AN ABSTRACT OF THE THESIS OF

Jung Ta Cheng (Name) for the Master of Science (Degree) in Biochemistry (Major)

Date thesis is presented January 14, 1965

Title: The Effect of Potassium Pentachlorophenate on Selected Enzymes in Fish

Abstract approved [redacted] (Major Professor)

Research on chemicals used as pesticides has been intensified because of current interest in the possible long range effects on the biota. It is vitally important to understand the action of these chemicals on the metabolism of animals and plants which have direct or indirect contact with pesticides.

It has been shown that pentachlorophenol (PCP) is a potent uncoupling agent of oxidative phosphorylation. The effect of PCP on four selected enzymes in the muscles of cichlids and goldfish was studied.

Aldolase, glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and lactic dehydrogenase (LD) were apparently inhibited in vitro by potassium pentachlorophenate (KPCP). The inhibition increased with the increasing of the concentration of KPCP. Isocitric dehydrogenase (ICD) in contrast

showed a gradual increase in activity with KPCP up to 150 ppm.

For in vivo studies, acetone powder extracts of cichlids exposed to 0.0, 0.1 and 0.2 ppm KPCP for 0, 1, 2 and 4 days and acetone powder extracts of goldfish having been exposed to 0.0, 0.1, 0.2 and 0.4 ppm KPCP for 0 and 4 days were used. The results showed that there was a dual effect of KPCP, giving stimulation or inhibition of aldolase, GOT and GPT in cichlids muscle, and aldolase, GOT and LD in goldfish muscle depending on the concentration.

The increasing inhibition of these enzymes in vivo is directly proportional to the concentration of KPCP. This might be an indication that the inhibition was non-competitive.

The lethal dosage of KPCP for goldfish has been found as 0.46 ppm or 1.43×10^{-6} M KPCP.

THE EFFECT OF POTASSIUM PENTACHLOROPHENATE ON SELECTED ENZYMES IN FISH

by

JUNG TA CHENG

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1965

APPROVED:

[redacted]

Professor of Chemistry
Agricultural Chemistry Department

in Charge of Major

[redacted]

Head of Chemistry Department

[redacted]

Dean of Graduate School

Date thesis is presented January 14, 1965

Typed by Betty Hostetter

ACKNOWLEDGEMENT

The writer expresses his sincere gratitude to Professor Virgil H. Freed for his guidance during the course of this study.

Especial appreciation is extended to Dr. Shih-Dzung Lu for her suggestions and help in the course of work, and to Dr. Charles E. Warren and Mr. George G. Chadwick for their supply of fish samples.

This investigation is supported in part by the U. S. Public Health Service.

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INTRODUCTION

During the last decade the widespread use of chemical agents for control of weeds and insects has been rapidly established. Consequently a problem of contamination of the environment of human beings has arisen.

The possible danger arising from compounds used in this manner is chronic toxicity from either the parent compound or a metabolite. These conditions are of concern not only for organisms against which they are primarily directed, but also for other organisms which may be exposed by the atmosphere or by runoff into receiving bodies of water.

The effectiveness of pentachlorophenol (PCP) and its sodium salt as preservative agent, molluscicide, herbicide and in several other industries to supress the growth of many types of undesirable micro-organisms is well known. Thus, surface water is particularly susceptible to contamination by this toxicant and fish are organisms which are frequently exposed to harmful levels. It is the object of this study to investigate the effect of this chemical on fish, particularly the biochemical effects.

A great deal of work has been conducted with the toxicity of

of PCP related to animals. It is a potent uncoupling agent (27, 31) of oxidative phosphorylation. Nevertheless, the information on the ability of this toxicant to alter enzymes of glycolysis and nitrogen metabolism of animal tissue, especially in fish, is still scanty. An understanding of this toxicant in chronic enzyme inhibition in fish tissues is vitally important.

The objectives for this investigation were as follows:

(1) To determine the lethal concentration of KPCP for goldfish and compare the tolerance of cichlid and goldfish to this toxicant.

(2) To study the degree of action of some key enzymes, such as aldolase, lactic dehydrogenase, glutamic oxalacetic transaminase, glutamic pyruvic transaminase and isocitric dehydrogenase in muscles of both cichlid and goldfish by PCP with various sublethal dosages at different lengths of time exposure.

REVIEW OF LITERATURE

The toxicity of pentachlorophenol (PCP) to Diphtheria bacillus was noted by Bechhold and Ehrlich (3) in 1906. Kehoe et al. (12) found in 1939 that the oil soluble PCP or the water soluble sodium or potassium pentachlorophenate, when dissolved, were capable of absorption through the healthy intact skin of rabbits, resulting in increased respiratory and cardiac activity. When it was absorbed by the tissue of experimental animals in sufficient quantity, it would produce an acute toxemia characterized by accelerated respiration, hyperpyrexia, hyperglycemia and glycosuria. At sublethal dosages, in rabbits, the fever ran almost parallel with the blood sugar concentration, and some diuresis was exhibited during the second and third hour after treatment. However, the total volume of urine excreted during the first 24 hours was reduced to almost one half.

In 1942, Deichmann et al. (9) studied extensively the acute and chronic effects of PCP and its sodium salt upon dogs, rabbits, rats, and guinea pigs. These studies confirmed the work of Kehoe et al. (12).

In 1941, McGavack et al. (14) pointed out that sodium pentachlorophenate had some cumulative effect when it was applied to rabbits in sublethal dosage. They found in rabbits

that the minimum lethal dose of the compound per kilogram of body weight for oral, cutaneous, subcutaneous and intraperitoneal routes of administration were 512.5, 275.0, 135.5, and 550.0 mg respectively.

Since Loomis (13) in 1948 established the well known uncoupling of phosphorylation from oxidation by 2, 4-dinitrophenol in enzyme preparations of rabbit kidney homogenate, a number of uncoupling agents have been found. Weinbach (27) in 1954 showed that in the rat liver mitochondria incubated with PCP in concentrations ranging from $1 \times 10^{-4} - 1 \times 10^{-6}$ M, there was a supression of the inorganic phosphate uptake while the oxidation of α-ketoglutarate to succinate was relatively unaffected. On the other hand, the liberation of inorganic phosphate from ATP in fresh mitochondria preparation was greatly enhanced by adding 5×10^{-5} M PCP. The enhancement of ATPase activity of fresh mitochondria preparation by PCP was depressed by adding fluoride or magnesium ions. The explanation offered was that the uncoupling of phosphorylation from oxidation by PCP might account, at least in part, for the toxic manifestations of this compound. Later, Weinbach (28, 31, 32) carefully worked on rat liver mitochondria and found that pentachlorophenol, in contrast to 2, 4-dinitrophenol, exerted a dual effect upon mitochondria ATPase. In low concentration (5×10^{-5} M), PCP elicited ATPase activity from fresh prepared, undamaged rat

liver mitochondria and enhanced the hydrolysis of ATP by soluble enzymes from acetone powder extract. In higher concentration (5×10^{-4} M), PCP inhibited the ATPase activity of both damaged or undamaged rat liver mitochondria.

PCP in concentrations as low as 3 ppm (corresponding to 10^{-5} M) was lethal dose to certain aquatic snails under controlled laboratory conditions (18, p. 743). Weinbach (31, 30, 26) studied the effect of PCP on the metabolism of the snail Australorbis glabratus and showed that aerobic exposure of living snails to low concentrations (7.5×10^{-6} M or 2 ppm) of PCP resulted in the accumulation of acetate, lactate, pyruvate and inorganic phosphate in their tissue. At low concentration (2×10^{-6} M), there was a definite acceleration of the oxygen consumption while at higher concentration (2×10^{-5} M) inhibition was observed. Similar results were found with the minced-snail tissue. These findings indicated that PCP increased glycolysis and disturbed inorganic phosphate metabolism. Also these results supported the hypothesis that the molluscicidal activity of PCP was due, at least partially, to its ability to uncouple oxidative phosphorylation.

Although PCP was a very effective uncoupler even at low concentration, it was interesting to note that glycolytic phosphorylation in the whole homogenate or the soluble fraction of snail tissue

was not interrupted by PCP in concentrations as high as 5×10^{-4} M (29). However, 5×10^{-3} M PCP completely inhibited glycolysis and the associated anaerobic phosphorylations. On the other hand, PCP in concentration of 2.5×10^{-5} M completely prevented the uptake of inorganic phosphate which was associated with the oxidation of β-hydroxybutyrate in snail tissue. This showed that PCP is a potent uncoupler.

In 1958, Parker (19) studied the similarities and differences of a series of nitro- and halogenophenols with respect to their action on the enzymes activity of rat liver mitochondria in vitro. The phenols could be divided into two groups based upon the characteristics of their behavior toward oxidative phosphorylation, oxidation of glutamic acid and release or activation of latent ATPase. The first group included 2, 4-dinitrophenol, p-nitrophenol, pentachlorophenol and trichlorophenol. The main features of this group were: a maximum stimulation of ATPase activity, a large stimulation of oxidation of glutamic acid and an inhibition of oxidative phosphorylation with no effect on the concurrent oxygen uptake except at concentrations which completely inhibit phosphorylation. The second group of phenols included 2, 4-dichlorophenol, o-chlorophenol, m-nitrophenol, o-nitrophenol and phenol. These compounds produced no maximum ATPase activity, stimulated oxidation only slightly and inhibition of oxidative phosphorylation was closely

followed by inhibition of the concurrent oxygen uptake.

Buffa et al. in 1959 (5) and 1960 (6) studied some biochemical lesion in rat-liver mitochondria produced by injection with PCP. In one minute after the intraperitoneal injection of 20 mg of PCP per kilogram of body weight, the liver mitochondria entirely lost respiratory activity. The P/O ratio fell more rapidly with increasing dose of PCP when mitochondria were incubated with pyruvate. The results showed a highly reduced phosphorylative capacity and a much increased ATPase activity. The tissue content of high-energy phosphate compounds decreased and was followed by an increase of body temperature. In 1963 Buffa et al. (7) reported their further work on the recovery tests in rats after the injection of PCP. The results showed that the return of liver mitochondria to the conditions of controls occurred gradually and lagged behind the return of the body temperature to the normal values. The explanation was that the altered function was due to a biochemical lesion caused by PCP and was not on some generic structural change induced by intoxication.

In 1963, Mitsuda et al. (16) studied the inhibition of oxidative phosphorylation in rat liver mitochondria caused by chlorophenols using polarographic technique. Pentachlorophenol, tetra-, tri-, di- and monochlorophenol were found to inhibit the oxidative phosphorylation in a similar manner. The concentration of inhibitor

and the length of reaction time affected the behavior of the inhibition. The mode of the inhibition was classified into the uncoupling reaction of oxidative phosphorylation at the lowest concentration, the inhibition of the formation of the high energy intermediate compound in the phosphorylation system at the intermediate concentration, and the inhibition of electron transport system at the highest concentration. The inhibitory activity of chlorophenol analogues varied with the number and the position of chlorine group, PCP being the strongest, 2-monochlorophenol being the weakest. The dissociation constant of the inhibitor correlated with the inhibitory activity of the inhibitor.

In 1963, Weinbach et al. (33, 34) and Garbus et al. (10) reported the ability of serum albumin to restore the capacity for oxidative phosphorylation to rat liver mitochondria uncoupled by various substituted phenols. Serum albumin was able to exercise the restorative effect by binding and removing the uncoupling compounds from the liver mitochondria. Later it was found that the addition of ATP to serum albumin was more effective in promoting rapid reversal of mitochondrial swelling induced by PCP.

Bargoni (2) in 1964 studied the effect of 2, 4-dinitrophenol and salicylate on purified enzymes: glucose phosphate isomerase, phosphoglucomutase, aldolase, glyceraldehyde phosphate dehydrogenase, phosphopyruvate hydratase, pyruvate kinase and lactic

dehydrogenase. The effect was inversely proportional to the enzyme concentration. The inhibition could be reduced by the addition of crystalline serum albumin to the enzyme. However, the mechanism of the inactivation produced by the two phenols remained unknown. The suggestion was that a very labile bond might be formed between the effector and the enzyme.

MATERIALS AND METHODS

Experimental Animals

The test animals used in the experiments were the tropical fish Cichlasoma bimaculatum and the goldfish Carassius auratus. The cichlids were obtained from Pacific Cooperative Pollution and Fisheries Research Laboratory, Oak Creek, Corvallis, Oregon. The goldfish were obtained from a hatchery in Hubbard, Oregon.

Chemical

Pentachlorophenol (PCP, C_6HCl_5O)

The pentachlorophenol used in this study was obtained from Monsanto Chemical Company and recrystallized in this laboratory.

The physical properties: the pure form is colorless crystals with phenolic odor, m. p. 191°C. The solubility in water is about 20 ppm and the solubility of the potassium salt is about 33 gm. per 100 gm. of water at 25°C. In these experiments, potassium pentachlorophenate (KPCP) was used.

Preparation of potassium pentachlorophenate stock solution:

One hundred mg of recrystallized KPCP were dissolved in 100 ml of redistilled water and kept in a cold room. This 1000 ppm stock solution was diluted to the proper concentration during the experiments.

Methods

Determination of Lethal Concentration

The lethal dosage of PCP for cichlids as determined in this laboratory by other workers is 0.24 ppm (or 9.0×10^{-7}M).

Studies were conducted to determine the lethal concentration for goldfish as follows:

Five-gallon enamel tanks were used and each contained 10 liters dechlorinated water. Varying amounts of KPCP solution were added to the water to achieve the desired concentration. A small stream of air was introduced into each tank. Two uniform size fish, 6 - 7 inches long, were selected for each dosage tolerance test. The fish were held without food during the 96 hour test period. In order to keep the toxicant at a constant concentration, the solution was changed every 24 hours. The dead fish were removed as soon as they were noticed. The results of this test are shown in Table 1.

Exposure Test in Sublethal Dosage of KPCP to Cichlid and Goldfish

Experimental exposures of cichlids, 2 to 4 inches long, to KPCP were carried out in a 5-gallon enamel tank, with 10 liters of dechlorinated water at room temperature. In each test, one fish tank without treatment was used as a control. Concentrations of

Table 1. Lethal Limit of KPCP to Goldfish.

Concentration of KPCP	No. of Fish per Tank	Survival Time	No. of Survival	Mortality
ppm		Hours		%
0.00	6		6	0
1.00	2	3, 3	0	100
0.80	2	3, 4	0	100
0.60	2	9, 10	0	100
0.50	2	12, 15	0	100
0.48	2	14, 20	0	100
0.47	4	10, 23, 30, 90	0	100
0.46	4	7, 8, 27, 89	0	100
0.45	4	7, 90, 95	1	75
0.40	4	16	3	25
0.30	2		2	0

KPCP of 0.1 and 0.2 ppm were used at exposure times of 24, 48 and 96 hours.

For goldfish, the same procedure was employed. Concentrations of KPCP at 0.1, 0.2 and 0.4 ppm were used.

Acetone Powder Preparation

Preparation of acetone powders of fish tissue was conducted as quickly as possible in a cold room at 4°C. The preparation was conducted according to the method of Morton (17, p. 37) with slight modifications. This preparation has shown a high enzyme activity.

