AN ABSTRACT OF THE THESIS OF

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in Fisheries and Wildlife (Major) presented on May 7, 1969 (Date)

Title: ALTERATIONS OF CARBOHYDRATE METABOLISM BY PENTACHLOROPHENOL IN CICHLID FISH

Abstract approved: Redacted for privacy
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Pentachlorophenol (PCP) is an important, biologically-active compound which has a wide variety of applications in agriculture and industry. It appears to affect all phases of metabolism in fish as evidenced by its ability to alter growth, food conversion efficiency, appetite, fat and protein deposition, swimming stamina, the level of ATP in tissues and the activities of certain enzymes. This investigation had as its purpose the determination of the nature and magnitude of the alterations produced by potassium pentachlorophenate (KPCP) upon the catabolism of carbohydrate in the cichlid fish, Cichlasoma bimaculatum.

The production of respiratory $^{14}CO_2$ from cichlids, injected intraperitoneally with ^{14}C-labelled substrates, was measured over a period of 24 hours. The test groups were: controls, cichlids exposed to 0.20 ppm KPCP, and cichlids exposed to 1.5 ppm KPCP.

In controls the percentage of radioactivity recovered as $^{14}CO_2$ from C-1 and C-2 of acetate were 98 and 70 percent respectively. Preferential conversion of C-1 over C-2 of acetate to $^{14}CO_2$ was considered presumptive evidence for the operation of the TCA cycle in cichlid fish. The addition of 0.20 ppm KPCP produced a delay in the appearance of $^{14}CO_2$ from C-1 and C-2 in the expired air. Over the 24-hour test period the cumulative yields from C-1 and C-2 were 95 and 68 percent respectively. Thus in the presence of 0.20 ppm KPCP the respective yields from both acetate carbons were very similar to the yields obtained from controls. Cichlids exposed to 1.5 ppm KPCP exhibited a marked increase in the rate of recovery of activity from both acetate carbons. The cumulative 24-hour yield from C-1 was 102 percent while the yield from C-2 was 98 percent. With cichlids exposed to 1.5 ppm KPCP the utilization of the TCA cycle as a respiratory mechanism rather than as a route for biosynthesis was implied.

The difference in the rate and extent of recovery of $^{14}CO_2$ derived from the individual carbons of glucose suggested that glucose may have been catabolized via several pathways. In controls the respective 24-hour cumulative yields of $^{14}CO_2$ from carbons 1, 2, 3(4) and 6 of glucose were 53, 47, 66, and 56 percent of the injected activity. The rapid and extensive recovery of C-3(4) as $^{14}CO_2$, presumably via the decarboxylation of pyruvate, identified the Embden-Meyerhof-Parnas (EMP) pathway as the major route of

glucose degradation in cichlids. Preferential conversion of C-1 over C-2 to $^{14}CO_2$ gave presumptive evidence for the operation of the pentose phosphate (PP) pathway; preferential conversion of C-6 over C-2 to $^{14}CO_2$ suggested the operation of the glucuronic acid (GA) pathway.

In cichlids exposed to 0.20 ppm KPCP the cumulative yields of $^{14}CO_2$ from carbons 1, 2, 3(4) and 6 of glucose were 57, 56, 80, and 56 percent respectively. The relatively larger increase in the extent of recovery from C-2 and C-3(4) suggested that the addition of 0.20 ppm KPCP increased the fraction of labelled glucose catabolized via the EMP-TCA pathway.

In cichlids exposed to 1.5 ppm KPCP the respective cumulative yields from carbons 1, 2, 3(4) and 6 of glucose were 81, 90, 100, and 88 percent. The rapid and extensive recovery of $^{14}CO_2$ from all glucose carbons indicated that the presence of 1.5 ppm KPCP markedly increased the rate of glucose degradation in cichlids. A comparison of the relative rates of recovery from the individual carbons of glucose indicated that the EMP-TCA pathway was used almost exclusively.

In an additional study made to determine the effect of KPCP upon the recovery of $^{14}CO_2$ from bicarbonate-^{14}C, the cumulative yield from controls was 91 percent of injected activity. Failure to obtain as much $^{14}CO_2$ from labelled bicarbonate as from C-1 of acetate suggested that due to the sudden increase in the concentration

of dissolved $^{14}CO_2$ resulting from the injection of bicarbonate, some of the injected bicarbonate may have been incorporated into relatively non-labile compounds such as bone. In cichlids exposed to 0.20 ppm KPCP, depletion of radiocarbon from injected bicarbonate-^{14}C occurred more slowly than in controls and only 80 percent of the injected radioactivity was recovered as $^{14}CO_2$ in 24 hours. This suggested that in the presence of 0.20 ppm KPCP, a larger amount of radiocarbon from bicarbonate-^{14}C may have been incorporated into non-labile compounds.

Alterations of Carbohydrate Metabolism by Pentachlorophenol in Cichlid Fish

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1969

APPROVED:

Redacted for privacy

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Date thesis is presented May 9, 1969

Typed by Muriel Davis for David Hyuk Wui Liu

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. Hugo M. Krueger for his constant and willing guidance throughout the course of this investigation.

Many thanks are due to Dr. C. H. Wang for his assistance in the design and provision of the radiorespirometer system and for his advice on the interpretation of the data.

To my wife, Kaye, goes my deepest gratitude for her patience, encouragement and understanding.

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ALTERATIONS OF CARBOHYDRATE METABOLISM BY PENTACHLOROPHENOL IN CICHLID FISH

INTRODUCTION

Pentachlorophenol is an important biologically active compound which has a wide variety of applications in agriculture and industry. The production of pentachlorophenol approached 40 million pounds in 1965 (45). Its potent fungicidal and bacteriocidal properties have been employed for the preservation of wood and wood products (13) and in the processing of adhesives, leather, starches, oils and rubber (14). In tropical counties, sodium pentachlorophenate (NaPCP) has been very effective in controlling snails acting as intermediate hosts for the schistosomes infecting man (1). Other applications include its use in the manufacture of polyvinyl chloride polymers and of closure-sealing gasket material for food containers, as a moth-proofing agent, an herbicide and a food preservative (2).

The use of pentachlorophenol and its salts has led to cases of accidental poisoning and sometimes death in fish, livestock and man (6, 28, 47, 53, 54). Toxic symptoms in man have included abdominal pain, nausea, vomiting, excessive sweating, elevated temperature, rapid pulse and increased respiration (28). Poisoning in laboratory mammals has been characterized by increased cardiac and respiratory rates, elevated temperature, hyperglycemia, glycosurea,

and rapidly-developing motor weakness which, in fatal cases, has terminated in cardiac and muscular collapse followed by immediate and profound rigor mortis (17, 35). Goodnight (22) observed that fish exposed to lethal concentrations of NaPCP developed increased respiration and bleeding from the gills, mouth and pectoral regions.

It has been suggested that pentachlorophenol exerts its toxic effects by uncoupling oxidative phosphorylation (15, 64). If pentachlorophenol interferes with the formation of adenosine triphosphate, it should alter carbohydrate metabolism. Therefore a series of experiments were designed to investigate the effects of pentachlorophenol on carbohydrate metabolism in the cichlid fish, Cichlasoma bimaculatum.

PERTINENT LITERATURE

In this section no attempt was made to include all of the literature on pentachlorophenol or on carbohydrate metabolism in fish. Extensive reviews of both subjects have been published. In 1967 Bevenue and Beckman (2) discussed the chemical and toxicological properties of pentachlorophenol, its uses in agriculture and industry and the various methods used to assay residual quantities of the toxicant in human and animal tissues. A review on intermediary carbohydrate metabolism in fish was written by Gumbman and coworkers (24) in 1958 and another by Black and associates (5) in 1961. This section thus includes only the articles which were deemed pertinent to the present problem. First pentachlorophenol will be considered and then carbohydrate metabolism in fish.

Pentachlorophenol

Early work by Goodnight (22) demonstrated that NaPCP is quite toxic to fish. Acute toxicity tests, conducted on 19 species of freshwater fish, showed that the sensitive fish species were incapable of surviving in water containing more than 0.20 ppm NaPCP; more tolerant fish succumbed when the concentration exceeded 0.60 ppm. The toxicity of NaPCP increased with decreasing water pH and with increasing water temperature.

More recently a considerable amount of effort was made to conduct a comprehensive investigation into the effects of pentachlorophenol on the physiology and biochemistry of the cichlid fish, Cichlasoma bimaculatum. In 1963 Brockway established the 24-hour median tolerance limit for cichlids exposed to NaPCP in standing water as 0.22 ppm; the 48-hour median tolerance limit was 0.16 ppm (10). The median tolerance limit is the estimated concentration which will kill 50 percent of a population during a stated time period. For potassium pentachlorophenate (KPCP) the 36-hour median tolerance limits were 0.37, 0.47 and 0.27 ppm for cichlids in running water, aerated standing water and unaerated standing water, respectively. When cichlids were exposed to 0.20 ppm KPCP for over a month, no mortalities were observed (15).

Chapman (15) reported that the body weight of control cichlids starved for 30 days increased 11 percent and the caloric content per gram of dry weight decreased 10 percent. When cichlids were exposed to 0.20 ppm KPCP, body weight decreased 12 percent and the caloric content per gram decreased 14 percent. Thus the decrease in body weight and in the caloric content per gram indicates that the energy loss in poisoned cichlids was 20 percent more rapid than in controls. Most of the loss occurred during the first ten days.

Daily food consumption in cichlids exposed to 0.20 ppm KPCP was sometimes more and sometimes less than that of controls (37). However, over a 42-day period, the poisoned fish consumed a total of 34.6 kilocalories of food whereas the controls consumed 23.0 kilocalories. It was calculated that the controls required 1929 calories of food to gain 1000 calories in body stores for a food conversion efficiency of 50.3 percent. Cichlids exposed to 0.20 ppm KPCP required 2443 calories of food to gain 1000 calories for a food conversion efficiency of 41.0 percent. From the average weight of the fish and the amount of calories lost in the process of metabolizing food, the metabolic rate for poisoned cichlids was 43.1 percent higher than that for controls. Brockway (10) observed no change in oxygen consumption at 0.16 ppm NaPCP, the 48-hour median tolerance limit for cichlids. On the other hand sublethal and lethal doses of NaPCP increased respiratory activity in snails, fish, rabbits and man (7, 22, 23, 66). The discrepancy is probably due to the greater reliability of determinations of 42-day caloric deficits over random hourly oxygen consumption determinations.

Growth in control cichlids was greater than in cichlids exposed to 0.20 ppm KPCP (15). Generally, the deposition of fat, phospholipids and protein was lower in poisoned cichlids than in controls; however, the ash content for both groups was similar.

Cichlids exposed to 0.20 ppm KPCP for one day were not able

to swim for ten minutes at the same high sustained velocities at which controls could swim. After seven days of exposure, there was no difference between the performance of poisoned and control cichlids and after 14-15 days of exposure, poisoned cichlids appeared to be better swimmers than controls (15). The swimming performance data was augmented by determining ATP (adenosine triphosphate) levels in controls and in KPCP-treated cichlids under conditions of exercise and no exercise. Unexercised controls contained 12.3 mg per kilogram of ATP. When cichlids were subjected to a very high water velocity, exhaustion usually occurred within five minutes. The level of ATP in these fish was the same as in controls. At a moderate water velocity at which exhaustion occurred at about 30 minutes, the ATP content increased to 13.7 mg per kilogram. At very low water velocity at which the fish were allowed to swim for three hours, ATP increased to 16.5 mg per kilogram.

Cichlids exposed to 0.23 ppm KPCP for 1, 2, 4 and 25 days and not exercised contained 16.7, 11.8, 13.8 and 6.2 mg per kilogram ATP compared with 12.3 mg per kilogram ATP in unexercised controls. When cichlids were exposed to KPCP for 15 days and exercised to exhaustion, the level of ATP was 11 mg per kilogram whereas exhausted control cichlids contained 13 mg per kilogram.

In an effort to explore possible effects of KPCP upon intermediary metabolism in cichlids, Cheng (16) measured the activity

of selected enzymes in cichlids which had been exposed to 0.10 and 0.20 ppm KPCP for 24 to 96 hours. In cichlids which had been exposed to 0.10 ppm KPCP for 24 hours, the activity of the glycolytic enzyme, aldolase, was reduced by 12 percent. The activity of lactic acid dehydrogenase, which is involved in the reversible conversion of pyruvate to lactate, was reduced by six percent. The activities of glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase were increased by ten and five percent, respectively. The transaminases are involved in the reversible conversion of carbohydrates to amino acids. At 0.20 ppm KPCP, reductions of 5, 30 and 12 percent were found in the respective activities of aldolase, the transaminases and lactic acid dehydrogenase in 24 hours.

A series of studies by Weinbach (64) led him to suggest that the toxicity of pentachlorophenol is probably due to its ability to uncouple oxidative phosphorylation. Sodium pentachlorophenate (NaPCP), at a concentration of 1×10^{-6} M, reduced inorganic phosphate uptake associated with the oxidation of alpha-ketoglutarate to succinate in rat liver mitochondria and enhanced oxygen uptake so that the P:O ratio was 1.5 (65). For control mitochondria the P:O ratio was 2.8. When the NaPCP concentration was increased to 1×10^{-5} and 1×10^{-4} M, phosphate uptake was further depressed; however, oxygen uptake was higher at 1×10^{-5} M and lower at 1×10^{-4} M than when NaPCP was absent from the mitochondrial preparation. At both concentrations the P:O ratio was 0.30. Oxidation and phosphorylation were abolished at 1×10^{-3} M NaPCP. Phosphate

uptake associated with the oxidation of beta-hydroxybutyrate to acetoacetate was completely inhibited at 2.5×10^{-4} M NaPCP in snail hepatopancreas preparations (67). However, oxygen uptake and the rate of acetoacetate formation were increased.

Pentachlorophenol also affected adenosine triphosphate levels in mitochondria by increasing ATP-ase activity at low concentrations and inhibiting the enzyme at high concentrations (68). At PCP concentrations of 5×10^{-5} and 5×10^{-6} M, inorganic phosphate levels were much higher in fresh mitochondrial preparations to which ATP was added than at concentrations of 5×10^{-4} and 5×10^{-3} M. In preparations to which ATP was added in the absence of PCP, phosphate levels were very low. Phosphate levels did not change when PCP was added to ATP in the absence of mitochondria indicating that PCP does not chemically hydrolyze ATP. The dual effect of PCP upon ATP-ase activity is unlike the effect of dinitrophenol whose ATP-ase-stimulating property is proportional to the concentration of dinitrophenol. Pentachlorophenol appears to be specific for mitochondrial ATP-ase for it had no effect upon human and rabbit alkaline serum phosphatase, acid phosphatase from rat liver and potato phosphatase.

Carbohydrate Metabolism in Fish

The principal carbohydrates that have been studied in fish are

glycogen, glucose, lactate, pyruvate, and certain pentose sugars. Resting levels of glycogen in the muscle and liver of rainbow trout vary considerably and in general the levels appear to be low in comparison with those for corresponding mammalian tissues. Black and associates (5) reported that in rainbow trout weighing from 29 to 120 grams, muscle glycogen levels varied from 0.01 to 0.171 mg percent (mean = 0.085) and liver glycogen varied from 0.50 to 3.22 mg percent (mean = 1.73). In larger trout with weights of 152 to 397 grams Black and co-workers (5) reported muscle and liver glycogen levels of 0.049 to 0.310 mg percent (mean = 0.123) and 0.29 to 7.79 mg percent (mean = 3.36), respectively. According to Black the values reported for glycogen by different authors depend upon variations inherent in the different methods of analysis, the history of the fish before the samples were taken, the amount of autolytic glycolysis that occurred between the time of sampling and fixing of the tissues, and for muscle, the particular muscle mass selected for analysis.

Blood glucose in rainbow trout is maintained at about 60 mg percent in resting fish (5). Under certain conditions blood glucose will increase markedly. Removal of dogfish from water caused blood glucose to increase from 66 mg percent to about 168 mg percent in three minutes, but the level returned to near-normal in about three minutes (42). In rainbow trout exercised strenuously for 15

minutes, blood glucose rose from 60 to 118 mg percent; however, the level of glucose did not rise until after exercise was stopped and unlike the rapid recovery observed in dogfish, the high level of blood glucose was maintained for at least 24 hours (5).

Blood lactate may vary among fish of a given species and between species of fish. Black and co-workers (4) reported an average of 8.6 mg percent and a range of 3.2 to 19.6 mg percent for blood lactate in Kamloops trout. In carp blood lactate ranged from 11.8 to 17.7 mg percent (43). Much higher levels of lactate may be found in muscle tissue which in rainbow trout may contain as much as 60 mg percent under resting conditions (5).

Because of the difficulty in measuring small amounts of pyruvate, reported levels may be questionable. According to Black and associates (5), blood, muscle and liver of rainbow trout contain less than 1.0 mg percent of pyruvate.

Prior to 1960 the pathways involved in the intermediary metabolism of carbohydrate in fish was a matter of conjecture. Evidence for defining the processes involved in carbohydrate metabolism was obtained indirectly. By studying changes in amount of various carbohydrates in fish in relation to various physiological and environmental factors and comparing the alterations with those found in mammalian systems, inferences on the processes involved in intermediary carbohydrate metabolism in fish were drawn.

Thus presumptive evidence for the glycolytic pathway in fish was obtained when it was observed that exercise brought on almost simultaneously an increase in lactate production and a decrease in glycogen stores (5). Strittmatter and co-workers (48) found that glucose added to the isolated swim bladder of the scup increased the amount of lactate in the preparation. More evidence was obtained by the discovery of certain glycolytic enzymes such as hexokinase (36) and pyruvate kinase (8) in fish.

According to Gumbmann and co-workers (25) evidence in fish for the pentose phosphate pathway, otherwise known as the hexose monophosphate pathway or the direct oxidative pathway, was almost completely lacking. The only clue to its existence in fish was that ribose could be cleaved from ribose-5-phosphate, ribonucleotides, ribonucleosides and ribonucleic acids when these substrates were added to muscle homogenates of fish (49, 50, 51, 52).

Oxidation of carbohydrate intermediates via the TCA or the citric acid cycle was inferred from measurements of oxygen consumption in fish and from some enzyme studies. In 1955 Sexton and Russell (44) observed that oxygen uptake by homogenized fish gills increased upon the addition of succinate and decreased upon the addition of mercuric chloride. Addition of citrate, malate, succinate, glutamate or pyruvate enhanced oxygen uptake in fish egg homogenates (29). In 1951 succinic and cytochrome oxidase as well as

malic dehydrogenase were isolated from carp muscle (55, 56, 57).

More recently studies have provided more substantial evidence for the reactions involved in intermediary carbohydrate metabolism in fish. In 1960 MacLeod and associates (39) identified in steelhead trout, all of the enzymes necessary for the conversion of glucose to lactate via glycolysis. The activities of the enzymes in cardiac muscle, skeletal muscle, liver and kidney were compared. Only cardiac muscle had the ability to convert glucose to lactate; addition of hexokinase to the homogenates of the other tisses was necessary before this conversion could be accomplished. All tissues readily converted fructose-6-phosphate and fructose-1,6-diphosphate to lactate. Thus, hexokinase actvitity is generally low in fish. In 1962 Gumbmann and Tappel (25) demonstrated the presence of the enzymes of the TCA cycle in carp.

Evidence for the operation of the pentose phosphate pathway in fish was obtained from radiotracer experiments involving the comparison of the yields of $^{14}CO_2$ from fish metabolizing glucose-1- and -6-^{14}C. Hoskin (31) discovered that the C-6/C-1 ratio approached 1.0 only in the brain tissue of the electric eel; in other tissues the ratio was considerably less. In carp Hoskin found that the C-6/C-1 ratio in tail muscle was only 0.07. Hoskin argued that preferential conversion of C-1 over C-6 to CO_2 would indicate the operation of the pentose phosphate pathway while equal yields of $^{14}CO_2$ from both

carbons would indicate exclusive utilization of the Embden-Meyerhof-Parnas and TCA pathways. From similar studies with live carp, Brown (17) concluded that the pentose phosphate pathway played a very small role in the overall degradation of glucose in carp.

Hochachka and Hayes (30) reasoned that if the EMP pathway were the major route of glucose degradation, the reversal of the reactions in the pathway would preferentially place labelled carbon, introduced as $Na_2{}^{14}CO_3$, on positions 3 and 4 of the hexose molecule in glycogen. Positions 1 and 2 would become labelled via recycling of the pentose phosphate system and position 5 and 6 through equilibration of triose phosphates via both the EMP and the pentose phosphate pathways. They found that in warm-acclimated fish, positions 3 and 4 contained a larger amount of radiocarbon than any other position. Out of 11,000,000 dpm injected, 3400 cpm were recovered per millimole of $BaCO_3$ derived from positions 3 and 4 of glycogen. In cold-acclimated fish labelling of positions 3 and 4 was reduced considerably, but there was no change in the amount of radioactivity on positions 1, 2, 5 and 6. The authors concluded that warm-acclimated fish utilize the EMP pathway more extensively than cold-acclimated fish and that in the latter more emphasis is placed on the pentose phosphate pathway. Because of the energy levels per carbon in glucose and its degradation products, it is unlikely that the pathways from glucose to CO_2 and from CO_2 to glucose would be the same only

with the directions reversed. In many cases where a single step seems reversible, different enzymes are required for the anabolic and the catabolic processes.

This survey on the present status of the problem concerning the effects of pentachlorophenol on fish has shown that the metabolism of cichlid fish can be altered measureably by sublethal concentrations. Pentachlorophenol appears to affect all phases of metabolism as evidenced by its ability to alter growth, food conversion efficiency, appetite, fat and protein deposition, the level of ATP in tissues, swimming stamina and the activities of certain enzymes. It seems desirable at this point to investigate more closely the effects of pentachlorophenol upon broad but limited phases of metabolism such as the metabolism of carbohydrates, fats and proteins. This investigation had its purpose the determination of the nature and magnitude of alterations produced by potassium pentachlorophenate upon the catabolism of carbohydrate in cichlid fish.

