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Polychlorinated biphenyls (PCBs) have been reported to cause alterations in the metabolism of phospholipids and glycerides in animals and humans. This project was undertaken in an attempt to determine the mode of action of PCBs and their primary metabolites on phospholipid and glyceride biosynthesis.

The activity of sn-glycerol-3 phosphate acyltransferase was inhibited by PCBs *in vitro*. The inhibition was noncompetitive suggesting a binding to a site other than the active site on the enzyme. The inhibition of the activity of this enzyme was accompanied by a general decline in the *in vitro* synthesis of phospholipids by microsomes, mitochondria and cell-free homogenates. Glyceride synthesis by mitochondria and cell-free homogenates decreased while it increased in the microsomes.

After *in vivo* treatment of rats with PCB for 30 days, the activity of microsomal sn-glycerol-3 phosphate acyltransferase declined. The decline in enzyme activity was accompanied by a decreased incorporation of radioactivity into microsomal phospholipids and glycerides. On the other hand, mitochondria and cell-free homogenates consistently showed apparent increases in the incorporation of radioactivity into

phospholipids and glycerides after the PCB treatment.

The purified isomer, 2,4,5,2',4',5'-hexachlorobiphenyl elicited the same kind of response as the commercial PCB preparation. It inhibited the activity of sn-glycerol-3 phosphate acyltransferase which caused a decrease in the synthesis of phospholipids *in vitro*. Glyceride synthesis again increased in microsomes but decreased in the cell-free homogenate.

The *in vitro* activities of glycerol kinase and diglyceride acyltransferase were not affected by PCB at concentrations as high as 1.5 mM. This concentration was more than three times that needed to inhibit sn-glycerol-3 phosphate acyltransferase.

Microsomal phosphatidate phosphatase activity was not affected during the initial stages of catalysis, but after prolonged incubation, there was a small increase (22 percent) or decrease (18 percent) in the activity under *in vitro* and *in vivo* conditions, respectively. The slight increase in the activity of this enzyme under the *in vitro* conditions was observed at 1.2 mM PCB which was more than two times that at which sn-glycerol-3 phosphate acyltransferase is significantly inhibited.

The activity of phosphorylcholine-glyceride transferase was inhibited at 1.5 mM PCB *in vitro* but the inhibition was much less than observed with sn-glycerol-3 phosphate acyltransferase.

Two primary metabolites of PCB, 4'-chloro-4-biphenylol and 2',3',4',5,5'-pentachloro-2-biphenylol elicited the same kind of response as PCBs *in vitro*. Both compounds inhibited the activity of sn-glycerol-3 phosphate acyltransferase, with large decreases in the *in vitro* synthesis of phospholipids by microsomes, mitochondria, and

cell-free homogenates. Glyceride synthesis by mitochondria and homogenates also decreased, but increased in the microsomes. The pentachlorobiphenylol was a more potent inhibitor of the activity of sn-glycerol-3 phosphate acyltransferase than PCBs. The inhibition of the enzyme by this compound was also noncompetitive. The 4'-chloro-4-biphenylol did not affect the activity of microsomal phosphatidate phosphatase, but the pentachlorobiphenylol slightly stimulated the enzyme activity (25 percent). The latter observation might in part account for the increased glyceride synthesis observed in the microsomes.

The overall results are consistent with the conclusion that PCBs and their metabolites inhibit phospholipid and glyceride synthesis at the site of the reaction catalyzed by sn-glycerol-3 phosphate acyltransferase. A model is proposed in this project which accounts for all the observed results and is consistent with the conclusion drawn.

Polychlorinated Biphenyls and Related Metabolites: Mode of Action on Phospholipid and Glyceride Biosynthesis

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DEDICATION

To Mawuena and Kafui

for their patience and understanding

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INTRODUCTION

Polychlorinated biphenyls (PCBs) are a class of relatively unreactive and thermally stable compounds that have found widespread industrial application as fire retardants, heat transfer fluids, plasticizers and dielectric fluids.

Commercial PCB products consist of mixtures of PCB isomers, differing from one another in the extent of chlorination. In the United States PCBs were marketed by Monsanto Chemical Company, St. Louis, under the tradename Aroclor, followed by a four-digit number with the last two digits indicating the percent chlorination. For example, Aroclor 1254 is a mixture of PCB preparation having 54% chlorination. Polychlorinated biphenyls are extremely stable and virtually insoluble in water and are thus very desirable as industrial chemicals. The very properties of PCBs that make them useful industrially also prevent them from being degraded once they reach the environment.

Polychlorinated biphenyls were first identified in the environment during the 1960s (1) and since that time have been found to be among the most ubiquitous and persistent chemical pollutants in the global ecosystem (2, 3, 4). Their presence at all levels of the food chain has been detected (5), thus posing a direct threat to both humans and wildlife. Due to their strong lipophilic properties they tend to accumulate in animal tissues of high lipid content. In fact,

tissue samples from a variety of wildlife have been found to contain PCB residues (3). Detectable concentrations (≥ 1 ppm wet weight) of PCBs in human adipose tissue within the general population of the United States have been reported (6). Presently, the use of PCBs has been restricted to electrical and electronic industries, in capacitors and transformers, especially because of their heat transfer and dielectric properties.

Due to their lipophilic nature and the fact that they accumulate in animal tissues with high lipid content, it is conceivable that PCBs will interfere with lipid metabolism in these tissues. In fact, increases in total phospholipid content of the rat liver endoplasmic reticulum following PCB administration has been reported (7). Large increases in liver triglyceride content following PCB administration in rats has also been reported (8).

In view of the biological importance of phospholipids and triglycerides for the proper maintenance of various hepatic functions such as the formation of very low density lipoproteins (VLDL) and the proliferation and repair of membranes, it was of interest to assess the extent to which PCBs interfere with phospholipid and triglyceride metabolism in the rat liver.

The purpose of this project was to investigate the extent to which PCBs alter phospholipid and glyceride biosynthesis in the rat liver. A few studies have been carried out (7, 9) on the effect of PCBs on phospholipid biosynthesis in the rat liver; however, these studies dealt only with the overall effect of PCBs on total phospholipid synthesis. The question, therefore, arises as to which key

enzymes in the biosynthetic pathway are being influenced by the presence of PCB. The gross effect seen in terms of amounts of products formed could result from differential effects of PCBs on the activities of the various enzymes in the pathway. Thus, part of this project was concerned with determining the effect of PCBs on the activities of some selected key enzymes in the pathway of phospholipid and triglyceride biosynthesis.

Alterations in the liver mitochondrial structure (10) and function (11) following PCB administration to rats have been reported. Mitochondria are the primary organelles responsible for providing all the cell's energy requirements in the form of ATP. Since the integrity of the mitochondrial membrane is dependent upon phospholipids, part of this project also assessed the extent to which PCBs alter the capacity of the mitochondria to synthesize phospholipids. In addition, PCBs are primarily metabolized by microsomal enzymes to hydroxylated derivatives (12-20), but the effect of these metabolites on phospholipid and glyceride metabolism, to our knowledge, has not been reported. Therefore, part of this project was also concerned with investigating the mode of action of PCB metabolites on phospholipid and glyceride biosynthesis.

Since the discovery of PCBs as additional environmental pollutants and toxicants, several studies on the various aspects of PCB toxicity have been reported. Below is a survey of the literature on the toxicological effects of PCBs in mammalian tissues.

The Toxicity of PCBs

Although the toxic effects of PCBs are diverse, in animals, hepatotoxicity appears to be the most widely recognized effect. The principal target organs of PCBs in man appear to be the liver and skin. However, in rodents skin disorders are generally not apparent and therefore toxicity studies in these animals have thus tended to concentrate on the liver. The toxicity studies on PCBs covers diverse areas of discipline, the most prevalent among them being morphological, biochemical, metabolic and electron microscopic studies. In addition, acute toxicity studies (LD 50) in animals have been reported.

Acute Toxicity for Experimental Animals

The oral LD_{50} (50% lethal dose) of PCBs for albino rats was reported to be several grams/kilogram body weight (21). It was also reported (21) that highly chlorinated biphenyls such as tetrachlorobiphenyl and higher homologues are slightly less toxic (ca 10 g/kg body weight) than trichlorobiphenyl (5-6 g/kg b.w.). Grant *et al.* (22) have, however, reported the LD_{50} of Aroclor 1254 to be 1.4 g/kg body weight in Wistar strain 30-day-old rats, and 2.0-2.5 g/kg body weight for 120-day-old rats. There were no major differences between the sexes. The reason for the discrepancy in the two sets of LD_{50} data has not been explained, but possible factors such as the difference in the strain of rats used as well as the mode of PCB administration could probably account for the differences.

The acute toxicity of a PCB mixture equivalent to Aroclor 1242 on the guinea pig, rabbit and the rat was conducted by Miller (23). The

results obtained gave the impression that the guinea pig was the most sensitive of the three species tested, followed by the rabbit and the rat in that order. A single subcutaneous dose of 345 mg was fatal to ten out of ten guinea pigs within 13 days. Taking the weights of the animals into consideration, this was calculated to represent 870 mg/kg body weight. Death also resulted in eight out of eight guinea pigs from the administration of two oral doses of 69 mg a week apart. All the animals died in 11 to 29 days following the initial dose. In a comparable oral-toxicity trial, 25 daily doses of 138 mg were apparently not fatal to rats within 90 days of the initial feeding. The validity of the above results is questionable, however, since the purity of the PCB mixture used for example, might not necessarily be comparable to the present-day Aroclors.

Tucker and Crabtree (24) reported that the acute oral LD_{50} for the Aroclor series 1242 through 1268 in mallard ducks were all in excess of 2000 mg/kg. The acute effects of Aroclor 1254 on the feline cardiovascular system was studied by Righter et al. (25). Aroclor 1254 was administered intravenously at 30-minute intervals to anesthetized cats at a dosage of 100 mg/kg body weight. The oxygen tension (pO_2), carbon dioxide tension (pCO_2) and pH of the arterial and venous blood samples were determined at ten-minute intervals. There was a rapid and marked reduction in pO_2 with concurrent changes in pCO_2 and pH characterizing a respiratory acidosis. Respiratory rate was reported to increase to a sizeable degree with development of cardiac arrhythmias. All the animals died within 60 minutes following initial dosing.

Baker et al. (26) reported that after feeding Aroclor 1254 daily to female and male rats for nine weeks at a dosage of 6.4 mg/kg in their drinking water, only the higher homologues, namely penta- and hexachlorobiphenyls persisted in the livers even up to the 19th week after termination of exposure. These findings are in agreement with the results obtained by Kikuchi and Masuda (27) from studies on human subjects who have accidentally ingested PCBs in their diet. Thus, since only the higher homologues of PCBs have been found to persist in the mammalian system for long periods after pretreatment, the chronic toxicity of these chlorobiphenyls appears to be of more importance than acute toxicity studies in evaluating health effects of the PCBs polluting the environment.

Subacute and Chronic Studies

As mentioned in the previous section, chronic toxicity studies on PCBs are of more importance than the acute toxicity studies since under normal exposure conditions, massive doses of PCBs are not encountered. However, as mentioned above, some PCB homologues are persistent in the animal system even long periods after exposure. Thus, a gradual build-up of these persistent PCBs in the system will ultimately produce manifestations of the chronic toxic effects of PCBs polluting the environment.

Allen et al. (28) fed Aroclor 1248 at a level of 25 ppm for two months to adult female rhesus monkeys. The total dosage of PCB was between 250 and 400 mg for an average body weight of 5-6 kg. Facial edema and acne developed within one month and one of the animals died

from the effects of PCB intoxication two months after removal from the experimental diet. The dead monkey showed symptoms of anemia, hypoproteinemia and bone-marrow hypoplasia. The surviving animals also showed apparent acne, subcutaneous edema and 34 µg/g of PCB was still present in the adipose tissues even eight months after removal from the experimental diet, thus indicating a persistence of toxic effects long after the termination of exposure.

Garthoff et al. (11) fed 0, 5, 50 and 500 ppm of either Aroclor 1254 or Firemaster (a commercial polybrominated biphenyl) to male rats for periods of two, three or five weeks. They did not observe any dose-related effect with either chemical at any level on relative kidney, liver or testis weights. However, there was a significant depression of growth and adipose tissue weight by both compounds after three weeks at the 500 ppm level. The growth effect they explained, appeared to be due primarily to decreased efficiency of food utilization. Carter and Cameron (10) administered ad libitum either 0, 200, 500 or 1000 mg/kg of 2,4,5,2',4',5'-hexachlorobiphenyl dissolved in peanut oil to sexually mature male mice for 28 days, and observed that their food and water consumption, urine and fecal (dry) output and body weights were not depressed or enhanced by the PCB treatment. However, the wet spleen weights of those animals receiving the 500 mg/kg were significantly depressed and the wet kidney weights of animals receiving 500 and 1000 mg/kg were also significantly depressed. In contrast, the wet and dry weights of livers were significantly elevated in animals treated with 500 and 1000 mg PCB/kg. It has been observed repeatedly in this laboratory that feeding 1000 ppm of Aroclor 1254

dissolved in corn oil to both male and female rats resulted in significant increases in the wet weight of the livers. Similar observations have been reported by other workers (7, 8, 29, 30).