At the end of each exposure, the fish were removed to the cold room and sacrificed by decapitation. The scales and fins were removed and the fish were eviscerated immediately. The muscle was removed and transferred to a chilled beaker. Each sample was a combination of the muscles of two fish. About twenty grams of wet muscle were obtained in most cases.

The muscle was added to 200 milliliters of purified acetone at -18° to -20°C in a 500 ml Waring Blender jar. The acetone temperature was reduced by adding a small amount of dry ice. The sample was then blended for three minutes, transferred to a Buchner funnel and filtered under vacuum through Whatman #1 filter paper. The powder was removed and again submitted to the same procedure three times for cichlid and four times for goldfish muscle. The suction was continued for two hours after the pad was dry. The acetone powder was stored in a vacuum desiccator at 4°C. The yield of dried acetone powder was about 3.4 grams (17%).

Measurement of Aldolase Activity

Reagents:

Tris buffer, 0.05 M, pH 8.9: 0.1 M solutions were prepared by dissolving 12.11 grams of tris (hydroxymethyl) amino-methane in one liter of redistilled water or 15.81 grams of tris (hydroxymethyl) amino methane hydrogen chloride

in one liter of redistilled water. They were diluted to half of the strength and adjusted to pH 8.9 and stored in a refrigerator.

Aldolase substrate, 0.05 M; 0.25 grams Fructose-1, 6-Diphosphate, sodium salt, dissolved in 10 ml redistilled water and stored in a freezer.

Hydrazine solution, 0.56 M, pH 8.6: Sigma reagent stock No. 750-3.

Trichloroacetic Acid (TCA), 10% : 20 grams of trichloroacetic acid dissolved in 200 ml of redistilled water.

Sodium Hydroxide, 0.75 N: 30 grams of NaOH dissolved in 1 liter of redistilled water.

2, 4-Dinitrophenylhydrazine in HCl: Sigma reagent stock No. 750-2. Stored in a refrigerator and protected from light.

Aldolase Calibration solution: Sigma reagent stock No. 750-11. Stored in a freezer.

Procedure:

This investigation included the tests of in vitro and in vivo activity.

in vitro: The acetone powder was made from control fish and the enzymes activity assayed by adding various amounts of KPCP in the homogenate.

in vivo: The acetone powder was made from fish exposed to

KPCP and assayed for enzymes activity.

Aldolase activity was measured by using a modification of Sibley and Lehninger's method (21, 23). This method was based on the determination of triose phosphate hydrolyzed from fructose-1, 6-diphosphate substrate.

One half gram of acetone powdered fish muscle was added to 10.0 ml of 0.05 M tris buffer of pH 8.9 at 4°C in a 30-ml Virtis flask. The mixture was blended in a Virtis homogenizer at the lowest speed for 30 minutes. The thick suspension was transferred to a 50-ml polyethylene centrifuge tube and centrifuged twice at 18,400 x g for 10 minutes in a model "A" Betafuge centrifuge. The supernatant was used as a 5% stock aldolase homogenate for the total nitrogen (1, 15) and the aldolase activity determinations (23, 21).

One ml of the 5% homogenate was diluted to 250 ml with 0.05 M tris buffer pH 8.9 as a 0.02% homogenate. To both the "Test" and "Blank" tubes, 0.2 ml of the 0.02% homogenate, 1.4 ml of 0.05 M tris buffer and 0.2 ml of hydrazine solution were added and incubated at 37°C for 3 to 5 minutes. Two-tenths ml of fructose-1, 6-diphosphate was added to each "Test" tube and both tubes were incubated at 37°C for 30 minutes. Two ml of 10% TCA was added to each tube and the reaction was stopped. To the "Blank" tube, 0.02 ml of aldolase substrate solution was added and mixed well.

The tubes were centrifuged at 2500 rpm for 5 minutes in a clinical centrifuge. To 1.0 ml of supernatant of each tube, 1.0 ml of 0.75 N NaOH was added and kept at room temperature for 15 minutes. One ml of 2, 4-dinitrophenylhydrazine reagent was added to each tube and the tubes were incubated in the 37°C water bath for one hour. They were removed from the bath and added to 7.0 ml of 0.75 N NaOH. The tubes were mixed well and allowed to stand at room temperature for 5 minutes. Color was developed and read at 540 mμ on Beckman DU Spectrophotometer. The readings should be completed within 10 minutes after the addition of the NaOH.

Aldolase activity of muscle of both untreated cichlid and goldfish homogenate were treated with different concentrations of KPCP. This is referred to as the in vitro tests.

Calibration Curve:

Two flasks were marked "Blank" and "Standard" which contained 1.0 ml of H_2O and 1.0 of aldolase calibration solution respectively. One-tenth ml hydrazine solution, 1.0 ml 10% TCA, 2.0 ml 0.75 N NaOH and 2.0 ml 2, 4-dinitrophenylhydrazine reagent were added to both flasks. They were mixed well by swirling after each addition. Both flasks were incubated for one hour in the 37°C water bath. After the addition of 14.0 ml 0.75 N NaOH to each flask, mixed well by swirling and placed at room temperature.

Pipetted the "Blank" and "Standard" solutions into six tubes

in the proportions shown in Table 2. The Calibration Curve is shown in Figure 1.

Measurement of the Activities of Glutamic-Oxalacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPT).

Reagents:

Tris buffer, 0.1 M, pH 7.5

Standardized Asparate-α-ketoglutarate Substrate, pH 7.5; Sigma Reagent Stock No. 505-1. Stored in refrigerator.

2, 4-Dinitrophenylhydrazine: 20 mg of 2, 4-Dinitrophenylhydrazine dissolved in 1 N HCl and made up to 100 ml.

Sodium Hydroxide, 0.40 N. This solution had to be accurately standardized and reasonably free of CO_2.

Alanine-α-ketoglutarate Substrate, pH 7.5: Sigma Reagent Stock No. 505-51. Stored in refrigerator.

Pyridoxal-5'-phosphate, 300 ppm: 36 mg of Pyridoxal-5'-phosphate dissolved in 120 ml of 0.1 M Tris buffer pH 7.5.

Calibration Standard Solution: Sigma Reagent Stock No. 505-10

Procedure:

The preparation of 5% homogenate for measurement of GOT and GPT activity was the same as described in aldolase except the tris buffer was 0.1 M, pH 7.5.

GOT and GPT activities were determined by using the method of Reitman and Frankel (20, 22) with slight modification.

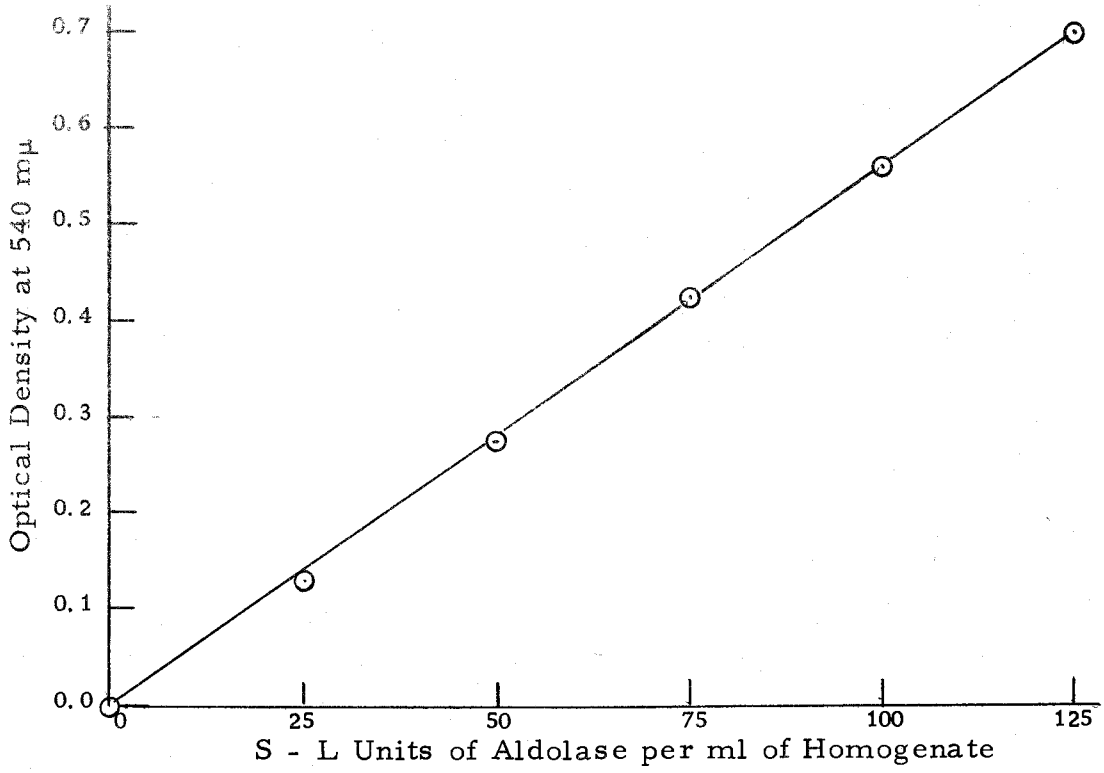


Figure 1. Calibration Curve of Aldolase Activity.

Table 2. Aldolase Standard Solution for Calibration Curve.

Tube No.	"Blank"	"Standard"	O. D. 540 mμ	S - L Units[1] of Aldolase Per ml of homogenate
1	5	0	0.000	0
2	4	1	0.130	25
3	3	2	0.278	50
4	2	3	0.425	75
5	1	4	0.561	100
6	0	5	0.700	125

[1] S - L Unit of Aldolase activity is defined as that amount of enzyme which will split 1 cubic millimeter of Fructose-1, 6-Diphosphate per hour at 37°C, under the assay conditions described in the procedure (23, p. 5).

GOT activity determination: For a 0.2% homogenate, 1.0 ml of 5% homogenate was diluted to 25 ml with 0.1 M Tris buffer, pH 7.5. To 1.0 ml Asparate-α-ketoglutarate substrate which was already at 37°C in water bath, was added 0.2 ml of the 0.2% homogenate and 0.05 ml of 300 ppm Pyridoxal-5'-phosphate mixed gently and replaced into the bath. Exactly 60 minutes after adding 0.2% homogenate, 1.0 ml 2, 4-dinitrophenylhydrazine reagent was added to terminate the reaction, the tube shaken gently and left at room temperature. At the end of 20 minutes 10 ml of 0.40 N NaOH was added and mixed well. After 5 minutes, the optical density was read on Beckman DU Spectrophotometer at 510 Mμ. Redistilled water was used as reference.

GPT activity determination: For 1% homogenate, 1.0 ml of 5% homogenate was diluted to 5 ml with 0.1 M Tris buffer, pH 7.5. 1% homogenate was found to be the optimum concentration for the test. To 1.0 ml of alanine-α-ketoglutarate, which was already at 37°C in water bath, 0.2 ml of 1% homogenate and 0.05 ml of 300 ppm Pyridoxal-5'-phosphate were added, shaken gently and replaced into the bath. Exactly after 30 minutes, 1.0 ml of the color reagent was added. The remaining procedure was the same as described for GOT determination.

Calibration Curve:

Into six test tubes, the proportional amount of solutions were

pipetted as indicated in Table 3. The entire calibration procedure was carried out at room temperature. One ml of 2, 4-dinitrophenylhydrazine was added to each tube, shaken gently and allowed to stand for 20 minutes. To each tube, 10 ml of 0.40 N NaOH was added, mixed well and transferred to cuvettes. Redistilled water was used as reference. After five minutes, the optical density was read at 510 mμ. The calibration curve was shown in Figure 2.

Measurement of Lactic Dehydrogenase (LD) Activity

Reagents:

Tris buffer, 0.1 M, pH 7.5

β-Nicotinamide adenine dinucleotide, reduced form Disodium salt (β-NADH), Sigma grade

Standardized Pyruvate Substrate, pH 7.5: Sigma Reagent Stock No. 500L-1

2,4-Dinitrophenylhydrazine Reagent: 20 mg of 2,4-Dinitrophenylhydrazine dissolved in 1 N HCl and made up to 100 ml.

Sodium Hydroxide, 0.40 N: 16 grams of sodium hydroxide pellets dissolved in redistilled water and made up to 1 liter. The 0.40 N NaOH had to be accurately standardized and reasonably free of CO_2.

Procedure:

LD activity was determined according to the method of Berger

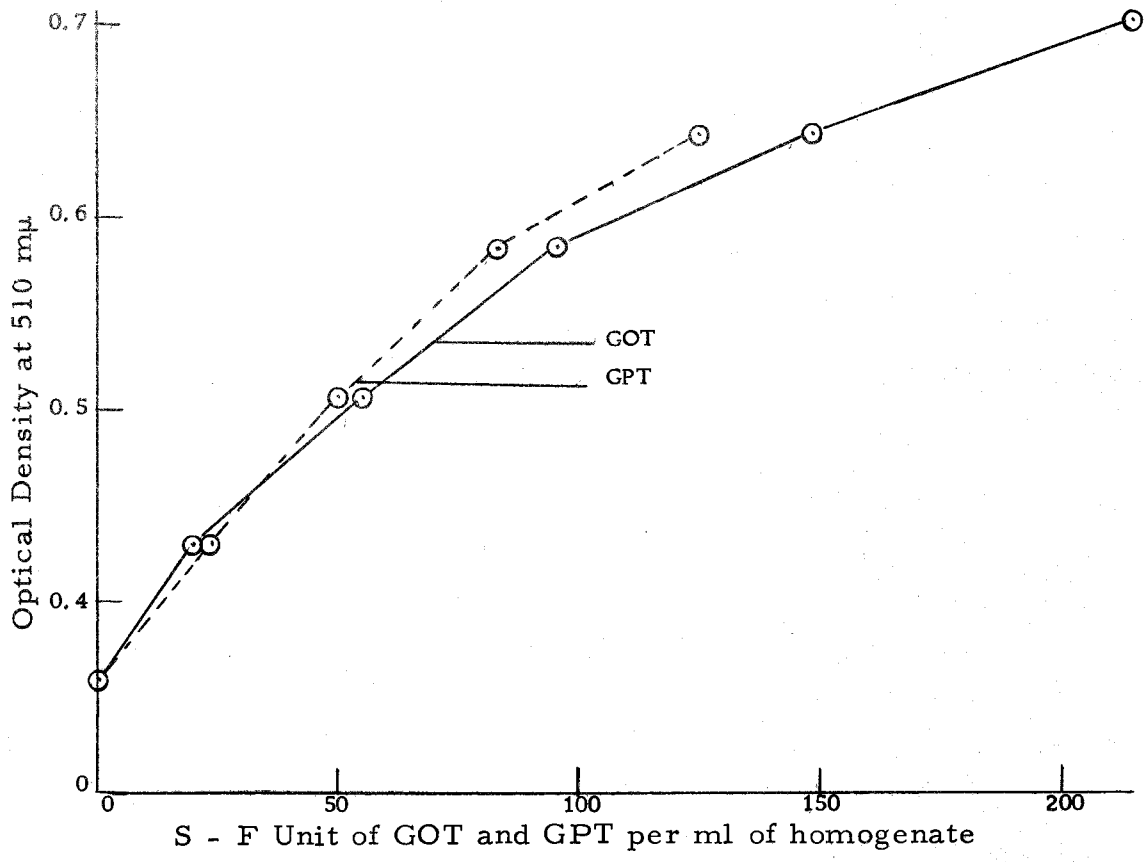


Figure 2. Calibration Curve of GOT and GPT Activities

Table 3. GOT and GPT Standard Solution for Calibration Curve.