MATERIALS AND METHODS

Experimental Fish

Cichlid fish (Cichlasoma bimaculatum), were obtained from the Oregon State University Oak Creek Laboratory located about five miles from the University campus. They were brought into the Radiation Center laboratory where they were kept in 15-gallon aquaria containing filtered, dechlorinated, tap water maintained at 25 degrees C. Commercially-prepared, dry trout pellets were provided once daily. At least two weeks were allowed for the fish to acclimate to the Radiation Center laboratory conditions. Cichlids selected for use in the study averaged 10 grams in weight and ranged from 8 to 13 grams. Both sexes were used since it is virtually impossible to distinguish the sexes by appearance alone.

Pentachlorophenol

Pure, crystalline pentachlorophenol was purchased from the Eastman Chemical Company. The potassium salt was obtained by treating the phenol with concentrated potassium hydroxide and subsequent crystallization. A fresh solution was prepared for each experiment by dissolving a weighed amount of salt in 100 ml distilled water.

Radiochemicals

All of the ^{14}C-labelled substrates were obtained from the New England Nuclear Corporation. A list of the radiochemicals used, the administered radioactivity, and the administered mass is given in Table 1.

Table 1. Radioactivity and Mass of Labelled Substrates Administered.

Substrate	Number of Fish Tested			Microcuries Injected	Milligrams Injected
	Control	0.20 ppm	1.50 ppm		
$NaH^{14}CO_3$	4	4	0	0.67	0.01
Acetate-1-^{14}C	6	6	3	1.01	0.30
Acetate-2-^{14}C	6	6	3	1.45	0.30
Glucose-1-^{14}C	6	6	3	1.02	0.30
Glucose-2-^{14}C	6	6	3	0.86	0.30
Glucose-3(4)-^{14}C	6	6	3	0.43	0.30
Glucose-6-^{14}C	6	6	3	0.88	0.30
Gluconate-1-^{14}C	2	0	0	0.91	0.05
Glucuronate-6-^{14}C	2	0	0	0.62	0.07

The activity per unit volume (cpm) of each substrate was determined by counting, in Bray's solution (9), replicate 1.0 ml aliquots of a working stock solution prepared by diluting a 0.1 ml aliquot of the original stock with a sufficient amount of distilled water to make a 10 ml total volume. All samples were counted for ten minutes on a Packard Tri-Carb liquid scintillation counter (Model 314).

The molar concentration of each substrate was calculated

from the specific activities provided by the vendor and the counts per minute per ml. To provide equivalent molar concentrations, non-radioactive carrier was added. In addition 7.0 mg of NaCl was added to each milliliter of substrate to approximate isotonicity with the body fluids of the fish. For a given substrate, e.g., glucose, all stock solutions were made up to the same molar concentration. This insured that the radioactivity, recovered as $^{14}CO_2$, varied mainly with the carbon labelled and did not vary significantly due to the mass of the substrate administered.

The Radiorespirometer

The radiorespirometric system was designed to measure the radioactivity of flowing gas over a continuous time period. The main components comprising the system and the direction of the air flow are shown in Figure 1. Components of the system are listed below:

Electrometer - Nuclear Chicago, Dynacon Model 6000
Ion Chamber - 250 ml capacity, Nuclear Chicago
Flow Meter - Manostat Corporation, Model 36-541-07
Air Pump - Neptune Dynapump, Model 2
Metering Valve - Nupro Fine Metering Valve, Model B-4MA, Nuclear Products Company
Recorder - Esterline-Angus Company, Model AW
Animal Chamber - all glass construction, fabricated at Oregon State University.
Drying Tower - fabricated at Oregon State University
Polyethylene Tube Fittings - Becton-Dickenson Company
Water Temperature Control - National Appliance Company, Model 730
Water Heater - 100 watt, Wil-Nes Company
Water Bath - 2-gallon glass aquarium

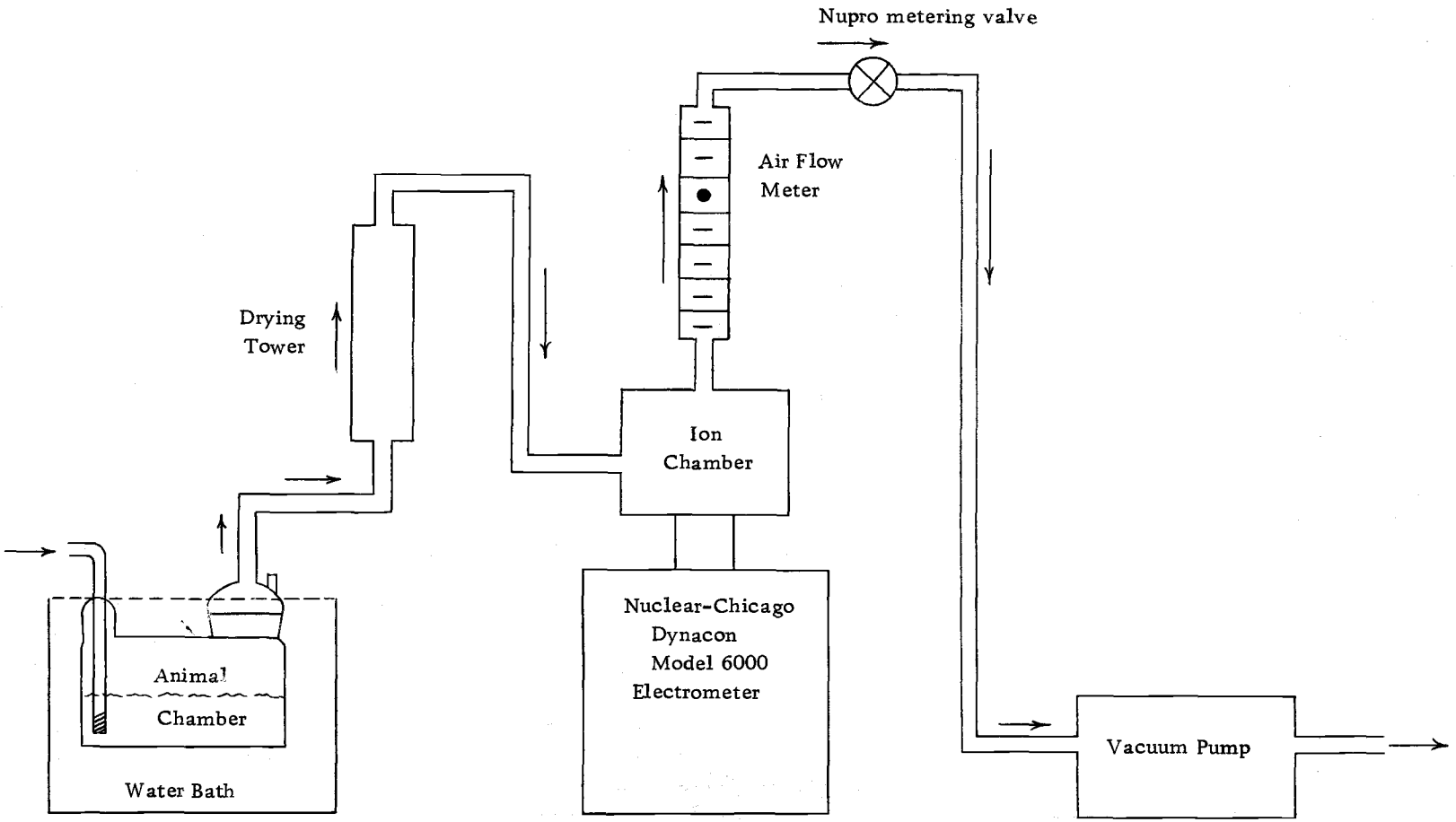


Figure 1. Ion chamber-electrometer system. Arrows indicate the direction of air flow.

Room air was pulled through the system by a vacuum pump located at the down-stream end of the one-pass system. The air entered the animal chamber through a glass tube, fritted at the blind end to insure rapid sparging of dissolved gases from the water in the animal chamber. Air leaving the chamber was dried upon passing through a $CaSO_4$-tower and directed into the ion chamber for radioanalysis. The flow rate of the air leaving the ion chamber was monitored by a flow meter and regulated by means of a fine metering valve connected near the pump intake nozzle.

The radioactivity of the gas entering the ion chamber was measured at a collection voltage of 90 volts. Current produced by electron collection in the ion chamber was rectified and amplified by the electrometer and the pulse produced was recorded on chart paper.

Electrometer Calibration

Calibration of the ion chamber-electrometer system was accomplished by recording the pulse produced by a known quantity of $^{14}CO_2$ liberated when a known volume of $NaH^{14}CO_3$ solution was injected into sulfuric acid in the animal chamber. The animal chamber was filled with 250 ml of 3N sulfuric acid. This volume of acid was equivalent to the amount of dechlorinated tap water used in the actual experiment. The chamber was positioned into the 25 degree

C water bath and the air flow through the system adjusted to 250 ml per minute. A period of 30 minutes was allowed for equilibration. Then through a small entry port fitted with a serum cap, standardized $NaH^{14}CO_3$ solution was injected into the chamber by means of a 1.0 ml plastic syringe, calibrated in one-hundredth ml subdivisions.

Radioactivity, measured by the electrometer system, was automatically recorded on the strip chart. By varying the volume of the injections and integrating the areas under each curve, the linearity of the measuring system with respect to different injected activities and the activity per unit area was obtained. The curves were integrated by counting the number of graph units under each curve to the nearest 0.1 unit. The chart paper (Chart No. 4310-X, Esterline-Angus Company) is divided into ten uniform one-half inch units by height. Each unit measured one-fourth inch in width. This one-fourth by one-half inch unit is further divided into five smaller units. The one-fourth by one-half inch unit was taken as a unit area. Calibration data are shown in Table 2.

Selection of KPCP Test Concentrations

Earlier work (10, 15, 16) showed that the slope of the dose-response curve for cichlids exposed to pentachlorophenol was very steep. Under flowing water conditions, the 36-hour median tolerance limit was 0.37 ppm, but at 0.20 ppm, no mortalities occurred

Table 2. Calibration of Radiorespirometer System. Radioactivity per Unit Area under $^{14}CO_2$ Calibration Curves.

Test Number	Microcuries $NaH^{14}CO_3$ Injected	Area Units	Microcuries Per Unit
1	0.0146	0.85	0.017
2	0.0146	0.90	0.016
3	0.0146	0.85	0.017
4	0.0219	1.35	0.016
5	0.0219	1.35	0.016
6	0.0219	1.30	0.017
7	0.0292	1.70	0.017
8	0.0292	1.70	0.017
9	0.0292	1.60	0.018
10	0.0438	2.60	0.017
11	0.0438	2.50	0.018
12	0.0438	2.50	0.018
		Mean =	0.017

over an exposure period of 40 days. In subsequent experiments at KPCP levels of less than 0.20 ppm, the effects on adult cichlid fish were slight. At 0.20 ppm, measureable effects were observed. Thus 0.20 ppm was the concentration chosen as a level satisfactory for the study of sublethal effects. A major part of this study was concerned with the determination of the effects of exposure to 0.20 ppm KPCP on carbohydrate metabolism.

Preliminary inspection of the data obtained at 0.20 ppm KPCP indicated that it might be difficult to determine with certainty the effect of KPCP upon the catabolism of carbohydrates in fish at this concentration and that additional experiments using a higher concentration might provide more definitive data. The higher concentration tested was 1.5 ppm KPCP. Under conditions of the standing aerated water bioassay tests made by Chapman (15), 1.5 ppm would have caused death within about six hours. However, in the experiments here described, none of the cichlids exposed to 1.5 ppm for up to 48 hours died or showed signs of abnormality. It should be noted that the volume of the test solutions used in Chapman's study was about 19 liters while only 250 ml were used in the present study. Thus although the bioassays involved ten fish per test, the absolute amount of KPCP available to each fish exposed to 0.20 ppm KPCP was more than the amount available to the single fish used in each test involved in the present study. The amount of KPCP available

to each fish exposed to 0.20 ppm in Chapman's study was 0.38 mg. This amount, dissolved in 250 ml of water, is equivalent to a concentration of 1.5 ppm. Thus the production of respiratory $^{14}CO_2$ from administered ^{14}C-labelled substrates was measured in three groups of cichlids: controls, cichlids exposed to 0.20 ppm KPCP and cichlids exposed to 1.5 ppm KPCP. The number of fish used in each test is shown in Table 1.

Test Procedure

Prior to the beginning of an experiment, the animal chamber was filled with 250 ml of dechlorinated tap water. The cichlid to be tested was starved for 48 hours, then placed in the chamber, which was then connected to the electrometer system and lowered into the 25 degree C water bath. Background activity was measured for about 40 minutes at an air flow rate of 250 ml per minute. Subsequently the fish was removed, injected intraperitoneally with 0.05 ml of labelled substrate and returned to the chamber. The air flow was again adjusted to 250 ml per minute and the experiment started. The duration of each experiment was 24 hours. At the end of each experiment the fish was removed from the chamber and killed by crushing the brain. The fish was then weighed, wrapped in a plastic bag and stored in a freezer. Poisoned fish were treated similarly except that after background measurement was completed, a solution

of potassium pentachlorophenate was added to the water in the test chamber.

Radioactivity Balance Study

Since the interpretation of the data from this study depended upon the accurate measurement of respired $^{14}CO_2$, it was important to determine the reliability of the yields. To accomplish this, radioanalysis was made on the water left in the animal chamber at the end of four tests on control fish injected with glucose-1-^{14}C and on the residual activity left in the fish carcass.

The radioactivity of the water was measured by transferring the water left in the animal chamber to a graduate cylinder and adding enough dechlorinated tap water to restore the original volume of 250 ml. A 1.0 ml aliquot was counted in Bray's solution for 30 minutes and quench corrected by adding a measured amount of ^{14}C-toluene to each sample after the initial count and counting the samples again.

Frozen fish carcasses were dehydrated under high vacuum for 20 hours; they were ground to a reasonably fine powder and radioanalyzed by the method of Mahin and Lofberg (40). Duplicate samples weighing about 50 mg were placed on the bottom of individual counting vials and thoroughly wetted with 0.2 ml of 60 percent perchloric acid. After the addition of 0.2 ml of 30 percent hydrogen

peroxide, the vials were tightly capped and warmed to 70 degrees C for one hour in a water bath with a shaker attachment. The samples were allowed to cool and then prepared for counting by adding 10 ml of a 1:2 mixture of ethyleneglycol-monoethylether and toluene phosphor. The toluene phosphor solution was prepared by adding six grams of 2, 5-diphenyloxazole (PPO) to a liter of toluene. Each sample was counted for ten minutes and quench corrected. The results are shown in Table III.

Table 3. Radioactivity Balance Study with Glucose-1-^{14}C.

Fish	Microcuries Recovered				Microcuries Injected	Percent Recovery
	CO_2	Carcass	Water	Total		
1	0.543	0.350	0.018	0.901	1.02	89
2	0.584	0.543	0.010	1.087	1.02	106
3	0.572	0.411	0.015	0.998	1.02	98
4	0.488	0.480	0.012	0.980	1.02	96

DATA AND DISCUSSION

Radioactivity Balance Study

In controls the percentage of radioactivity recovered in 24 hours from C-1 and C-2 of acetate was 98 and 70 percent, respectively. From labelled bicarbonate 91 percent of the injected activity was recovered in 24 hours. Over the same time period yields of $^{14}CO_2$ from carbons 1, 2, 3(4) and 6 of ^{14}C-labelled glucose were 53, 47, 66 and 56 percent, respectively. The recovery of at least 90 percent of the injected activity as respiratory $^{14}CO_2$ occurred only in the case of C-1 of acetate and of $NaH^{14}CO_3$. Recovery from acetate-2-^{14}C and from the labelled carbons in glucose was considerably less than 90 percent. The variability in the yields of labelled CO_2 from the different substrates raised the question of the adequacy of carbon dioxide recovery. Hence the radioactivity remaining in the cichlids which had been injected with glucose-1-^{14}C was measured. The amount of radioactivity recovered from the fish carcasses, the water in the respiration chamber, and as respiratory $^{14}CO_2$ averaged 97 percent of injected radioactivity in the four experiments with glucose-1-^{14}C (Table 3). Fifty-three percent was recovered as $^{14}CO_2$, 43 percent was recovered from the carcasses and about 1.5 percent from the water. Since an average of 97 percent of injected activity

was recovered, the activity measured as $^{14}CO_2$ was considered to represent quantitatively the portion of radiocarbon converted to CO_2.

Recovery of $^{14}CO_2$ from Injected Substrates

In order to illustrate the respiratory $^{14}CO_2$ recovery pattern from each carbon of a given substrate over the time course of 24 hours, the data are presented graphically in terms of the average percent of administered radioactivity recovered hourly. In general the recovery of radiocarbon as respiratory CO_2 was rapid during the early phase of the experimental period. Yields per hour reached a maximum within ten hours then declined to a low level by the end of the test. The ascending portions of the curves presumably involve substrate absorption and transport as well as the rate of catabolism of the substrate. During the descending portion of the curves, presumably absorption has been completed and the decreasing yields represent the exhaustion of the labelled substrate through catabolism and anabolism; in addition, there is a dilution of the labelled substrate through continuous neogenesis and replacement of the substrate initially injected by non-labelled substrate (58).

Without homeostatic replacement of the injected substrate and with a unidirectional movement of the substrate, the recovery of radioactivity would follow a simple logarithmic law:

$$dq/dt = .Kq \quad \text{or} \quad \text{Log } q = \text{Log } q_o - Kt \qquad (1)$$

where dq/dt is the time rate of change of q, q is the remaining radioactivity, q_o is the radioactivity initially administered and K is the decrease in Log q per unit of time. The antilog of K gives the rate of decay of radioactivity at any moment. Over a finite period of time, equation 1 may be rewritten:

$$\Delta q / \Delta t = -K_A q. \qquad (2)$$

If Δq is chosen as one hour, then $\Delta q/\Delta t$ is the fraction of q lost in one hour, q is the quantity of radioactivity at the beginning of the hour, and K_A is the average rate of loss or the average fraction of q lost per hour. A plot of Log q against time would give a straight line with a negative slope. If the plot of Log q against time is not a straight line, the interpretation of the curve of retention of Log q vs time becomes complex.

Deviation of the depletion curve from linearity may be attributed to at least three factors: 1) the fraction of remaining activity recovered per hour as $^{14}CO_2$ can be expected to increase in proportion to the amount of radioactive substrate absorbed per hour if absorption is still occurring, 2) if the substrate is being used in anabolic reactions, the fraction recovered can be expected to decrease as the radioactive carbons move beyond the influence of the labile pool of which they were a part, 3) if, as happens in stable biological steady states, the

substrate is replaced essentially molecule by molecule for each molecule destroyed, there will be a continual decrease in the concentration of radioactive molecules due to replacement of lost radioactive molecules by non-radioactive molecules as well as a decrease due to radioactivity lost as $^{14}CO_2$. All three of the factors may be operating simultaneously.

To visualize the extent to which these factors affected the recovery of the labelled carbons as $^{14}CO_2$, some of the data are presented in terms of the logarithm of the amount of radiocarbon remaining in the fish over the experimental period. The residual values were obtained by subtraction of the percentage recovered as $^{14}CO_2$ from 100 percent which represents the amount of radioactivity injected. Calculations were based on the assumption that the amount of radioactivity injected was 100 percent of the attempted amount. Various errors in the measurement of weights, volumes or radioactivity are possible. The relative interference of these errors on a percentage or logarithmic basis will increase as the calculated remainder becomes less and less. Hence the log plots were terminated whenever the calculated percentage of activity remaining in the fish reached a level of around 10 percent since an initial error up to $\pm$ 8 percent of the amount injected might occasionally have been expected in individual experiments (See Table 3). As the errors could have been sometimes positive and sometimes negative, the average expected error in the

mean of the injected amounts would be much less than 2 percent.

Bicarbonate

Labelled CO_2 administered as bicarbonate-^{14}C was recovered very rapidly and extensively (Figure 2). Over 50 percent of the administered activity of 0.8 microcuries was recovered from controls and from cichlids exposed to 0.20 ppm KPCP within three hours. After 18 hours $^{14}CO_2$ could not be detected by the electrometer system. The respective cumulative yields from controls and from cichlids exposed to 0.20 ppm of KPCP were 90 and 80 percent of injected radioactivity. From the 1st to the 20th hour the mean hourly yields from the KPCP-treated cichlids were less than that of the controls. Although the differences between the mean yield for each hour were not significant, the consistently lower recovery values for the KPCP-treated fish indicate that the difference in the overall recovery of radioactive carbon was not due to chance. A sequence of ten measureably lower recovery percentages in the KPCP-poisoned fish as compared with controls could be expected only once in a thousand times by chance. The differences in the recovery curves are brought out more clearly when the percent of injected activity recovered per hour is divided by the activity remaining in the fish and this fractional rate of disappearance of radioactivity is plotted against time (Figure 3).