In 1968 over 1000 persons in Fukuoka Prefecture, Southern Japan, suffered from an epidemic of a peculiar skin disease similar to chloracne. The cause of the epidemic was quickly demonstrated to be the ingestion of a specific brand of rice oil contaminated with over 2000 ppm of a commercial brand of PCB, Kanechlor 400 (31). The most common initial symptoms experienced by some of the patients include increased eye discharge, swelling of the upper eyelids, acne-form eruptions and pigmentation of the skin. Jaundice, transient visual disturbance, headache, vomiting and diarrhea were also experienced by some of the affected persons (31).

Gas chromatographic analysis of the PCB content of the visceral organs was carried out on these patients by Kikuchi and Masuda (27). The results showed that one year after the onset of symptoms, the PCB present in the body consisted mainly of ingredients of higher boiling point, corresponding to those of Kanechlor 500 or 600 (commercial PCB preparations containing about 55% and 60% chlorine respectively). These findings, the authors (27) reported, suggested that the patients had ingested large amounts of PCBs mainly with four chlorine atoms per molecule, but that most was discharged within one year leaving only PCBs with five to seven chlorine atoms per molecule in the body. The gas chromatographic pattern of a stillborn (to an affected mother) (27) was found to be almost identical with that of the rice oil which his mother had used. This was suggested to result from the short

interval between the mother's use of the oil and the death of the child.

As indicated earlier, the liver is the target organ of PCBs in rodents. Itokawa et al. (29) fed 500 ppm Kanechlor 500-supplemented diet alone or 500 ppm Kanechlor 500-supplemented diet and water containing 1000 ppm alkylbenzene sulfonic acid salt to male Wistar rats for periods of one, three and seven months. Light microscopic examination of the livers after these treatment periods revealed swelling of the individual hepatic cells as well as numerous vacuoles and degenerative changes in scattered areas of the liver. Carter and Cameron (10) reported large increases in the volumes of lipid bodies, secondary lysosomes, mitochondria, rough and smooth endoplasmic reticulum and cytoplasm of livers from mice orally dosed with 1000 mg/kg of 2,4,5,2',4',5'-hexachlorobiphenyl for 28 days. Mishizumi (32) has reported an increase in the smooth-surfaced endoplasmic reticulum (SER) together with a decrease in rough-surfaced endoplasmic reticulum (RER) in liver cells of mice given Kanechlor 400 for 13 weeks. Similar observations have been reported in rats dosed with Aroclor 1254 at 25 and 50 mg/kg body weight (9). Vos and Notemboom-Ram (33) have also reported proliferation of the SER which resulted in perinuclear and peripheral displacement of the mitochondria and RER.

Thus, in general, the subacute chronic toxic effects of PCBs in mammals is manifested by both pathological and morphological changes such as weight loss, liver enlargement, facial edema, acne skin eruptions, skin pigmentation (in humans), increases in cell volume, cell vacuolation and proliferation of the smooth endoplasmic reticulum. Presumably, these changes are a reflection of changes occurring at the molecular level in the cell's biochemical apparatus.

Effect of PCB on Drug-Metabolizing Enzymes

The effect of PCBs on the activities of drug-metabolizing enzymes represents the most widely studied area on the toxicological aspects of PCBs in animal systems. This was largely due to the known fact that chlorinated organic pesticides such as DDT or dieldrin are strong inducers of detoxicating enzymes in the liver. Thus, the possibility that PCBs may also display similar effects was naturally very attractive. The literature on enzyme inducing properties of PCBs is rather vast and no attempt is made in this review to touch on everything reported in the literature.

Villeneuve et al. (34) reported that the no-effect level of Aroclor 1254 for enzyme induction in pregnant rabbits is 1.0-10 mg/kg body weight when administered over a 28-day period during gestation. Induction of aniline hydroxylase and aminopyrine N-demethylase was observed at 10 mg/kg. Shimada and Ugawa (35) reported significant increases in the activities of cytochrome P-450, aminopyrine N-demethylase and benzpyrene hydroxylase in Sprague-Dawley rats fed Kanechlor 500 and prepared PCBs, the gas chromatographic profile of these PCBs have much in common with that in human milk. Ecobichon et al. (36) reported no effect of 4,4'-difluoro- and 4,4'-diiodobiphenyl on the activities of p-nitroanisole O-demethylase and aniline hydroxylase in the 12,000 xg, 20 minute supernatant. On the other hand, 4,4'-dichloro- and 4,4'-dibromobiphenyl caused marked inducation of these enzymes. Goldstein et al. (37) also reported marked increases in cytochrome P-450, N-demethylase, nitroreductase, aniline hydroxylase and glucuronyl transferase by Aroclor 1242 when the latter was fed in 100 or 500 ppm level to female rats for one week. Hansell

et al. (38) reported marked induction of p-nitroanisole O-demethylase and aniline hydroxylase by 2,4,2',4'-tetrachloro- and 2,4,5,2',4',5'-hexachlorobiphenyls as early as three days after the PCB treatment.

Schmoldt et al. (39) studied the in vitro metabolism of p-nitroanisole, aminopyrine and aniline by rat liver microsomal monooxygenases in the presence of different PCB mixtures and some related hydroxybiphenyls. All the PCB mixtures used, containing preferably di-, tetra-, or hexachlorobiphenyl competitively inhibited aminopyrine demethylation by normal microsomes. When microsomes prepared from PCB treated rats were used, the aminopyrine demethylation was inhibited non-competitively by the di- and hexachlorobiphenyl while the tetrachlorobiphenyl remained a competitive inhibitor. After PCB pretreatment, all the PCBs were competitive inhibitors of p-nitroanisole demethylation. The hydroxybiphenyls competitively inhibited aminopyrine demethylation and aniline hydroxylation but failed to inhibit p-nitroanisole metabolism.

Thus, in general, PCB administration to laboratory animals results in the induction of drug-metabolizing enzymes in the liver. This induction is actually thought to be an adaptive reaction of organisms for enhancing detoxification of the foreign compound. However, if the induction occurs too strongly and of long duration then it is possible that essential components of the cell such as the steroid hormones may be metabolized and thus lose their physiological activity. The activities of these drug-metabolizing enzymes have been found to be inhibited by PCBs in vitro, however.

Effect on Lipid Metabolism

In PCB-poisoned humans, alterations in general lipid metabolism were found to be the most important metabolic change to occur in the patients (40). These findings stimulated a great interest among researchers to determine the effect of PCBs on lipid metabolism in experimental animals. Elevated phospholipid and triglyceride levels in the serum (41) and liver (7, 8, 30, 41) in laboratory animals following PCB administration over a period of time has been reported.

The effect of polychlorinated biphenyls on phospholipid biosynthesis in microsomes from rat liver was studied by Ishidate and Nakazawa (7). Rats were orally administered a homogeneous suspension of PCB in 2% (w/v) sodium carboxymethyl cellulose (CMC) daily for three days at a dosage of 100 mg/kg/day. At various times afterwards, the animals were injected i.p. with [^{32}P]orthophosphate, [Me - ^{14}C] choline or [^{3}H]glycerol. The incorporation of [^{32}P] and [^{14}C] into microsomal choline-containing phospholipids was decreased by PCB, but little or no effect on other phospholipids was observed. The incorporation of [^{3}H]glycerol into total phospholipids was decreased with no significant change in the distribution of radioactivity after 15 minutes. After 60 minutes incubation, the total radioactivity in phospholipids was only slightly decreased (12%). After two days administration of PCB, a large increase in microsomal phospholipid content was observed. The authors thus suggested that the increase in microsomal phospholipid following PCB administration is not due to stimulation of synthesis, but rather to the inhibition of catabolism of membrane phospholipids.

The effect of 4-chlorobiphenyl on the incorporation of [^{14}C]ethanolamine and [^{14}C]choline into phosphatidyl ethanolamine and phosphatidyl choline, respectively, by mouse L5178Y lymphoma cells in vitro was studied by Spalding and coworkers (42). Inhibition of [^{14}C]ethanolamine incorporation into phosphatidyl ethanolamine was observed, whereas a two- to three-fold stimulation of [^{14}C]choline into phosphatidyl choline occurred. There was no apparent increase in the total phospholipid content of the cells after the 60 minute incubation period. The 4-chlorobiphenyl was not taken up by the cells, but remained bound to the cell surface. Desorption of the 4-chlorobiphenyl eliminated the observed effects. It was concluded that the stimulation of incorporation of [^{14}C]choline into phosphatidyl choline was due to increased turnover of phosphatidyl choline. Nagai et al. (43) reported that oral administration of PCB reduced the free fatty acids and triglyceride in the skin lipids of the rat. Incorporation of acetate into triglyceride and free fatty acids was decreased, whereas that into total sterols was increased.

Holub et al. (44) studied the effect of 2,4,2',4'-tetrachlorobiphenyl in vitro and in vivo on lipid synthesizing enzymes in rat liver microsomes. For the in vivo treatment, male Wistar rats were administered the PCB intraperitoneally at a dosage of 50 mg/kg body weight as a peanut oil solution successively for three days and rats were sacrificed 96 hours after the final injection. The effect of the PCB treatment on the activities of acyl-CoA:sn-glycerol-3 phsophate acyltransferase, CDP-choline:1,2-diacyl-sn-glycerol cholinephosphotransferase, acyl-CoA:1,2-diacyl-sn-glycerol acyltransferase and

acyl-CoA:1 acyl-sn-glycerol-3 phsophorylcholine acyltransferase was determined in the microsomal preparations. No significant difference in the activities of any of the four enzymes was observed between the PCB-treated rats and controls. When the effect of added PCB to the incubation medium on the activities of these enzymes was studied, however, it was found that all the enzymes were significantly affected by the added PCB in such a way that acyl-CoA:sn-glycerol-3 phosphate acyltransferase and acyl-CoA:1-acyl-sn-glycerol-3 phsophorylcholine acyltransferase were inhibited (>40%) at 40 ppm PCB, but CDP-choline: 1,2-diacyl-sn-glycerol cholinephosphotransferase was stimulated (>40%) at the same PCB concentration. It was thus concluded that the induction of these enzymes may not be the primary cause for the changes in lipid concentration due to PCB administration because of the failure of PCB to stimulate these enzymes when administered in vivo.

The in vivo administration of Aroclor 1254 on the synthesis and turnover of microsomal and cytoplasmic lipids of rat liver was studied by Hinton et al. (9). Rats were given intraperitoneal injection of the Aroclor emulsified by Ringers solution at a dosage of 20 and 50 mg/kg for three or six days. The effect of the treatment of lipid synthesis was determined by following the radioactivity of [^{14}C]glycerol incorporated into phospholipids in the homogenate and microsomes at various time periods. The incorporation of [^{14}C]glycerol into total phospholipids in the homogenate and microsomes was found to decline over a ten-minute period when results were claculated as dpm incorporated/mg phospholipid. When results were calculated as dpm/liver, however, there was no difference between the control and PCB treated

experiments for the homogenate, but in the microsomes the PCB-treated rats showed higher incorporation. Similar results were obtained with respect to triglyceride synthesis.

In order to determine if PCB caused any change in the turnover of liver lipids, the decrease in radioactivity of phospholipids and triglycerides after a single injection of [^{14}C]glycerol was followed. It was observed from such experiments that the specific activity of the total phospholipid pool from PCB-treated livers demonstrated a prolonged half-life from about ten hours for the controls to about 20 hours after exposure to PCB in the homogenate, and in the microsomes the corresponding values were eight and thirteen respectively. With respect to triglyceride turnover, similar prolonged half-life after exposure to PCB was observed both in the homogenate and microsomes. The authors (9) therefore concluded that PCB slows down the utilization of these lipids and consequently increases their apparent half-life. It was therefore suggested that the PCB-induced fatty liver is not a result of increased net synthesis of phospholipids and triglycerides, but rather the result of a decreased turnover. It should be noted however, that all these studies have been carried out on rats pre-treated with PCB for short periods only, i.e. three to six days at most.

The question therefore arises as to whether these short-term treatments are sufficient in themselves to reveal the actual chronic toxic effects of PCB with respect to lipid synthesis. In this project therefore, a longer pretreatment period, namely 30 days, has been used since it has been shown (26) that after three weeks of pretreatment of rats with Aroclor 1254 only the higher chlorinated homologues which

are probably responsible for the chronic toxic effects acculumate in the liver.

Metabolism of PCBs in Animals

It has been very well extablished that numerous foreign compounds, such as drugs, pesticides and other industrial chemicals once they have penetrated into the body of animals by different mechanisms are metabolized to more polar derivatives (45). This biotransformation tends to produce alterations in the biological activity and thus leads to rapid elimination from the body. Polychlorinated biphenyls are known to be biologically stable, but in order for them to be excreted from the body they also need to be metabolized to polar compounds. Secondly, in order to fully explore the toxic properties of PCBs as well as understand the dynamic aspects of their environmental pollution, it is necessary to understand the kind of transformation they undergo in the body before they are finally excreted. Hence, literature on the metabolic fate of PCBs in animals is gradually accumulating.

Grant et al. (46) suggested that in the rat, components of Aroclor 1254 with a lower chlorine content were metabolized to a greater extent than those with a higher chlorine content. Furthermore, they also reported that the tissue contents of PCB residues were significantly greater in rats with a carbon tetrachloride-damaged liver than in rats with normal liver after the oral administration of the Aroclor, indicating that the liver is a major site of Aroclor 1254 metabolism. Yoshimura et al. (12) investigated the distribution and elimination of PCBs in animals using ^{3}H-labelled Kanechlor 400 and they obtained

similar results to those of Grant et al. (46). In addition, they also found evidence to suggest that the radioactivity eliminated in the feces of rats, although composed mainly of unchanged Kanechlor 400, also included phenolic metabolites after the oral administration of Kanechlor 400. This observation is consistent with the fact that the major metabolic pathway of aromatic compounds is their hydroxylation (45). It has been observed that many of the lower chlorinated biphenyls are preferentially metabolized to their mono- and dihydroxy derivatives (13, 14). Thus, 4-chlorobiphenyl was shown to be metabolized to 4'-hydroxy-4-chlorobiphenyl and its glucuronide in rabbits (14).