Tube No.	Std. Soln. ml	Aspartate-glutarate Substrate ml	Pyridoxal phosphate ml	Water ml	O. D. 510 mμ	S-F Unit[1] for GOT per ml of Homogenate 37°C	S-F Unit[1] for GPT per ml of Homogenate 37°C
1	0	1.0	0.05	0.15	0.360	0	0
2	0.1	0.9	0.05	0.15	0.430	20	23
3	0.2	0.8	0.05	0.15	0.506	55	50
4	0.3	0.7	0.05	0.15	0.585	95	83
5	0.4	0.6	0.05	0.15	0.643	148	125
6	0.5	0.5	0.05	0.15	0.703	216	

[1] One theoretical Sigma-Frankel (S-F) unit of GOT or GPT will form 4.82×10^{-4} μM of glutamate per minute at pH 7.5, 25° C (22, p. 2).

and Brodia (4) and Cabaud, and Wroblewski (8).

For a 0.0167% homogenate, 1.0 ml of 5% homogenate was diluted to 300 ml with 0.1 M Tris buffer, pH 7.5. Fifty mg of 95% pure β-NADH dissolved in 47.5 ml of pyruvate substrate. One ml of the β-NADH and substrate mixture was pipetted to a tube and placed into a water bath at 37°C for five minutes. One-tenth ml of 0.0167% homogenate was added and shaken gently. The incubation was continued for 30 minutes. One ml of the color reagent was added to the tube, mixed well by swirling and removed from the bath. After standing at room temperature for 20 minutes, 10 ml of 0.40 N NaOH was added, mixed and transferred to cuvette. After five minutes the optical density was read at 460 mμ on Beckman DU Spectrophotometer. Redistilled water was used as reference.

Calibration Curve:

The calibration curve of LD activity was prepared from six tubes as indicated in Table 4. One ml of the color reagent was added to each tube and left at room temperature for 20 minutes. Ten ml of 0.40 N NaOH added to each tube and shaken well. After five minutes they were read and recorded. The calibration curve is shown in Figure 3.

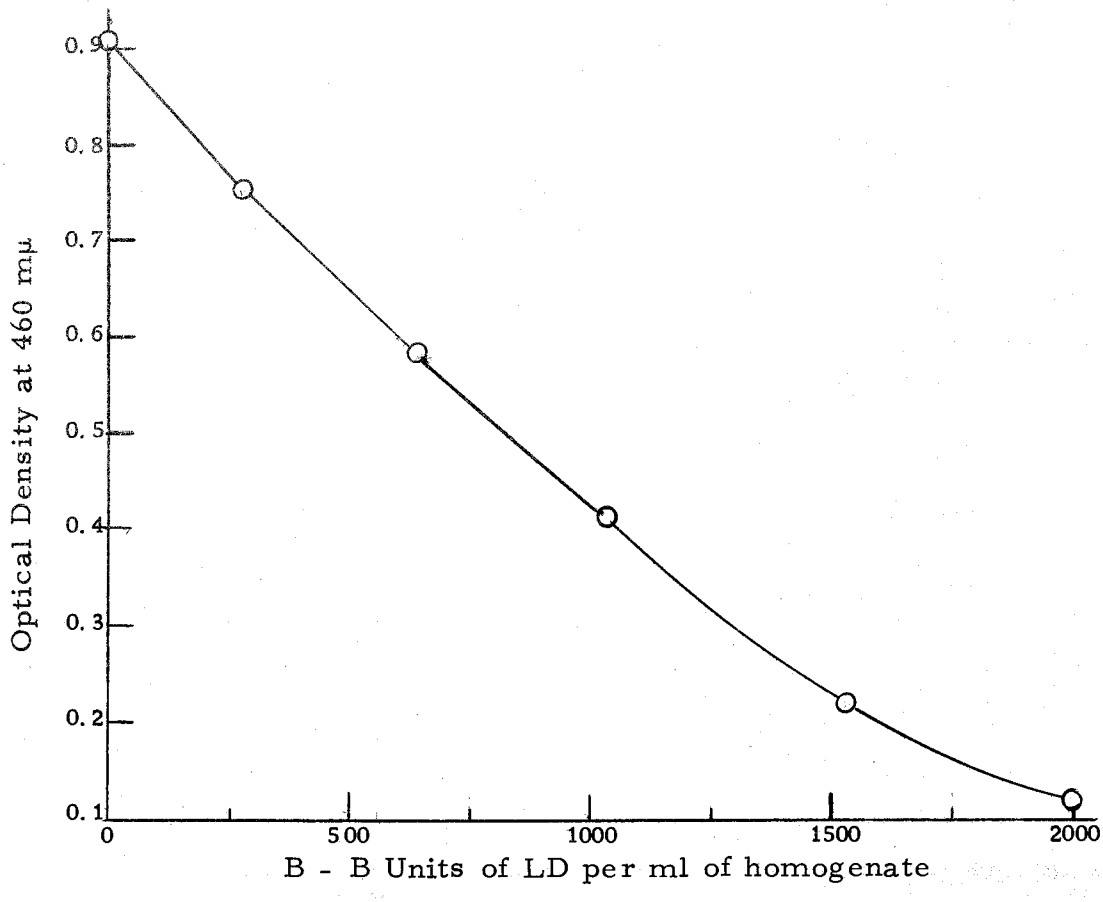


Figure 3. Calibration Curve of LD Activity

Table 4. LD Standard Solution for Calibration Curve.

Tube No.	Pyruvate Substrate ml	Water ml	O. D. at 460 mμ	B-B Units[1] of LD per ml of Homogenate
1	1.0	0.1	0.910	0
2	0.8	0.3	0.756	280
3	0.6	0.5	0.588	640
4	0.4	0.7	0.420	1,040
5	0.2	0.9	0.222	1,530
6	0.1	1.0	0.123	2,000

[1]Each B - B unit is equivalent to that amount of enzyme that would cause a decrease in O. D. at 340 mμ of 0.001 per minute in a reaction mix of 3 ml volume per Wroblewski (4, p. 6, 36).

Measurement of Isocitric Dehydrogenase (ICD) Activity

Reagents:

Tris buffer, 0.1 M, pH 7.5

Manganese Chloride Solution, 0.01 M: Sigma Reagent Stock No. 175-4.

Sodium Isocitrate Substrate, 0.1 M: Sigma Reagent Stock No. 175-1.

Ethylenediaminetetraacetate Solution (EDTA), 0.1 M: Sigma Reagent Stock No. 175-3.

2,4-Dinitrophenylhydrazine Reagent: 20 mg 2,4-Dinitrophenylhydrazine dissolved in 1 N HCl and made up to 100 ml.

NADP Solution: 30 mg of Nicotinamide adenine dineucleotide phosphate, Monosodium, Sigma Grade, Stock No. 201-30, dissolved in 10 ml of redistilled water.

Sodium Hydroxide, 0.40 N: 16 grams of NaOH pellets dissolved in redistilled water and made up to 1 liter. It was accurately standardized and reasonably free of CO_2.

ICD Calibration Standard Solution, (α-ketoglutaric Acid): Sigma Reagent Stock No. 175-10.

Procedure:

Isocitric dehydrogenase activity was measured according to the colorimetric procedure which was developed by the Sigma Research Laboratory (24). The procedure was based on the work of

Taylor and Friedman (25).

For a 6.67% homogenate, 15 ml of 0.1 M Tris buffer, pH 7.5 was added to one gram fish muscle acetone powder. To both "Test" and "Blank" tubes, 0.2 ml of 6.67% homogenate, 0.3 ml of Tris buffer and 0.2 ml of $MnCl_2$ solution were added and placed in a 37°C water bath for 3-5 minutes. One-tenth of isocitrate substrate and 0.2 ml of 0.004 M NADP solution were added to the "Test" tube, mixed gently and replaced in water bath. Three-tenths of redistilled water was added to the "Blank" tube. Exactly after 60 minutes, 0.5 ml of the color reagent and 0.2 ml of EDTA solution were added to both tubes, mixed and left at room temperature for 20 minutes. Five ml of 0.40 N NaOH was added to each tube, mixed well and transferred to cuvettes. After five minutes, they were read at 395 mμ on Beckman DU Spectrophotometer. Redistilled water was used as reference and the readings should be completed within ten minutes.

Calibration Curve:

The amount of ICD standard solutions and water were pipetted into each tube, as indicated in Table 5. Three-tenths of Tris buffer, 0.2 ml of $MnCl_2$ solution, 0.2 ml of EDTA solution and 0.5 ml of the color reagent were added to each tube, mixed gently and allowed to stand at room temperature for 20 minutes. Five ml of 0.40 N NaOH was added to each tube and mixed well.

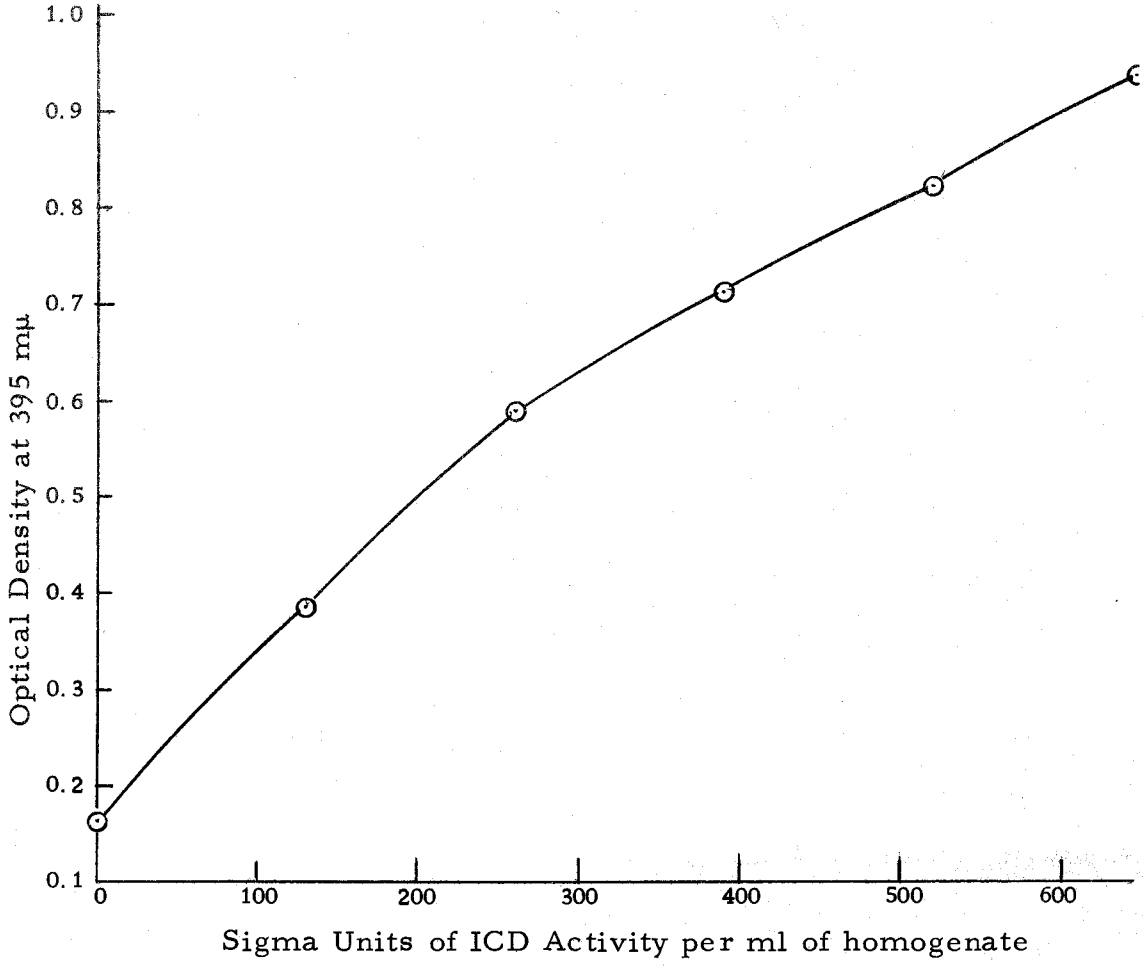


Figure 4. Calibration Curve of ICD Activity.

Table 5. ICD Standard Solution for Calibration Curve.

Tube No.	α-ketoglutaric Acid ml	Water ml	O. D. at 395 mμ	Sigma Units[1] of ICD Per ml of Homogenate
1	0.0	0.5	0.166	0
2	0.1	0.4	0.391	130
3	0.2	0.3	0.594	260
4	0.3	0.2	0.715	390
5	0.4	0.1	0.825	520
6	0.5	0.0	0.940	650

[1] One Sigma unit of ICD activity is defined as that amount of enzyme which causes the formation of one millimicromole of TPNH or α - KG per hour at 25° C (24, p. 6).

After the tubes stood at least five minutes, the optical densities were read at 395 mμ on Beckman DU Spectrophotometer and the readings completed within ten minutes. The calibration curve is shown in Figure 4.

Protein Determination

Reagent:

Mercuric Oxide, Reagent grade

Sucrose, Reagent grade

Potassium Sulfate, Reagent grade

Concentrated Sulfuric Acid, Reagent grade

50% Sodium Hydroxide, Reagent grade

25% Sodium Thiosulfate, Reagent grade

Boric Acid, saturated solution, Reagent grade

Methyl Red-Methylene blue indicator: mixed 2 parts 0.2% ethanol methyl red solution with 1 part 0.2% ethanol methylene blue solution.

HCl, 0.02 N.

Procedure:

Micro-Kjeldahl method (1, 15) was used to determine the total nitrogen content of the acetone powder extract of fish muscle.

Micro-Kjeldahl digesting mixture contained 2 parts of mercuric oxide, 5 parts of sucrose and 25 parts of potassium sulfate.

Two milliliter of concentrated sulfuric acid and about 0.5 grams of micro-Kjeldahl digesting mixture were added to each 30-ml micro-Kjeldahl digesting flask which contained 0.5 ml of 5% homogenate. The digestion was carried out on an electrical micro-Kjeldahl digestion rack for about five hours and continued for about 30 minutes after the content was colorless. The digested sample was transferred to a micro-Kjeldahl distillation flask and rinsed twice with 2-3 ml of distilled water. Ten milliliter of sodium hydroxide and sodium thiosulfate mixture - 200 ml of 25% sodium thiosulfate and 800 ml of 50% sodium hydroxide - was added to the sample. The receiving 100- ml beaker contained 5 ml of saturated boric acid, 10 ml of distilled water and 2 drops of methyl red - methylene blue indicator. The distillation was about 15 minutes. The process was stopped when the total volume of solution in the beaker collected at the amount of 40 ml. The samples were titrated immediately with 0.02 N HCl by using Beckman Expanded Scale pH meter to the end point of pH 3.8.

EXPERIMENTAL RESULTS

Test of KPCP Toxicity to Goldfish

The toxicity of KPCP to goldfish expressed in percentage of mortality is shown in Table 1. The tests with the concentration of toxicant between 0.47 - 0.40 ppm were repeated on a second batch of goldfish. The final mortality counts were made at the end of 96 hours observation. At the concentration of 0.46 ppm KPCP all goldfish were killed within 96 hours while at the concentration of 0.45 ppm, one fish remained alive more than ten days. This test indicated that the toxicity of KPCP to goldfish was almost half that to the cichlid.

