

The seemingly greater effect of CCl_4 in producing liver damage in trout may be attributed to several factors. Because CCl_4 was not administered in a corn oil vehicle it may have been absorbed more readily and had greater access to body compartments than did MCB. However, when

That serum GPT activity increased in a dose-dependent manner in trout both exposed to MCB is consistent with our impression that elevations in serum activity of this enzyme do reflect some specific organ toxicity. In laboratory mammals, halogenated benzene compounds, administered orally, produce consistent hepatic lesions presumably through highly reactive intermediate metabolites (Jerina and Daly, 1974). This hepatotoxicity, as well as that mediated through CCl_4, does cause an elevation in the serum activity of GPT and in most instances this elevated enzyme activity does correlate closely with the pathological state of the organ. We have not been able to demonstrate consistently these correlations in trout treated either acutely or subacutely with known mammalian hepatotoxic agents and therefore we can only speculate that the source of the increased GPT activity is from the liver.

Serum alkaline phosphatase activity of trout exposed to subacute levels of MCB was variable. A significant increase in AP activity was observed in fish exposed to low MCB concentrations after 15 days but this increase could not be related to dose. The serum AP activity after 30 days was not different from that of the paired controls. Fed control fish consistently had higher serum AP activity than either the treated or non-fed control group suggesting that serum AP activity may be mediated either by diet or nutritional state of the animal.

The relative decrease in the concentration of serum proteins noted in the subacute exposure study was related to the dose of MCB to which the fish were exposed. While these changes were not statistically significant the results suggest that some specific alteration in the constituent serum proteins had occurred. This is supported by the decrease observed in the ratio of fast to slow migrating zones identified on the serum electropherograms of fish sampled after 15 days of exposure. Because the liver functions to synthesize albumin decrease in the albumin to globulin ratio has been used as an index of liver function in mammalian toxicology and clinical medicine (Harper, 1975). Similarly, serum electrophoresis has been used to evaluate the responses of fish to such general conditions of stress as hypoxia (Bouck and Ball, 1965) and disease (Pesch, 1970) and to stress induced by exposure to sublethal levels of such pollutants as pulp mill effluent and industrial chemicals (Fujiya, 1961) and copper (Thurston, 1967). In all cases the response has been a decrease in the rapidly migrating fraction or fractions of the serum protein constituents. Whether this response is the result of a decrease in the rate of synthesis of these fractions or a preferential utilization of these fractions by the fish during food deprivation is not known.

Gross Pathology and Histology

The development of pericentral liver necrosis in trout following acute CCl_4 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 CCl_4 metabolism to critical cellular elements (Castro, et al., 1972; Klaassen and Plaa, 1969) 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 CCl_4 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 trout (Stanton and Khan, 1975; Chan, et al., 1967; Ludke, et al., 1972), and that rainbow trout are capable of hepatic biotransformations by this mixed function oxidase system (Petersen et al., 1976), it is conceivable that the pericentral liver necrosis that develops in trout following CCl_4 treatment is the result of its metabolism to an active intermediate or intermediates.

Only one animal in four from both the transected and non-transected fish treated with CCl_4 developed necrotic lesions in the pericentral regions of the liver, even though minor degenerative changes were found in 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 CCl_4 and the degree of hepatotoxicity it produces 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 et al. (1978a) have reported that rainbow trout dosed with undiluted $^{14}CCl_4$ (1.0 ml/kg i.p.) accumulated highest levels in the mesenteric fat surrounding the G.I. tract followed by intermediate concentrations in the heart, lvier, and gills. In the present study, large amounts of visceral fat may have reduced the effective dose of CCl_4 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 that periportal hepatocytes also would be damaged since cells in this region should be exposed to levels of CCl_4 sooner and in higher concentrations than those in the pericentral region. No evidence of periportal necrosis was found in the liver of any animal receiving CCl_4.