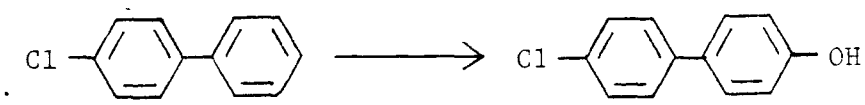

Figure 1. Scheme for metabolism of 4-chlorobiphenyl in rabbits.

It has been suggested that the vicinal hydrogen atoms of PCB are preferred for metabolic breakdown of the molecule (47). The hydroxylation step is thought to proceed via a reactive epoxide (arene oxide) which then yields a phenol by rearrangement as well as a dihydrodiol by hydration with epoxide hydrase and a glutathione conjugate with glutathione-S-epoxidetransferase. The mechanism of 4-chlorobiphenyl metabolism in rabbits was studied by Safe et al. (15). The major urinary metabolites identified were 4'-chloro-4-biphenylol and 4'-chloro-3,4-biphenyldiol. By using 4'-[^{2}H]-4-chlorobiphenyl as substrate, they demonstrated that the 4-chloro-4-biphenylol metabolite retained 79% of the deuterium which is consistent with the intermediacy of an arene oxide in the first hydroxylation reaction. On the other

hand, the diol metabolite was found to retain about one-half of the deuterium found in the phenol (39%) and it was therefore concluded that the diol was not formed directly from the arene oxide, but rather by a direct hydroxylation of the phenolic metabolite. The intermediacy of an arene oxide with an NIH shift (48) in the metabolism of 4-chlorobiphenyl was shown to occur in rat liver microsomes also (16).

Gardner et al. (17) have determined the complete structure of 2,5,2',5'-tetrachlorobiphenyl metabolites in rabbits. They found that animals fed with this tetrachlorobiphenyl excreted three hydroxylated metabolites in their urine of which two were identified as 3-hydroxy- and 4-hydroxytetrachlorobiphenyl. The third metabolite was shown to be trans-3,4-dihydroxytetrachlorobiphenyl. Based on these results it was suggested that the arene oxide, 2,5,2',5'-tetrachlorobiphenyl-3,4-oxide was their intermediate precursor and that this was transformed to dihydroxydiol via hydration and to monohydroxy metabolites by rearrangement. The general scheme for metabolism of PCBs through an arene oxide intermediate is shown in Figure 2.

Goto et al. (18) also reported in their series of studies that 2,3,5,6-tetrachlorobiphenyl may be metabolized to 4'- and 3'-hydroxy, 3',4'-dihydroxy and 4'-hydroxy-3'-methoxy or 3'-hydroxy-4'-methoxy derivatives in rats. Most of the metabolites were found to be excreted into the feces preferentially in the free form, although a small part was excreted into the urine exclusively as conjugates.

Only very few studies have been reported on the metabolism of biphenyls with more than four chlorine atoms per molecule. Goto et al. (19) investigated the metabolism of a pentachlorobiphenyl, an isomer

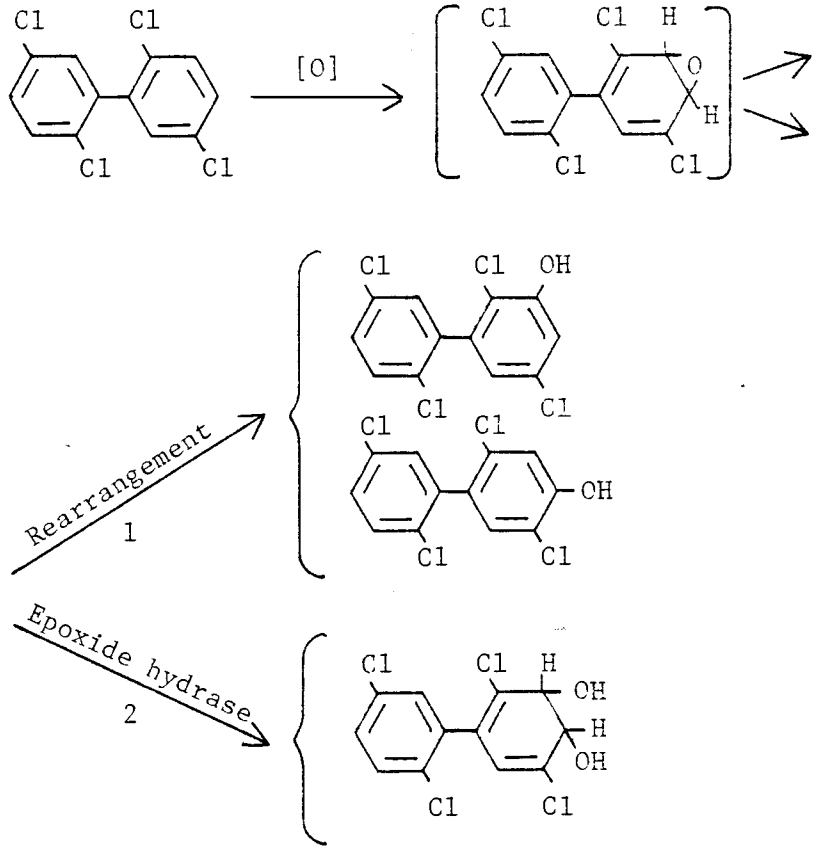

Figure 2. Metabolism of PCBs through an arene oxide intermediate.

in which all the chlorine atoms were located on one side of the phenyl rings, in the rat. Mono- and dihydroxy metabolites as well as methoxy derivatives were identified. The metabolism in mice of 2,4,5,2',4',5'-hexachlorobiphenyl (HCB), a PCB isomer containing only isolated unsubstituted positions was reported by Jensen and Sundstrom (20). It was found that this isomer was metabolized very slowly to 3-hydroxy-2,4,5,2',4',5'-hexachlorobiphenyl as shown below.

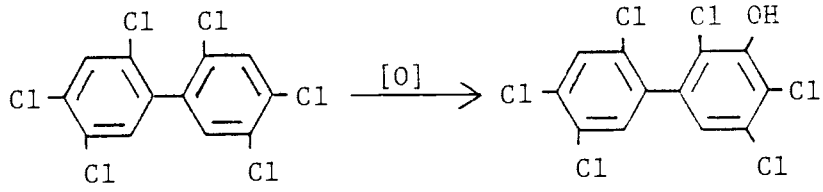

Figure 3. Scheme for metabolism of 2,4,5,2',4',5'-hexachlorobiphenyl in mice.

The total amount of metabolite excreted into the feces over a period of seven days was estimated to be 1.3% of the oral dose. However, no metabolite or its conjugate was detected in the urine. In a previous study, Hutzinger et al. (13) were unable to detect any metabolite of this isomer in the rat, pigeon and trout. Very recently, the metabolism of this same hexachlorobiphenyl isomer was again studied by Hutzinger et al. (49) in rabbits. Rabbits were fed a diet of food pellet containing 1% 2,4,5,2',4',5'-hexachlorobiphenyl for seven days. The concentration of total PCB administered was 1 g/kg. Two major metabolites in the urine, i.e. a hydroxy hexachlorobiphenyl and another metabolite formed by hydroxylation with concomitant dechlorination were identified.

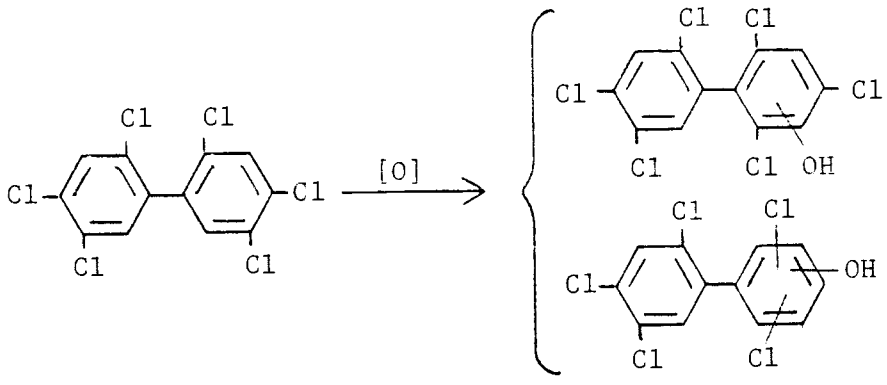

Figure 4. Urinary metabolites of 2,4,5,2',4',5'-hexachlorobiphenyl in rabbits.

The metabolism of 2,4,6,2',4',6'-hexachlorobiphenyl in rats has also been studied (50). The metabolite identified was reported to be 5-hydroxy-2,4,6 2',4',6'-hexachlorobiphenyl. In addition to these observations, Hansell et al. (38) reported that the 2,4,5,2',4',5'-hexachlorobiphenyl disappears rather very slowly from the livers of rats dosed with a single i.p. injection (0.2 mmole/kg) of the hexachlorobiphenyl over a 35-day period. Taken all together, it can be concluded that chlorobiphenyls with four or less chlorine atoms per molecule are rapidly metabolized through an arene oxide intermediate to give the hydroxy and dihydroxy chlorobiphenyls. On the other hand, the metabolism of the more highly chlorinated biphenyls becomes increasingly difficult as the number of chlorine atoms in the molecule increases.

Although hydroxylation of PCBs appears to be their major metabolic product, other kinds of metabolites have also been identified in animal tissues. As mentioned earlier in this section (49), a dechlorinated hydroxy metabolite of 2,4,5,2',4',5'-hexachlorobiphenyl was reported

in the urine of rabbits. The isolation of dechlorinated products from the urine of rats administered chlorobiphenylols, the primary hydroxylated metabolites of PCBs in mammals, has also been reported (51). Very recently, Mizutani (52) has reported the presence and identification of sulfur-containing metabolites of 2,4,2',4'-tetrachlorobiphenyl in the feces of mice given i.p. injection of the biphenyl dissolved in soybean oil and the mice killed six days after the injection. The metabolites identified were 6-methylsulfonyl-2,4,2',4'-tetrachlorobiphenyl and 5-methylsulfonyl-2,4,2',4'-tetrachlorobiphenyl. In addition, two methylthio derivatives of the biphenyl were also reported to be present. The total of all these metabolites excreted over the six-day period was calculated to be only 0.12% of the dose. Hesse et al. (53) investigating the activation of [^{14}C]chlorobiphenyls to protein-binding metabolites by rat liver microsomes observed that the majority of reactive metabolites of 2,2'-dichlorobiphenyl arise from secondary metabolism, i.e. the subsequent oxidation of the phenolic metabolites. They suggested that arene oxides, the primary products, appear to play a minor role in the protein binding of the dichlorobiphenyl used. From their results it was hypothesized that semiquinones and quinones are the possible secondary metabolites of PCB and that these are primarily responsible for protein binding. The formation of glutathione conjugates arising primarily from the arene oxide intermediates are also possible metabolites likely to be present in animal tissues.

In conclusion, PCBs are metabolized in animal tissues primarily by hydroxylation. With lower chlorinated biphenyls having adjacent unsubstituted carbon atoms, hydroxylation proceeds rapidly through an

arene oxide intermediate to give monohydroxy derivatives by rearrangement and dihydroxy derivates by epoxide hydrase. The higher chlorinated PCBs, especially those with only isolated unsubstituted carbon atoms, are metabolized rather slowly by direct hydroxylation and it appears the monohydroxy derivatives are the primary products. In addition to the hydroxylated metabolites of PCB, other kinds of metabolites presumably arising from further metabolism of the hydroxylated derivatives have been reported. Thus, sulfonated derivatives, dechlorinated hydroxy products, methoxy derivatives, semiquinones and quinones as well as glutathione and glucuronide conjugates have all been reported to be possible metabolic products of PCBs in animal tissues. Although most of these additional metabolites are present in minor quantities as opposed to the hydroxylated metabolites, their presence is nevertheless real, and possible interference of these other metabolites with the normal biochemical processes in the cell has to be kept in mind.

Scope of This Work

The work described in this thesis has been designed primarily in an effort to elucidate the mechanism of action of polychlorinated biphenyls and their related metabolites on phospholipid and glyceride biosynthesis. Several lines of approach were taken to accomplish this task. First, the effect of PCBs on the *in vitro* synthesis of phospholipids and glycerides was studied in different subcellular fractions in order to compare their similarities and differences. Secondly, the results obtained *in vitro* were compared to those obtained after *in vivo*

treatment of the animals with PCB so as to fully assess the significance of the direct effects observed *in vitro*. Commercial PCB preparations are a heterogeneous mixture of different isomers, therefore, a purified isomer, 2,4,5,2',4',5'-hexachlorobiphenyl was also used in these studies to determine whether it elicits the same kind of response as the commercial PCBs. In order to determine the site(s) of action of PCBs on the biosynthetic process, the activities of some key enzymes in the pathway were determined in the absence and presence of PCBs. Finally, the mode of action of the primary metabolites of PCB on phospholipid and glyceride biosynthesis was studied *in vitro*.