Effect of KPCP on the Activity of Aldolase *in vitro*

Table 6 and Figure 5 show that the aldolase activity of cichlid homogenate decreases rapidly with increasing concentrations of KPCP up to 100 ppm and only slowly at higher concentration. Thus one might be able to conclude that the aldolase was possibly inhibited by KPCP *in vivo*.

Effect of KPCP on the Activity of Aldolase *in vivo*.

In Table 7 and Figures 6-A, 6-B and 17, for both cichlid and goldfish, the aldolase activities were expressed as specific activity in S-L units per mg of protein. At the concentration of 0.1 ppm,

Table 6. Effect of KPCP on Aldolase Activity of Cichlid Muscle Homogenate in vitro.

No. of Fish	Tube No.	Tris Buffer 0.005 M pH 8.9	KPCP in 0.05 M Tris Buffer, pH 8.9 1000 ppm	Total KPCP	Hydrazine	Homogenate 0.08%	Aldolase Substrate	Activity
		ml	ml	ppm	ml	ml	ml	S - L Unit
2	1	1.4	0.0	0	0.2	0.2	0.2	95.00
	2	1.3	0.1	50	0.2	0.2	0.2	43.80
	3	0.9	0.5	250	0.2	0.2	0.2	12.15
	4	0.4	1.0	500	0.2	0.2	0.2	10.06
	5	0.0	1.4	700	0.2	0.2	0.2	4.56

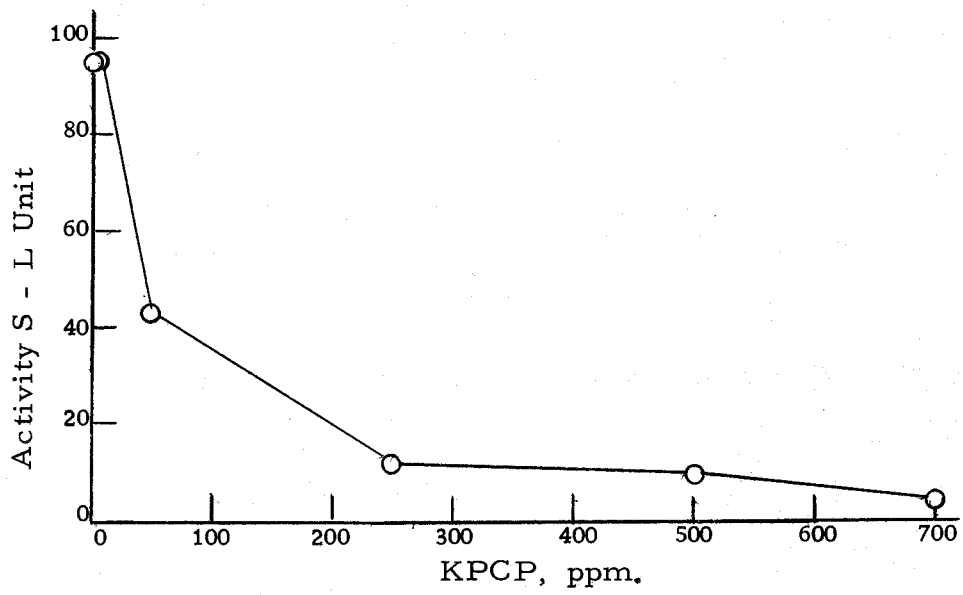
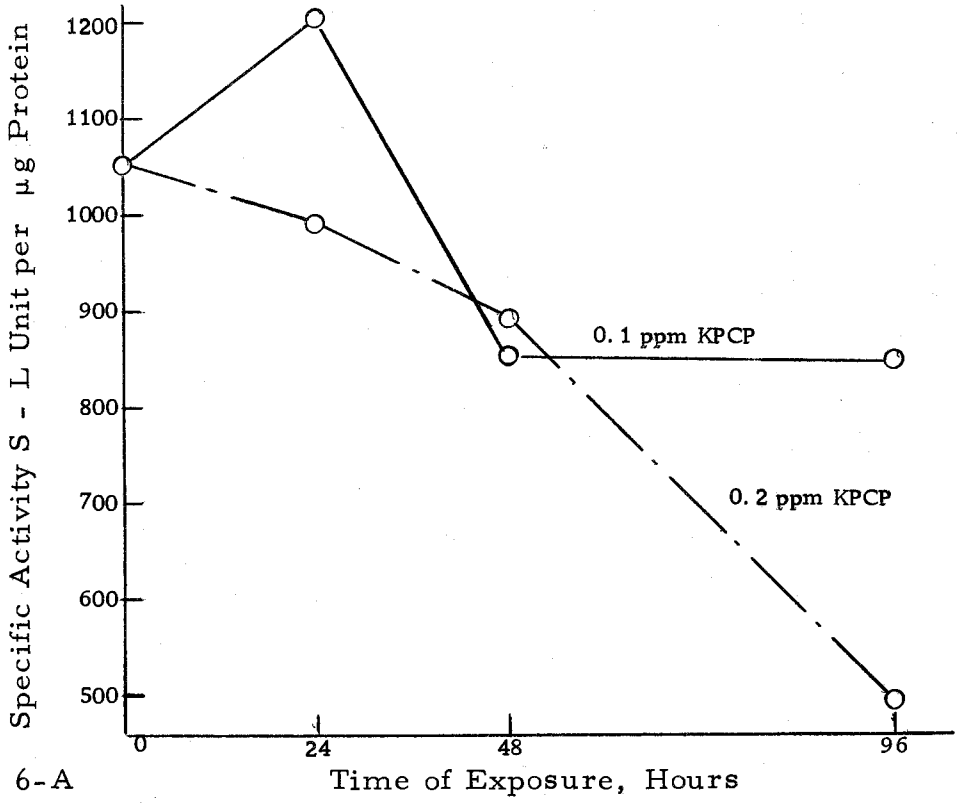


Figure 5. Aldolase Activity in vitro.

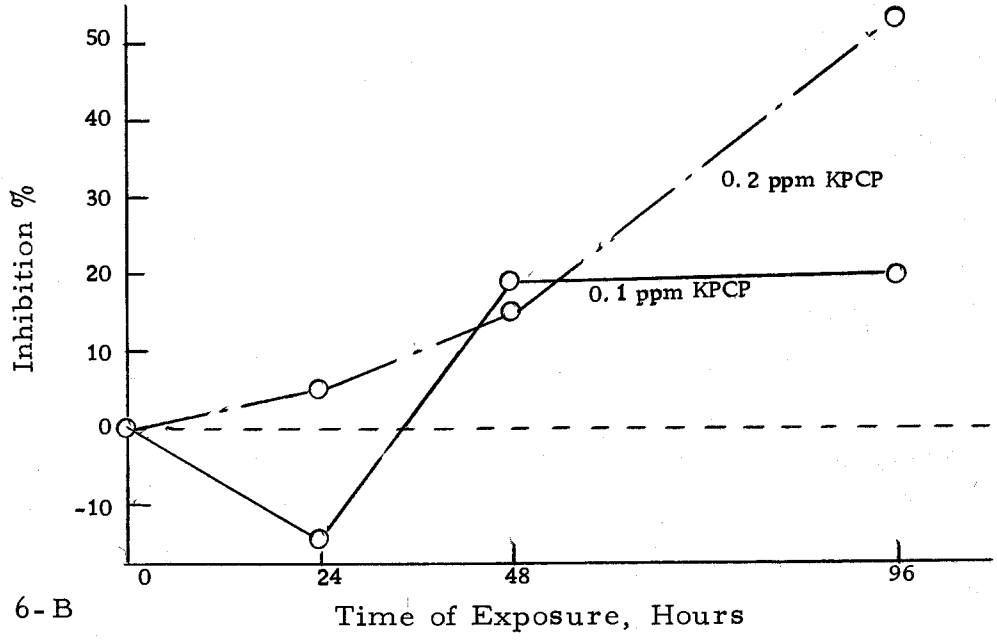
the aldolase activity of cichlid was stimulated in 24 hours exposure and decreased slowly upon exposures of 48 and 96 hours. An inhibition of about 19% occurred on 2 and 4 days exposure to 0.1 ppm KPCP. This shows that there was no cumulative effect on the aldolase level at 0.1 ppm KPCP at 4 days of exposure. On the other hand, aldolase activity showed rapid decrease with increasing exposure time with 0.2 ppm KPCP. The specific activity of aldolase after 96 hours exposure to 0.2 ppm KPCP was about one half that compared to the control sample. For the goldfish, the aldolase specific activities were measured after 96 hours exposure at concentrations of 0.0, 0.1, 0.2 and 0.4 ppm of KPCP. There was 40% inhibition at 0.4 ppm KPCP. A slight stimulation of 6.4% was found

Table 7. Effect of KPCP on the Activity of Aldolase *in vivo*.

Species	Conc. of KPCP ppm	Length of Exposure hours	No. of Fish	Aldolase Activity S-L Unit Ave.	μg of Protein per ml of Homogenate Ave.	Specific Activity S - L Unit Per mg. of Protein	Inhibition %
Cichlid	0.0	0	16	65.4	62.40	1048	
	0.1	24	8	78.8	65.52	1202	-14.7
	0.1	48	12	51.8	60.74	853	18.7
	0.1	96	4	50.3	59.29	848	19.1
	0.2	24	8	54.0	54.42	992	5.3
	0.2	48	12	55.0	61.78	890	15.1
	0.2	96	4	30.3	61.57	491	53.1
Goldfish	0.0	0	1	66.5	135.70	490	
	0.1	96	1	71.5	137.10	522	- 6.4
	0.2	96	1	51.0	136.40	374	23.7
	0.4	96	1	42.0	142.88	294	40.0



6-A



6-B

Figure 6, A and B, Inhibition of Aldolase Activity in vivo.

at 0.1 ppm KPCP.

Effect of KPCP on the Activities of Glutamic Oxalacetic (GOT) and Glutamic Pyruvic Transaminases (GPT).

The effect of KPCP on GOT and GPT activity was determined using homogenates of control fish with varying concentrations of chemical. The results are shown in Tables 8 and 9, and Figures 7 and 8. Activity of both transaminases decreased rapidly with increasing concentration of KPCP. After the concentration of KPCP reached 1000 and 123 ppm, there were no measurable activities of GOT and of GPT respectively. With the demonstration of *in vitro* inhibition of these enzymes, additional studies were undertaken to determine whether these enzymes were inhibited *in vivo*. Table 10 and Figures 9-A and 9-B indicate that the maximum percentages of inhibition of GOT activity for cichlid homogenate was 30.6 at 0.2 ppm KPCP with 24 hours exposure and 49.3 at 0.4 ppm with 96 hours exposure for goldfish homogenate. There was no increase of inhibition at 0.2 ppm KPCP when the exposure time was extended to double and fourfold. At 0.1 ppm KPCP, with 24 hours exposure for cichlid and 96 hours exposure for goldfish, the activity of GOT of both species was increased. This is similar to what was found for the activity of aldolase at the same level of dosage of KPCP and time of exposure. There was slight inhibition at both 48 and 96 hours exposure to 0.1 ppm KPCP but there was no significant

Table 8. Effect of KPCP on GOT Activity of Cichlid Muscle Homogenate in vitro.

Tube No.	No. of Fish	Aspartate α-ketoglutarate	KPCP 1600 ppm in 0.1 M Tris Buffer pH 7.5	Total KPCP	Homogenate 0.5%	2, 4-Dinitrophenyl-hydrazine	NaOH 0.4 N	GOT Activity
		ml	ml	ppm	ml	ml	ml	S - F Unit
1	2	1.0	0.0	0.0	0.2	1.0	10.0	145
2		1.0	0.1	123.1	0.2	1.0	9.9	132
3		1.0	0.2	228.6	0.2	1.0	9.8	108
4		1.0	0.4	400.0	0.2	1.0	9.6	89
5		1.0	0.6	533.0	0.2	1.0	9.4	66
6		1.0	0.8	640.0	0.2	1.0	9.2	26
7		1.0	1.3	832.0	0.2	1.0	8.7	22
8		1.0	2.0	1000.0	0.2	1.0	8.0	0

Table 9. Effect of KPCP on GPT Activity of Cichlid Muscle Homogenate *in vitro*.

Tube No.	No. of Fish	Alanine - α - ketoglutarate	KPCP 160 ppm and 1600 ppm in 0.1 M Tris Buffer, pH 7.5	Total KPCP	Homogenate 0.5%	2,4-Dinitrophenyl-hydrazine	NaOH 0.4 N	GPT Activity
		ml	ml	ppm	ml	ml	ml	S - F Unit
1	2	1.0	0.00	0.0	0.2	1.0	10.00	17.0
2		1.0	0.10*	12.3	0.2	1.0	9.90	14.0
3		1.0	0.20*	22.9	0.2	1.0	9.80	9.5
4		1.0	0.30*	32.0	0.2	1.0	9.70	5.0
5		1.0	0.05**	64.0	0.2	1.0	9.95	4.0
6		1.0	0.10**	123.0	0.2	1.0	9.90	0.0

* 160 ppm KPCP per ml
** 1600 ppm KPCP per ml

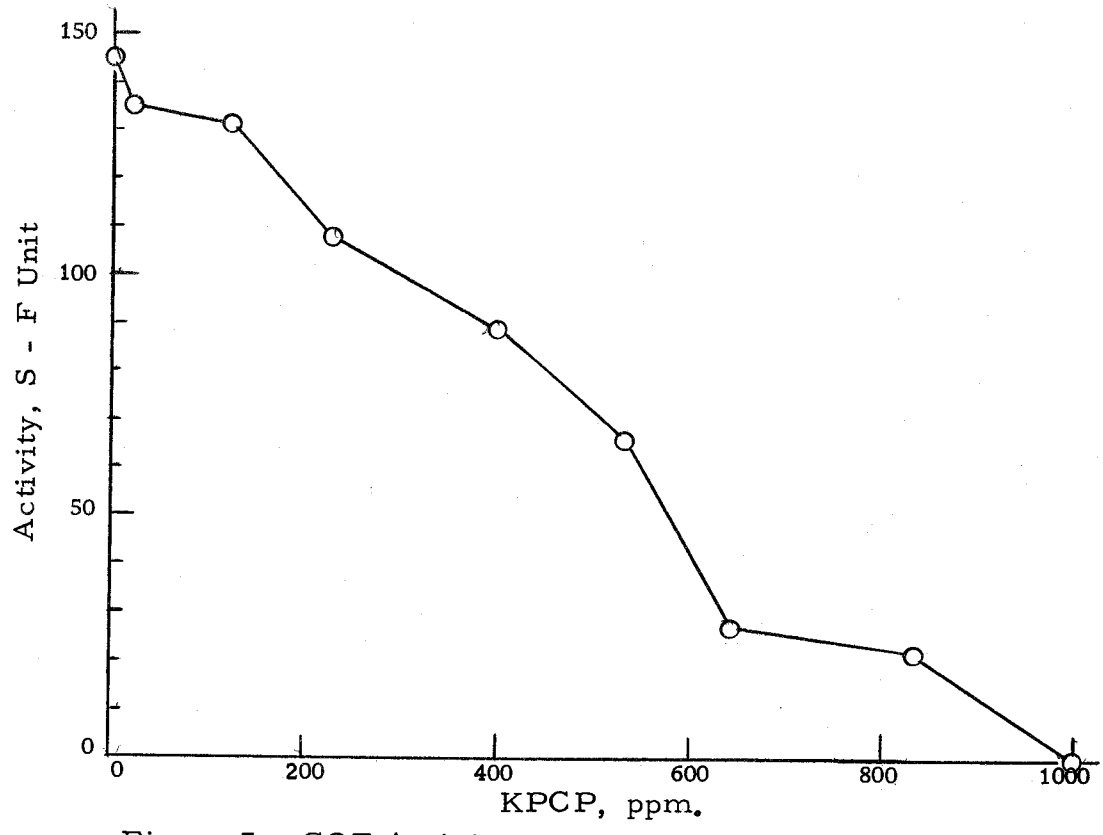


Figure 7. GOT Activity in vitro.