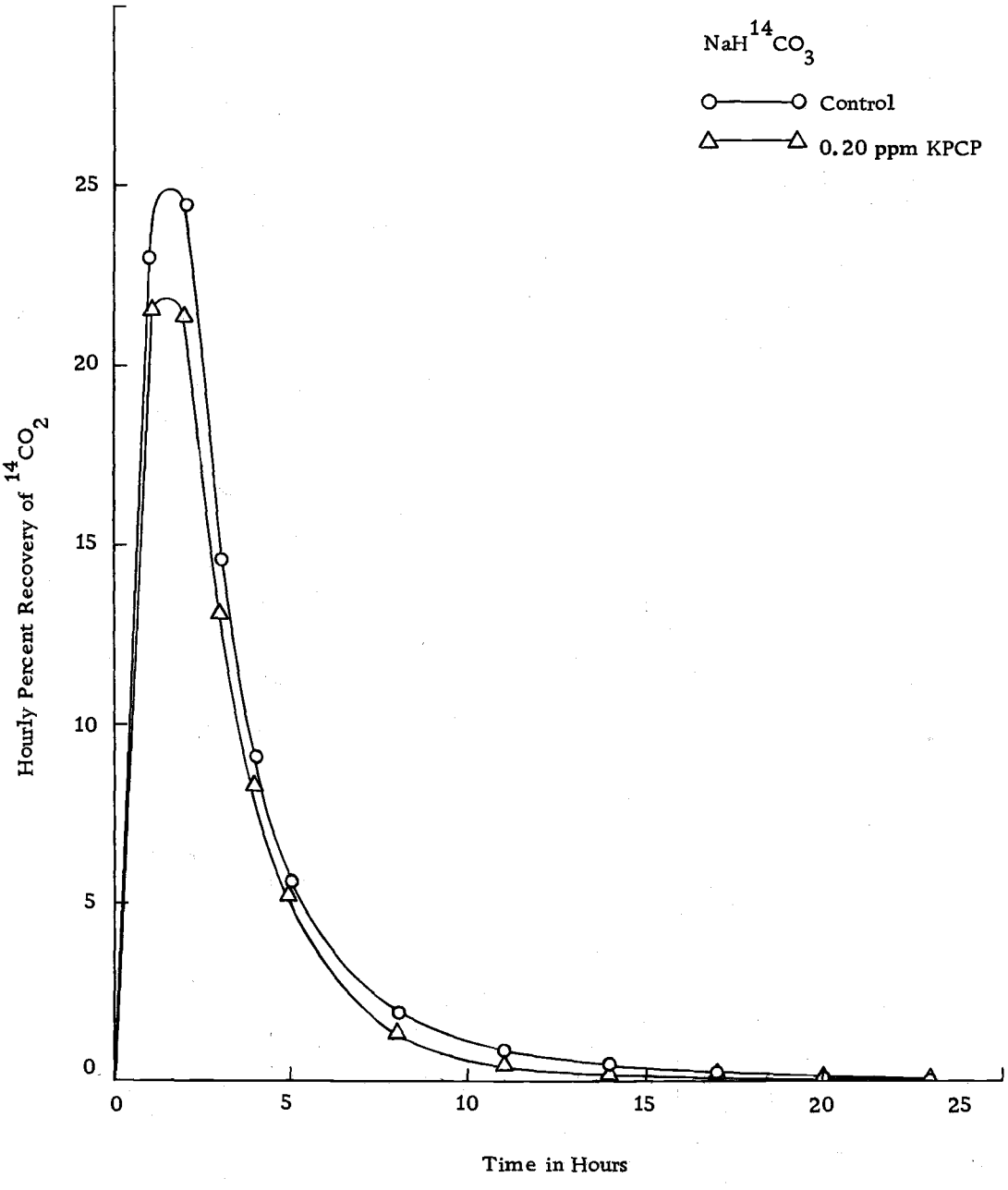


Figure 2. Effect of 0.20 ppm KPCP upon the hourly recovery of $^{14}CO_2$ from cichlids injected with $NaH^{14}CO_3$. Presence of 0.20 ppm KPCP reduced the rate and extent of recovery from labelled bicarbonate.

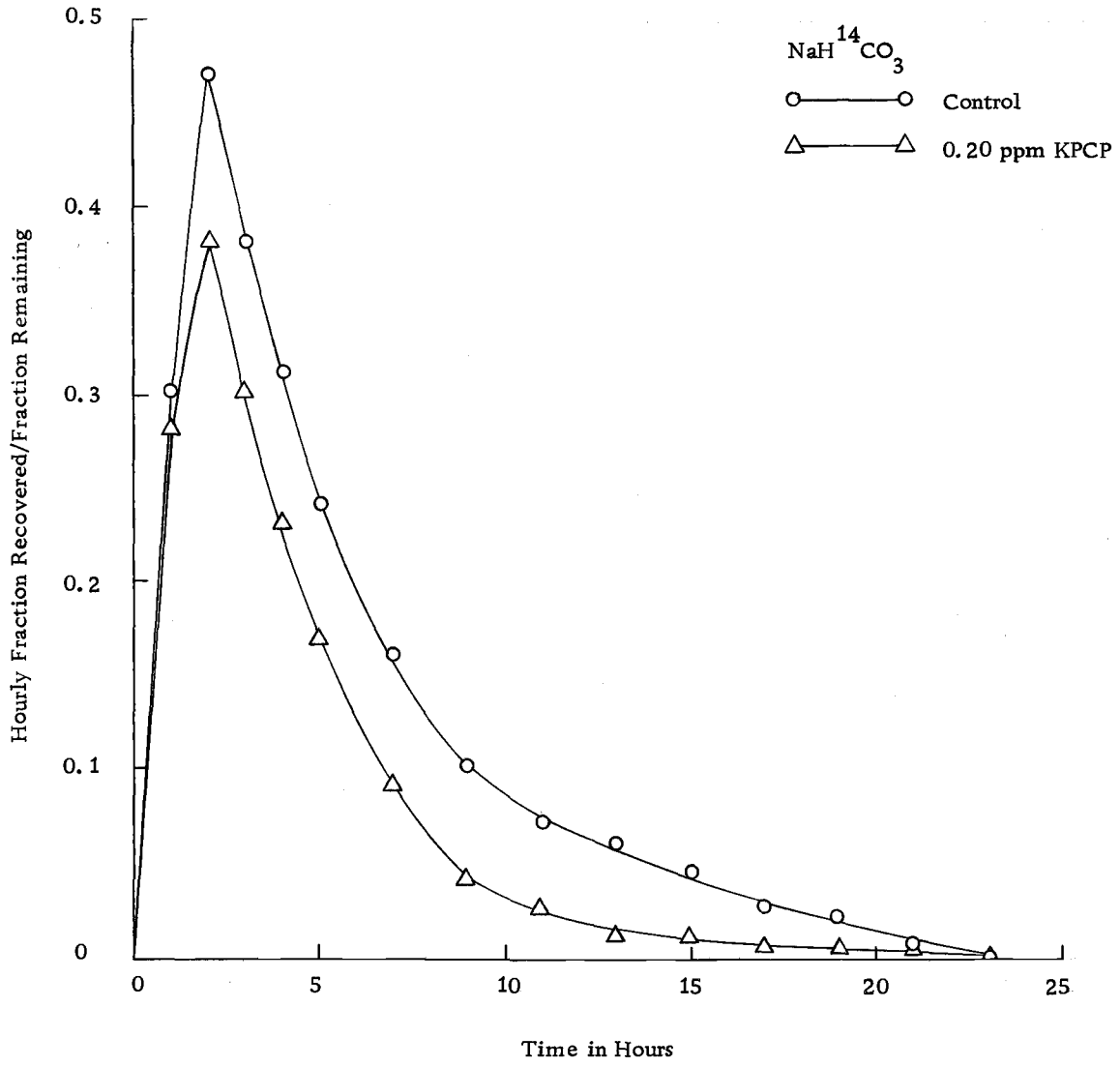


Figure 3. Effect of 0.20 ppm KPCP upon the hourly recovery of radiocarbon remaining in cichlids injected with $NaH^{14}CO_3$. Hour by hour the poisoned cichlids converted a smaller fraction of radiocarbon to $^{14}CO_2$ than controls.

The 24-hour $^{14}CO_2$ yield of 91 percent from $NaH^{14}CO_3$ was relatively low compared with the yield of 98 percent from C-1 of acetate in control cichlids. As expected the rate of depletion of radiocarbon derived from bicarbonate administered to control fish (Figure 4) was considerably faster than the depletion of radiocarbon derived from C-1 of acetate (Figure 8). The rate at which injected bicarbonate enters the pool of metabolically-derived bicarbonate depends primarily upon its rate of absorption while the rate at which bicarbonate from acetate enters the pool depends not only upon the absorption rate of acetate but also upon the rate of acetate degradation.

The depletion curves also showed that the fraction of labelled bicarbonate converted to $^{14}CO_2$ decreased more rapidly in each successive hour than the fraction of C-1 of acetate converted to $^{14}CO_2$. Also that after 18 hours, labelled CO_2 derived from bicarbonate could not be detected although only 91 percent of the injected activity was recovered. Failure to recover as much $^{14}CO_2$ from injected bicarbonate as from injected acetate suggested that some of the injected labelled bicarbonate became fixed.

Work by Irving and co-workers (21, 32) indicated that the source of CO_2 retention, noted in the present experiments with labelled bicarbonate, may have been bone. Irving and Chute (33) reported that the ratio of phosphorus, calcium and carbon dioxide in bone changed constantly. Carbon dioxide was more labile than either phsophorus

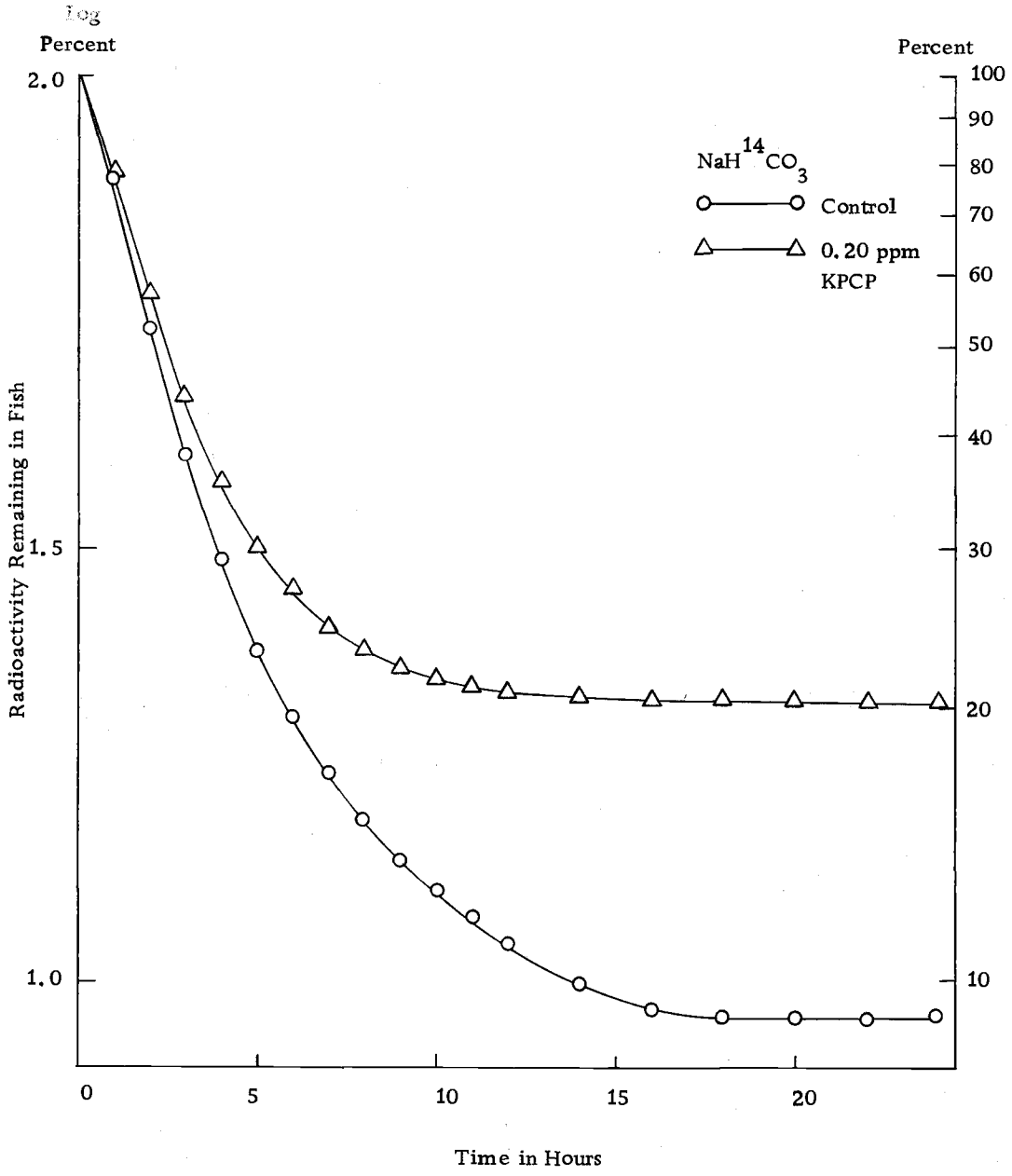


Figure 4. Logarithmic curves of the percentage of radiocarbon remaining in control and KPCP-treated cichlids after injection of $NaH^{14}CO_3$. Note slower rate of radiocarbon depletion and greater extent of bicarbonate conservation in poisoned cichlids.

or calcium. The authors suggested that CO_2 in bone may play a prominant role in acid-base balance. On the basis of mammalian data from Irving and Chute, the labile CO_2 pool may undergo continuous exchange with CO_2 in bone. The pool of CO_2 may be considered relatively non-labile and is known to contain some 10 to 20 times the mass of CO_2 found in the labile pool, viz., CO_2 and $NaHCO_3$ in solution in tissue fluids. When radioactive bicarbonate was injected, presumably the concentration of dissolved $^{14}CO_2$ increased almost instantly and considerable amounts may have disappeared into bone. However the amount incorporated would have been only a small fraction of the total. Hence there would be a relatively low rate of return of the labelled carbon dioxide from bone to tissue carbon dioxide or bicarbonate. Smaller and perhaps negligible quantities of labelled CO_2 developing from the metabolism of acetate-1-^{14}C was incorporated into bone since $^{14}CO_2$ presumably produced at a relatively slow rate.

In cichlids exposed to 0.20 ppm of KPCP depletion of radiocarbon derived from labelled bicarbonate occurred more slowly than in controls, and the fraction of radiocarbon delivered as $^{14}CO_2$ in each successive hour diminished more rapidly, reaching zero after 16 hours. Only 80 percent of the injected activity was recovered as $^{14}CO_2$. This suggested that much more radiocarbon was incorporated into non-labile compounds in the presence of KPCP. The slower

rate of depletion and the rapid decline in the fraction of remaining activity recovered as labelled CO_2 suggested an enhanced dilution effect from greater carbon dioxide production in the poisoned cichlids. Another possible explanation is that KPCP may have interfered with respiratory gas exchange related to the movement of water through the gills or to the circulation of the blood, thus partially arresting the rate of CO_2 removal from the cichlid. These effects of KPCP would not be constant but would vary with time as the amount of KPCP absorbed increased and further affected gas exchange or circulation. Secondarily, cellular activities interfering with the actions of KPCP and correcting the damage already done by the toxicant would appear. Since the presence of KPCP appeared to increase the rate of metabolism, there is a possibility that the resulting increase in CO_2 production could have upset the normal balance between bicarbonate and carbonic acid, causing the fish to become acidotic. Under this condition conservation of bicarbonate could have aided in combating acidosis. The mechanisms by which the KPCP-induced alterations in $^{14}CO_2$ loss after administration of labelled bicarbonate occur require additional investigation.

Acetate

In controls C-1 of acetate was converted to $^{14}CO_2$ more rapidly and extensively than C-2 of acetate (Figure 5). For C-1 the hourly

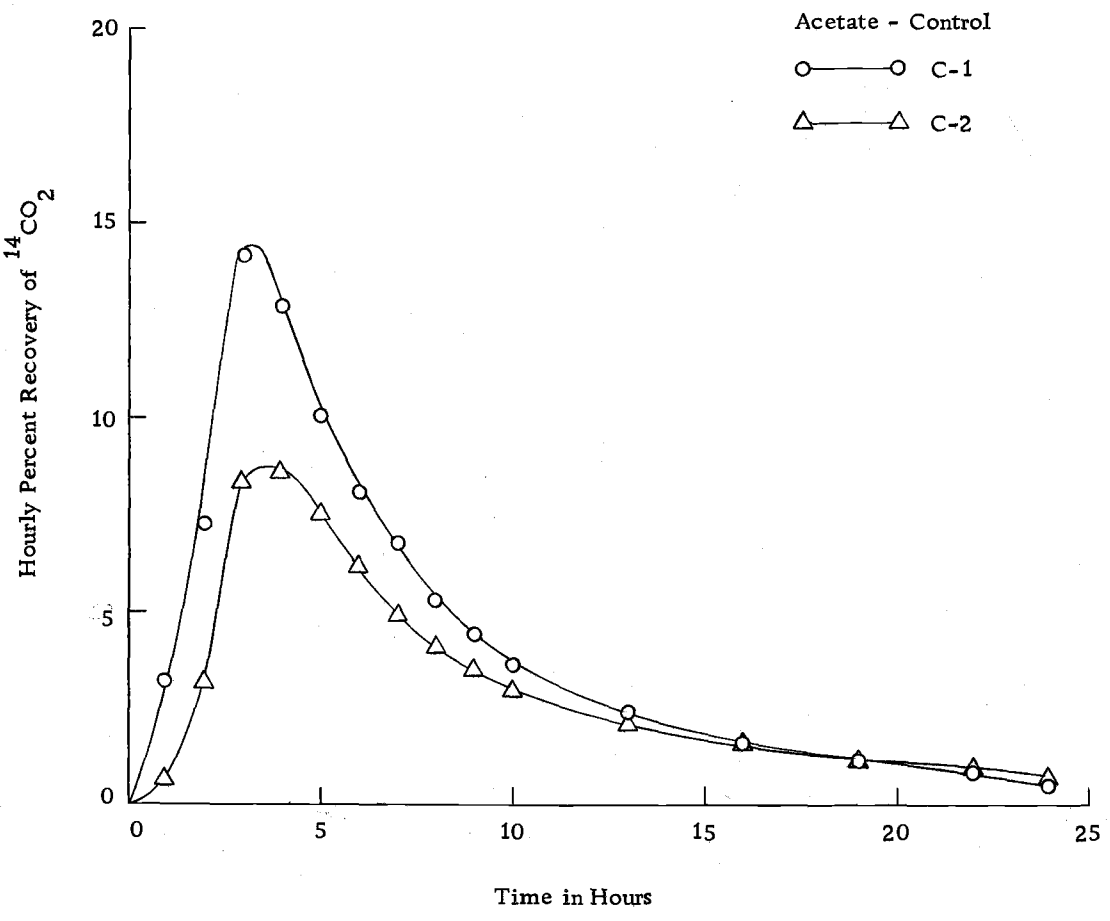


Figure 5. Hourly recovery of $^{14}CO_2$ from control cichlids injected with specifically-labelled ^{14}C-acetate. Operation of the TCA cycle is indicated by higher rate and extent of recovery from C-1 than from C-2.

yields increased to a maximum of 14 percent of injected activity and gave rise to a cumulative 24-hour yield of 98 percent. The maximum hourly yield from C-2 was only eight percent and the cumulative yield was 70 percent. Preferential conversion of C-1 over C-2 to CO_2 also occurred in the presence of 0.20 ppm and 1.5 ppm KPCP (Figures 6 and 7). However, at 1.5 ppm the differences were markedly reduced below the differences from controls and at 0.20 ppm.

Preferential conversion of C-1 over C-2 of acetate to $^{14}CO_2$ is considered presumptive evidence for the operation of the TCA cycle. In studies with alligators, crayfish, pepper fruit and several species of Pseudomonas, similar $^{14}CO_2$ patterns were obtained after administration of specifically-labelled ^{14}C-acetate (3, 18, 41, 62). These studies and the present one indicated that when acetate was utilized by the TCA cycle, the methyl carbon was conserved. Theoretically, preferential conservation of the methyl carbon over the carboxyl carbon of acetate would be expected. Acetate is a key intermediate in the degradation of glucose. Upon conversion to acetyl-CoA, acetate may be involved in the formation of lipids or may be further catabolized via the TCA cycle. Here it is important to note that lipid formation would not account for the difference in the disposition of the two acetate carbons since the process involves the acetate molecule. Degradation of acetate via the TCA cycle produces CO_2 from C-1 of acetate more rapidly and extensively than from C-2 since the

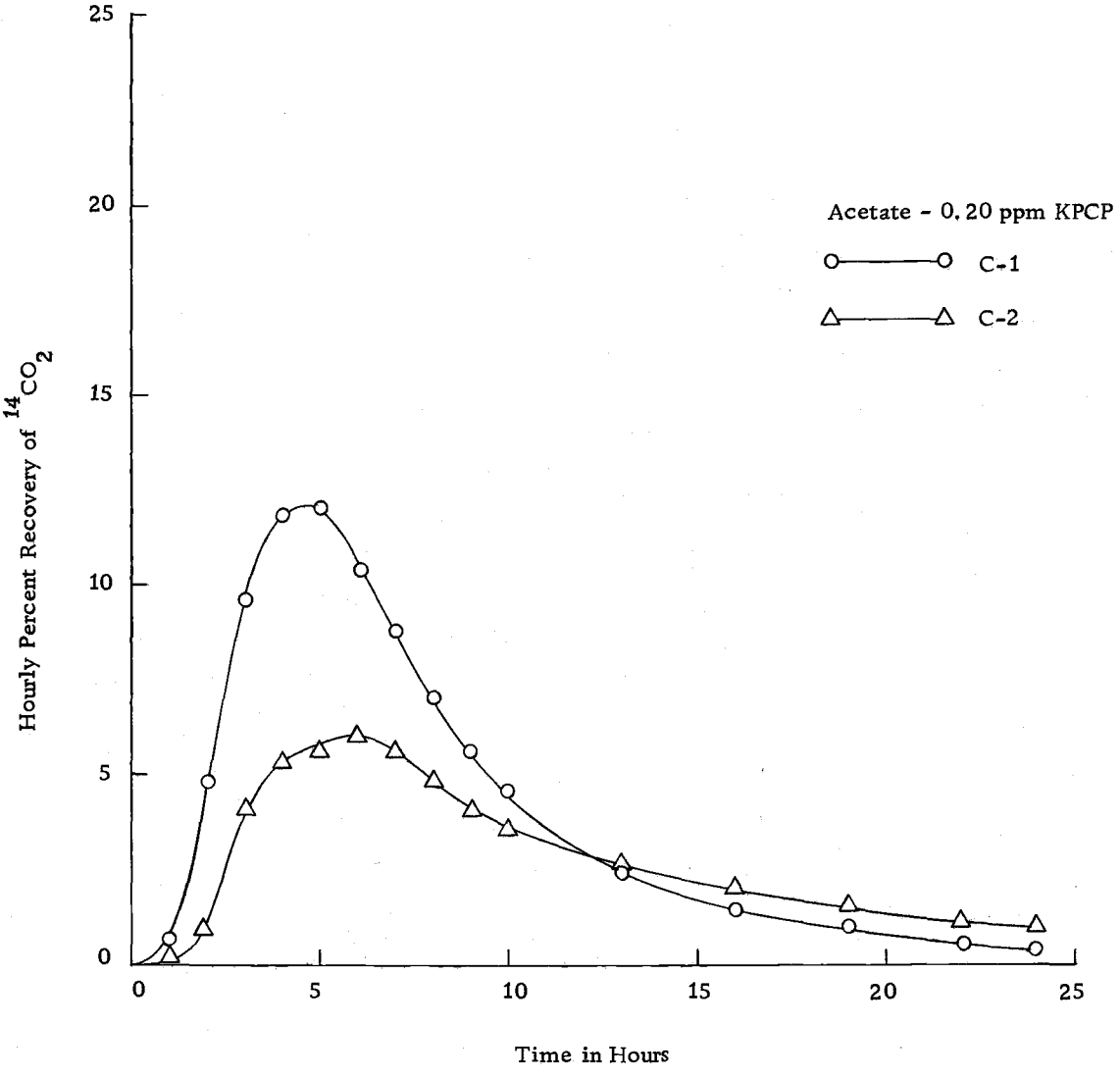


Figure 6. Hourly recovery of $^{14}CO_2$ from cichlids exposed to 0.20 ppm KPCP and injected with specifically-labelled ^{14}C-acetate. At this concentration of KPCP the initial rate of recovery from C-1 and C-2 was reduced; however, the respective 24-hour yields were similar to those from controls.