The overall results indicate that PCBs and their metabolites cause a severe inhibition in the synthesis of phospholipids and glycerides at the site of reaction catalyzed by sn-glycerol-3 phosphate acyltransferase, with little or no effect on the activities of other enzymes studied in the pathway. The major part of the work reported in this thesis has already been published. The mode of action of PCBs on phospholipid and glyceride biosynthesis was reported in an abstract form (54) and formed part of the article by Dzogbefia *et al.* (55). The results of the effects of the primary metabolites of PCBs on phospholipid and glyceride synthesis was also reported in an abstract form (56) pending its later publication. The biosynthetic pathway being studied is illustrated in Figure 5 with the enzymes of interest indicated.

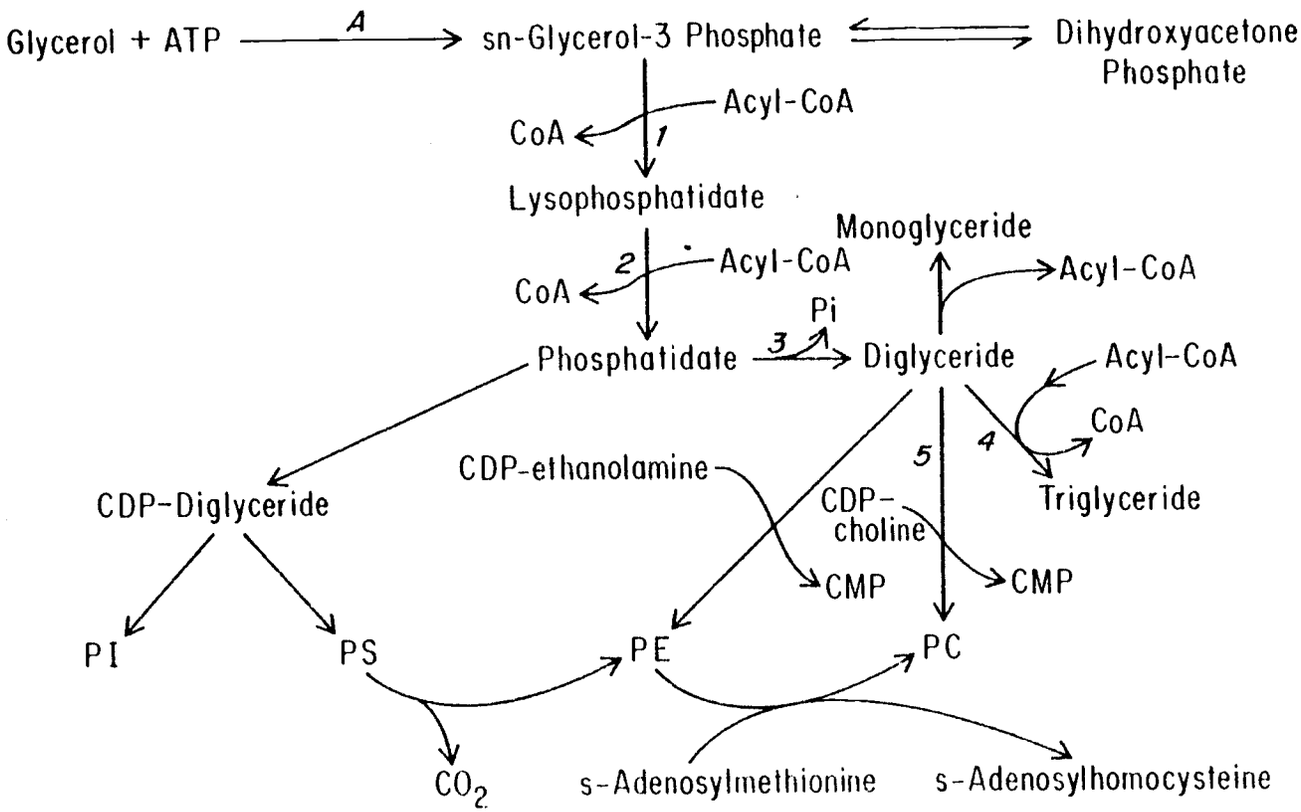

Figure 5. Pathway of phospholipid and glyceride biosynthesis from sn-glycerol-3 phosphate.

Key: A, glycerol kinase; 1, sn-glycerol-3 phosphate acyltransferase; 2, lysophosphatidate acyltransferase; 3, phosphatidate phosphatase; 4, diglyceride acyltransferase; 5, phosphorylcholine-glyceride transferase.

MATERIALS AND METHODS

Chemicals

Potassium chloride, ATP, dithiothreitol, Tris(hydroxymethyl) amino ethane (Tris), bovine serum albumin, D,L-α-glycerol-3 phosphate (hexahydrate), phenol reagent, cytidine 5'-diphosphocholine (sodium salt), 1-amino-2-naththol-4-sulfonic acid, NAD, cysteine hydrochloride, sucrose, and glycerol phosphate dehydrogenase were purchased from Sigma Biochemical Corporation, St. Louis. Sodium carbonate, sodium sulfite, sodium bisulfite, sodium chloride, glycerol, potassium hydroxide, potassium monobasic phosphate, chloroform, methanol, ethanol (95% and absolute), diethyl ether, petroleum ether, benzene, n-propanol, ethyl acetate and hexane were obtained from Mallinckrodt Chemical Works, St. Louis. Magnesium chloride, sodium hydroxide, glacial acetic acid, concentrated hydrochloric acid, EDTA, diphenylamine, magnesium sulfate, 2,5-diphenyloxazole (PPO), hydrazine sulfate, and potassium carbonate were obtained from J. T. Baker Company, Phillisburg. Coenzyme A, palmitoyl coenzyme A, triolein, 1,3-diolein, α-monoolein, oleic acid, phosphatidic acid, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine and lysolecithin were purchased from P-L Biochemicals, Inc., Millwaukee. Ammonium molybdate, sodium potassium tartrate and copper sulfate were purchased from Baker and Adamson (Allied Chemicals), New York. Radioactive compounds such as L-[^{14}C(U)]glycerol-3 phosphate, palmitic acid [^{14}C(U)], palmitoyl-coenzyme A-1-^{14}C, glycerol[1,3-^{14}C] and cytidine diphosphocholine-[methyl-^{14}C] were purchased from New England Nuclear, Boston. The

isomer, 2,4,5,2',4',5'-hexachlorobiphenyl was purchased from Analabs, New Haven. Trichloroacetic acid and sodium fluoride were obtained from Matheson, Coleman and Bell, Rutherford, N.J. D,L-α-tocopherol was purchased from Pfaltz & Bauer, Inc., Stamford, Conn. Acetaldehyde was obtained from Eastman Organic Chemicals, Rochester. Concentrated sulfuric acid was purchased from VW&R. Toluene (Scintillar grade) and diethylamino ethyl (DEAE) cellulose disc filter papers were purchased from Scientific Products. Perchloric acid (70%) was purchased from G. Frederick Smith Chemical Company, Columbus. Calf thymus DNA was a gift from Dr. K. E. Van Holde of the Department of Biochemistry and Biophysics at Oregon State University. Mercury was a gift from Dr. R. R. Becker of the Department of Biochemistry and Biophysics at Oregon State University. Adsorbosil-5, Adsorbosil-1 and 1,2-diolein-sn-glycerol were purchased from Applied Science Laboratories, State College, Pa. Silica gels H and G were purchased from E. M. Laboratories, Inc., Amsford, N.Y. 4'-Chloro-4-biphenylol and 2',3',4',5,5'-pentachloro-2-biphenylol were obtained from R. F. R. Corporation, Rhode Island. Aroclor 1254 was a gift from Monsanto Chemical Company, St. Louis. 1,4-Bis-[2-(5-phenyloxazoyl)]-benzene (POPOP) was purchased from Research Products International Corporation, Ill.

In vivo Pretreatment of Rats with Aroclor 1254

In order to determine the effect of Aroclor 1254 (PCB), in vivo, on phospholipid and glyceride biosynthesis, female Wistar rats were fed a standard diet [dextrose, 67% (w/w); casein, 22% (w/w); Hubbel-Mendel-Wickman salts vitamin mix; minerals, 6.0% (w/w); and Mazola

corn oil, 5% (w/w)]. Experimental rats received the standard diet containing 0.1% (w/w) Aroclor 1254 while the control rats received the standard diet only. The rats were maintained ad libitum on this diet for 30 days. At the end of the 30 days the rats were sacrificed, subcellular fractions prepared from the livers and used as required.

Preparation of Liver Homogenate

Female Wistar rats, age between 2 1/2 to 3 months and weighing 200-300 grams were used throughout this project. The rats were decapitated with scissors and the livers quickly removed and placed in a beaker containing a few milliliters of buffer which was previously chilled on ice. The livers were minced with scissors and homogenized in four volumes of 0.25M sucrose containing 0.01 M Tris-HCl buffer, pH 7.5 using a Potter Elhvejem homogenizer at 4°C. The homogenate was then centrifuged at 2,400 rpm for ten minutes in a Sorvall refrigerated centrifuge to remove nuclei and cell debris. The supernatant was filtered through a piece of muslin cloth to get rid of the fat layer formed on the surface. The filtrate was used as a crude homogenate for following the incorporation of radioactivity into phospholipids and glycerides. This crude homogenate was stable for at least four weeks with less than 20% loss in activity, when the homogenate was quickly frozen in liquid nitrogen and stored at -40°C. The homogenate varied somewhat in activity from one preparation to another.

Preparation of Mitochondria

For the preparation of mitochondria, the livers were homogenized in four volumes of 0.25 M sucrose-Tris buffer, pH 7.5. The homogenate was first centrifuged at 800 xg for 15 mintues and the supernatant saved. The nuclear pellet was rehomogenized in the same buffer, centrifuged and the supernatants combined. The resulting supernatant was then centrifuged at 10,000 xg for 15 minutes. The supernatant was discarded and the mitochondrial pellet resuspended twice in sucrose-Tris buffer and centrifuged to remove contaminating microsomes. The isolated mitochondria were then suspended in one-third the original volume of buffer.

Preparation of Microsomes

For the preparation of microsomes, which was the preparation used in most experiments, the procedure described by Fallon and Lamb (57) was adopted. The livers were homogenized in sucrose-Tris HCl buffer, pH 6.5, containing 1 mg/liter of D,L-α-tocopherol. The homogenate was first centrifuged at 400 xg for ten minutes and the supernatant saved. The supernatant was centrifuged twice at 16,000 xg for 15 minutes to remove mitochondria. The resulting 16,000 xg supernatant was then centrifuged at 105,000 xg for 30 minutes in a Spinco Model L ultracentrifuge maintained at 4°C. The microsomes were washed and resuspended in the same buffer. The microsomes were then rehomogenized and centrifuged again at 105,000 xg for 30 minutes. The resulting microsomal pellets were resuspended in one-third the original volume

of buffer and carefully homogenized by hand. When microsomes were quickly frozen in liquid nitrogen and stored at -40°C they retained over 80% of the original activity for more than three months. There was a large variation in the activity from one preparation to another. The protein concentration of each microsomal preparation was determined before being used.

Standard Assay Procedure in Homogenate and Mitochondria

For the incorporation of ^{14}C-sn-glycerol-3 phosphate into phospholipids and glycerides by liver homogenate, the incubation medium contained 0.01 ml KCl (50 mM), 0.04 ml Tris-HCl, pH 7.5 (20 mM), 0.001 ml $MgCl_2$ (3.6 mM), 0.1 ml ATP (3.6 mM), 0.02 ml dithiothreitol (0.7 mM), 0.028 ml Coenzyme A (40 µM), 0.1 ml BSA (2.5 mg), 0.1 ml sodium palmitate (1.42 mM) and 0.01 ml glycerol-3 phosphate (0.12 mM, sp. act. 10 µCi/µmole) in a total volume of 0.7 ml. The reaction was initiated by the addition of 0.2 ml of the liver homogenate and the tubes incubated at 37°C for 15 minutes. Incubations were carried out with and without Aroclor 1254 dissolved in ethanol. Control incubations indicated that ethanol at the levels employed had no inhibitory effect on the reaction. The incubation conditions described above are those established by Adams *et al*. (58) as being optimal for ^{14}C-glycerol-3 phosphate incorporation into lipids by liver homogenate. At the end of the incubation the reactions were stopped with 10 ml of chloroform-methanol 2:1 (v/v). Zero time reactions were carried out routinely and these values always substracted from the 15 minute values. When mitochondria were used as the enzyme source for the reaction, the

incubation conditions were exactly as those described for the liver homogenate except that 0.2 ml of mitochondrial preparation was used instead of 0.2 ml homogenate.

Standard Assay Procedure in Microsomes

When microsomal preparations were used to carry out the reaction, the incubation medium contained 0.05 ml Tris-HCl, pH 6.5 (70 mM), 0.08 mg dithiothreitol (1.5 mM), 0.1 ml palmitoyl-CoA (40 nmoles), 2.5 mg of fatty acid poor BSA, 4 µl ^{14}C-glycerol-3 phosphate (0.17 mM, Sp. act. 6.7 µCi/µmole) and 0.3 mg microsomal protein in a total reaction volume of 0.34 ml. The reaction was routinely initiated by the addition of microsomes and after 15 minutes incubation at 37°C, 10 ml chloroform-methanol 2:1 (v/v) was added to each tube in order to stop the reaction. Lipids were extracted from the incubation medium as described in the next section. The assay system described above is that of Fallon and Lamb (54) for essentially following the activity of microsomal Acyl CoA-sn-glycerol-3 phosphate acyltransferase.