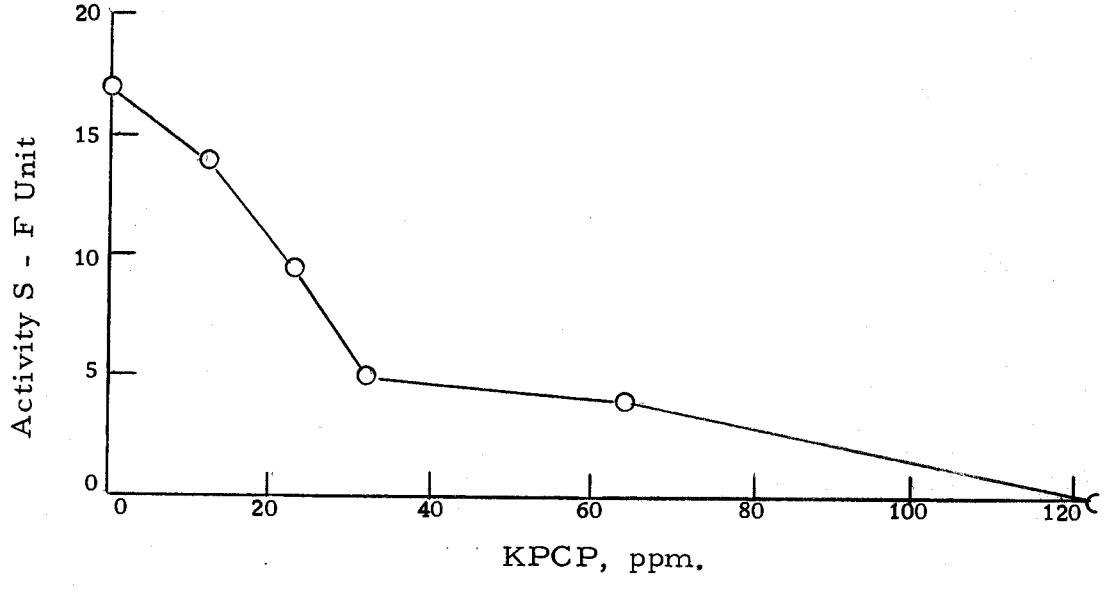
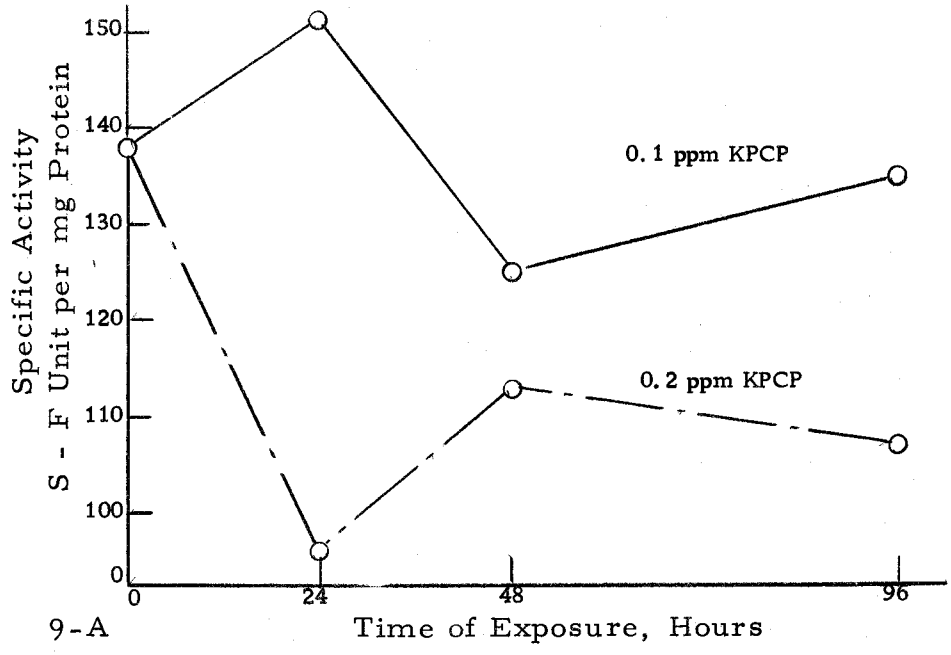


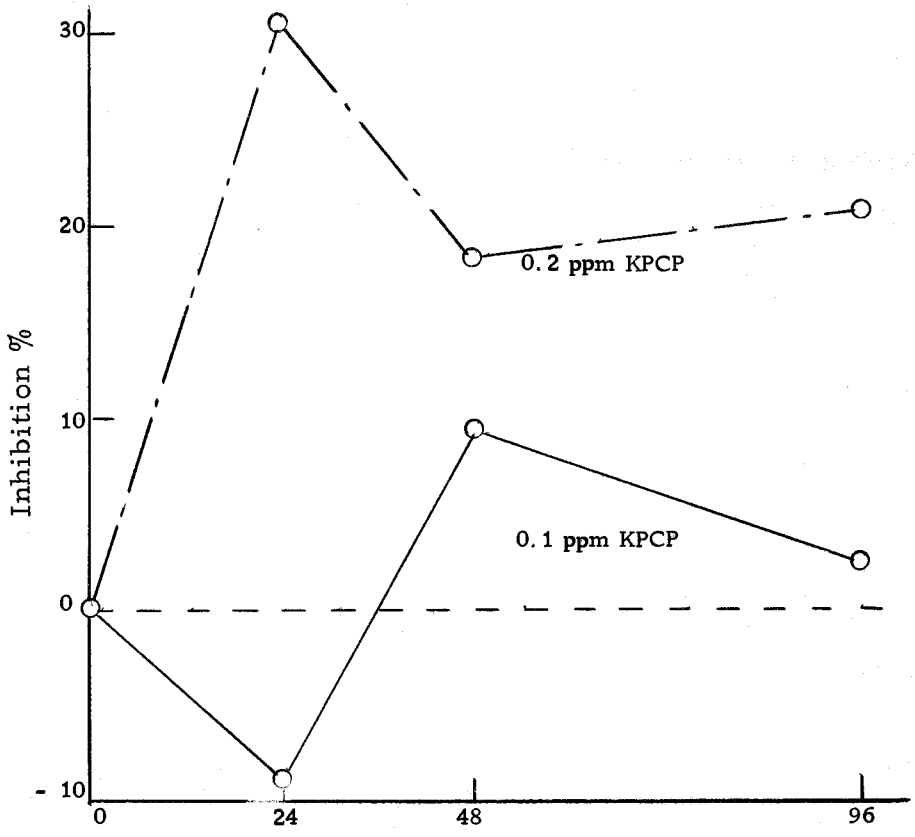
Figure 8. GPT Activity in vitro.

Table 10. Effect of KPCP on the Activity of GOT in vivo.

Species	No. of Fish	KPCP	Exposure	Activity	µg of Protein per ml of Homogenate	Specific Activity	Inhibition
		ppm	Hours	Ave. S - F Unit	Ave. µg	S - F Unit per mg of Protein	%
Cichlid	14	0.0	0	69.7	503.8	138	
	4	0.1	24	78.6	521.1	151	-9.0
	8	0.1	48	62.4	497.5	125	9.4
	4	0.1	96	65.8	487.1	135	2.4
	4	0.2	24	42.5	442.7	96	30.6
	8	0.2	48	54.7	485.0	113	18.5
	4	0.2	96	52.0	486.1	107	22.7
Goldfish	1	0.0	0	93	204.5	455	
	1	0.1	96	137	235.0	583	-28.2
	1	0.2	96	78	229.0	341	25.1
	1	0.4	96	51	221.3	231	49.3



9-A



9-B

Figure 9, A and B, GOT Activity in vivo.

difference in percentage of inhibition between the two exposure times. No increase of inhibition at 0.2 ppm with prolonged exposure for GOT activity of cichlid homogenate was noted. For goldfish, the inhibition of GOT activity at 0.4 ppm KPCP was about double that at 0.2 ppm upon 96 hours exposure.

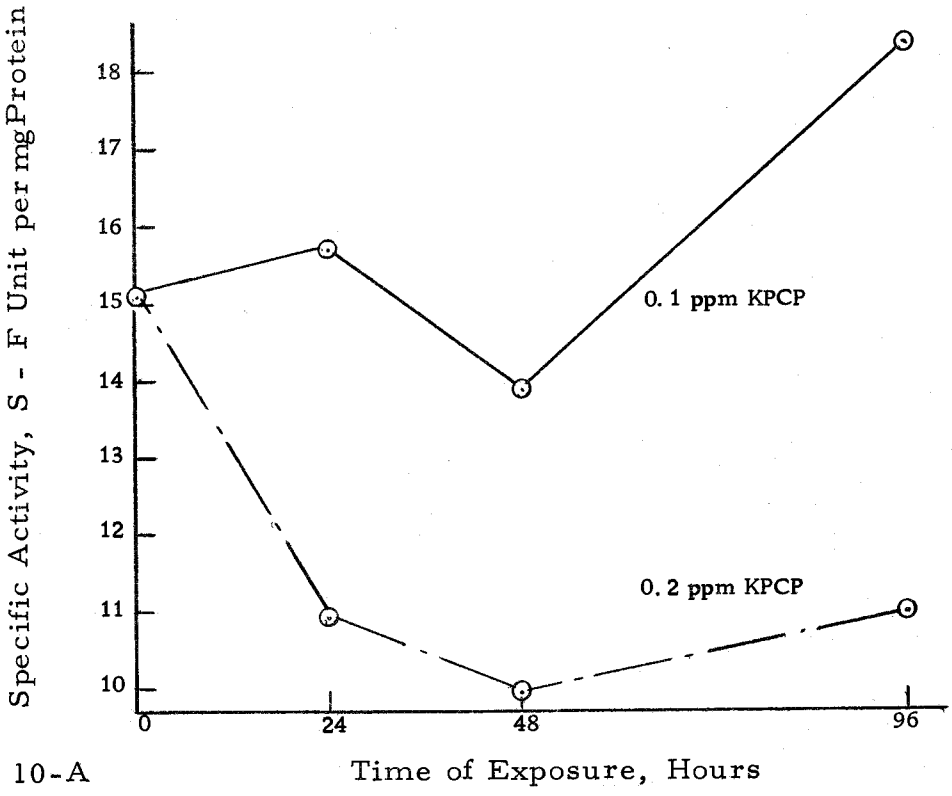
GPT activity is shown in the cichlid homogenate in Table 11 and Figures 10-A and 10-B. Maximum inhibition occurred at 48 hours exposure (34%) and with 28% and 27% inhibition at 24 and 96 hours exposure respectively using 0.2 ppm KPCP. However, 0.1 ppm KPCP with 24 and 96 hours exposure gave no inhibition of GPT activity of cichlid homogenate but resulted in a stimulation of 4% and 21% respectively. The inhibition of GPT activity of goldfish homogenate at 0.4 ppm KPCP was about six times greater than at 0.1 ppm although the fish were exposed for 96 hours. The inhibition of GPT activity increased with increasing concentrations of KPCP over a four day exposure.

The Residual Activities of GOT and GPT in vivo and in vitro

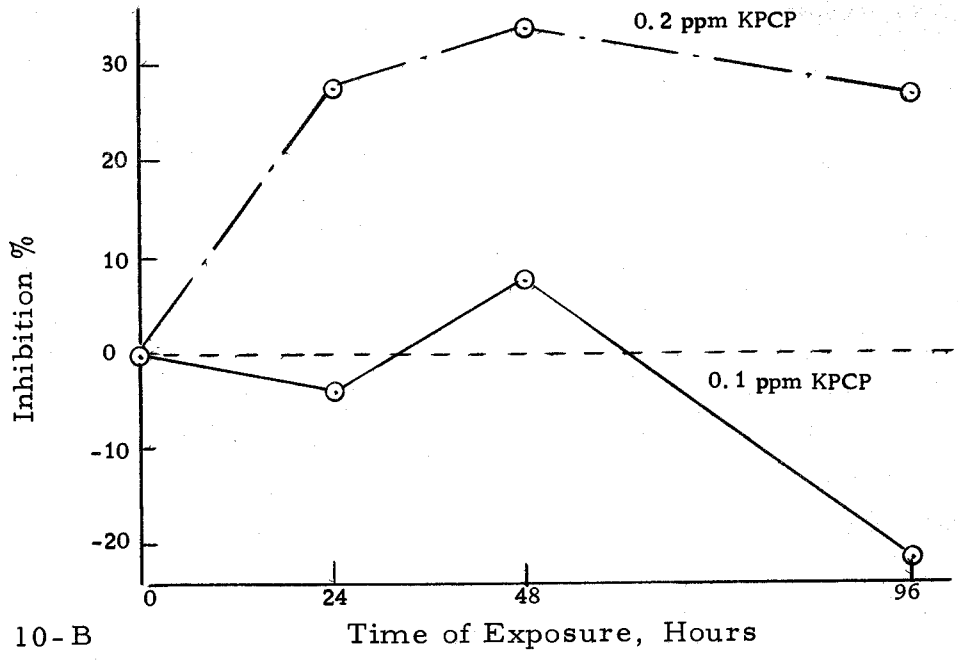
Data presented in Tables 12 and 13 showed that activity of transaminases in homogenates of fish which were treated for varying lengths of time with the different levels of toxicant, may be further reduced by additional amounts of KPCP added to the preparations. Rapid and complete inhibition is obtained at high concentrations of

Table 11. Effect of KPCP on the Activity of GPT *in vivo*.

Species	No. of Fish	KPCP	Exposure	Activity	mg of Protein per ml of Homogenate	Specific Activity	Inhibition
		ppm	Hours	Ave. S-F Unit	Ave. mg	S - F Unit per mg of Protein	%
Cichlid	14	0.0	0	38.3	2.53	15.2	
	4	0.1	24	41.0	2.61	15.7	-3.9
	8	0.1	48	34.6	2.49	13.9	8.1
	4	0.1	96	44.8	2.44	18.4	-21.4
	4	0.2	24	24.3	2.21	11.0	27.7
	8	0.2	48	24.3	2.43	10.0	34.0
	4	0.2	96	26.8	2.43	11.0	27.2
Goldfish	1	0.0	0	27.0	2.05	13.2	
	1	0.1	96	28.0	2.35	11.9	9.7
	1	0.2	96	13.0	2.29	5.7	57.0
	1	0.4	96	8.5	2.21	3.8	70.9



10-A



10-B

Figure 10, A and B, GPT Activity in vivo.