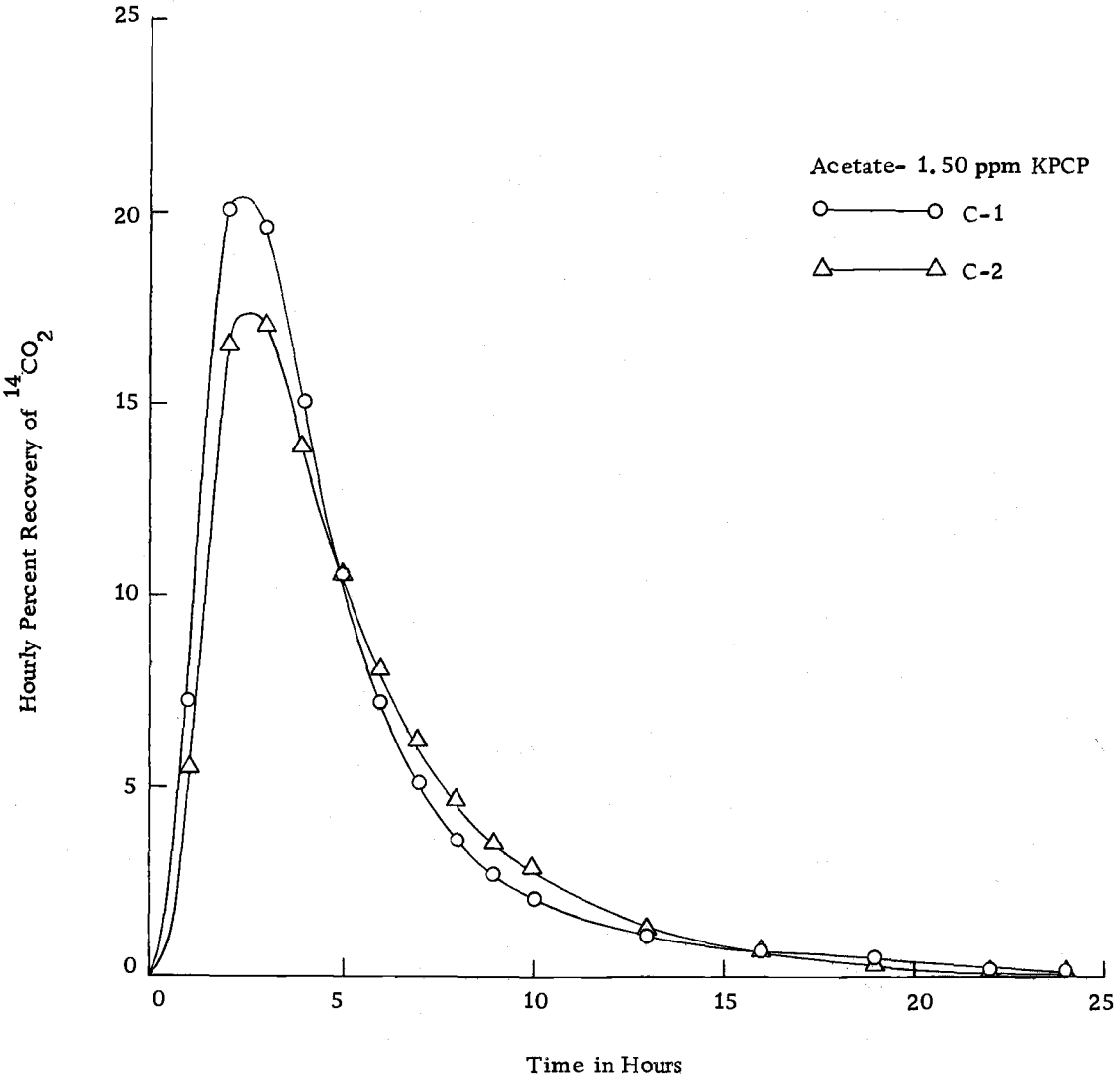


Figure 7. Hourly recovery of $^{14}CO_2$ from cichlids exposed to 1.5 ppm KPCP and injected with specifically-labelled ^{14}C-acetate. Here the rate and extent of recovery from C-1 and C-2 are almost equal.

former carbon initially occupies a carboxyl position in the molecular structure of TCA intermediates and is thus located in a prime position for conversion to CO_2 upon decarboxylation of the intermediate. The methyl carbon (C-2) initially takes an internal position and thus cannot be converted to CO_2 until molecular rearrangement of TCA intermediate places it in a carboxyl position. Since the TCA cycle serves not only as a respiratory route for converting acetyl-CoA to CO_2 and water, but also as a mechanism for the biosynthesis of a large number of amino acids, the delay in the conversion of C-2 to CO_2 would allow more of this carbon to be incorporated into amino acids and other substrates.

The differences in the metabolic fates of C-1 and C-2 of acetate were shown quite clearly by the depletion curves in Figure 8 and 9. It was apparent that the depletion curves for C-1 and C-2 from controls (defined by open circles) followed different paths. The curve for C-1 had a greater slope and departed less from linearity than the curve for C-2. Using the same reasoning used for the interpretation of the depletion curves for bicarbonate, it seemed clear that more C-2 of acetate left the catabolic route than C-1.

The presence of 0.20 ppm KPCP produced a delay in the appearance of $^{14}CO_2$ from C-1 and C-2 in the expired air and a reduction in the maximum hourly yields (Figures 10 and 11). The maximum yields for one hour from each carbon were obtained two hours later than those in controls and for C-1 the maximum yield was 16 percent less

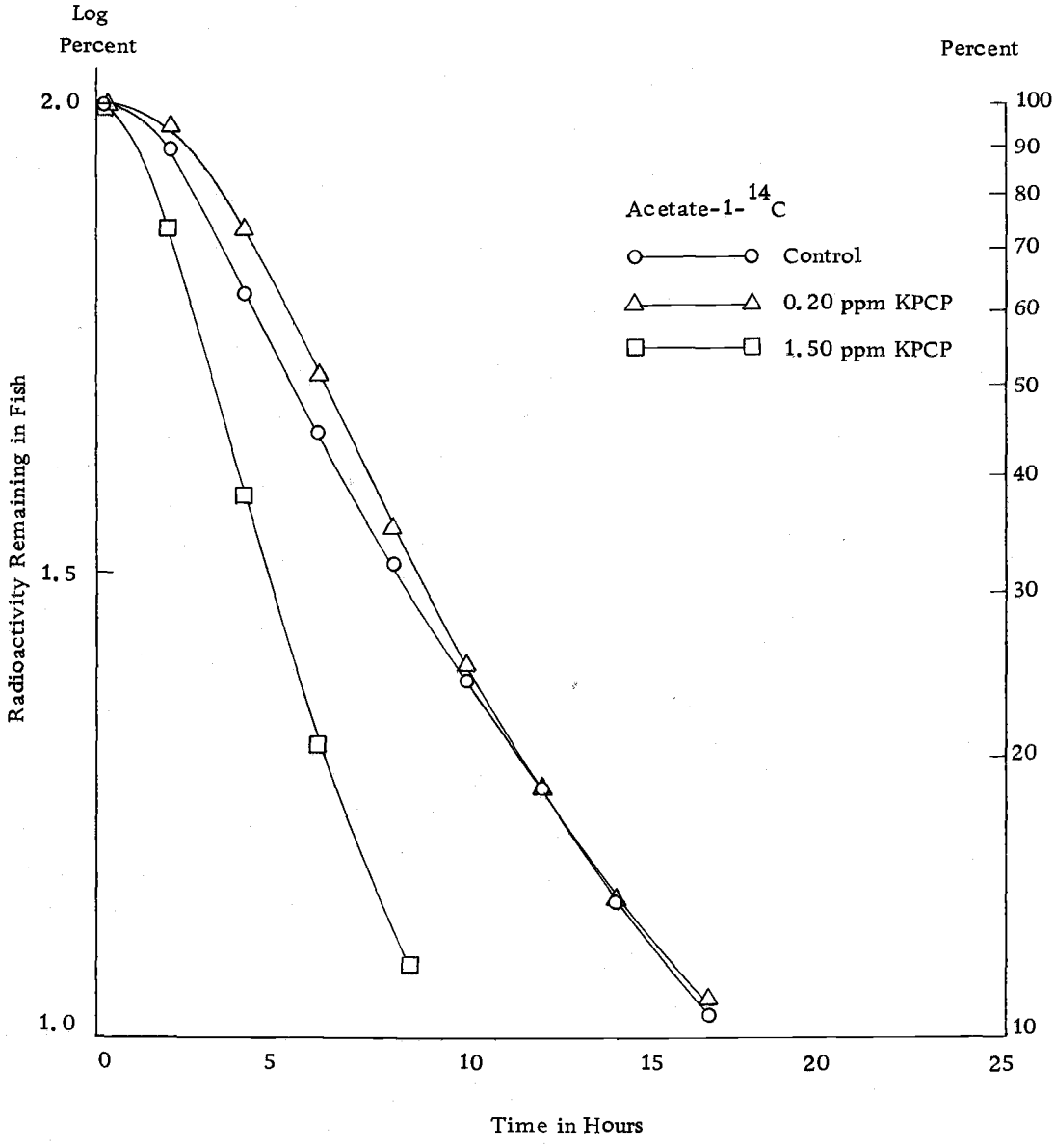


Figure 8. Logarithmic curves of the percentage of radiocarbon remaining in cichlids after injection with acetate-1-^{14}C. Reduction and subsequent increase in recovery at 0.20 ppm and rapid rate of acetate catabolism at 1.5 ppm show clearly.

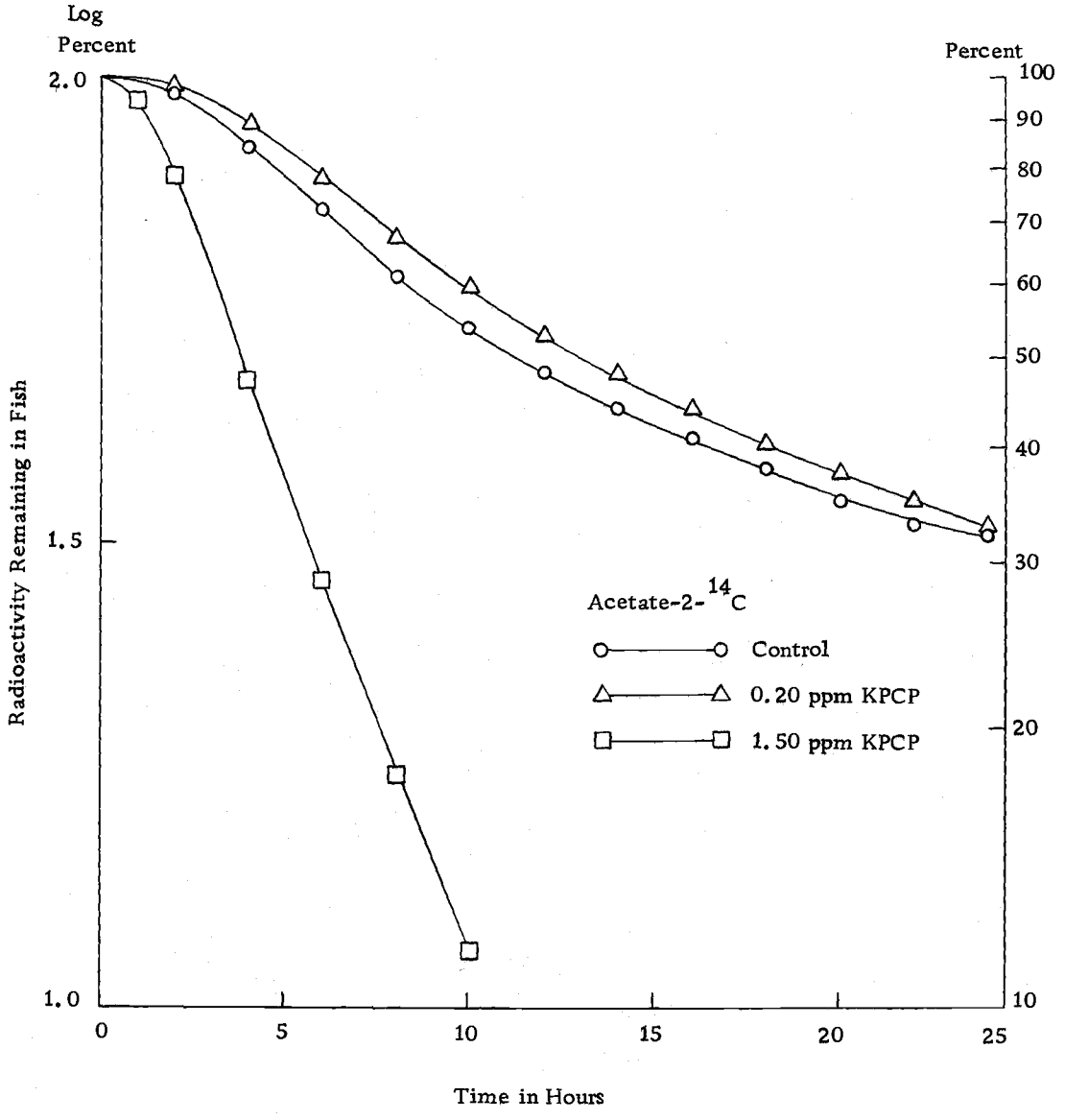


Figure 9. Logarithmic curves of the percentage of radiocarbon remaining in cichlids after injection with acetate-2-^{14}C. Note considerable conservation of C-2 at 0 and 0.20 ppm and rapid conversion to CO_2 at 1.5 ppm.

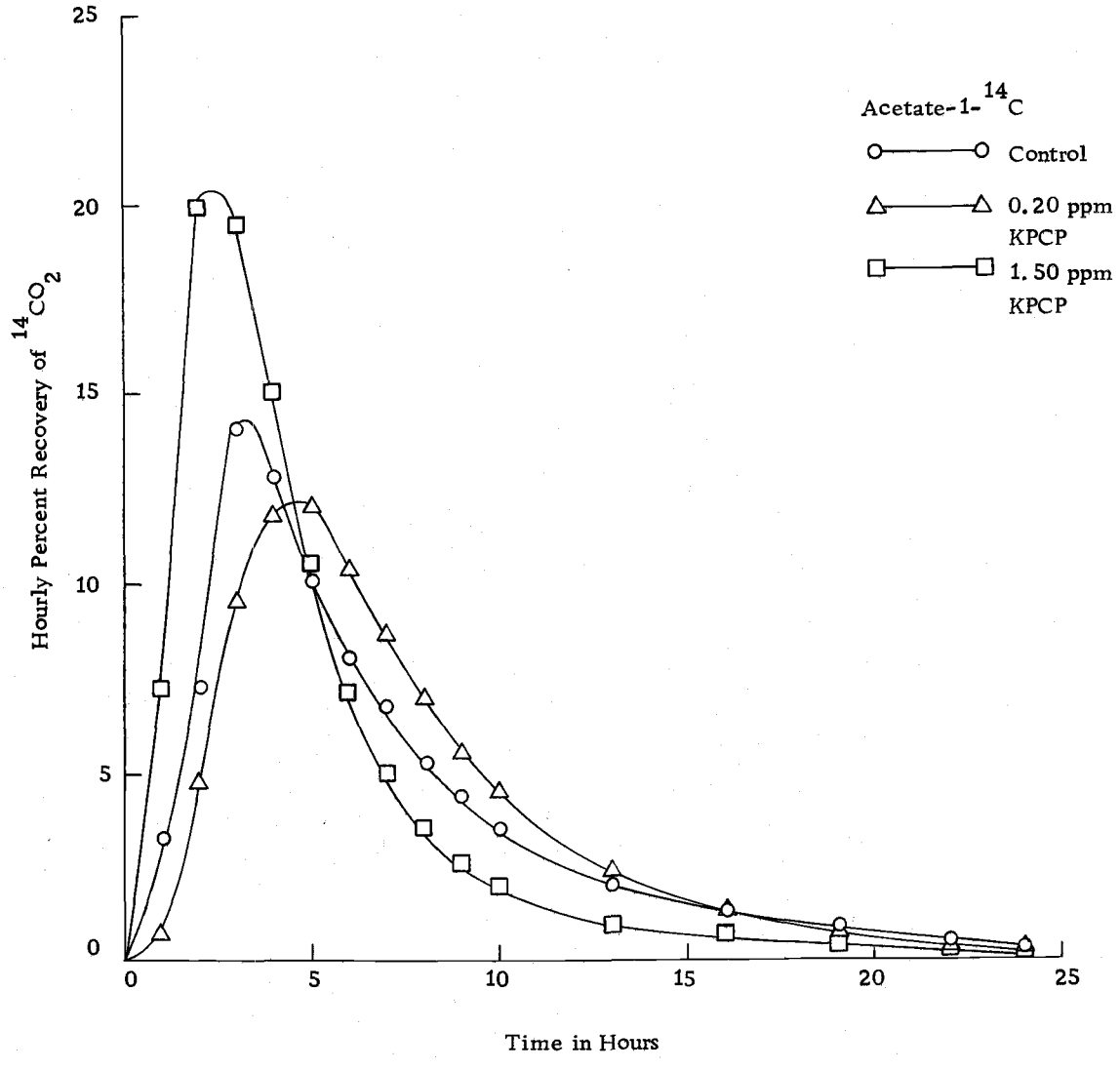


Figure 10. Effect of KPCP upon the hourly recovery of $^{14}CO_2$ from cichlids injected with acetate-1-^{14}C. Note the reduced initial recovery at 0.20 ppm and enhanced initial recovery at 1.5 ppm KPCP.

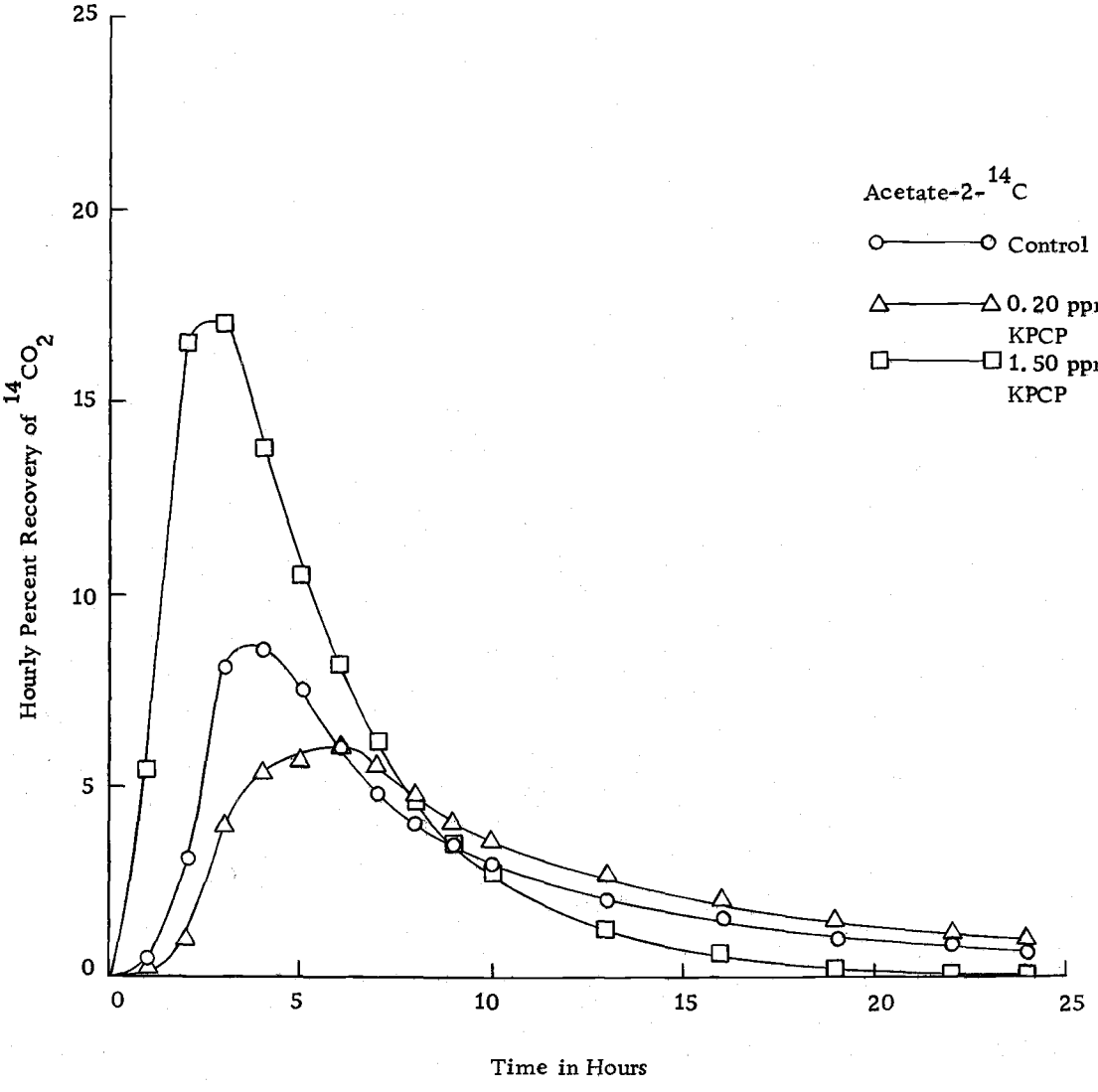


Figure 11. Effect of KPCP upon the hourly recovery of $^{14}CO_2$ from cichlids injected with acetate-2-^{14}C. Effect appears similar to that for C-1 of acetate.

than that for controls; for C-2 the maximum yield was 29 percent less than controls. Over the 24-hour test period the cumulative yield from C-1 was 95 percent and from C-2, 68 percent. Yields from both carbons were very similar to the respective yields from controls (Table 4).