Extraction of Total Lipids from Incubation Medium

After the reactions were stopped with 10 ml chloroform-methanol 2:1 (v/v), the total volume in each tube was transferred separately into a homogenizing tube and homogenized with two strokes of the pestle. The homogenized sample was then filtered through a Whatman No. 1 filter paper into 30 ml corex centrifuge tubes. The homogenizing tube and the pestle were rinsed with 5 ml of the same chloroform-methanol and this was used to rinse the filter paper as well. To the

filtrate in the 30 ml centrifuge tube, 3 ml of glass distilled water was added and then mixed vigorously on a vortex mixer. The samples were then centrifuged at 3000 rpm for 10 minutes in a Sorvall centrifuge. At the end of the centrifugation, the aqueous upper phase was carefully aspirated off. To the bottom chloroform layer, 0.2 ml of methanol-water 1:1 (v/v) was added and the contents again mixed, centrifuged and the upper phase removed as described above. The chloroform layer containing the total lipids was evaporated under nitrogen and the lipids picked up in 1 ml of chloroform. Ten percent of the total lipid extract, i.e. 0.1 ml from above was routinely counted by liquid scintillation counting to ascertain that there was significant enzymatic activity before proceeding to separate the individual radioactive lipids by thin layer chromatography (TLC). This extraction procedure is that established by Folch et al. (59).

Purification of Silica Gel H

For the separation of individual phospholipis, either silica gel H (type 60) without $CaSO_4$ binder or Adsorbosil-5 prekoted plates purchased from Applied Science Laboratories was routinely used. The silica gel H was always purified before being used for preparing thin layer plates. The silica gel H (E M Laboratories, Inc.) was purified according to the method of Parker and Peterson (60). One hundred and twenty-five grams of silica gel H were purified at one time. All solvents were of reagent grade and distilled prior to use. The silica gel H was placed in a Buchner #4 funnel which was first lined with two discs of Whatman #2 filter paper and the funnel attached to a suction

flask. One liter of methanol-formic acid-chloroform 2:1:1(v/v/v) was carefully poured over the silica gel to thoroughly wash it. The solvent was then filtered with suction under vacuum. This was then followed by washing the silica gel with two liters of distilled water. The wet gel was then placed in a Pyrex dish lined with aluminum foil and dried in the oven for 48 hours at 110°C. At the end of the drying process, the tiny lumps left in the gel were carefully broken by grinding in a mortar and the gel stored in a covered container until needed.

Separation of Individual Phospholipids from Total Glycerides by Thin Layer Chromatography (TLC)

In order to separate individual phospholipids from total neutral lipids, different solvent systems of varying polarity were employed. Separations were carried out on 20 x 20 cm silica gel H coated plates or on Adsorbosil-5 prekoted plates. The routine procedure for carrying out the TLC separations was as described below.

The thin layer plates were first activated at 105°C for 45 minutes before use. The chromatographic tanks were lined with Whatman paper and the tanks presaturated with the solvent which was to be used for the separation. Saturation of the tanks was carried out for at least 40 minutes before the plates were placed in. After the plates had been activated they were allowed to cool to room temperature before being used.

The lipid samples which were evaporated to dryness under nitrogen were redissolved in 0.05 ml of distilled chloroform. This was then

applied to the plates using a 50 µl Hamilton syringe. The samples were spotted in bands of 1 cm length and 2 cm apart with the help of a template. Each spot, upon application was dried under a stream of hot air. Standard phospholipids (50 µg) were simultaneously spotted to help in identifying the radioactive phospholipid spots. Each chromatographic tank contained 200 ml of the developing solvent. The plates were supported on plate holders and carefully lowered into the tanks making sure that the sides did not touch the linings. The tanks were first sealed with a polyethylene food wrap, followed by a glass cover plate and then a number of heavy books. These precuations were necessary in order to minimize solvent evaporation during the chromatography so that a superior separation could be attained. The chromatograms were allowed to develop until the solvent front was 2 cm from the top of the plates. When this distance was attained, the plates were removed from the tank, the solvent front marked and then the solvent evaporated under a stream of hot air.

The solvent systems used for phospholipid separation were (1) chloroform-methanol-7 N NH_4OH 60:35:5 (v/v), (2) chloroform-methanol-water 65:25:4 (v/v), (3) chloroform-methanol-acetic acid-water 25:15:4:2 (v/v). All the above solvent systems separated the phospholipids and moved the neutral lipids with the solvent front. It was, however, discovered that when chloroform-methanol-acetic acid-water 25:15:4:2 (v/v) was used, it hydrolyzed all the phosphatidic acid, which turned out to be one of the major radioactive products in our system to neutral lipids, most likely diglyceride. Thus, since the discovery of this phenomenon, the use of this solvent was discontinued.

At the end of the TLC separation, the plates were exposed to iodine vapor and the lipid spots which were stained yellow were quickly marked with a needle. The iodine was then allowed to evaporate before the spots were scraped into scintillation vials for radioactivity determination.

Separation of Individual Glycerides from Total Phospholipids

When indiviudal glycerides were to be separated from total phospholipids, the method of Freeman and West (61) was used. Thin layer plates (20 cm x 40 cm) and coated with 0.25 mm thickness of silica gel G was used. The lipids in chloroform were spotted on the plates as described for phospholipids. The separation of the lipids was achieved using a double development procedure. The plates were first developed in diethyl ether-benzene-ethanol-acetic acid 40:50:2:0.2 (v/v) until the solvent front reached the 25 cm mark from the origin. The plates were removed from this solvent and gently dried under a warm stream of air in order to get rid of all traces of acetic acid from the plates. The plates were then transferred to the second solvent system - diethyl ether-hexane 6:94 (v/v). This solvent system was run up to 2 cm from the top of the plate. At the end of the run, the plates were first air dried and then given a final drying at 60°C for 30 minutes. Lipid spots were identified as described in the earlier section. The spots were scraped into scintillation vials and their radioactivity determined.

Assay for Radioactivity

The radioactivity of the phospholipids and glycerides was assayed by liquid scintillation counting in a Packard "Tri-Carb" liquid scintillation spectrometer Model 3003. The suitability of the method stems from the fact that both the phospholipids and glycerides are readily soluble in the solvents used for preparing the scintillation fluid. Liquid scintillation counting is advantageous because of the ease of use and its high efficiency. It, however, has one disadvantage in that once the material has been dissolved in the scintillation fluid it cannot be recovered for any further analysis.

The scintillation fluid used for counting was made up of 4 g of 2,5-diphenyloxazole (PPO) and 30 mg of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) dissolved in one liter of scintillar grade toluene. Ten milliliters of this fluid was added to each vial. Radioactivity was determined without prior elution of the lipids from the silica gel since control experiments indicated that the silica gel did not interfere significantly with the counting efficiency. The spectrometer with a window setting of 50 to 1000 and a gain of 8% has an efficiency of 85%. All data reported in this study has been corrected for background counting.

Determination of Total Phospholipid Phosphorus Content

When data were to be normalized on the basis of lipid phosphorus, the total phospholipid phosphorus content of the reaction medium was determined. The method employed was that of Raheja *et al.* (62) in

which the phospholipid phosphorus was determined with a chromogenic reagent without prior acid digestion.

Reagents: Mercury, ammonium molybdate, concentrated HCl, concentreated H_2SO_4, chloroform, methanol.

Preparation of chromogenic solution: First, 16 g of ammonium molybdate was dissolved in 120 ml of water to give solution I. Solution II was prepared by shaking 40 ml of concentrated HCl and 10 ml of mercury with 80 ml of sulution I for 30 minutes followed by filtration through Whatman No. 1 filter paper. To the remainder of solution I, 200 ml of concentrated H_2SO_4 was carefully added. To this resultant solution, solution II was added to give, after careful mixing, the chromogenic solution III. This solution was stable for at least three months when stored at 4°C.

Procedure for Phosphorus Determination

The reactions were carried out in small 10 ml test tubes. The lipid samples in chloroform or an aliquot of the total lipid samples was evaporated to dryness under nitrogen. To each tube, 0.4 ml of chloroform and 0.1 ml of the chromogenic solution were added. The tubes were placed in boiling water for 1.5 minutes. After cooling the tubes to room temperature they were allowed to stand for five minutes then 5 ml of chloroform was added to each tube and the tubes gently shaken. The resulting blue colored solution was carefully decanted into 3 ml cuvettes and the absorbance at 710 nm measured against a distilled water reference in a Beckman Model DB spectrophotometer. For the preparation of the calibration curve, standard

phosphatidyl ethanolamine (0-10 µg) was used. Blank tubes contained 0.4 ml chloroform plus reagent. The lipid phosphorus content of the samples was determined from the calibration curve shown in Figure 6.

Determination of Protein Content of Liver Preparations

The method of Lowry et al. (63) was employed in determining the protein concnetration of the liver preparations.

Reagent A: 2% Na_2CO_3 prepared in 0.1 M NaOH.

Reagent B: 0.5% copper sulfate in 1% sodium potassium tartrate.

Reagent C: Mix 50 ml reagent A with 1 ml of reagent B. Discard after one day.

Folin Reagent: Dilute phenol reagent (Folin-Ciocalteu) 1:1 with distilled water.

Procedure: One milliliter of the liver preparations, homogenate, mitochrondria, microsomes or an aliquot of each diluted to 1 ml with distilled water was pipetted into each test tube. Bovine serum albumin (BSA, 0-100 µg/ml) was used for preparation of the calibration curve. All determinations were carried out in duplicate. Blank tubes contained 1 ml distilled water. To the samples and standards, 5.0 ml of reagent C was added and the contents immediately mixed on a vortex mixer. The tubes were then allowed to stand for ten minutes. After ten minutes, 0.5 ml of Folin reagent (diluted 1:1) was added to each tube and the contents again well mixed. The tubes were then placed in the dark for 30 minutes to allow color development. The absorbance of the resulting blue color was read in a Beckman double beam spectorphotometer. The protein content of the liver preparations was determined from the calibration curve shown in Figure 7.

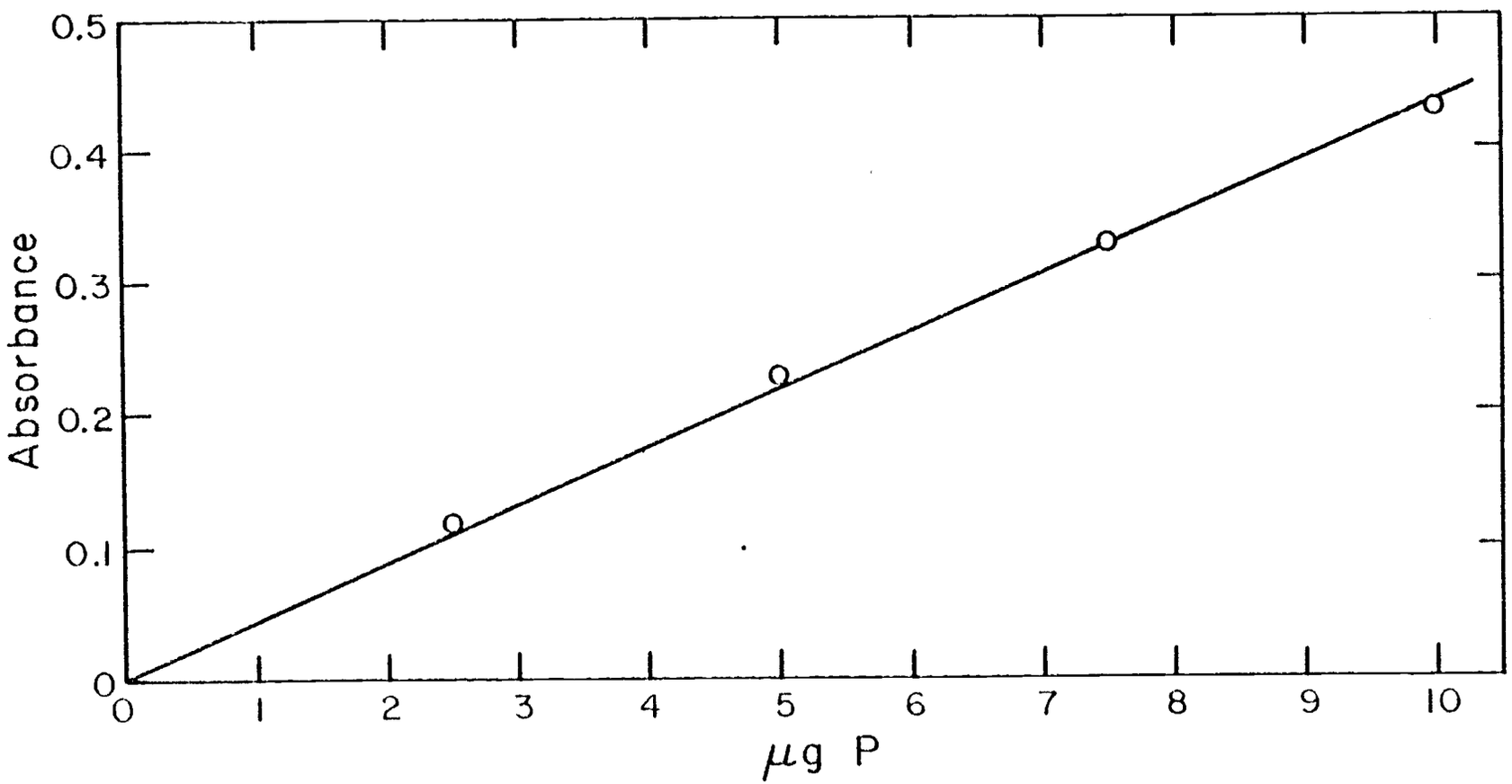

Figure 6. Standard curve for phospholipid phosphorus.