Table 12. Specific Activity of GOT of Cichlid Muscle Homogenates in vivo and in vitro.

Tube No.	Asparate-α-ketoglutarate	KPCP 1000 ppm	Total KPCP	Homo-genate 0.2%	2, 4-DPH	NaOH 0.40 N	Specific Activity S - F Unit per mg of Protein						
							Control 10*	0.1 ppm 24 Hours 4*	0.1 ppm 48 Hours 4*	0.1 ppm 96 Hours 2*	0.2 ppm 24 Hours 2*	0.2 ppm 48 Hours 4*	0.2 ppm 96 Hours 2*
	ml	ml	ppm	ml	ml	ml	Ave. S - F Unit per mg of Protein						
1	1.0	0.00	0	0.2	1.0	10.00	138	151	125	135	96	113	107
2	1.0	0.15	107	0.2	1.0	9.85	117	118	103	102	43	84	90
3	1.0	0.35	219	0.2	1.0	9.65	90	101	72	84	25	58	60
4	1.0	0.80	390	0.2	1.0	9.20	44	65	30	25	7	16	21
5	1.0	1.70	576	0.2	1.0	8.30	14	19	10	0	0	6	0
6	1.0	2.00	615	0.2	1.0	8.00	0	0	0	0	0	0	0

*Number of fish.

Table 13. Specific Activity of GPT of Cichlid Muscle Homogenates in vivo and in vitro.

Tube No.	Alanine - α - ketoglutarate	KPCP 1000 ppm	Total KPCP	Homogenate 1%	2, 4-DPH	NaOH 0.40 N	Specific Activity S - F Unit per mg of Protein				
							Control 8*	0.1 ppm 24 Hours 4*	0.1 ppm 48 Hours 4*	0.1 ppm 96 Hours 2*	0.2 ppm 48 Hours 4*
	ml	ml	ppm	ml	ml	ml	Ave. S - F Unit per mg of Protein				
1	1.0	0.00	0.0	0.2	1.0	10.00	15.2	15.7	13.9	18.4	10.0
2	1.0	0.20**	13.8	0.2	1.0	9.80	12.3	9.7	10.9	14.7	7.4
3	1.0	0.04	31.0	0.2	1.0	9.96	10.3	9.2	10.0	11.0	5.6
4	1.0	0.08	60.0	0.2	1.0	9.92	7.8	7.6	7.6	10.0	3.7
5	1.0	0.17	120.0	0.2	1.0	9.83	3.7	1.6	3.4	4.1	0
6	1.0	0.35	219.0	0.2	1.0	9.65	0	0	0	0	0

*Number of fish

** From 100 ppm KPCP

toxicant. Figures 11 and 12 show that the activities of GOT and GPT were finally reduced to zero and intersected at one point.

Effect of KPCP on the Activity of Lactic Dehydrogenase (LD)

The data found in Table 14 and Figure 13 indicate that the LD activity in vitro was inhibited as the concentration of KPCP increased.

The inhibition of activity of LD from in vivo exposure in cichlids increased slowly with increasing concentrations of toxicant. In Table 15 and Figures 14-A and 14-B, it is seen that at both 0.1 ppm and 0.2 ppm KPCP, a maximum of 14% and 17% of inhibition respectively is exhibited at 48 hours exposure. There was a rapid decrease of inhibition at 96 hours exposure in 0.1 ppm KPCP and a slight decrease at the same time of exposure in 0.2 ppm KPCP. The inhibition of LD activity by KPCP in cichlid homogenate was comparatively less than that of aldolase, GOT and GPT. In goldfish homogenate, there was stimulation of LD activity at 0.1 ppm KPCP and the inhibition at 0.4 ppm was increased twice as much as at 0.2 ppm although all were at four days exposure.

The Residual Activities of LD in vivo and in vitro

The tests of LD residual activity of cichlid homogenates are shown on Table 16 and Figure 15. The inhibition was not quite complete even at the high concentration of KPCP. This indicates

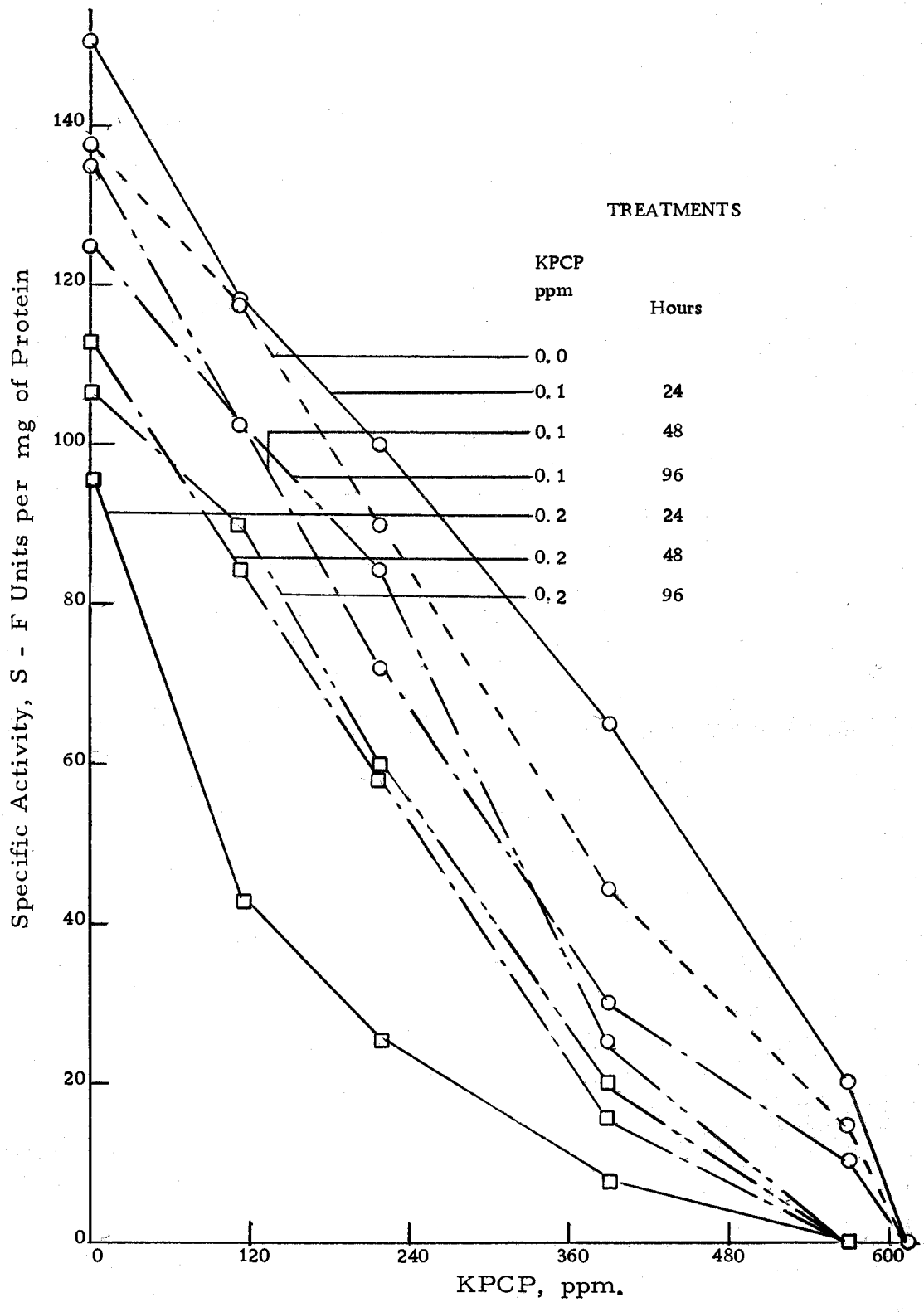


Figure 11. The Residual Activity of GOT.

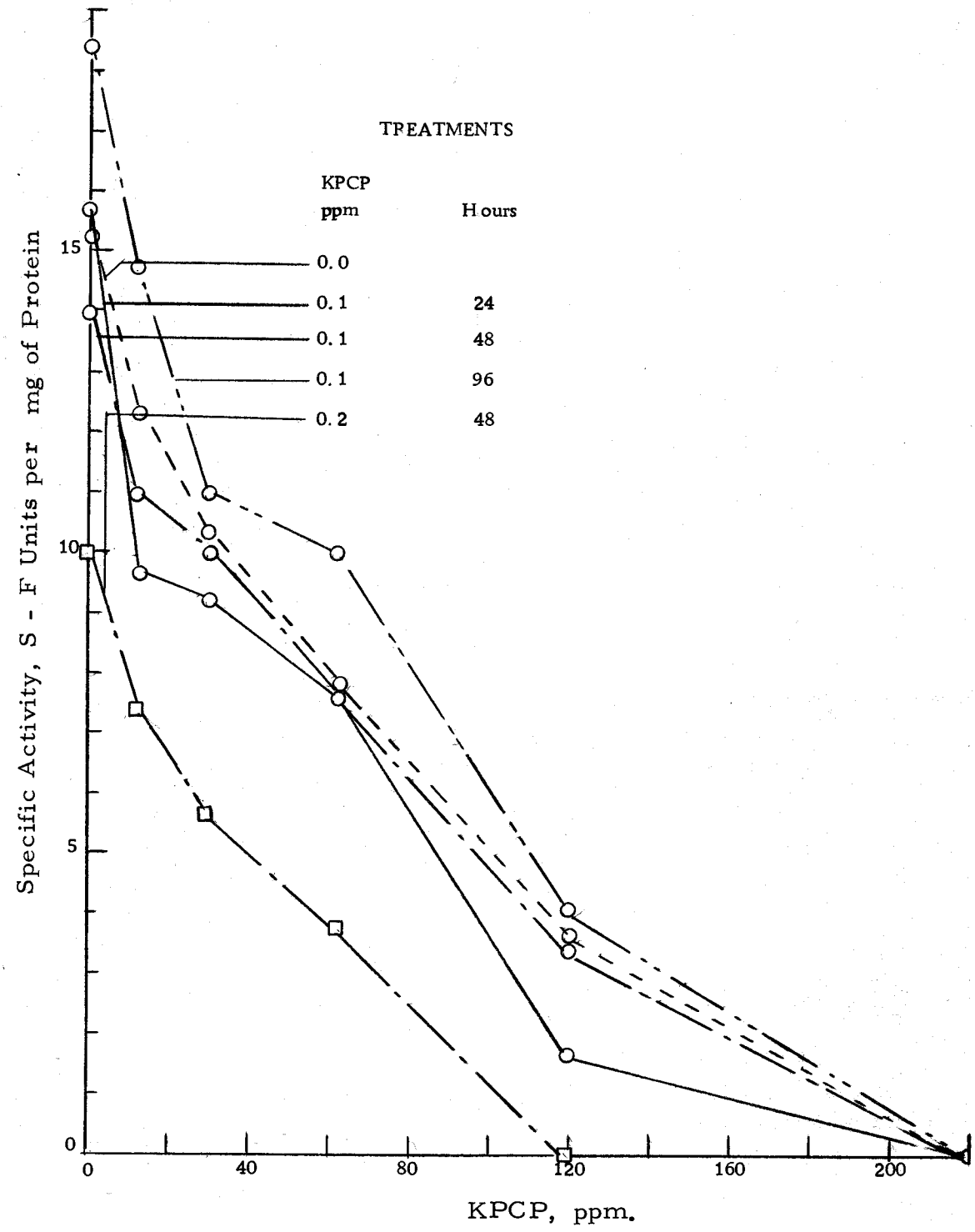


Figure 12. The Residual Activity of GPT

Table 14. Effect of KPCP on LD Activity of Cichlid Muscle Homogenate in vitro.

Tube No.	No. of Fish	β-NADH and Pyruvate Substrate*	KPCP 1600 ppm	Total KPCP	NaOH 0.40 N	Homogenate 0.025%	2,4-DPH	Activity
		ml	ml	ppm	ml	ml	ml	B - B Unit
1	2	1.0	0.00	0	10.00	0.1	1.0	910
2		1.0	0.05	70	9.95	0.1	1.0	750
3		1.0	0.10	133	9.90	0.1	1.0	650
4		1.0	0.40	427	9.60	0.1	1.0	295
5		1.0	0.80	674	9.20	0.1	1.0	250
6		1.0	1.20	835	8.80	0.1	1.0	240

*One milligram of β-NADH per milliliter of pyruvate substrate.

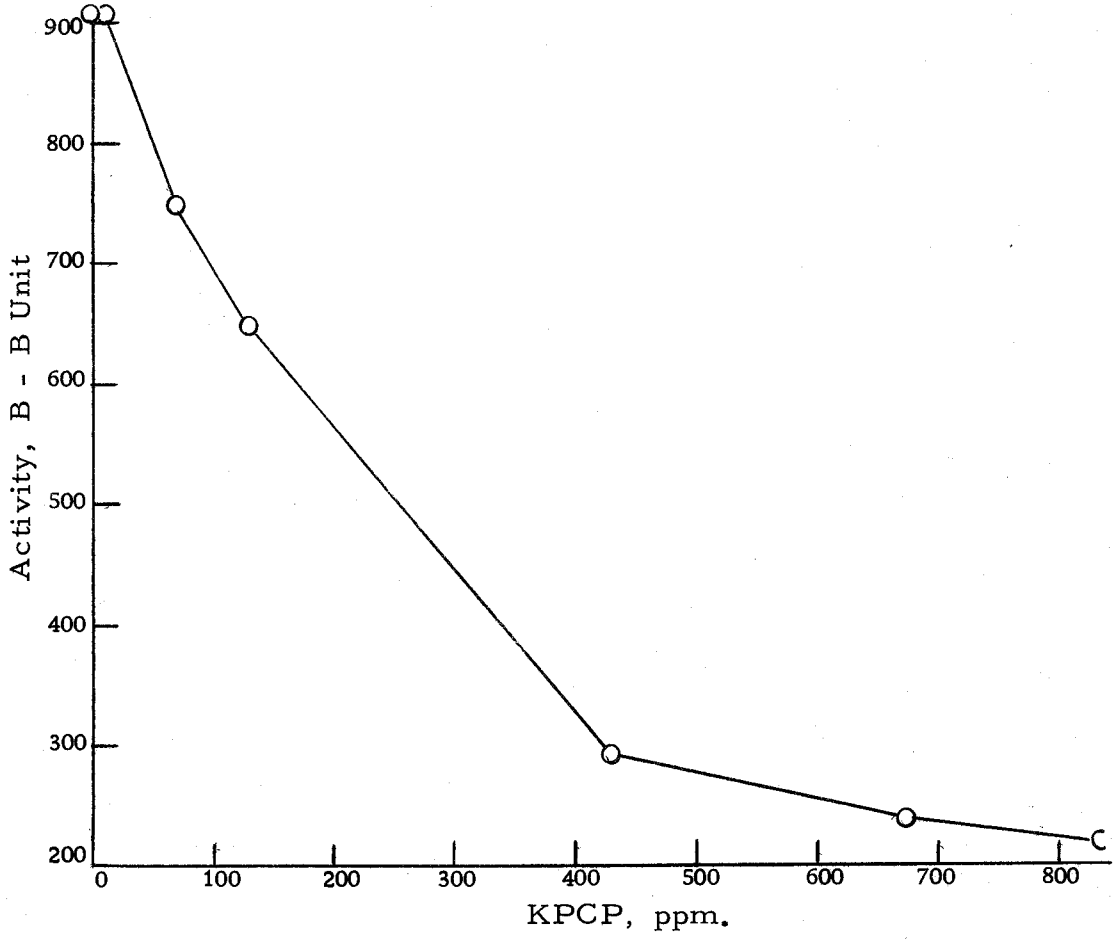


Figure 13. LD Activity in vitro.