Table 4. Cumulative Yields of $^{14}CO_2$ Recovered from $NaH^{14}CO_3$ and Specifically-labelled Acetate-^{14}C Injected into Controls + KPCP-treated Cichlids. (Percent of Injected Radioactivity)

Hours	Control		0.20 ppm		1.50 ppm	
	C-1	C-2	C-1	C-2	C-1	C-2
			ACETATE			
5	49	28	39	16	72	63
10	77	50	75	41	93	88
15	89	60	88	56	99	95
20	95	66	93	63	101	97
24	98	70	95	68	102	98

Hours	Control	0.20 ppm
	SODIUM BICARBONATE	
5	77	70
10	88	78
15	90	80
20	91	80
24	91	80

Cichlids exposed to 1.5 ppm KPCP exhibited a marked increase in the rate of recovery of both acetate carbons (Figures 10 and 11). The magnitude of effect was greater with C-2 than with C-1. The maximum hourly yield from C-1 was 41 percent greater than the maximum hourly yield from controls, but from C-2 the maximum hourly yield was 100 percent greater. The differences in effect resulted in the relative $^{14}CO_2$ recovery pattern shown in Figure 7. The cumulative 24-hour yields from C-1 and C-2 were 102 and 98 percent, respectively.

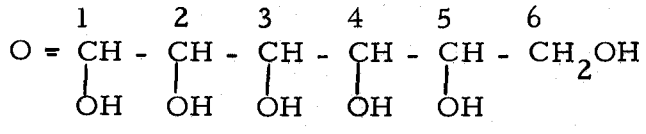
At 0.20 ppm KPCP the delay in the appearance of $^{14}CO_2$ from the acetate carbons could have been due to one or more of several factors, each of which would require verification before a proper explanation can be made. It was apparent that the presence of this concentration of KPCP altered the rate of recovery of $^{14}CO_2$ from acetate without significantly altering the extent over 24 hours. The data suggested that KPCP may have reduced the rate of acetate absorption from the peritoneal cavity or the rate of acetate transport into the mitochondria. It is also possible that KPCP may have had a dual effect upon the rate of acetate turnover in cichlids, reducing the rate at low concentrations and increasing it at higher levels. Interpretation of the acetate data was made more complex by the fact that the chemical mass of injected acetate exceeded the estimated tissue level about tenfold. The actual effect of such a large increase in the level of acetate cannot be determined from examination of the present data. However,

it appears that the fish were able to readily metabolize the excess, very likely without enzyme saturation as evidenced by the sharp peaks in the recovery curves. The mechanism that would account for the lower percentage of $^{14}CO_2$ delivery during the first few hours after the cichlids were exposed to 0.20 ppm KPCP cannot be ascertained from the data on hand.

With cichlids exposed to 1.5 ppm KPCP the utilization of the TCA cycle as respiratory mechanism rather than as a route for biosynthesis seems evident. This is shown by the very extensive conversion of C-2 of acetate to $^{14}CO_2$. Whereas the ratio of the 24-hour cumulative yields from C-2 and C-1 at 0 and 0.20 ppm KPCP was 0.71 and 0.72, respectively, the C-2/C-1 ratio from cichlids exposed to 1.5 ppm KPCP was 0.95. The fact that the logarithmic curves shown in Figures 10 and 11 are essentially linear for cichlids exposed to 1.5 ppm (open triangles) indicate that the conversion of C-1 and C-2 of acetate to CO_2 depended almost entirely upon the rate of catabolsim.

Glucose

The positions of the labelled carbons within the glucose molecule are as follows:



Specifically-labelled glucose is commercially available with radioactive carbons in the 1, 2, 3, 3(4) or 6 positions. Glucose-3(4)-^{14}C is a mixture of glucose molecules labelled in the 3 or the 4 position or in both positions. The percentage of each labelled carbon is isotopically equal. All of the available types except glucose-3-^{14}C were used in this study.

In controls the conversion of the individual glucose carbons to $^{14}CO_2$ occurred at different rates (Figure 12). The differences among the rates were most marked during the first five hours. During this period of time the recovery of $^{14}CO_2$ derived from C-3(4) exceeded that from any other glucose carbon. At the same time the recovery from C-1 was greater than that from C-6 and the recovery from C-2 was the lowest. At the end of 24 hours the respective cumulative yields from C-1, C-2, C-3(4) and C-6 were 53, 47, 66 and 56 percent of injected radioactivity (Table 5).

Since absorption involves the entire glucose molecule, the differences in the rate and extent at which the individual carbons of glucose were converted to carbon dioxide must be attributed to the differences in the history or the fate of the six skeletal carbons. The implications are that glucose may have been catabolized via several sets of reactions sequences, each metabolizing glucose at a different rate and converting the individual carbons to carbon dioxide at different times. There are several known metabolic

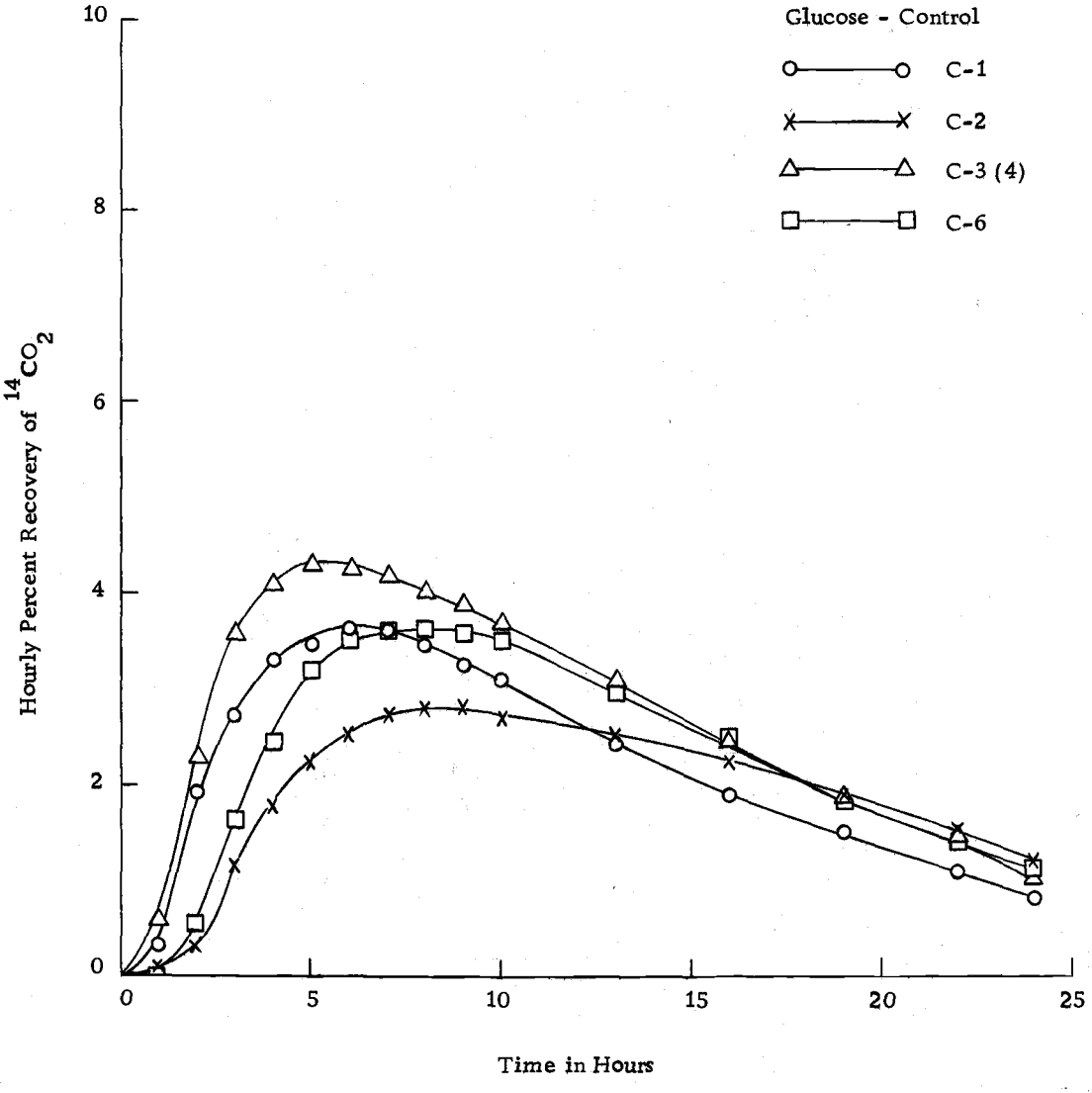


Figure 12. Hourly recovery of $^{14}CO_2$ from controls cichlids injected with specifically-labelled glucose-^{14}C. Recovery pattern used for pathway identification. Note relative position of curves for C-2 and C-3(4).

Table 5. Cumulative Yields of $^{14}CO_2$ Recovered from Specifically-labelled Glucose-^{14}C Injected into Controls and KPCP-treated Cichlids. (Percent of Injected Activity).

Hours	C-1	C-2	C-3(4)	C-6
		CONTROL		
5	12	5	15	8
10	29	19	35	26
15	41	31	50	40
20	49	41	61	50
24	53	47	66	56
		0.20 PPM KPCP		
5	10	7	19	9
10	30	25	44	26
15	44	39	61	40
20	52	50	73	50
24	57	56	80	56
		1.50 PPM KPCP		
5	20	21	37	17
10	46	50	67	46
15	62	68	83	67
20	74	82	94	81
24	81	90	100	88

pathways by which decarboxylation of glucose derivatives leads to the formation of carbon dioxide from different carbons of the original glucose molecule. Using this information, Wang and co-workers (59) introduced the radiorespirometric method. According to the authors pathways involved in glucose degradation can be identified and in some cases compared quantitatively by comparing the rate and extent of conversion of specifically-labelled glucose-^{14}C to $^{14}CO_2$. Analysis of the data obtained from control cichlids by this method revealed that cichlids normally catabolize glucose primarily by way of the Embden-Meyerhof-Parnas (EMP) pathway and to a lesser extent via the pentose phosphate (PP) pathway and the glucuronic acid (GA) pathway.

The EMP pathway is a sequence of reactions which converts one mole of glucose to two moles of pyruvate. It is probably the most important pathway involved in the degradation of glucose and with the TCA cycle comprises the most important system providing energy for biological work.

The PP pathway converts glucose-6-phosphate to phosphogluconate which in turn is converted to pentose phosphate and ultimately to fructose-6-phosphate. Fructose-6-phosphate may then enter the EMP pathway for further degradation. Although this pathway generates ATP, it is generally considered to function more importantly in biosynthesis, particularly of nucleic acids and nucleotides (58).

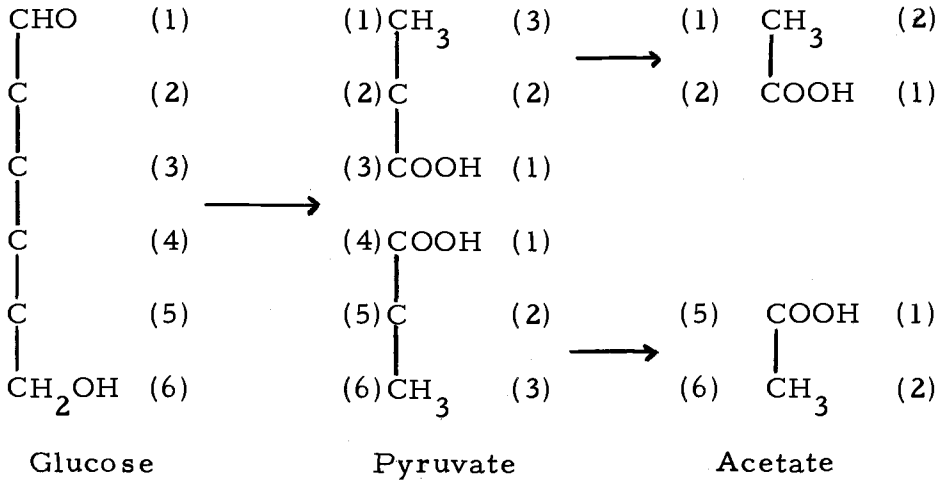
It also serves as a source of reduced NADP which is essential in several reactions in fatty acid and steroid synthesis (27).

Glucose-6-phosphate may be converted to pentose phosphate via formation of glucuronic acid. The reactions responsible for this process comprise the glucuronic acid (GA) pathway which has been worked out in detail by Eisenberg and associates (20). Pentose phosphates formed via the GA pathway may be converted to fructose-6-phosphate by way of the PP pathway. The GA pathway serves as a route for the synthesis of ascorbic acid in some organisms and is not considered an important mechanism for the generation of energy (58).

According to Wang and co-workers (61) the cumulative yields of $^{14}CO_2$, derived from the individual carbons of glucose metabolized via the EMP-TCA pathway alone, should follow the order: C-3 = C-4> C-2 = C-5 > C-1 = C-6, or in terms of the specifically-labelled carbons used in this study : C-3(4)> C-2 > C-1 = C-6. The inequalities develop primarily from drainage of glucose metabolites into non-catabolic cellular constituents. If the data on the rate or extent of recovery do not meet this order, other pathways or mechanisms involved in glucose degradation must exist.

The primary end-product of the EMP pathway is pyruvic acid. Glucose carbons are conserved via this route. Each mole of glucose forms two moles of pyruvic acid and no carbon dioxide is produced.

Carbons 1, 2, and 3 of glucose become carbons 3, 2, and 1 of pyruvate and carbons 4, 5, and 6 of glucose become carbons 1, 2, and 3 of pyruvate. Pyruvate, derived from glucose, is oxidatively decarboxylated, forming acetyl-CoA and carbon dioxide. The two moles of carbon dioxide derived from two moles of pyruvate are made from C-3 and C-4 of the original glucose molecule. Carbons 2 and 5 of glucose become the carboxyl carbons of acetyl-CoA and carbons 1 and 6 the methyl carbons of acetyl-CoA. Note that the respective carbon pairs, C-2 and C-5 from glucose, will be converted to carbon dioxide as C-1 of acetyl-CoA and the carbon pairs, C-1 and C-6 of glucose, as C-2 of acetyl-CoA. The relationship and interchange of glucose carbons to the carbons of pyruvate and acetyl-CoA (shown as acetate) are given below. The numbers representing carbons derived from glucose are shown to the left of each skeletal structure and the conventional numbering for the carbons of each compound is shown on the right.



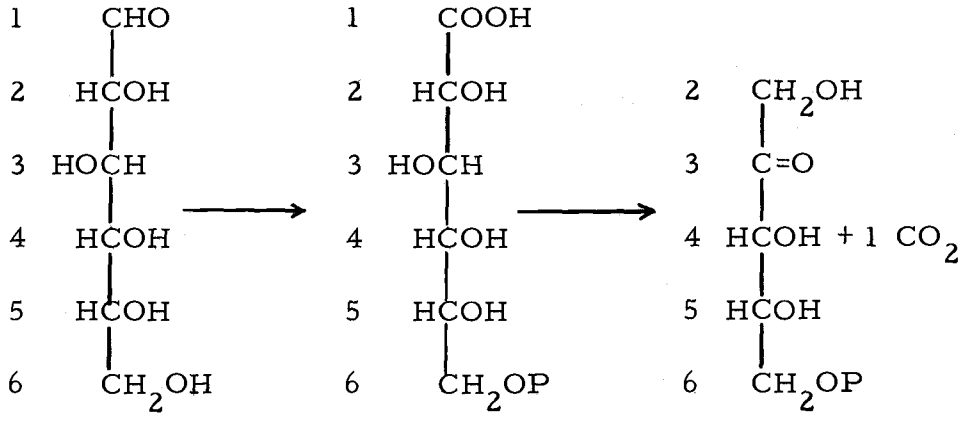
Degradation of acetyl-CoA, from the EMP pathway, via the TCA cycle gives an equal production of CO_2 from C-2 and C-5 of glucose, followed by a lower, but again equal production of CO_2 from C-1 and C-6. The acetyl-CoA carboxyl position, taken by carbons 2 and 5 of glucose, allows formation of CO_2 from these carbons to be more rapid and extensive than the formation of CO_2 from carbons 1 and 6 of glucose since the latter will initially occupy internal molecular positions in the various TCA intermediates and will thus more readily be lost via biosynthetic pathways associated with the TCA cycle. Complete recovery of glucose carbons as CO_2 via the EMP-TCA pathway is rare because of biosynthetic reactions such as the formation of glycogen from glucose, lipids from acetyl-CoA and certain amino acids from a number of TCA intermediates. The formation of lipids from acetyl-CoA would provide a smaller recovery of activity from glucose labelled on carbons 2 and 5 than from glucose labelled on carbons 3 or 4. Anabolism involving 4 or 5-carbon TCA intermediates would lead to a smaller recovery of activity from glucose labelled on carbons 1 or 6.

The rapid and extensive recovery of C-3(4) as $^{14}CO_2$ from control cichlids, above the recovery from C-1, C-2 or C-6 of glucose, identified the EMP pathway as the major route of glucose degradation. The preferential conversion of C-3(4) to CO_2, presumably by decarboxylation of pyruvate, has been observed in a number of

microorganisms, the pepper fruit, the American cockroach, crayfish, and rats (18, 41, 46, 61, 63).

The relative yields of $^{14}CO_2$ from C-1, C-2 and C-6 of glucose deviated from that expected on the assumption of exclusive utilization of the EMP and TCA pathways. Conversion to $^{14}CO_2$ was greater from labelled C-1 and C-6 than from labelled C-2 of glucose. Further the amounts of $^{14}CO_2$ derived from labelled C-1 and C-6 were not equal.

A metabolic reaction which leads to a relatively prompt formation of CO_2 from C-1 of glucose is the decarboxylation of phosphogluconate via the pentose phosphate pathway. The scheme for this mechanism is as follows:

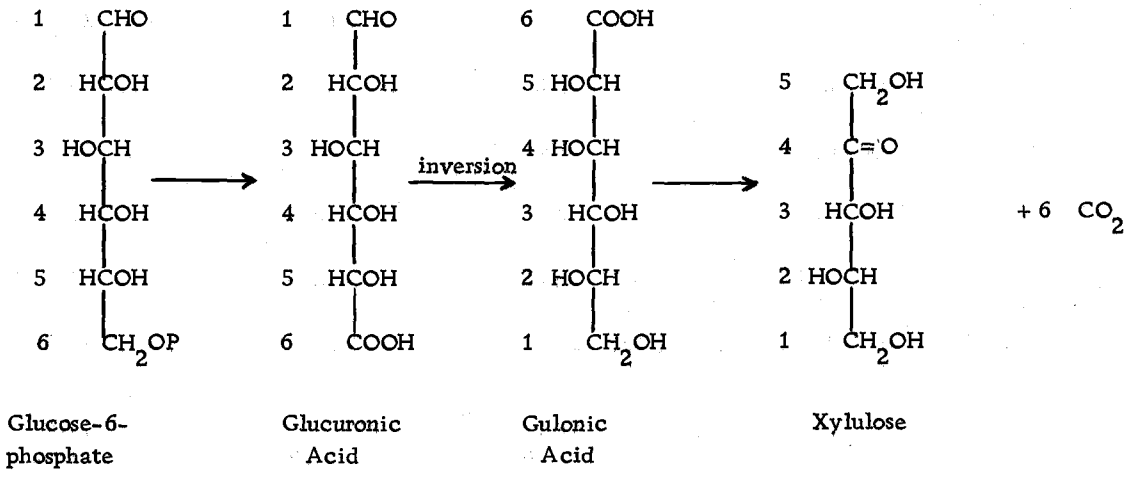


Glucose-6-phosphate 6-phosphogluconate Ribulose-5-phosphate

The greater recovery of activity as $^{14}CO_2$ from labelled C-1 than from labelled C-2 of glucose was considered presumptive evidence of the presence of the PP pathway. More direct evidence for

the presence of the PP pathway was obtained by injecting gluconate-1-^{14}C into two cichlids. After 24 hours, yields of 41 and 53 percent of injected activity were obtained. According to Gunsalus and co-workers (26) the conversion of glucose to gluconate is essentially irreversible. Unless fish are unique by possessing a pathway other than the PP pathway for the utilization of gluconate, the relatively extensive yields of $^{14}CO_2$ from injected gluconate-1-^{14}C were thus considered as additional evidence for the PP pathway in cichlids.