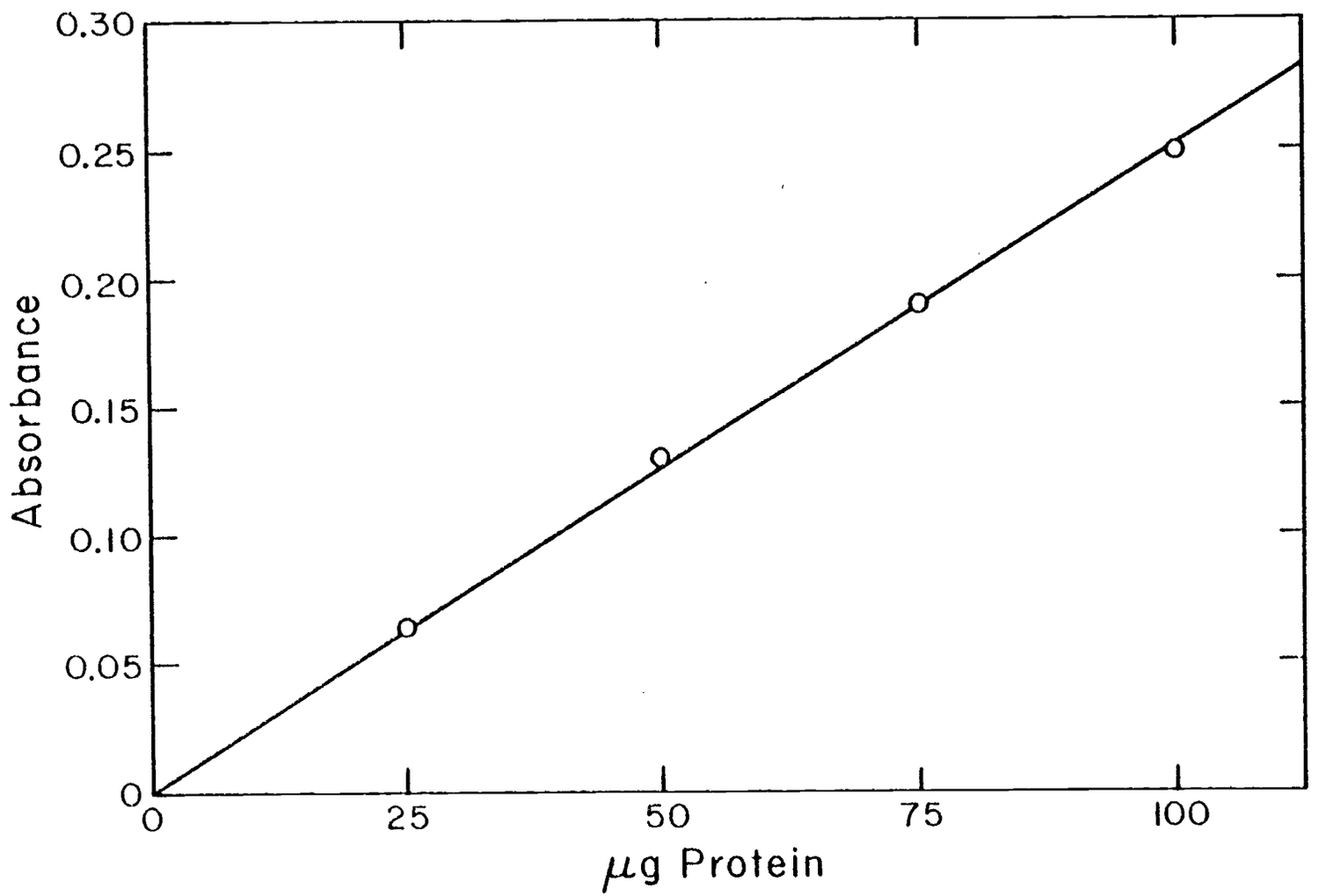

Figure 7. Standard curve for protein.

Determination of DNA Content of Rat Liver Preparations

In all the *in vivo* experiments the results obtained were normalized, where appropriate, on the basis of the DNA content of the livers. This is necessary so as to correct for any changes in cell number that might arise as a result of the PCB treatment. DNA was determined as described by Burton (64).

Reagents:

1. Aqueous acetaldehyde (1.6%): Prepare by first cooling the acetaldehyde and transfer 1 ml in a cooled pipette into 50 ml of distilled water.

2. Diphenylamine reagent: Dissolve 1.5 g in 100 ml of glacial acetic acid. Then add 1.5 ml of concentrated sulfuric acid. Just before use, add 0.1 ml of 1.6% aqueous acetaldehyde per 20 ml of reagent. Reagent is stable without the acetaldehyde for at least three months at 4°C.

3. DNA standards: Dissolve an amount of calf thymus DNA in 5 mM NaOH to give a concentration of 0.3 mg/ml. Prepare working standards from stock by mixing equal volumes of stock standard and 1 N perchloric acid and heat at 70°C for 15 minutes. Store at 4°C.

4. 0.5 M perchloric acid: Prepare from 70% perchloric acid solution by diluting 7.18 ml to 100 ml with glass distilled water.

5. 0.2 N perchloric acid: Prepare from 0.5 M solution above.

6. 0.3 M KOH:

Procedure: Five milliliters of a 20% liver homogenate (w/v) was measured into a 30 ml Corex glass centrifuge tube. Then 5 ml of 0.3 M KOH was added to each and the tubes incubated at 37°C for one hour.

the tubes were then cooled on ice after the incubation and the acidity adjusted to 0.2 M with 70% perchloric acid to precipitate DNA and proteins. The precipitates were centrifuged at 10,000 xg for ten minutes, the supernatant discarded and the precipitates washed once with 4 ml of 0.2 M perchloric acid. DNA was then extracted from the precipitates with 4 ml of 0.5 M perchloric acid and incubated at 90°C for 30 minutes (these conditions were used for DNA extraction from animal tissues only). At the end of the incubation, the tubes were cooled and then centrifuged at 10,000 xg for ten minutes and the supernatant saved for analysis.

One milliliter of the extracted DNA samples was pipetted into test tubes and 2 ml of diphenylamine reagent added. One milliliter of the DNA working standards (0-24 µg) were also pipetted into test tubes and 2 ml reagent added. The blank tubes contained 1 ml of 0.5 M perchloric acid and 2 ml reagent. All determinations were carried out in duplicate. The tubes were incubated at 25-30°C for 17 hours and the absorbance at 600 nm was determined in a Beckman spectorphotometer. The DNA content of the samples was determined from the calibration curve. It can be seen in Figure 8 that there is a linear response between 6-24 µg DNA.

Assay for Glycerol Kinase Activity

The activity of glycerol kinase (ATP: glycerol phosphotransferase, EC 2.7.1.30) was measured by following the conversion of glycerol [1,3-^{14}C] to glycerol-3 phosphate by liver homogenate. The reaction involved is shown below.

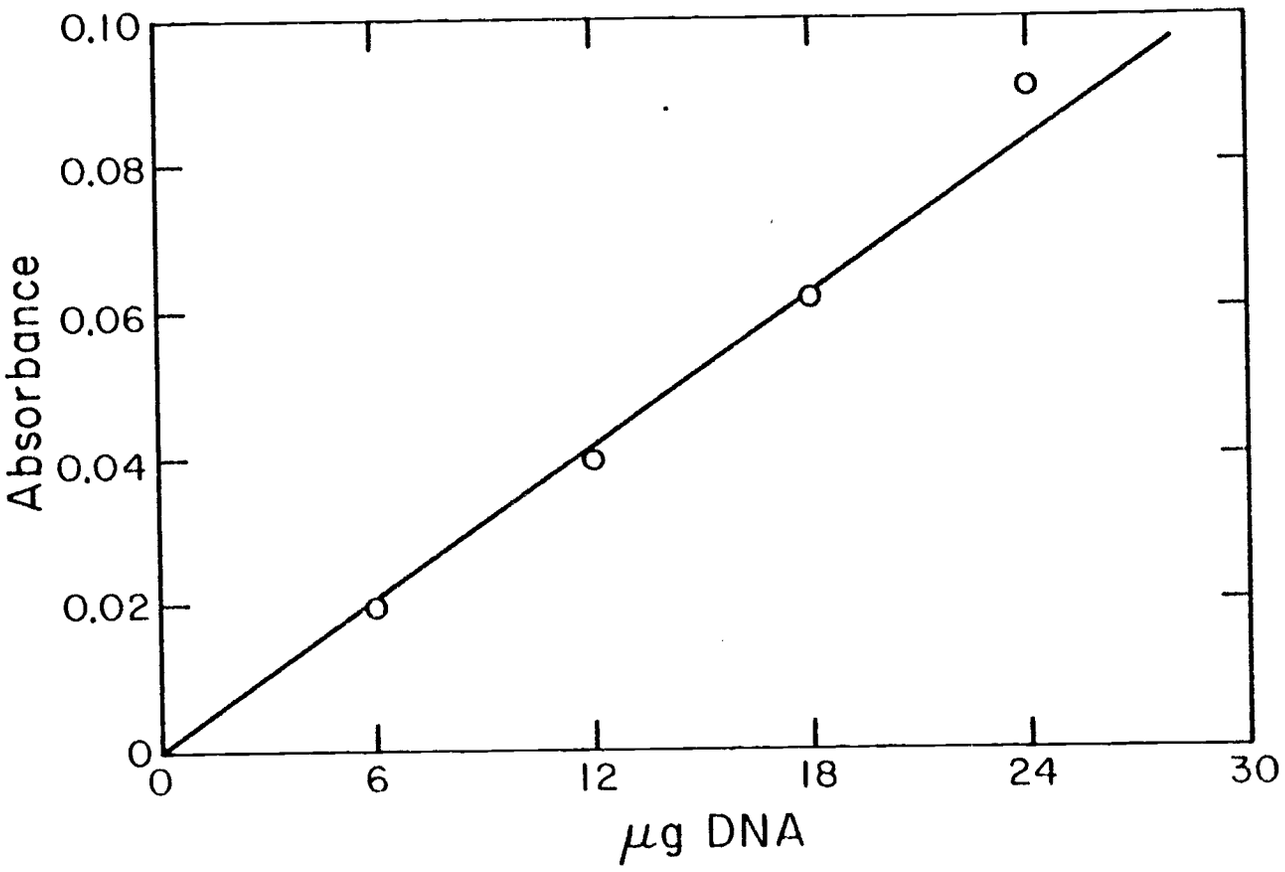

Figure 8. Standard curve for DNA.

$$\text{Glycerol} + \text{ATP} \xrightarrow[\text{Kinase}]{\text{Glycerol}} \text{Glycerol-3 phosphate} + \text{ADP}$$

After the enzymic conversion, radioactive glycerol and glycerol-3 phosphate were separated using DEAE-cellulose paper. The uncharged glycerol was washed off the paper with water while the anionic product remained adsorbed onto the paper. The radioactivity adhering to the paper was measured by liquid scintillation counting giving an estimate of the enzyme activity. The advantages of this method are the simplicity of the assay procedure as well as the high degree of sensitivity of the estimation. The procedure described here is essentially that of Robinson and Newsholme (65) and Newsholme *et al.* (66) as modified by Adams *et al.* (58).

Procedure: Rat livers were homogenized in four volumes of 1% KCl containing 1 mM EDTA and centrifuged at 3,000 rpm to remove nuclei and cell debris. This homogenate was diluted 1:4 with incubation medium (minus substrate) before assay. The homogenate (0.05 ml) was incubated with 0.1 ml Tris-HCl, pH 7.5 (85 mM), 0.01 ml EDTA (1.25 mM), 0.001 ml $MgSO_4$ (3.4 mM), 0.294 mg NaF (21 mM), 0.74 mg dithiothreitol (17 mM), 0.16 mg ATP (3.6 mM) and 0.103 mg glycerol (4 mM, sp. act. 0.3 µCi/µmole) in a total reaction volume of 0.28 ml. Incubations were carried out with and without Aroclor dissolved in ethanol. The reaction tubes were incubated at 28-30°C for 5 minutes and stopped with 0.23 ml of 98% ethanol. Zero time reactions were routinely carried out and the final results were corrected for these.

The radioactive glycerol-3 phosphate was separated from the unreacted glycerol as follows: at the end of the incubation 40 µl of the samples were carefully pipetted onto the centers of discs of

DEAE-cellulose paper with continuous drying under a stream of hot air from a hair dryer. This was necessary so that the samples remained as discreet spots at the centers of the discs without spreading to the edges. The discs were placed on filter holders that were lined with two unused discs and washed slowly with 200 ml distilled water with suction under vacuum. At the end of the washing process, the discs, still on the filter holders were dried in the oven at 100°C for 25-30 minutes. The three discs on each filter holder were dried together. After drying, the radioactive disc was sandwiched between the other two discs, placed in scintillation vials and counted in 10 ml of regular scintillation fluid. The efficiency of the method, as pointed out by Newsholme et al. (66) was more than 90% with the rate of product formation being the same whether measured radiochemically or enzymatically.