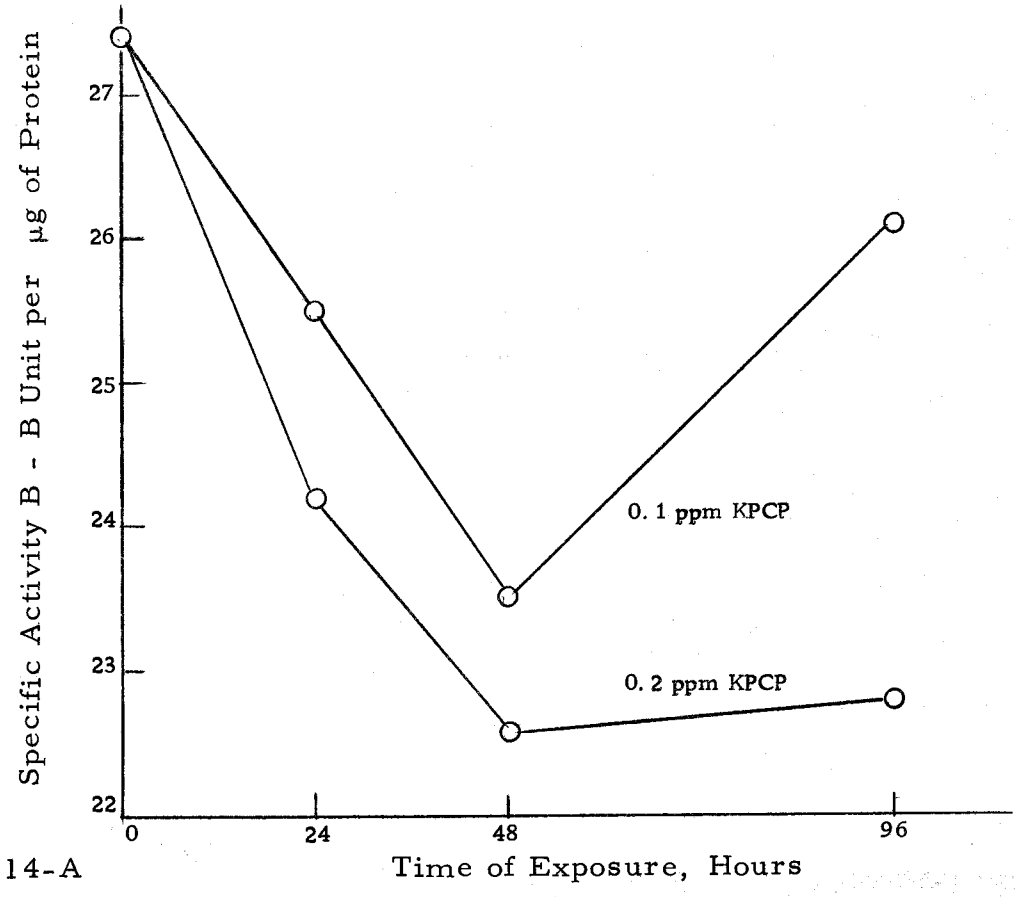
that after the treatment of the fish with KPCP, the LD still retained a residual activity resistant to in vitro additions of KPCP.

Effect of KPCP on the Activity of Isocitric Dehydrogenase (ICD)

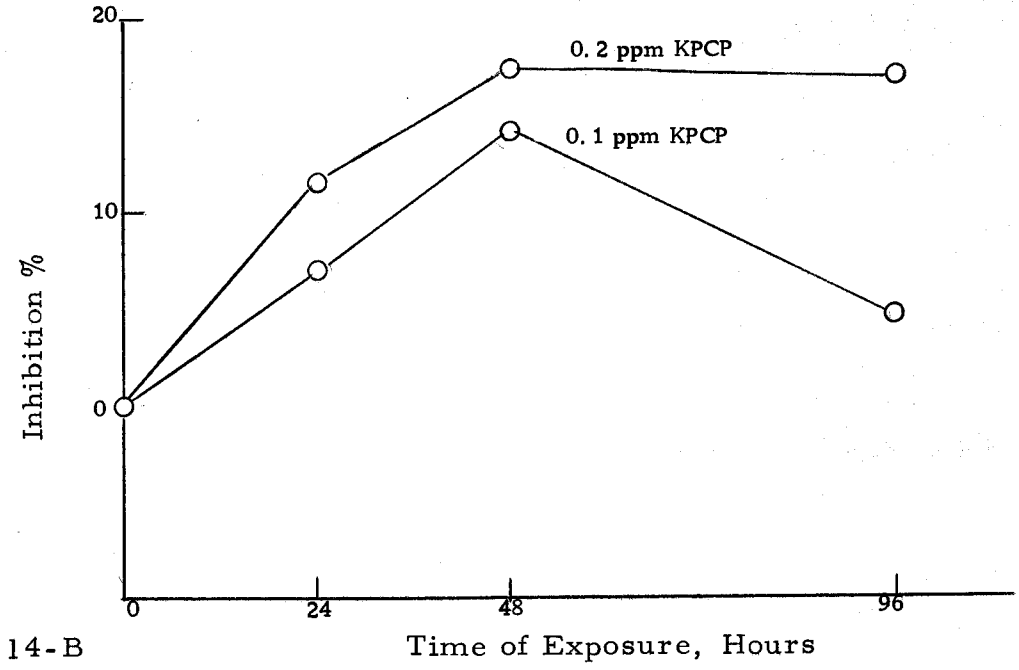
Table 17 and Figure 16 indicated that the activity of ICD of cichlid homogenate was increased with the increasing concentration of KPCP. It did not show any inhibition in vitro, therefore, no test was conducted with this enzyme in vivo.

Table 15. Effect of KPCP on the activity of LD *in vivo*.

Species	No. of Fish	KPCP	Exposure	Activity	µg of Protein per ml of Homogenate	Specific Activity	Inhibition
		ppm	Hrs.	Ave. B-B Unit	Ave. µg	B-B unit per µg of Protein	%
Cichlid	12	0.0	0	1233	45.0	27.4	
	4	0.1	24	1295	50.8	25.5	7.0
	8	0.1	48	1115	47.4	23.5	14.2
	4	0.1	96	1083	41.5	26.1	4.8
	4	0.2	24	990	40.9	24.2	11.6
	8	0.2	48	1019	45.1	22.6	17.4
	4	0.2	96	949	41.7	22.8	17.0
Goldfish	1	0.0	0	950	34.1	27.9	
	1	0.1	96	1185	39.2	30.3	-8.6
	1	0.2	96	910	38.2	23.8	14.5
	1	0.4	96	725	38.9	19.7	29.5



14-A



14-B

Figure 14, A and B, LD Activity in vivo.

Table 16. Specific Activity of LD of Cichlid Muscle Homogenates in vivo and in vitro.

Tube No.	β-NADH and Pyruvate Substrate*	KPCP 1000 ppm	Total KPCP	Homogenate 0.0167 %	2,4-DPH	NaOH 0.40 N	Specific Activity B - B Unit per µg of Protein					
							Control 8**	0.1 ppm 24 hours 2**	0.1 ppm 48 hours 6**	0.1 ppm 96 hours 2**	0.2 ppm 48 hours 6**	0.2 ppm 96 hours 2**
	ml	ml	ppm	ml	ml	ml	Average B - B unit per µg of protein					
1	1.0	0.0	0	0.2	1.0	10.0	27.4	25.5	23.5	26.1	22.6	22.8
2	1.0	0.1	83	0.2	1.0	9.9	23.3	18.3	20.4	19.0	18.2	15.4
3	1.0	0.4	267	0.2	1.0	9.6	13.4	10.2	12.5	11.8	11.3	8.9
4	1.0	0.9	450	0.2	1.0	9.1	8.1	6.4	7.8	6.1	7.3	5.8
5	1.0	2.0	645	0.2	1.0	8.0	6.2	4.5	5.8	5.0	5.6	5.5

*One milligram of β-NADH per milliliter of pyruvate substrate.

**Number of fish.

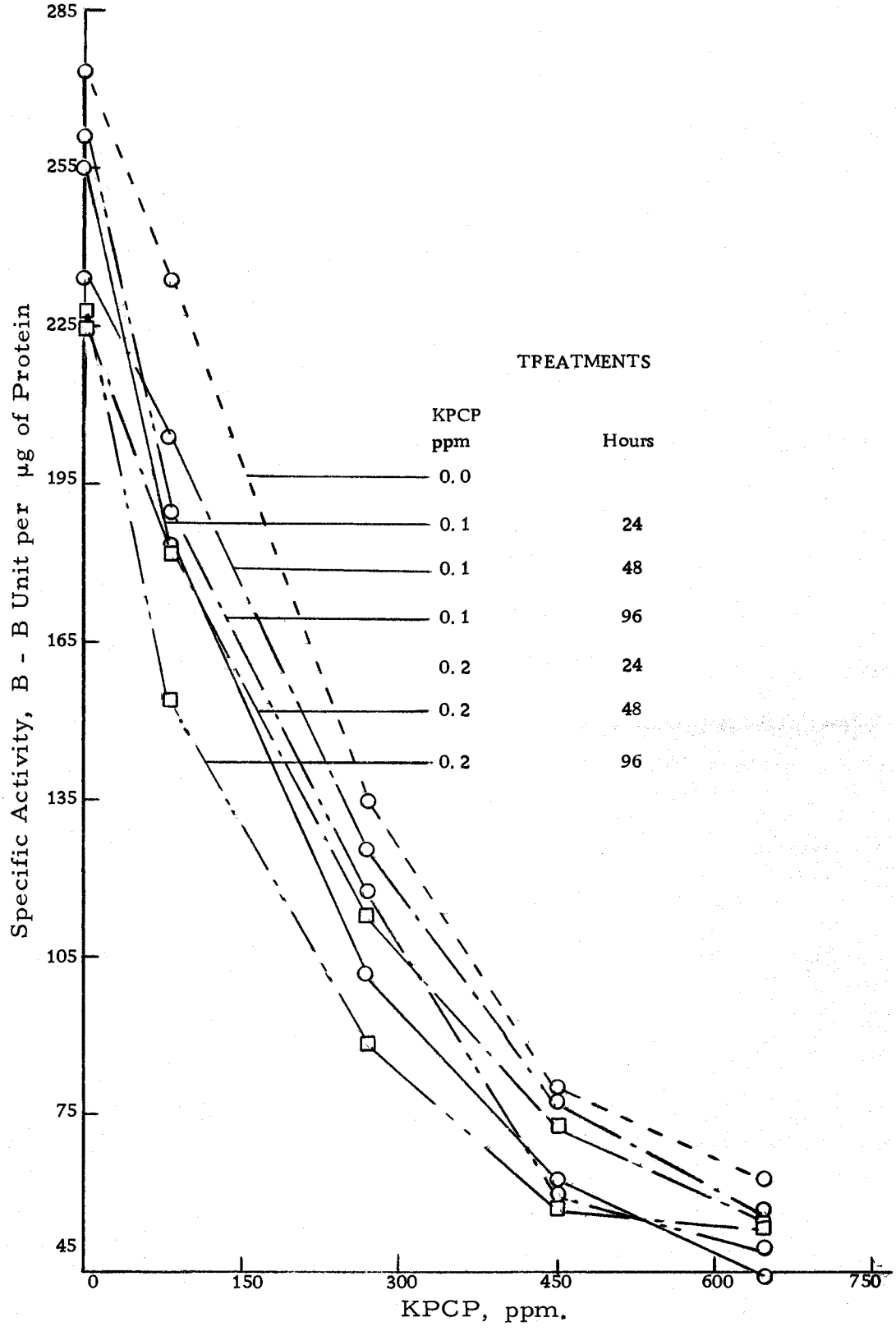


Figure 15. The Residual Activity of LD.

Table 17. Effect of KPCP on ICD Activity of Cichlid Muscle Homogenate *in vitro*.

Tube No.	No. of Fish	Tris Buffer 0.1 M, pH 7.5	KPCP in 0.1 M Tris Buffer, pH 7.5, 200 or 500 ppm	Total KPCP	Homogenate 6.67%	$MnCl_2$	TPN 0.004 M	Na-isocitrate substrate	Activity
	ml	ml	ml	ppm	ml	ml	ml	ml	Sigma Unit
1	2	0.30	0.00	0	0.2	0.2	0.2	0.1	335
2		0.28	0.02*	4	0.2	0.2	0.2	0.1	360
3		0.25	0.05*	10	0.2	0.2	0.2	0.1	381
4		0.20	0.10*	20	0.2	0.2	0.2	0.1	375
5		0.15	0.15**	30	0.2	0.2	0.2	0.1	385
6		0.20	0.10**	50	0.2	0.2	0.2	0.1	395
7		0.10	0.20**	100	0.2	0.2	0.2	0.1	410
8		0.00	0.30**	150	0.2	0.2	0.2	0.1	434

*From 200 ppm KPCP.

**From 500 ppm KPCP.

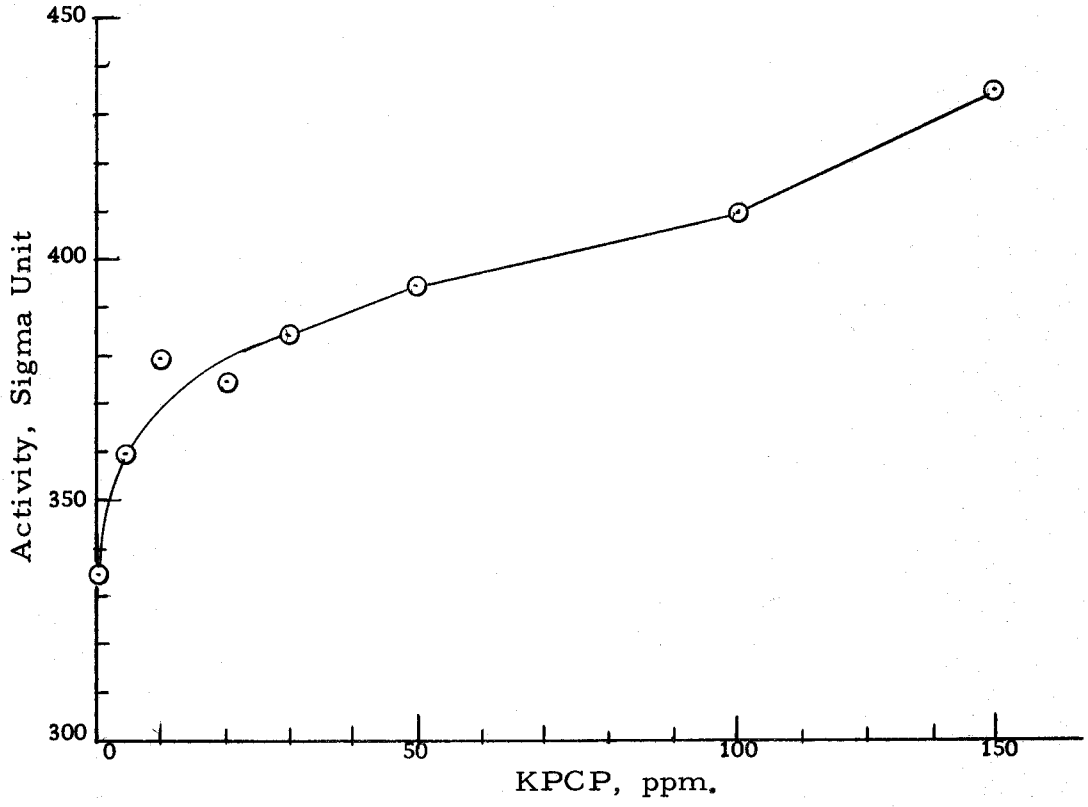


Figure 16. ICD Activity in vitro.