Preferential conversion of C-6 over C-2 to CO_2 suggested that cichlids may possess an active mechanism for triose recombination resulting in the randomization of C-6 to the C-1 position. However, the delay in the appearance of the maximum hourly yield of $^{14}CO_2$ from C-6 of glucose placed doubt upon the extensive operation of this mechanism if present. It is more likely that C-6 of glucose was converted to CO_2 via the glucuronic acid pathway. The scheme for this mechanism is as follows:



Yields of 85 and 94 percent of injected activity as $^{14}CO_2$ were obtained within 24 hours from two cichlids injected with glucuronate-6-^{14}C. According to Burns and co-workers (12), the conversion of glucose to glucuronate is an irreversible reaction. Presumably glucuronate can be metabolized only if the glucuronic acid pathway is present. Hence the high recovery of $^{14}CO_2$ from glucuronate-6-^{14}C as well as the greater recovery of $^{14}CO_2$ from C-6 of glucose than from C-2 of glucose were considered evidence for the presence of the GA pathway in cichlids.

Recovery of 66 percent of glucose-3(4)-^{14}C as $^{14}CO_2$ from controls in 24 hours indicated that about 34 percent of the injected radioactivity was left in the fish. This residual amount could have represented, in part, injected glucose which had been routed through the PP and GA pathways where carbons 3 and 4 would have been subject to dilution and loss via biosynthetic and exchange reactions. It also could have represented, in part, injected glucose which had not been utilized or glucose which had become engaged in anabolism such as in the formation of glucogen. Finally it could have represented, in part, C-3(4)-derived $^{14}CO_2$ still circulating, but much too diluted by non-labelled carbon dioxide to be detected by the electrometer system or a decrease in the specific activity of labelled glucose and its derivatives due to the presence of non-labelled counterparts. That one or more of these factors may have affected the extent of

recovery of all labelled glucose carbons was indicated by the decreasing rate of radiocarbon depletion shown by the log curves in Figure 13.

Recovery of 66 percent from C-3(4) of glucose was also indicative of the fraction of glucose converted to acetyl-CoA. If acetyl-CoA derived via the EMP pathway were completely oxidized after entering the TCA cycle, one would expect a recovery of about 66 percent from glucose-2-^{14}C since the carboxyl carbon (C-1) of acetyl-CoA would be derived from C-2 of glucose. However, only 47 percent was recovered from C-2 of glucose. It thus appeared that after glucose was converted to acetyl-CoA, a considerable amount of C-2 and possibly C-1, C-5 and C-6 may have been engaged in biosynthetic or exchange reactions.

Note that the data from control cichlids injected with acetate-1-^{14}C indicated that very little of C-1 of acetate was involved in such side reactions. This suggested that injected acetate was metabolized differently than acetyl-CoA derived from glucose traversing the EMP pathway.

The presence of 0.20 ppm KPCP altered the rate of conversion of all glucose carbons to carbon dioxide. The percentages of injected activity recovered per hour from each carbon are shown in Figure 14. The relationship among the initial rates of $^{14}CO_2$ recovery from the individual carbons remained the same as that found in controls, i.e., C-3(4) > C-1 > C-6 > C-2. However, the initial rate of recovery from

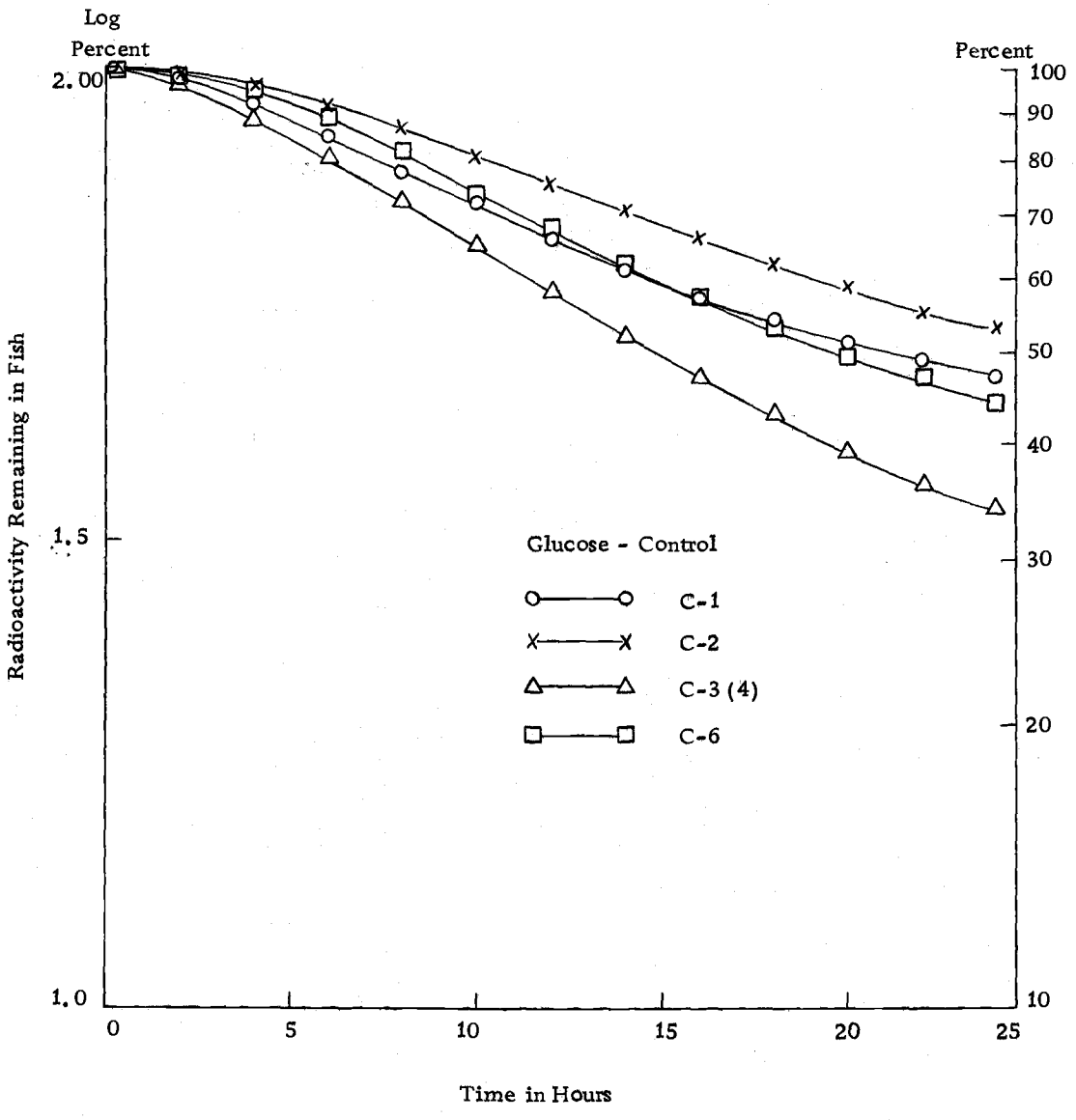


Figure 13. Logarithmic curves of the percentage of radiocarbon remaining in control cichlids after injection of specifically-labelled glucose-^{14}C. Curves illustrate relative rate of radiocarbon depletion.

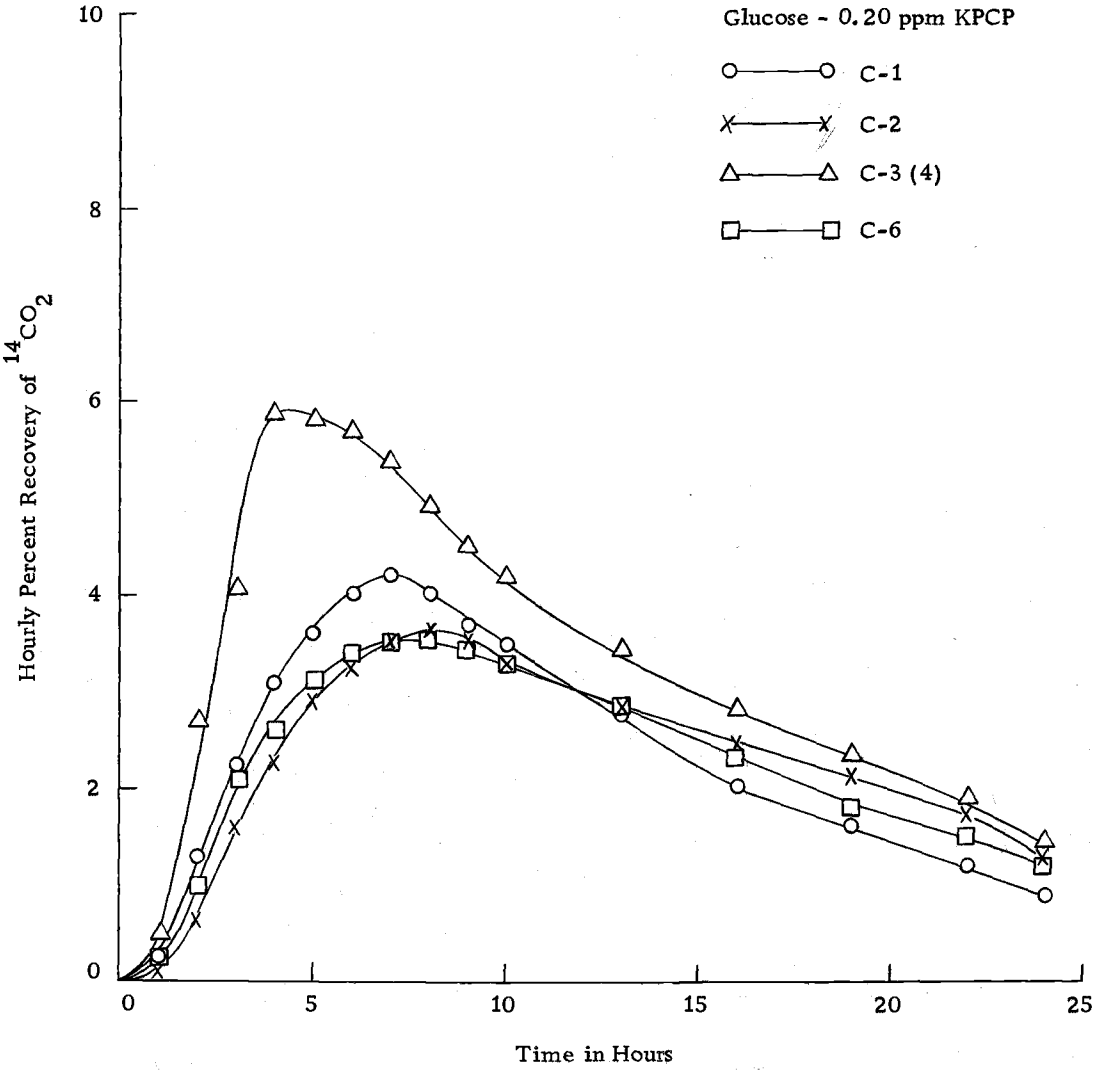


Figure 14. Hourly recovery of $^{14}CO_2$ from cichlids exposed to 0.20 ppm KPCP and injected with specifically-labelled glucose-^{14}C. Note the change in the relative positions of C-2 and C-3(4) to those of C-1 and C-6.

C-3(4), relative to that from the other labelled carbons, was increased while the differences among the initial rates of recovery from C-1, C-2, and C-6 were decreased. This trend was magnified with time so that at the end of the 24-hour experiments, the percentage yield from C-3(4) was markedly greater than that from any other glucose carbon and the percentage yield from C-1, C-2 and C-6 were essentially equal (Table 5).

The events leading to this pattern of $^{14}CO_2$ recovery were shown more clearly by examining the effects of 0.20 ppm KPCP on the recovery of $^{14}CO_2$ from the individual carbons. KPCP-induced alterations in the conversion of C-1 to $^{14}CO_2$ are shown in Figure 15. During the first four hours, less labelled carbon dioxide was recovered from cichlids exposed to 0.20 ppm KPCP than from controls. However, from the 5th to the 24th hour the hourly yields from the poisoned cichlids were greater than those from controls. As a result the cumulative 24-hour yield from the poisoned fish was slightly greater than that from controls.

With C-6 of glucose the presence of 0.20 ppm KPCP increased its rate of recovery during the first four hours (Figure 16). From the fifth hour on, the hourly yields from C-6 from the poisoned cichlids appeared slightly less than the hourly yields from controls. In spite of these alterations, equal amounts of $^{14}CO_2$ were recovered from controls and from cichlids exposed to 0.20 ppm KPCP.

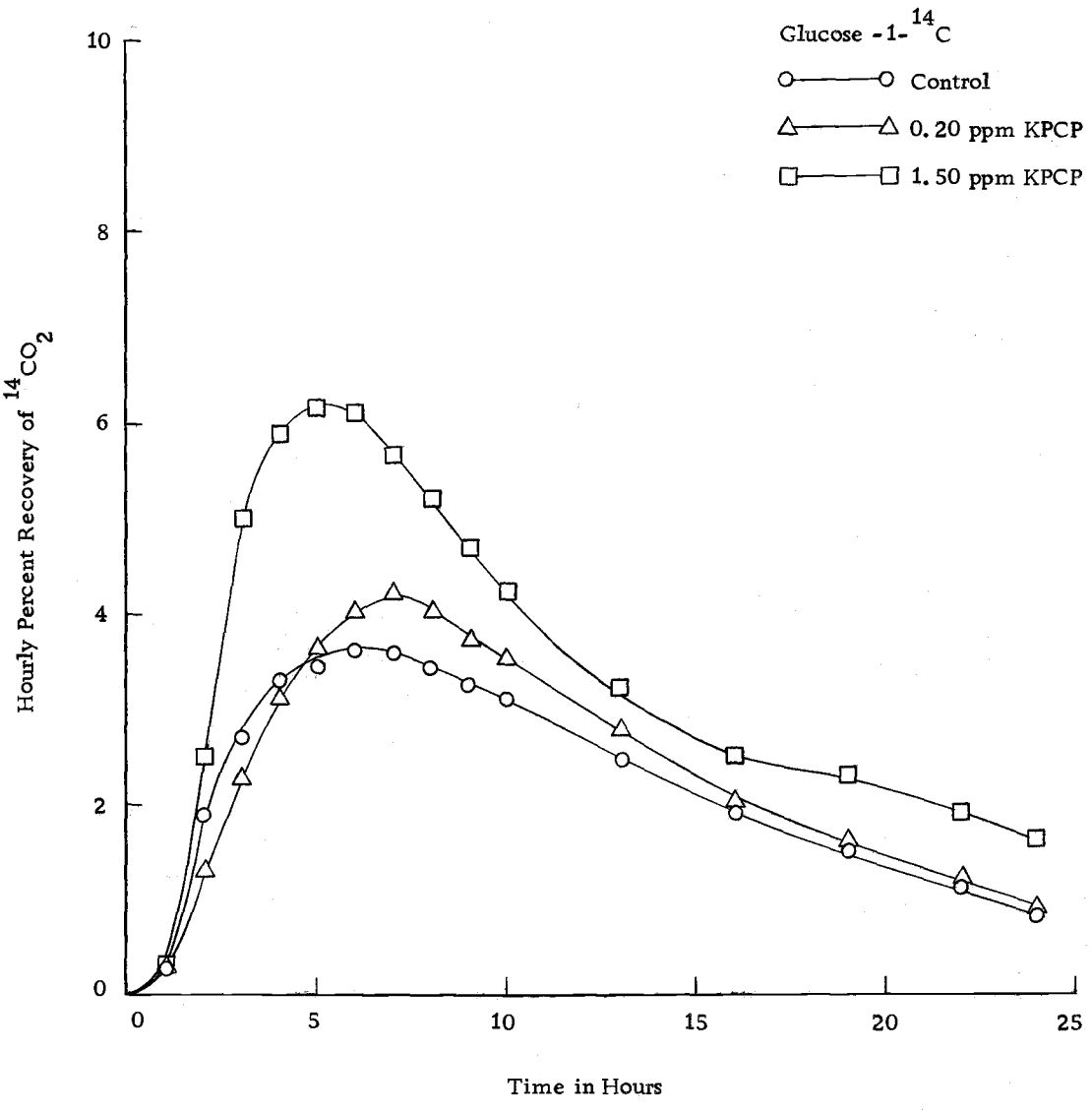


Figure 15. Effect of KPCP upon the hourly recovery of $^{14}CO_2$ from cichlids injected with glucose-1-^{14}C. Effect of 0.20 ppm upon recovery is relatively slight compared to effect at 1.5 ppm. Note the initial reduction and subsequent increase in recovery at 0.20 ppm.

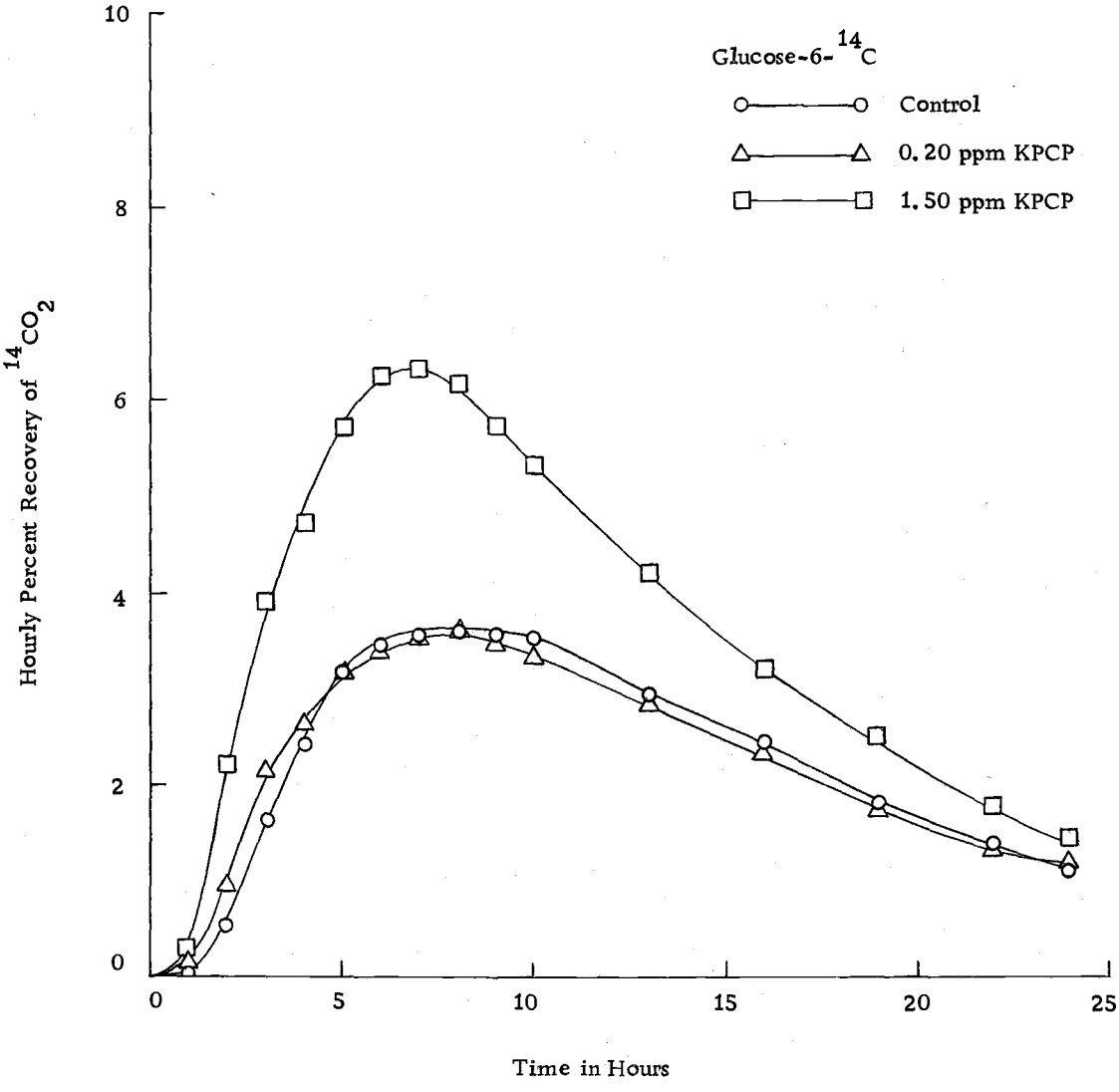


Figure 16. Effect of KPCP upon the hourly recovery of $^{14}CO_2$ from cichlids injected with glucose-6-^{14}C. Overall change in the recovery pattern at 0.20 ppm is slight. Compare with those for C-2 and C-3(4).

The effects of 0.20 ppm KPCP upon the conversion of C-2 and C-3(4) to $^{14}CO_2$ are illustrated by the recovery curves in Figures 17 and 18. Exposure of the cichlids to this concentration of the toxicant markedly increased the hourly yields of $^{14}CO_2$ from both carbons. The 24-hour cumulative yield from C-2 increased from 47 to 56 percent. From C-3(4) the cumulative yield increased from 66 to 80 percent.

The marked increase in the rate and extent of conversion of C-3(4) to $^{14}CO_2$ suggested that a considerably larger amount of labelled glucose was catabolized via the EMP pathway in cichlids exposed to 0.20 ppm KPCP. The increase in the recovery of $^{14}CO_2$ from C-2 of glucose suggested that the molecules of acetyl-CoA, derived by way of the decarboxylation of pyruvate, were oxidized more rapidly and extensively upon entering the TCA cycle.