Assay for Phosphatidate Phosphatase Activity

The activity of phosphatidate phosphatase (a microsomal particulate preparation) was measured by determining the amount of inorganic phosphorus released from the hydrolysis of phosphatidic acid. The activity of the enzyme was measured in the presence and absence of PCB. Enzyme activity was measured by the method of Lamb and Fallon (67).

Reagents: 0.07 M Tris-malate, pH 6.5; phosphatidic acid

Procedure: First, the benzene in which the phosphatidic acid was purchased was evaporated under nitrogen. Then the solid phosphatidic acid left in the tube was carefully made into a fine suspension in

Tris-malate, pH 6.5 by vortexing. The incubation medium contained 0.3 ml of Tris-malate, pH 6.5 (35 mM) and 0.01 ml phosphatidic acid (1.3 mM). The reaction was initiated by the addition of microsomes (0.9-1.2 mg protein) in a total reaction volume of 0.6 ml. The tubes were incubated at 37°C for periods of 1-3 hours in a Research Specialties oscillating water bath. At the end of each incubation period. 0.4 ml of 5% trichloro acetic acid was added to each tube to stop the reaction. The tubes were cooled on ice for five minutes and then centrifuged at 600 xg for ten minutes in a clinical centrifuge to sediment protein precipitates. The inorganic phosphorus content of the supernatant was determined by the standard method of Fiske and Subbarow.

Determination of Inorganic Phosphorus

Reagents: 15% sodium bisulfite - dissolve 15 g sodium bisulfite in 100 ml distilled water; 20% sodium sulfite - dissolve 20 g in 100 ml distilled water; 5 M H_2SO_4; 0.073 M H_2SO_4; 2.5% ammonium molybdate; monobasic potassium phosphate 12.9 mM (400 µgPi/ml); 0.1 M H_2SO_4; 1-amino-2-naphthol-4-sulfonic acid.

Preparation of the reducing agent: One hundred milligrams of 1-amino-2-naphthol-4-sulfonic acid was dissolved in 39 ml of 15% sodium bisulfite and 1 ml of 20% sodium sulfite. This solution was then filtered to remove undissolved material.

Procedure: The supernatant obtained after the phosphatase assay was carefully pipetted into test tubes. The approximate yield of supernatant was 0.8 ml. To this 0.8 ml sample, 6.8 ml of 0.073 M H_2SO_4 was

added. (Note: the concentration of H_2SO_4 must be adjusted when starting with smaller or larger volume of sample). This was well mixed by vortexing and then 0.9 ml of 5 M H_2SO_4, 1.0 ml of 2.5% ammonium molybdate, and 0.4 ml of the reducing agent were added in the order listed with thorough mixing upon each addition. The tubes were allowed to stand for ten minutes, after which a blue color developed. The absorbance was measured by means of a Beckman spectrophotometer at 660 nm against a reagent blank in which 0.8 ml distilled water replaced the 0.8 ml sample. The inorganic phosphorus content of each tube was estimated from a calibration curve.

Standard Curve for Inorganic Phosphorus Determination

Reagent: 12.9 mM monobasic potassium phosphate. This was prepared by dissolving 1.75 g in 0.1 M H_2SO_4. This standard contains 400 µg Pi/ml. The stock solution was diluted to various concentrations (0-60 µg/ml) and 0.8 ml of each was used for the determination of Pi as described above. Figure 9 shows that a linear response is obtained up to 60 µg Pi.

Assay for Diglyceride Acyltransferase Activity

The final reaction step in the biosynthesis of triacylglycerol is catalyzed by acyl-CoA:1,2,-diglyceride O-acyltransferase (EC 2.3.1.d) (diglyceride acyltransferase), a microsomal particulate preparation. The reaction involved is:

1,2-diacyl-sn-glycerol + Acyl-CoA ⟶ Triacylglycerol + CoA

Thus, one can measure the activity of this enzyme by following the

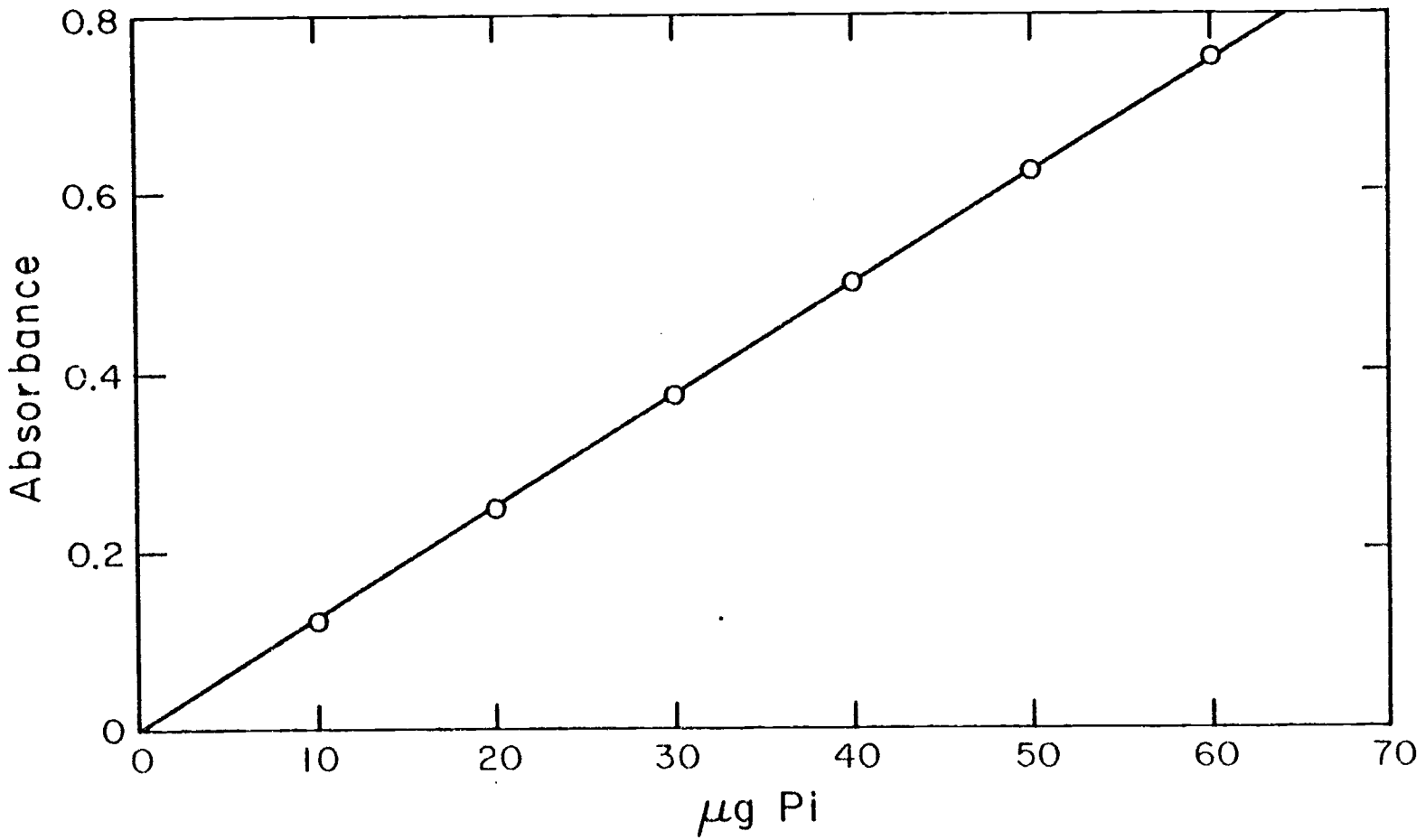

Figure 9. Standard curve for orthophosphate.

radioactivity of ^{14}C-Acyl-CoA incorporated into triacylglycerol under appropriate conditions. The procedure described here is a modification of the method of Young and Lynen (68).

Preparation of the enzyme system: Rat livers were homogenized in four volumes of 0.25 M sucrose containing 0.02 M Tris-HCl, pH 7.4 and 0.001 M EDTA by means of a Potter-Elvehjem homogenizer. The crude homogenate was centrifuged at 10,000 xg for ten minutes in a Sorvall refrigerated centrifuge to remove mitochondria and larger fragments. The 10,000 xg supernatant was then centrifuged at 100,000 xg for 60 minutes in a Spinco Model L ultracentrifuge and the pellet was used as microsomes. The microsomes were resuspended in Tris-HCl buffer and dilluted to a microsomal protein concentration of 2.5 mg/ml before use.

Enzyme assay: The incubation medium (total volume 1.0 ml) contained 0.5 ml Tris-HCl, pH 7.4 (50 µmoles), 0.1 ml freshly neutralized cysteine-HCl (10 µmoles), 0.04 ml 1,2-diolein-sn-glycerol (3 µmoles), 0.9 mg Tween 20, 0.1 ml BSA (0.5 mg), 0.06 ml ^{14}C palmitoyl-CoA (0.3 µmole, sp. act. 0.5 µCi/µmole) and 0.61 mg $MgCl_2$ (3 µmoles). Due to the insolubility of 1,2-diolein-sn-glycerol in water, an emulsion was prepared by pipetting the desired concentration of 1,2-diolein-sn-glycerol in benzene into a tube and evaporating off the benzene under nitrogen. Then the reagents described above were added and the contents of the tube sonicated using a Branson sonifier until a uniform emulsion was obtained. The reaction was initiated by addition of microsomes (0.5 mg protein) and the tubes incubated at 37°C for 30 minutes. At the end of the incubation, the reaction was stopped with

10 ml of chloroform-methanol 2:1 (v/v) and the lipids extracted as previously described.

Isolation of Labelled Triglyceride

For the isolation of radioactive triglyceride from the other lipids, Adsorbosil-1 prekoted plates were used. The plates, 20 x 20 cm and coated with 250 μ thickness of silica gel were first activated at 105°C for 30 minutes. The lipid samples dissolved in 0.05 ml chloroform were spotted on the plates as described in a previous section. The plates were developed in a presaturated chromatographic tank containing 200 ml of petroleum ether-diethyl ether-glacial acetic acid 90:15:1.5 (v/v). The chromatogram was run until the solvent front was 2 cm from the top. The plates were removed and after drying in air, the lipid spots were detected by a brief exposure of the plates to iodine vapor. The lipid spots were quickly marked with the tip of a needle and the iodine allowed to evaporate. The spots were scraped into scintillation vials and the radioactivity determined as previously described.

Assay for Phosphorylcholine-Glyceride Transferase

In the biosynthesis of phosphatidyl choline from 1,2-diacyl-sn-glycerol, the last enzymatic step in the pathway is catalyzed by the microsomal enzyme CDP-choline: 1,2-diglyceride choline-phosphotransferase (EC 2.7.8.2) (phosphorylcholine-glyceride transferase). The reaction

$$\text{CDP-choline} + \text{1,2-diacyl-sn-glycerol} \rightleftharpoons$$

$$\text{3-sn-phosphatidyl choline} + \text{CMP}$$

can be followed by measuring the amount of radioactivity of ^{14}C-CDP-choline incorporated into phosphatidyl choline when the former is incubated with a microsomal preparation under optimum reaction conditions. This will give an estimate of the activity of phsophorylcholine-glyceride transferase. The procedure described here is a modification of that of Young and Lynen (68).

Enzyme assay: Microsomes were prepared as described for diacyl-sn-glycerol acyltransferase. The incubation medium contained 0.5 ml Tris-HCl, pH 7.4 (50 µmoles), 0.1 ml freshly neutralized cysteine-HCl (10 µmoles), 0.037 ml 1,2-diolein-sn-glycerol (7.4 µmoles), Tween 20 (0.3 mg), 0.1 ml BSA (0.5 mg), 4.1 mg $MgCl_2$ (20 µmoles) and 0.06 ml CDP-choline (0.3 µmole, sp. act. 3 µCi/µmole). The 1,2-diolein-sn-glycerol was emulsified as described earlier. Incubations were carried out with and without Aroclor. The reaction was initiated by addition of microsomes (0.5 mg protein) in a total reaction volume of 1.0 ml and incubated at 37°C for 30 minutes. The reaction was stopped with 10 ml chloroform-methanol 2:1 (v/v) and lipids extracted as previously described. The separation of phosphatidyl choline was carried out as described in an earlier section for phospholipid separation.

Determination of Glycerol-3 Phosphate in Liver Preparations

L-(-)-glycerol-3 phosphate can be oxidized by glycerol-3 phosphate dehydrogenase (GDH) in the presence of NAD^+ to form dihydroxyacetone phosphate (DHAP) + NADH + H^+. The formation of NADH as measured at 340 nm is proportional to the quantity of glycerol-3 phosphate present in the sample.

(1) L-(-)-glycerol-3-P + NAD^+ $\overset{GDH}{\rightleftharpoons}$ dihydroxyacetone-P + NADH + H^+

Optimum conditions for measurement: The equilibrium of the reaction lies well to the left, but glycerol-3-P is quantitatively oxidized by NAD if the reaction products are removed from the equilibrium. The H^+ are bound by an alkaline reaction medium and the dihydroxyacetone-P is trapped as the hydrazone with the use of hydrazine.

(2) Glycerol-3-P + NAD^+ + hydrazine $\xrightarrow[\text{pH } 9.5]{GDH}$ DHAP-hydrazone + NADH + $H_3{}^+O$

thus, glycerol-3-P concentration can be measured enzymatically by coupling it with reduction of NAD^+ by glycerol-3 phosphate dehydrogenase. The method is described by Gerhard and Lang (69).