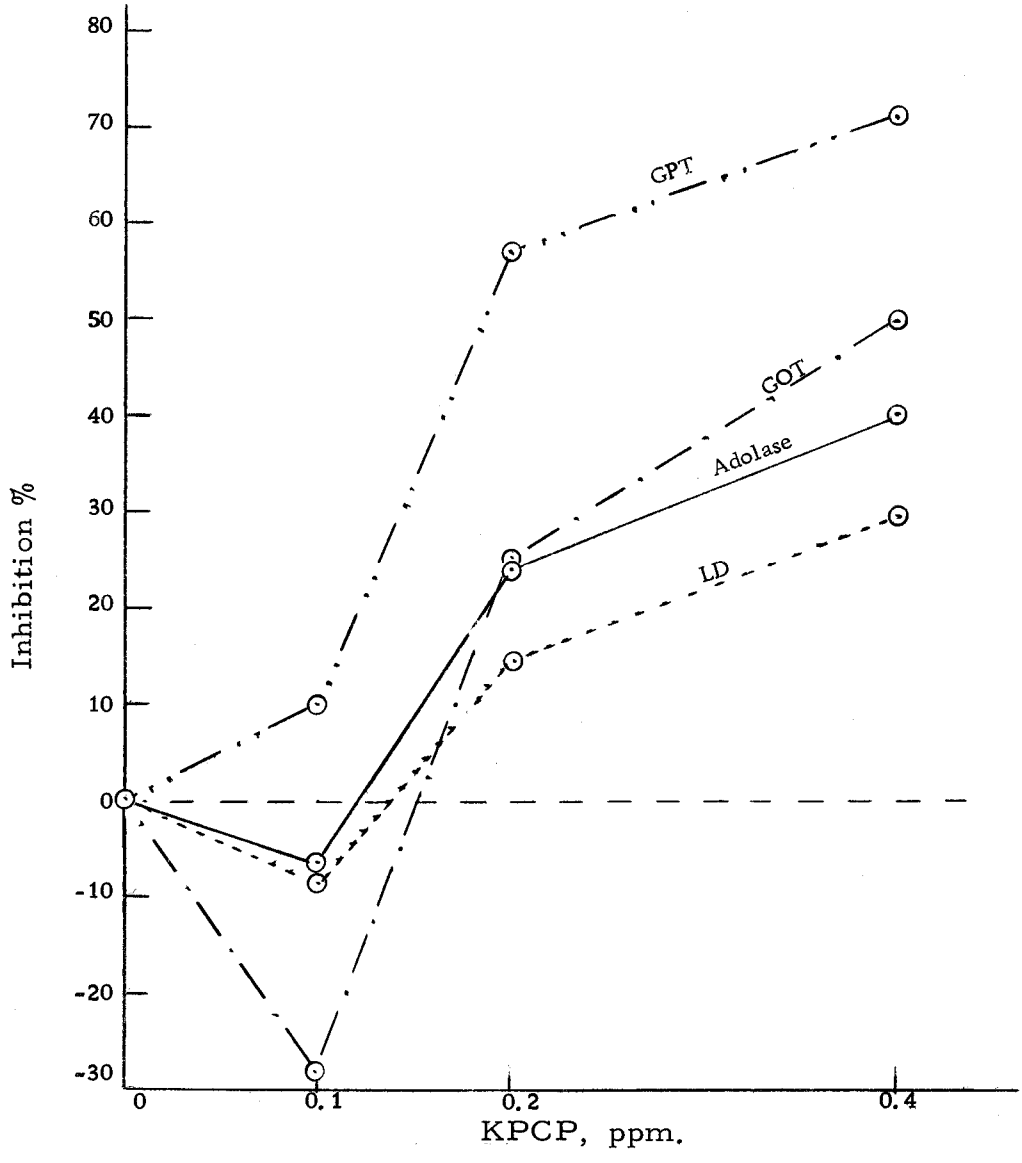


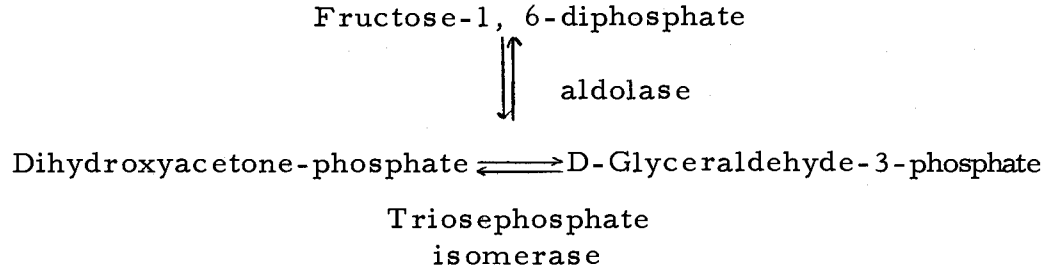
Figure 17. Effect of KPCP on enzyme activity of goldfish in vivo with 96 hour exposure.

DISCUSSION

Experimental tests revealed that concentrations of 0.24 ppm PCP was the lethal level for cichlids and 0.46 ppm KPCP for goldfish. Thus, it appears that KPCP is much more toxic to cichlids than to goldfish. On the other hand, KPCP shows less toxicity to goldfish than does DDT or the pure gamma isomer of benzene hexachloride which have been reported to give 100% mortality at 0.25 ppm and 0.125 respectively (11, p. 133).

The concentrations of KPCP in in vitro tests were very much higher than in in vivo tests. The activity of aldolase was almost completely inhibited above 700 ppm KPCP in in vitro tests. Only sublethal levels of KPCP were used in in vivo studies.

Aldolase catalyzes the following reaction:



The purpose of hydrazine in the reaction mixture was to combine with the triosephosphate in order to prevent the interconversion. The triose phosphates were hydrolyzed at the room temperature with alkali to the corresponding trioses and were converted to "osazones" by the 2, 4-dinitrophenylhydrazine reagent. The intensity

of the color of "osazones" is proportional to the amount of triose present. The amount of triose produced was related to the amount of aldolase activity in the sample.

From the in vitro and in vivo studies it was apparent that the KPCP exerted a dual effect upon aldolase in the muscles of cichlid and goldfish. In low concentration - 0.1 ppm - KPCP elicited aldolase activity of fish muscle and in higher concentrations -- 0.2 ppm for cichlid and 0.4 ppm for goldfish -- KPCP strongly inhibited the aldolase activity. These findings seemed similar to the Weinbach's work (28, 32) on the effect of PCP on ATPase in which he showed that PCP at low concentration (5×10^{-5} M) stimulated ATPase activity from rat liver mitochondria while at higher concentration (5×10^{-4} M or higher) PCP inhibited the ATPase activity. Thus, the increase in the concentration of toxicant or prolonged time of exposure resulted in lower enzyme activity. It is interesting that upon prolonged exposure of the fish, the inhibition was irreversible. Further work is needed to be done for the actual kinetics of the reaction in the fish muscle.

In the nitrogen metabolism of all living systems, the reaction between L-glutamic acid and the pyridine nucleotides play an important role. The reversible conversion of L-glutamic acid to α-ketoglutaric acid or L-alanine to pyruvic acid, which is a member of the citric acid cycle, serves as a link between the

metabolism of amino acids with the metabolism of carbohydrates. The enzymes -- transaminases -- catalyze the following reactions:

$$\alpha\text{ - Ketoglutaric acid + L-aspartic acid} \xrightleftharpoons{\text{GOT}} \text{L-Glutamic acid + oxaloacetic acid}$$

$$\alpha\text{ - Ketoglutaric acid + L-alanine} \xrightleftharpoons{\text{GPT}} \text{L-Glutamic acid + pyruvic acid}$$

The transamination system is important not only in the breakdown of amino acid but also in their biosynthesis. The amount of oxalacetate or pyruvate formed can be determined colorimetrically by the formation of a "hydrazone" which is highly colored.

The effect of KPCP on GOT and GPT activity also showed a dual effect. T he slowly increasing or decreasing percentage of inhibition at 0. 2 ppm KPCP for both GOT and GPT might indicate that the absorption of toxicant by the fish is limiting at this level of KPCP with no cumulative effect in vivo.

In vivo studies with goldfish show a proportional increase of inhibition with the increasing concentration of KPCP with four days exposure. This probably indicates either an irreversible effect on the enzyme present or induction of biosynthesis of a modified enzyme or inhibition of the synthesis of this enzyme.

The residual activity of transaminase of homogenate of treated fish was further inhibited by additional amounts of KPCP in vitro. This indicated that during the treatment of the fish with 0. 1 and

0.2 ppm of KPCP, the transaminases were still present in amounts sufficient to carry on metabolism. The fish could survive within these levels of toxicant.

In the process of metabolism, biological oxidation and reduction must take place in the living system. Lactic dehydrogenase (LD) participates in the process of dehydrogenation. It removes hydrogens from lactic acid and transfers them to its own co-enzyme, as is seen in the following reaction:

$$\text{Lactic acid} + \text{Co-enzyme} \overset{\text{LD}}{\rightleftharpoons} \text{Pyruvic acid} + \text{reduced co-enzyme}$$

Lactic dehydrogenase is made up of two distinct components, a protein and a co-enzyme. The co-enzyme of lactic dehydrogenase is diphosphopyridine nucleotide (NAD), lactic dehydrogenase catalyzes the following reaction:

$$\text{Pyruvic acid} + \text{NADH} \overset{\text{LD}}{\rightleftharpoons} \text{Lactic acid} + \text{NAD}$$

The speed of the reaction is proportional to the amount of lactic dehydrogenase present. Pyruvic acid reacts with 2,4-dinitrophenylhydrazine to form an intensely colored "hydrazone." The amount of pyruvate remaining after the reaction is inversely proportional to the activity of the lactic dehydrogenase present in the reaction.

The activity of LD was rapidly inhibited with increasing concentrations of KPCP in in vitro experiments. Above the hundreds fold concentration of KPCP, the activity of LD decreased only slightly. In the residual activity tests, the activity of LD also

showed the similar pattern. In vivo, tests of LD activity in treated cichlid homogenates showed only limited inhibition at the sublethal dose of KPCP. This might indicate that the inhibition was non-competitive with no cumulative effect of the toxicant. At the sub-lethal concentration of KPCP, the biological oxidation and reduction in cichlid tissues did not appear to be so impaired as to influence survival.

In goldfish, however, the higher concentration of KPCP with prolonged exposure gave slightly higher inhibition of LD activity. A greater inhibition may have been obtained with goldfish if the time of exposure were increased.

In the metabolism of fats and carbohydrates, the citric acid cycle acts as a central metabolic site. Isocitric dehydrogenase (ICD) is a tricarboxylic acid cycle enzyme which catalizes the reversible dehydrogenation and decarboxylation of isocitrate, one of the metabolites in this cycle:

$$\text{d-Isocitrate} + \text{NADP} \underset{Mn^{++}}{\overset{ICD}{\rightleftharpoons}} \alpha\text{-Ketoglutarate} + CO_2 + \text{NADPH}$$

The immediate product of the reaction is very likely oxalosuccinate which undergoes spontaneous (or enzymatic) decarboxylation to α-Ketoglutarate and CO_2. The amount of α-ketoglutarate formed is proportional to the amount of isocitric dehydrogenase activity. α-ketoglutarate reacts with 2, 4-dinitrophenylhydrazine to form a

"hydrazone" which is highly colored in alkaline solution.

The presence of Mn^{++} as an activator necessitates the use of EDTA as a chelating agent. In the absence of chelation, the final color is unstable and too intense to be of practical use.

In vitro, the activity of ICD of cichlid homogenate was stimulated by increasing the concentration of KPCP up to 150 ppm. Since this enzyme appeared to be tolerant of KPCP, it did not appear profitable to study ICD in treated fish at the levels of toxicant used. It is possible, however, that the activity of ICD might be inhibited by increasing the concentration of KPCP.

SUMMARY

Pentachlorophenol (PCP) has been shown to be a potent uncoupling agent to oxidative phosphorylation in the rat-liver mitochondria (27, 31). At low concentration, it produced stimulation to the ATPase activity (28, 32).

The present investigation was to study: (1) the lethal concentration of KPCP for goldfish; (2) the toxicity of PCP toward four selected enzymes in both cichlid and goldfish in vitro and in vivo. The cichlids were treated with 0.0, 0.1 and 0.2 ppm KPCP under 0, 24, 48 and 96 hours exposure while goldfish were exposed to 0.0, 0.1, 0.2 and 0.4 ppm KPCP for 0 and 96 hours.

The results indicated that the lethal concentration of KPCP for goldfish was 0.46 ppm or 1.43×10^{-6} M. The degree of toxicity of KPCP to the four enzymes were different but all were apparently inhibited by it. In vitro, aldolase, GOT, GPT and LD all displayed decreased activity with increasing concentrations of KPCP. In vivo, KPCP produced a dual effect, stimulation being observed at low concentration and inhibition at high concentration, with the activity of aldolase, GOT and GPT in cichlid muscle. The activity of aldolase and GOT were stimulated at 0.1 ppm KPCP with 24 hours exposure and for GPT at 0.1 ppm with 96 hours exposure. The activity of aldolase was rapidly decreased at 0.2 ppm of KPCP with increasing

time. Aldolase showed 53.1% inhibition at 96 hours exposure while the activities of GOT and GPT reached maximum inhibition of 30.6% at 24 hours exposure and 34% at 48 hours exposure respectively.

The activity of LD exhibited no dual effect from KPCP intoxication. The minimum 7% inhibition was at 0.1 ppm KPCP upon 24 hours exposure and the maximum inhibition, 17.4% was found at 0.2 ppm with 48 hours exposure.

KPCP produced both stimulation and inhibition, a dual effect, on the activities of aldolase, GOT and LD in goldfish muscle. At 0.1 ppm KPCP, the percentage of stimulation was 6.4 for aldolase, 28.2 for GOT and 8.6 for LD. The percentage of inhibition was 40.0 for aldolase, 49.3 for GOT and 29.5 for LD at 0.4 ppm KPCP. For GPT, the percent inhibition was 9.7 at 0.1 ppm and 70.9 at 0.4 ppm KPCP.

As the concentration of KPCP increased the activities of the four enzymes exhibited different degrees of inhibition. This might be an indication that these inhibitions were noncompetitive.

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