Intoxication of laboratory mammals with CCl_4 results in vacuolization of hepatocytes and triglyceride accumulation (Cornish, 1975). Previous studies with trout have demonstrated intense vacuolization of hepatocytes in both control fish and fish treated with CCl_4 (Racicot et al., 1975). These observations were similar to those made in this study, however histochemical staining confirmed that the vacuoles contained no lipid material but did contain glycogen. In support of these findings, Statham et al. (1978a) found that CCl_4 had no effect on liver triglyceride accumulation in rainbow trout and Sakaguchi and Hamaguchi (1975) reported no effect of CCl_4 on the hepatopancreas lipids of the yellowtail (Seriola dorsalis). Additionally, Statham et al. (1978a) noted vacuolization in control trout and intense vacuolization as well as focal and laminar necrosis in CCl_4-treated trout at 6 h post treatment (1.0 ml/kg i.p.).

Histopathological examination of liver and kidney sections of trout treated with CCl_4 indicate that there was no definitive correlation between the degree of hepatocyte or nephron damage and the dose of CCl_4 given, the diet fed or the time after treatment. Furthermore, it was not possible to correlate plasma GPT activity with the degree of hepatocelluar damage. The central vein necrosis, a characteristic lesion in mammals given CCl_4, was not a consistent histopathological feature of CCl_4 intoxication in the trout used in these studies.

The results of acute exposure studies suggest that monochlorobenzene intoxication may cause some hepatotoxicity in the rainbow trout. After 8 h moderate pericentral necrosis was evident in the liver of one treated fish and less dramatic degenerative changes were seen in pericentral hepatocytes of several other treated fish sampled at 24 and 48 h. The development of such lesions in pericentral regions of the trout liver is a response not unlike the centrilobular necrosis observed in laboratory mammals following MCB intoxication (Reid and Krishna, 1973; Brodie et al., 1971). In view of reports indicating that active metabolites are responsible for the hepatotoxicity observed in mammals following MCB intoxication (Reid and Krishna, 1973) and that rainbow trout are capable of hepatic biotransformations by a mixed function oxidase system (Petersen et al. 1976), it is possible that the morphological changes observed in the pericentral hepatocytes of the trout are mediated through active intermediates.

The value of assessing the effects of subacute or chronic exposure to pollutants on the function and performance of individual organ systems in fish in some sense lies in the successful adaptation of these techniques to field studies. Because of the considerable variation that exists in the clinical indices of liver function between individual fish and groups of fish these methods seem somewhat impractical for general field use. Such variations make inter-group comparison of field populations increasingly difficult. Observations of acutely and subacutely treated fish revealed gross behavioral changes even though alterations of clinical indices were not evident. For significant alterations to be apparent in these indices treated animals were severely stressed in most cases, suggesting that fish in the field would have to be similarly incapacitated before differences could be detected. The investment of large amounts of time and effort to describe such obviously deleterious effects seems impractical. Therefore, while these methods do have value in comparative toxicological research their relevance seems restricted to precisely controlled laboratory studies.

PUBLICATIONS RESULTING FROM PROJECT

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15. SUPPLEMENTARY NOTES

16. ABSTRACT

Procedures were developed to clinically evaluate liver damage and liver function in rainbow trout following either acute intraperitoneal (i.p.) treatment or subacute bath exposure to selected mammalian hepatotoxic agents. Elevations in serum of liver specific enzymes such as aspartate aminotransferase (GOT), alanine aminotransferase (GPT) and alkaline phosphatase (AP) were investigated as potential indicators of hepatocellular damage. An exogenous test of liver function, plasma clearance of the organic anion sulfobromophthalein (BSP), also was investigated as a potentially useful test of overall liver function in the trout.

The application of clinical tests to diagnose liver dysfunction in fishes following their exposure to environmental toxicants may be practical in controlled laboratory facilities. Despite the considerable variation that exists between groups of fish, significant differences could be demonstrated between control and treated fish. Variation among groups of fish make intergroup comparison of the field populations increasingly difficult by these methods. Therefore, the use of such techniques should be employed to evaluate liver toxicity under precisely controlled laboratory studies. Their application to field studies does not seem advisable.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Biochemistry Physiology Fishes Aquatic biology Enzymes	Rainbow trout Liver toxicity Clinical tests Hepatic	06/A 06/C 06/P 06/T

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