Besides indicating more rapid and extensive glucose degradation via the EMP pathway, the higher yield from C-3(4) also indicated that before glucose was converted to acetyl-CoA, a smaller fraction of C-3(4) was engaged in side processes than in controls. These two events would naturally be related; however, it was interesting to find that the ratios of the yields of $^{14}CO_2$ from C-2 and C-3(4) from controls and from cichlids exposed to 0.20 ppm KPCP were 0.71 and 0.70, respectively. The similarity of the ratios suggested that after glucose was converted into acetyl-CoA, the proportion of glucose

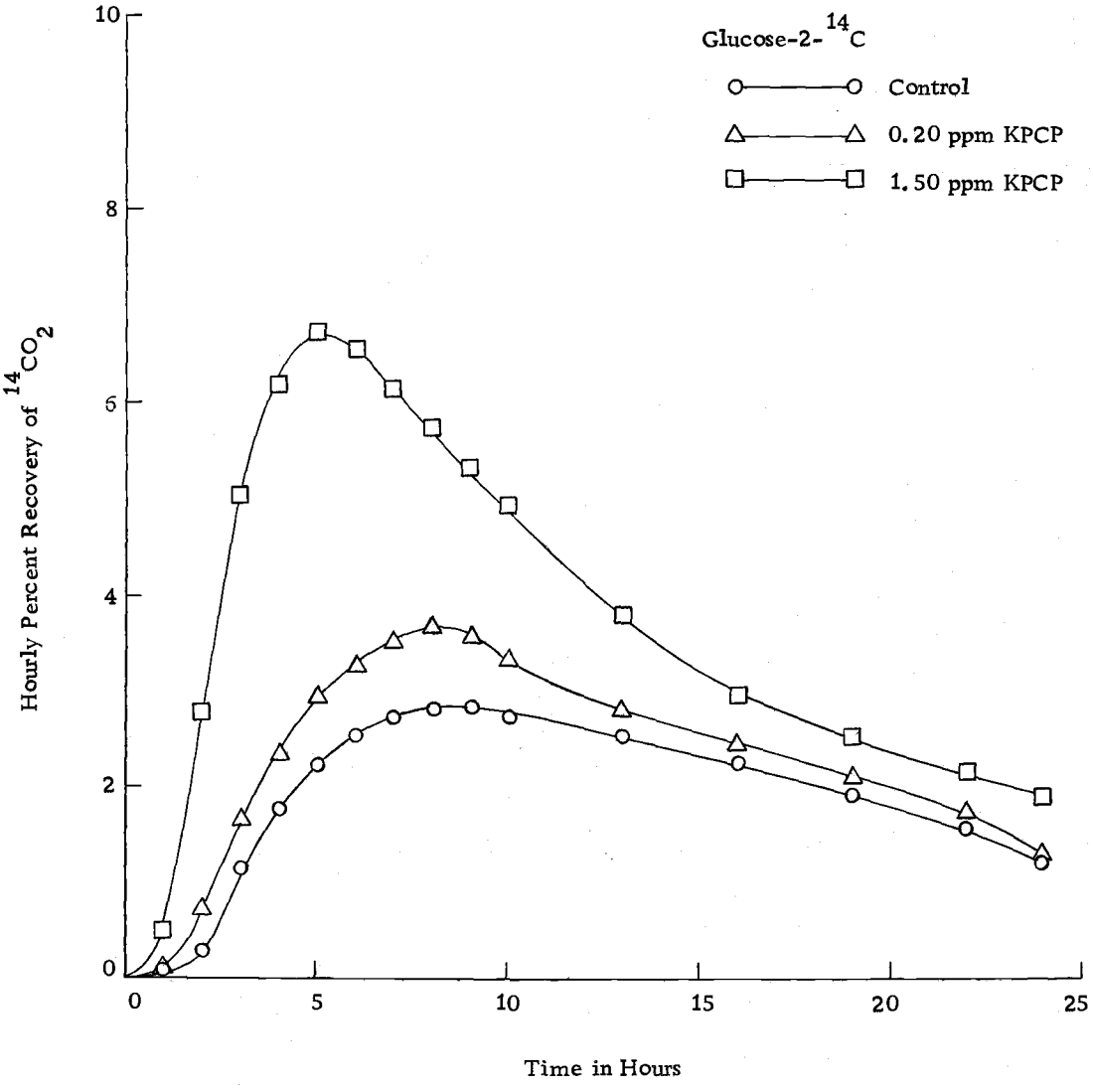


Figure 17. Effect of KPCP upon the hourly recovery of $^{14}CO_2$ from cichlids injected with glucose-2-^{14}C. Presence of 0.20 ppm increases recovery from C-2 more than it does from C-1 or C-6.

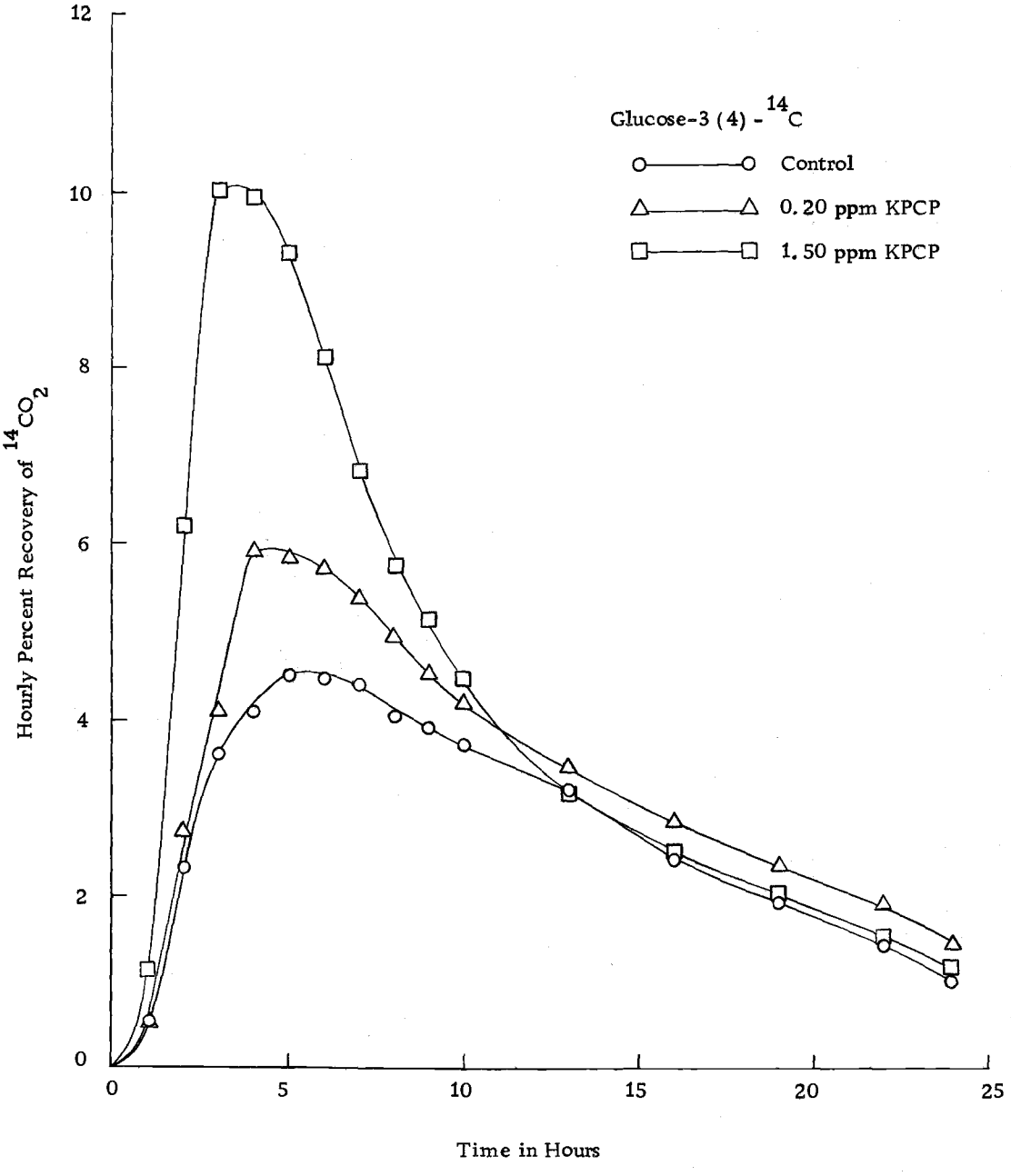


Figure 18. Effect of KPCP upon the hourly recovery of $^{14}CO_2$ from cichlids injected with glucose-3 (4)-^{14}C. Effect of KPCP at different levels follows the same trend as for C-2 of glucose.

carbons involved in biosynthesis and other processes was not altered.

The depletion curves in Figure 19 showed that after the initial absorption phase, the curve for C-3(4) of glucose was essentially linear. This suggested that the degradation of labelled glucose by way of the EMP pathway was dependent primarily upon the rate of glucose turnover through the EMP pathway and that non-catabolic and dilution factors had little if any effect upon the conversion of labelled C-3(4) to $^{14}CO_2$. If the same applied to the formation of $^{14}CO_2$ from C-2 of glucose, the depletion curve for C-2 should have also been linear. However, the curve showed that the rate of depletion of C-2 decreased with time, thus suggesting the influence of other factors.

Interpretation of the alterations in the recovery curves for C-1 and C-6 was more difficult. Presumably the recovery curve for C-1 represented the amount of $^{14}CO_2$ derived by way of the PP and the EMP-TCA pathways and the recovery curve for C-6 represented the amount of $^{14}CO_2$ derived by way of the GA and the EMP-TCA pathways. Since two pathways may simultaneously give rise to $^{14}CO_2$ from C-1 or C-6, there was no way to distinguish from the respective curves for C-1 or C-6 whether the alterations were due to effects on the PP, the GA or the EMP-TCA pathways. However, a comparison of the relationship between the respective recovery curves for C-1 and C-6 with the curve for C-2, led to a reasonable suggestion as to which pathway had been altered.

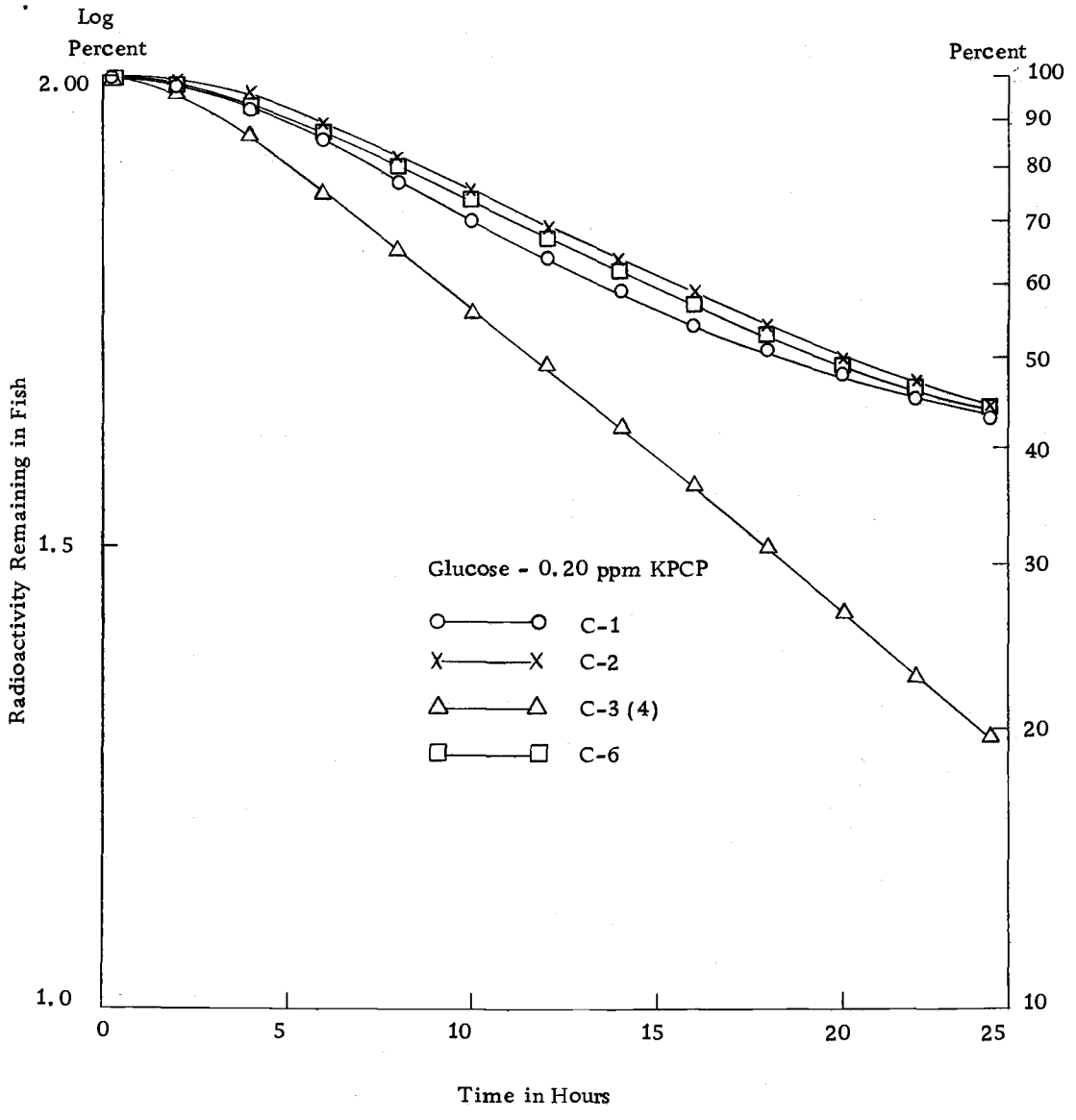


Figure 19. Logarithmic curves of the percentage of radiocarbon remaining in cichlids exposed to 0.20 ppm KPCP and injected with specifically-labelled glucose-^{14}C. Curves illustrate relative rate of radiocarbon depletion in the presence of 0.20 ppm KPCP.

Presumably the recovery curve for C-2 represented $^{14}CO_2$ derived from C-2 of glucose by way of the TCA cycle alone. The scheme presented earlier for the relative recovery pattern for each carbon of glucose called for the rate and extent of recovery from C-1 and C-6 to be less than that from C-2 when glucose is catabolized only by way of the EMP-TCA pathway. Evidence for the presence of the minor pathways was obtained by noting that the rate and extent of recovery from C-1 and C-6 exceeded that from C-2. The higher rate of recovery from C-1 and C-6 was evident during the first few hours after administration of the labelled glucose. This presumably represented the relatively prompt conversion of C-1 and C-6 to carbon dioxide by way of the PP and the GA pathways, respectively. It is thus conceivable that the early decrease in the recovery from C-1 may have reflected a delay in the conversion of glucose to pentose or a reduction in the amount of glucose traversing the PP pathway. Similarly, the increase in the early recovery from C-6 may have reflected enhancement of the rate of pentose formation from glucose or an increase in the amount of glucose catabolized via the GA pathway.

When cichlids were exposed to 1.5 ppm KPCP the rate and extent of recovery of $^{14}CO_2$ from all labelled carbons of glucose were strikingly enhanced (Figure 15 to 18). As observed in cichlids exposed to 0.20 ppm KPCP, the increase in the radiochemical

recovery of carbon dioxide was most marked from C-2 and C-3(4). The maximum rates of recovery (yields per hour at peak) from C-1 and C-6 were greater by 72 and 75 percent respectively, than the maximum rates for the corresponding carbons in controls. However the maximum rates from C-2 and C-3(4) were 139 and 132 percent higher. The respective 24-hour cumulative yields from C-1, C-2, C-3(4) and C-6 were 81, 90, 100 and 88 percent.

The relative rate and extent of recovery of $^{14}CO_2$ from the individual carbons of glucose from cichlids exposed to 1.5 ppm KPCP indicated that the EMP-TCA system may have been used exclusively since the relative order of recovery was C-3(4) > C-2 > C-1 or C-6 (Figure 20). However, although the initial recovery from C-1 and C-6 of glucose was less than the initial recovery from C-2, the difference was very slight. This suggested the involvement of the PP and GA pathways since exclusive utilization of the EMP-TCA pathway calls for a considerably higher rate of recovery from C-2 than from C-1 or C-6. Identification of the minor pathways was made difficult since it appeared that the conversion of all glucose carbons to carbon dioxide in cichlids exposed to 1.5 ppm KPCP was dependent almost entirely upon the rate of glucose degradation and the time that each carbon was converted to carbon dioxide along the catabolic sequence of reactions. The influence of non-catabolic processes appeared to be relatively minor. The value of 0.90 for the ratio of

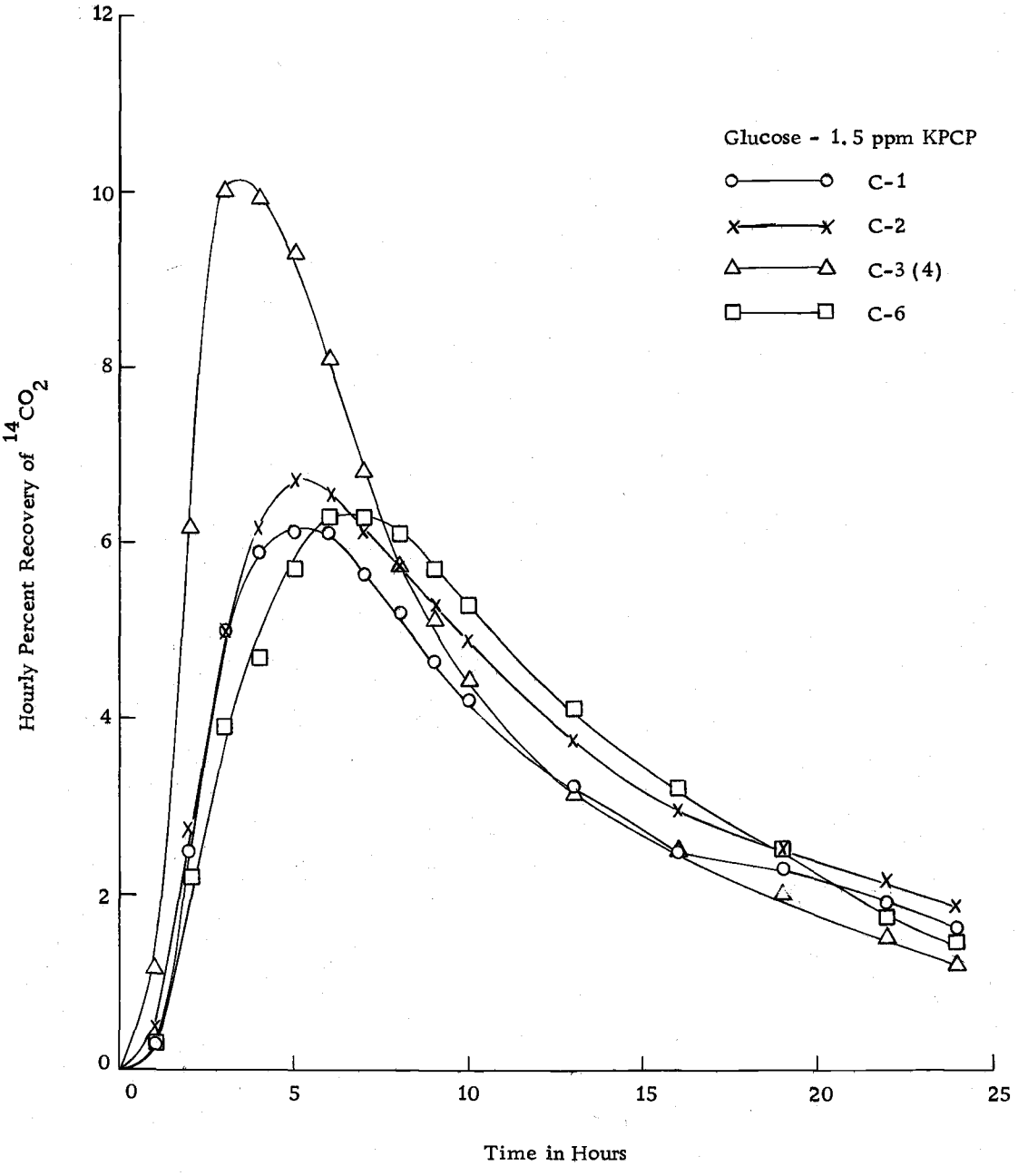


Figure 20. Hourly recovery of $^{14}CO_2$ from cichlids exposed to 1.5 ppm KPCP and injected with specifically-labelled glucose-^{14}C. Here recovery from C-3(4) is very rapid and extensive. Note that the maximum yield per hour from C-2 exceeds those from C-1 and C-6.

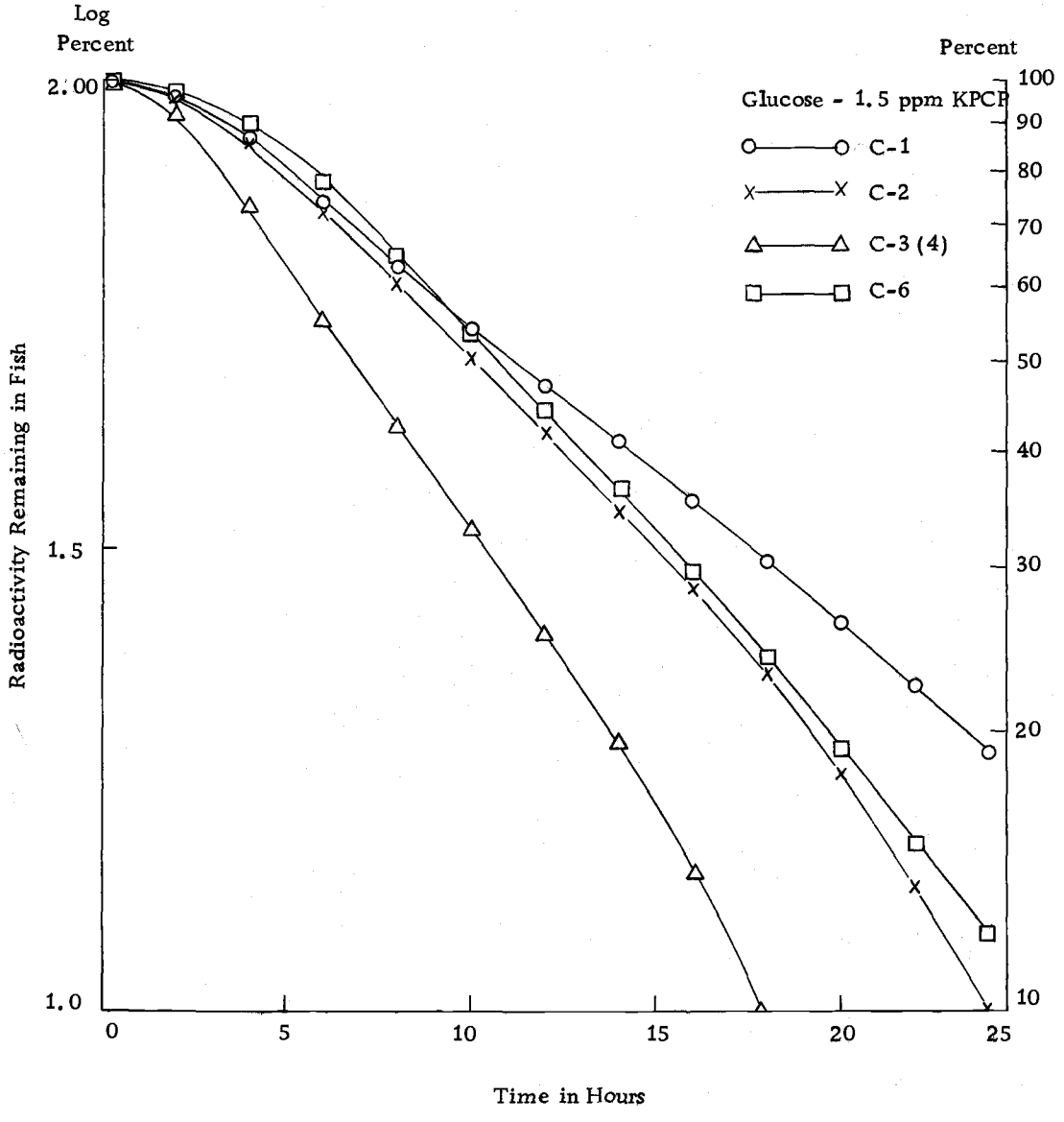


Figure 21. Logarithmic curves of the percentage of radiocarbon remaining in cichlids exposed to 1.5 ppm KPCP and injected with specifically-labelled glucose-^{14}C. Curves illustrate the relative rate of radiocarbon depletion in the presence of 1.5 ppm KPCP.

the respective 24-hour cumulative yields from C-2 and from C-3(4) suggested that a considerably higher percentage of acetyl-CoA, derived from glucose in the poisoned fish, was oxidized rather than converted into lipids and that only a small amount of the carboxyl carbon of acetyl-CoA was involved in biosynthetic or exchange reactions related to the TCA cycle. Further the curves in Figure 21 showed that the rate of radiocarbon depletion did not diminish, but increased with time. The increase in the depletion rate was probably due to a time-related accumulation of KPCP in the cichlids. Thus, within the TCA cycle it was conceivable that a reduction in non-catabolic activities and an increase in catabolic activities could have caused the rate and extent of recovery from C-1 and C-6 of glucose to approach that from C-2. Whether or not the catabolism of glucose occurred by way of the PP or the GA pathways in cichlids exposed to 1.5 ppm KPCP may thus be questioned. A closer examination of the data in Table 5 (to be discussed later) indicated that in the early period of intoxication, both pathways were in operation and were being utilized. Later very little glucose traversed the PP pathway and somewhat larger amounts moved through the GA pathway.

Pathway Estimation

At present there is no method by which the relative amount of glucose catabolized by each of three concurrently-operating pathways

can be estimated precisely from data obtained from single-dose experiments of the type used in this study. However, reasonable estimates can be made. The estimates presented support and are consistent with the qualitative statements made earlier about the effects of KPCP upon the catabolism of glucose in cichlid fish.

The principal difficulty in assessing the relative contribution of each pathway to glucose catabolism from the yields of $^{14}CO_2$ from the individual carbons of glucose is that the yield from each carbon is possibly made up of three components. These are the yield from the EMP-TCA pathway, the yield from the PP pathway and the yield from the GA pathway. This is true since reconstructed hexoses derived from the degradation of glucose via the PP pathway and via the combination of the GA and PP pathways may possibly be recycled through the PP or the GA pathway. If glucose enters the PP pathway, C-1 would be converted to CO_2 promptly and C-2 of the original glucose molecule would then be located on the C-1 position of the reconstructed hexose, be it fructose or glucose. Recycling of the hexose through the PP pathway would then give rise to the formation of CO_2 from C-2 of the original glucose molecule. Carbon-3 of glucose would be converted to CO_2 on the third time around. Thus all of the $^{14}CO_2$ obtained from C-2 or C-3 of glucose need not necessarily come from the EMP-TCA pathway. Also the reconstructed hexose could enter the EMP-TCA pathway directly and thereby give rise to the

formation of CO_2 from all glucose carbons.

According to Dr. C. H. Wang (Oregon State University), who was one of the first to discover and develop the use of $^{14}CO_2$ pattern analysis or "radiorespirometry" for the identification and estimation of the relative participation of catabolic pathways for carbohydrates, the net contribution of some of the pathways to the observed yields of $^{14}CO_2$ from some of the glucose carbons may be considered very small. Thus in the calculation of first estimates of relative pathway participation, it is necessary to consider only those pathways which contribute most significantly to the cumulative yield of $^{14}CO_2$ from each glucose carbon.

The EMP-TCA pathway has been established as the single major route for glucose catabolism in cichlid fish; more $^{14}CO_2$ was derived from labelled C-3(4) of glucose than from C-1, C-2 or C-6. Hence a significant fraction of the observed cumulative yields from each carbon of glucose would be derived from this pathway. All glucose molecules routed through the PP pathway undergo a relatively prompt 1-5 cleavage resulting in the formation of CO_2 from C-1. Before the formation of pentose phosphate, there is no drainage of hexose derivatives of glucose. Hence the PP pathway would contribute a considerable fraction of $^{14}CO_2$ derived from C-1 of glucose. The conversion of C-6 of glucose to CO_2 via the GA pathway is also relatively prompt and the drainage of six-carbon derivatives such as

ascorbic acid, glucuronic acid or gulonic acid is generally considered to be very small. Thus the GA pathway could contribute significantly to the yield of $^{14}CO_2$ from C-6 of glucose.

The pentose derived by way of the PP pathway become involved in a long series of reactions, some of which are biosynthetic or exchange types which could markedly reduce the amount of labelled glucose derivatives reconverted into hexose and again be able to enter the EMP pathway or recycle through the PP pathway. The pentoses derived by way of the GA pathway are further metabolized via the PP pathway and are thus subject to the same fate as those derived via the PP pathway.

The net contribution of the PP pathway to the yield from C-6 and the net contribution of the GA pathway to the yield from C-1 can be considered negligible. Likewise the GA and PP pathways can only contribute negligible amounts of $^{14}CO_2$ from C-2 and from C-3(4) of glucose. Although the data indicated that about 20 percent of the injected glucose enters the PP pathway (details to be given later), the amount of drainage of glucose metabolites into synthetic and other pathways as well as the complexities of the pathways involved in reconversion of pentose to fructose or to glucose indicated that only minor quantities of C-2 or C-3(4) could have been involved in a second trip through the EMP, PP or GA pathways. Thus the observed yields from C-1 can be considered to have been derived primarily by way of

the EMP-TCA and the PP pathways; the yield from C-6, via the EMP-TCA and the GA pathways; and the yield from C-2 and C-3(4), via the EMP-TCA pathway.

In order to estimate the net contribution of the EMP-TCA pathway to the observed yields from C-1 and C-6, the yields from C-1 and C-2 of acetate were used as a reference on the assumption that the conversion of acetate carbons to CO_2 via the TCA cycle simulated that of glucose-derived acetyl-CoA. The methyl carbon (C-2) of acetyl-CoA derived from the degradation of glucose via the EMP pathway is equivalent to C-1 or C-6 of glucose and the carboxyl carbon (C-1) of acetyl-CoA is equivalent to C-2 or C-5. Hence, the yield from C-1 of acetate should be proportional to the yield from C-2 or C-5 of glucose and the yield from C-2 of acetate should be proportional to the yield from C-1 or C-6 of glucose. It was recognized that injected acetate may not have been metabolized like acetyl-CoA; however, it is conceivable that once acetate was converted to acetyl-CoA, its metabolic fate would have been no different from that of acetyl-CoA derived from glucose.

To obtain estimates of over-all pathway participation, the cumulative yields of $^{14}CO_2$ observed at the end of the time course for complete substrate utilization (i.e., the originally labelled substrate is no longer present with a label but the labelled carbon atom may have moved and be present in other compounds) have been found to

give good estimates (19, 34, 60). This is so because the net effects of each pathway can be established only after all of the injected glucose destined to be catabolized via each pathway have yielded $^{14}CO_2$ and because the catabolism of glucose may proceed at different rates via the various pathways. It has been shown that the ratio of the yields of $^{14}CO_2$ from the individual carbons of glucose change significantly during the early phase of glucose utilization but become stable after a period of time. Thus in a study on the catabolism of glucose by Bacillus subtilus (60), it was demonstrated that the apparent relative participation of the two catabolic pathways in this organism changed remarkably during the early phase of substrate utilization, but became stable after the administered glucose was in essence completely utilized. Thus use of yields before complete substrate utilization may produce a distorted view of the over-all utilization of the various pathways for glucose catabolism.

Three approximations are presented for the fraction of labelled glucose catabolized via the three pathways (Table 6). The first was based on the assumption that the observed cumulative yield from C-3(4) represented the percentage of labelled glucose catabolized exclusively via the EMP pathway. It was noted however, that the sum of the fractions of glucose traversing each pathway exceeded 100 percent when this assumption was used. Thus a second approximation was made. The second approximation was based on the assumption

that since the experimental fish were starved for 48 to 72 hours, virtually all of the injected glucose was utilized. It was also based upon the hypothesis that the estimated fraction of glucose traversing only the EMP pathway may have been too high when calculated from the cumulative yield from C-3(4).

Table 6. Relative Percent Participation of Pathways for the Catabolism of Glucose in Controls and in Cichlids Exposed to KPCP.

	First Approximation PPM KPCP						Second Approximation PPM KPCP			Third Approximation PPM KPCP		
	0		0.20		1.50		0	0.20	1.50	0	0.20	1.50
EMP	66	(62)	80	(71)	100	(98)	58	67	98	58	67	83
PP	20	(18)	17	(15)	0	(0)	20	17	0	20	17	3
GA	22	(20)	16	(14)	2	(2)	22	16	2	22	16	11

Reconstructed hexoses may be derived from pentoses developing from the degradation of glucose by way of the PP pathway alone. They may also be formed from GA pathway-derived pentoses which had traversed the PP pathway. The carbons of the reconstructed hexoses, derived by way of the PP pathway only, would be numbered in the following manner with respect to the original glucose molecule (38):

$$\overset{2}{OHC}-\overset{3}{C}-\overset{2}{C}-\overset{4}{C}-\overset{5}{C}-\overset{6}{CH_2OH} \quad \text{and} \quad \overset{2}{OHC}-\overset{3}{C}-\overset{3}{C}-\overset{4}{C}-\overset{5}{C}-\overset{6}{CH_2OH}.$$

Pentoses derived by way of the GA pathway and further metabolized via the PP pathway form reconstructed hexoses with carbons

numbered in the following manner:

$$\overset{1}{OHC}-\overset{2}{C}-\overset{1}{C}-\overset{3}{C}-\overset{4}{C}-\overset{5}{CH_2OH} \quad \text{and} \quad \overset{1}{OHC}-\overset{2}{C}-\overset{2}{C}-\overset{3}{C}-\overset{4}{C}-\overset{5}{CH_2OH}$$

Since the EMP pathway is the single dominant route for glucose catabolism, it is highly likely that these reconstructed hexoses entered the EMP pathway rather than recycled through the PP pathway. Since an estimated 42 percent of the injected glucose was routed through the minor pathways, the reformed hexoses could have given rise to some $^{14}CO_2$ derived especially from C-2, C-3 and C-4 of glucose when pyruvate formed from these hexoses was decarboxylated. Another net effect of the formation of reconstructed hexoses is that more than 100 percent of the number of labelled hexose molecules injected were available to traverse the three pathways. If the values in the first approximation are reduced proportionally to a total of 100 percent, the first approximation may possibly give the best values of the relative use of the three pathways. The reduced values are given in parentheses under the first approximation in Table 6.

The first approximation was calculated as follows:

Let,

Gp = the percentage of glucose catabolized via the PP pathway

Ga = the percentage of glucose catabolized via the GA pathway

Ge = the percentage of glucose catabolized via the EMP pathway

R = the net contribution of the TCA cycle to the respective yields from C-1 and C-6 of glucose.

The percentage R was calculated from the proportion:

$$\frac{\text{Yield from C-1 of acetate}}{\text{Yield from C-2 of acetate}} = \frac{\text{Yield from C-2 of glucose}}{R}$$

Then,

Yield from C-1 of glucose - R = Gp

Yield from C-6 of glucose - R = Ga

Yield from C-3(4) of glucose = Ge

For the second approximation the estimate for the EMP pathway was obtained by subtracting the sum, Gp + Ga, from 100 percent. Both approximations are presented in Table 6.

Although the estimates listed as first and second approximations support earlier statements regarding the effect of KPCP upon the utilization of the various pathways for the catabolism of glucose, the estimates for the percentage of glucose catabolized by way of the three pathways in cichlids exposed to 1.5 ppm KPCP should be qualified. An important and basic assumption applying to all scientific experimentation is that the test substance or subject will not differ from the standard or control until the test has begun. Thus, with cichlids exposed to 1.5 ppm KPCP all pathways involved in glucose metabolism must have been operating normally prior to exposure of the fish to the toxicant. Unless the action of KPCP was immediate and maximal once the fish were placed into the solution

containing KPCP, the pathways should have been operating to some extent for some time following initial exposure even if total interference developed subsequently.

If the conversion of C-1 of glucose to CO_2 via the TCA cycle occurs at an equal rate and to the same extent as that of C-6 of glucose, any inequality in the rate or extent of CO_2 formation from these two carbons must be taken as evidence of the existence of some other pathway or mechanism that would favor the conversion of one over the other. The other possible explanation is that there was no statistically significant difference among the parameters measured. The yields from C-1 exceeded those of C-6 until the tenth hour after which the yields from C-6 exceeded those from C-1. This was the same pattern observed in controls except that the cross-over in controls did not occur until the 16th hour. In both cases the data suggested that it required more time for glucose to be converted to pentose via the GA pathway than via the PP pathway. The maximum difference between the yields from C-1 and C-6 before the 10th hour was three percent. Thus if the GA pathway were in fact catabolizing glucose during this time, at least three percent more glucose must have been routed through the PP pathway. However the first and second estimates indicated that no glucose was routed through the PP pathway in cichlids exposed to 1.5 ppm KPCP. The estimates were thus contrary to the available information and required revision.

The revised estimates are thus given as third approximations of the relative fraction of glucose traversing the three pathways.

Some lack of precision can develop from the use of cumulative $^{14}CO_2$ yields for the estimation of the relative participation of three concurrently-operating pathways. The estimates provide only an approximation of the distribution of glucose through the three pathways in normal and in KPCP-treated cichlids. Whether one is dealing with an organism with a two or three pathway system, the estimates of relative pathway participation, calculated from $^{14}CO_2$ yields obtained before complete substrate utilization, do not permit an accurate, quantitative evaluation of the effects of a biologically-active compound such as KPCP while it is being absorbed or being transported to its sites of action. However, $^{14}CO_2$ data from single-dose experiments can give a valuable first approximation of the intensity of the effect of a biologically-active compound on metabolic processes, certainly areas requiring further exploration are exposed.

SUMMARY

The effects of 0.20 and 1.5 ppm KPCP upon the catabolism of carbohydrate in the cichlid fish, Cichlasoma bimaculatum, were investigated by measuring the rate of extent of recovery of respiratory $^{14}CO_2$ from specifically-labelled acetate- and glucose-^{14}C. In addition a study was made to determine the effects of 0.20 ppm KPCP upon the delivery of $^{14}CO_2$ in the expired air in cichlids injected with $NaH^{14}CO_3$.

To determine the adequacy of the ion chamber-electrometer system in measuring quantitatively the amount of radioactivity in the expired air, an inventory was taken of the amount of injected activity recovered as $^{14}CO_2$, the amount remaining in the fish and the amount remaining in the aqueous medium in four control experiments with glucose-1-^{14}C. An average of 97 percent of the injected activity was accounted for. Of this percentage, $^{14}CO_2$ accounted for 53 percent, 43 percent came from the fish carcasses and about 1.5 percent from the water. It was concluded that the amount of radiocarbon recovered as $^{14}CO_2$ represented quantitatively the percentage of radiocarbon converted to CO_2.

In controls the 24-hour cumulative yield from injected bicarbonate-^{14}C was 91 percent of injected activity; however, 98 percent of the injected radiocarbon from acetate-1-^{14}C was recovered during

the same time period. Failure to obtain as much $^{14}CO_2$ from injected bicarbonate as from C-1 of acetate suggested that due to the sudden increase in the concentration of bicarbonate brought about by injection, some of the labelled bicarbonate may have been incorporated into relatively non-labile compounds such as bone. Recovery of only 80 percent of the injected activity from labelled bicarbonate injected into cichlids exposed to 0.20 ppm KPCP indicated a tendancy towards conserving bicarbonate. It was suggested that KPCP may have increased the movement of bicarbonate into bone or interfered with circulation or gaseous exchange at the gills. Conservation of bicarbonate to compensate for possible acidosis was also suggested.

In controls the respective 24-hour cumulative yields from C-1 and C-2 of acetate were 98 and 70 percent on injected activity. Preferential conversion of C-1 over C-2 to $^{14}CO_2$ was considered presumptive evidence for the operation of the TCA cycle in cichlids. The initial recovery of $^{14}CO_2$ from both acetate carbon atoms was delayed in cichlids exposed to 0.20 ppm KPCP; however, the 24-hour cumulative yields from C-1 and C-2 were almost equal to the respective yields from controls. The reason for the delay could not be ascertained from the data. In the presence of 1.5 ppm KPCP the recovery from C-1 and C-2 of acetate was very rapid and extensive. The most marked change occurred in the extent of recovery of C-2 which approached that of C-1. It was concluded that in the presence of 1.5

ppm KPCP cichlids may have used the TCA cycle primarily for the generation of energy rather than as a route for biosynthesis.

The pathways used by cichlids for the catabolism of glucose were identified by comparing the relative rate and extent of recovery of $^{14}CO_2$ from the individual carbons of specifically-labelled ^{14}C-glucose. In controls the respective 24-hour cumulative yields from C-1, C-2, C-3(4) and C-6 of glucose were 53, 47, 66, and 56 percent of injected radioactivity. Recovery from C-3(4) was most rapid and extensive and presumably $^{14}CO_2$ derived from C-3 or C-4 developed via decarboxylation of pyruvate. It was concluded that the EMP pathway was the major route of glucose catabolism in cichlids. Preferential conversion of C-1 of glucose over C-2 to $^{14}CO_2$ provided presumptive evidence for the operation of the pentose phosphate (PP) pathway with CO_2 being derived from C-1 via the decarboxylation of phosphogluconate. Preferential conversion of C-6 over C-2 to $^{14}CO_2$ suggested the operation of the glucuronic acid (GA) pathway with CO_2 being derived from C-6 presumably via decarboxylation of gulonic acid.

In cichlids exposed to 0.20 ppm KPCP the cumulative yields of $^{14}CO_2$ from C-1, C-2, C-3(4) and C-6 of glucose were 57, 56, 80, and 56 percent, respectively. The relatively larger increase in the extent of recovery from C-2 and C-3(4) suggested that the addition of 0.20 ppm KPCP increased the fraction of labelled

glucose catabolized via the EMP-TCA pathway.

In cichlids exposed to 1.5 ppm KPCP the respective cumulative yields from C-1, C-2, C-3(4) and C-6 of glucose were 81, 90, 100, and 88 percent of the injected radioactivity. The rapid and extensive recovery of $^{14}CO_2$ from all glucose carbons indicated that the rate of glucose catabolism was markedly enhanced by the presence of 1.5 ppm KPCP. A comparison of the relative rate and extent of recovery from the individual carbons of glucose indicated that the EMP-TCA pathway was used almost exclusively for the degradation of glucose in cichlids exposed to 1.5 ppm KPCP.

Estimates of the relative participation of the EMP, PP and GA pathways for glucose catabolism were made from the yields of $^{14}CO_2$ obtained after 24 hours from the individual carbon atoms of injected glucose- and acetate-^{14}C. The estimated percentage of labelled glucose catabolized via the EMP pathway in controls was 58 to 62 percent; in cichlids exposed to 0.20 ppm KPCP, 67 to 80 percent traversed the EMP pathway; in cichlids exposed to 1.5 ppm KPCP the estimated amount of glucose traversing the EMP pathway was 83 to 100 percent. From 18 to 20 percent of the injected glucose was catabolized via the PP pathway in controls. Addition of 0.20 ppm reduced the percentage catabolized via the PP pathway to an estimated 15 to 17 percent and at 1.5 ppm KPCP about 3 percent traversed the PP pathway. In controls an estimated 20 to 22 percent

of the injected glucose was routed through the GA pathway while only 14 to 16 percent traversed this pathway in cichlids exposed to 0.20 ppm KPCP. From 2 to 11 percent was catabolized via the GA pathway in cichlids exposed to 1.5 ppm KPCP.

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