Reagents: Glycerol-3 phosphate dehydrogenase-enzyme suspension containing 10 mg protein/ml was used without further dilution.

2. Perchloric acid, 6 N - dilute 7.7 ml of 70% perchloric acid (sp. gr. 1.67) to 150 ml with distilled water.

3. Potassium carbonate (5 M).

4. Hydrazine-glycine buffer: (0.4 M hydrazine, 1 M glycine, pH 9.5) - suspend 5.2 g hydrazine sulfate and 7.5 g glycine plus 0.2 g EDTA-$Na_2H_2 \cdot H_2O$ in a little water and add 5 ml of 2 M NaOH. Then make up to 100 ml with distilled water.

5. NAD^+ solution - 40 mg per ml H_2O.

Preparation of sample (deproteinization): Five milliliters of 20% liver homogenate (w/v) prepared from control and PCB-treated rats was pipetted into 30 ml Corex glass centrifuge tubes. Then 5 ml of 12M perchloric acid was added to each, the contents well mixed and then centrifuged at 3,000 xg for ten minutes. The precipitate formed

was washed once with 1 ml of perchloric acid plus 1 ml water and the supernatants combined. The combined supernatant was adjusted to pH 3.5 with 5 M K_2CO_3. This was then brought to a final volume of 16 ml with water, allowed to stand on ice for 15 minutes and then the supernatant pipetted off the precipitate. Portions of the resulting extract were used for glycerol-3 phosphate determination.

Assay: All reactions were carried out in 3 ml cuvettes. First, 1.0 ml of the hydrazine/glycine buffer, final concentration (0.189 M hydrazine, 0.47 M glycine, 2.7 mM EDTA) was pipetted into the cuvette. Then 0.10 ml NAD solution (2.31 mM) was added followed by 1.0 ml of the protein-free extract. The contents of the cuvette were then mixed thoroughly with plumpers (a small plastic stirrer, product of Calbiochem) and the initial extinction E_1 at 340 nm was measured twice at 3-minute intervals. When the initial extinction E_1 remained constant after two readings, 0.02 ml (100 µg/ml) of the glycerol-3-P dehydrogenase suspension was added and the contents again mixed. The reaction was allowed to proceed for 15 minutes after the addition of the enzyme and the extinction E_2 read twice at 3-minute intervals. The change in extinction $\Delta E = E_2 - E_1$ was porportional to glycerol-3-P concentration and was calculated from the formula:

at 340 nm: concentration = $\Delta E \times 0.054$ mg/ml or

$\Delta E \times 0.341$ µmole/ml

The dilution due to extraction and neutralization was corrected for by the factor

$$F_{tissue} = 16/w$$

where w = weight of tissue and 16 the final volume of extract.

Determination of Glycerol-3 Phosphate Concentration of Rat Liver Mitochondria

The assay for glycerol-3 phosphate concentration in the mitochondria was essentially carried out as described for the liver homogenate, except that 5 ml of mitochondrial preparation isolated by differential centrifugation of the homogenate was used in place of the liver homogenate. The calculation for glycerol-3 phosphate concentration per gram of liver tissue was the same as used for calculating that of the homogenate.

Gas Chromatographic (GC) Analysis for PCB Residues

In order to ascertain whether any significant amounts of PCBs accumulate in the livers of rats fed a 0.1% (w/w) PCB diet for 30 days, GC analysis for the PCB content of whole livers, mitochondrial and microsomal fractions were carried out. The GC analysis was performed by Ms. Anna Marin and Mr. Donald A. Griffin of the Department of Agricultural Chemistry at Oregon State University. The analysis was carried out with a Hewlett-Packard 5700A series chromatograph with an Ni^{63} electron capture detector. For the determination of Aroclor 1254 standard, a nickel column measuring 10' x 1/8" and packed with 3% OV-101 on 80/100 chromosorb HPW was used. The column was operated at 200°C oven temperature, 300°C injection port and 350°C detector with a carrier gas flow of approximately 30 cc/min. 95% argon and 5% methane. For the determination of PCBs in the whole livers and subcellular fractions, a Pyrex column measuring 4' x 1/8" and packed with 1% Dexsill 300 on 100/120 chromosorb HPW was used. The determinations

were carried out at 200°C oven temperature, 300°C injection port and 350°C detector with a carrier gas flow of about 30 cc/min 95% argon, 5% methane.

EXPERIMENTAL RESULTS

In vitro Alterations in Phospholipid and Glyceride Biosynthesis by Aroclor 1254 (PCB) and 2,4,5,2',4',5'-Hexachlorobiphenyl (HCB)

Preliminary Studies

In order to determine whether PCBs have any effect at all on the biosynthesis of lipids in the rat liver and as well as to determine the PCB concentrations at which a significant effect is observed, a preliminary experiment was performed. Varying concentrations of PCB were added to the reaction mixture and the capacity of a cell-free homogenate to incorporate [U^{14}C]glycerol-3 phosphate into lipids was compared to that of the controls. The results of such an experiment are summarized in Table 1. It can be seen that there was an inhibitory effect of PCB on the radioactivity incorporated into total lipids but this inhibition was not significant until concentrations of 0.3 mM and above were used. At 0.8 mM PCB, there was a 45% decline in the radioactivity incorporated into total lipids.

A similar preliminary experiment using a microsomal preparation showed that between 40 and 75% inhibition of the incorporation of ^{14}C-glycerol-3 phosphate into total lipids occurred at PCB concentrations ranging from 0.33 mM to 2.2 mM respectively. Therefore, in most of the subsequent experiments, PCB concentrations were chosen to fall within this range.

Table 1. Effect of varying concentrations of PCB on [^{14}C]glycerol-3 phosphate incorporation into total lipids by rat liver.*

	CPM in Total Lipids	% Change
Control	3,225 ± 610	-
PCB, 0.03 mM	3,190 ± 350	-1
PCB, 0.15 mM	2,940 ± 315	-9
PCB, 0.3 mM	2,495 ± 310	-23
PCB, 0.6 mM	2,075 ± 195	-36
PCB, 0.8 mM	1,815 ± 140	-45

*Homogenates were prepared and incubations carried out as described under Materials and Methods. Values are the averages of three determinations ± standard deviation.

sn-Glycerol-3 Phosphate Acyltransferase and Microsomal Lipids

The effect of PCB, *in vitro*, on the activity of microsomal sn-glycerol-3 phosphate acyltransferase was studied at pH 6.5. At 1.32 mM and 0.66 mM, PCB inhibited the incorporation of [U-^{14}C]glycerol-3 phosphate into total lipids 77 and 67% respectively (Tables 2a and 2b). Upon separation of the total lipids by thin-layer chromatography (TLC), the majority of the radioactivity was found to be in lysophosphatidate in both controls and experimentals. The radioactivity incorporated into this major product was significantly inhibited 84 and 77% at 1.32 mM and 0.66 mM PCB respectively (Tables 2a and 2b).

The percent distribution of radioactivity was also altered in such a way that radioactivity in lysophosphatidate decreased whereas

Table 2a. Incorporation of [^{14}C]glycerol-3 phosphate into microsomal lipids by sn-glycerol-3 phosphate acyltransferase in the presence of PCB *in vitro*.*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
Compound	Control	PCB	Control	PCB
Lysolecithin	650 ± 112	183 ± 10	3 ± 0.4	3 ± 0.2
Lysophosphatidate	20,463 ± 2,800	3,233 ± 1,400	89 ± 10	59 ± 25
PC + PE	1,176 ± 150	1,017 ± 116	5 ± 0.5	18 ± 2
Glycerides	638 ± 50	1,183 ± 200	3 ± 0.2	21 ± 4
Total	22,927 ± 2,912	5,512 ± 1,523		

*The concentration of PCB was 1.32×10^{-3} M. Microsomes were prepared and enzyme activity assayed as described under Materials and Methods.

Values are averages of three determinations ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Table 2b. Incorporation of [^{14}C]glycerol-3 phosphate into microsomal lipids by sn-glycerol-3 phosphate acyltransferase in the presence of PCB *in vitro*.*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
Compound	Control	PCB	Control	PCB
Lysolecithin	529 ± 114	380 ± 160	1 ± 0.3	3 ± 1
Lysophosphatidate	39,943 ± 3,643	9,180 ± 1,200	90 ± 9	71 ± 9
PC + PE	971 ± 57	620 ± 80	2 ± 0.1	5 ± 1
Glycerides	986 ± 86	1,500 ± 100	2 ± 0.2	13 ± 1
Unidentified	2,129 ± 257	1,320 ± 260	5 ± 1	10 ± 2
Total lipid	63,857 ± 6,429	21,200 ± 3,400		

*The concentration of PCB was 6.6×10^{-4} M. The assay procedure and techniques employed are described under Materials and Methods.

Values are the average of three determinations ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

that of glycerides increased. As can be seen in Tables 2a and 2b, there was a consistent increase in the radioactivity incorporated into the glyceride fraction at both PCB concentrations tested. When the total glyceride fraction was separated into the individual glycerides by TLC (results not shown) it was observed that most of the radioactivity was in the diglyceride band. This latter observation suggests that there is probably an increase in the activity of microsomal phosphatidate phosphatase, or an inhibition of diglyceride acyltransferase or both by PCB *in vitro*. An increase in monoglyceride acyltransferase activity by PCB can also account for the increased glyceride synthesis.

Synthesis and Composition of Mitochondrial Lipids in Presence of PCB *in vitro*

Table 3 summarizes the results obtained when the capacity of mitochondria to synthesize phospholipids and glycerides from [U-^{14}C]glycerol-3 phosphate was determined in the presence and absence of PCB *in vitro*. As can be seen, at 1.5 mM PCB, there was a 57% decline in the radioactivity incorporated into the total lipids. Upon TLC separation of the lipids into their various fractions, a general decline in the radioactivity incorporated into all fractions was observed, with the highest decline being in phosphatidate (88%). The percent distribution of the total radioactivity in the lipid fractions was also altered. The percent of the total radioactivity incorporated into phosphatidate was decreased relative to the controls, but with glycerides and phosphatidyl choline plus phosphatidyl ethanolamine, there was a slight increase by PCB.

Table 3. In vitro effect of PCB on [^{14}C]glycerol-3 phosphate incorporation into phospholipids and glycerides by rat liver mitochondria.*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
Compound	Control	PCB	Control	PCB
Lysolecithin	74 ± 10	43 ± 12	2 ± 0.3	3 ± 1
Phosphatidate	997 ± 240	119 ± 9	26 ± 6	8 ± 0.8
PC + PE	465 ± 40	250 ± 51	12 ± 1	17 ± 3
Glycerides	2,249 ± 551	1,069 ± 94	59 ± 15	72 ± 6
Total lipid	7,333 ± 560	3,133 ± 1,000		

*The concentration of PCB was 1.5×10^{-4} M. The assay procedure and techniques employed are described under Materials and Methods.

Values are the average of three determinations ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Effect of PCB on the Incorporation of [U-^{14}C]Glycerol-3 Phosphate Into Lipids by Cell-free Homogenates

The in vitro effect of PCB on the synthesis of phospholipids and glycerides from [U-^{14}C]glycerol-3 phosphate by cell-free homogenates, a system that contains both mitochondrial and microsomal fractions was determined. The results are shown in Tables 4a and 4b. As can be seen in these tables, PCB caused a decrease in the radioactivity of ^{14}C-glycerol-3 phosphate incorporated into the total lipids 40 and 27% at 0.86 mM and 0.43 mM respectively. The inhibition was apparently concentration-dependent, but not linear. An important observation in this system was the finding that the distribution of radioactivity in the various lipid fractions was not significantly altered by PCB,

Table 4a. *In vitro* effect of PCB on [^{14}C]glycerol-3 phosphate incorporation into phospholipids and glycerides by rat liver.*

	CPM/umole Lipid Phosphorus		% of Total CPM	
Compound	Control	PCB	Control	PCB
Lysolecithin	106 ± 6	154 ± 11	4 ± 0.3	10 ± 1.0
Phosphatidate	414 ± 31	197 ± 54	17 ± 1.0	13 ± 4
PC + PE	128 ± 18	94 ± 5	5 ± 1.0	6 ± 0.3
Glycerides	1,756 ± 50	1,043 ± 78	67 ± 2	62 ± 5
Unidentified	178 ± 39	137 ± 11	7 ± 1	8 ± 0.9
Total lipid	3,555 ± 306	2,135 ± 297		

*Concentration of PCB was 8.6×10^{-4} M. Homogenate was prepared and incorporation of radioactivity determined as indicated under Materials and Methods.

Values are the average of three determinations ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Table 4b. *In vitro* effect of PCB on [^{14}C]glycerol-3 phosphate incorporation into phospholipids and glycerides by rat liver.*

	CPM/umole Lipid Phosphorous		% of Total CPM	
Compound	Control	PCB	Control	PCB
Lysolecithin	81 ± 17	37 ± 6	3 ± 0.6	3 ± 0.4
Phosphatidate	331 ± 53	231 ± 40	16 ± 2.3	17 ± 2.7
PC + PE	161 ± 44	114 ± 17	8 ± 1.9	9 ± 1.2
Glycerides	1,533 ± 69	971 ± 100	67 ± 3.0	66 ± 6.7
Unidentified	119 ± 19	77 ± 17	5 ± 0.7	5 ± 1.2
Total lipid	3,555 ± 250	2,486 ± 286		

*The concentration of PCB was 4.3×10^{-4} M. Homogenates were prepared and incorporation of radioactivity determined as indicated under Materials and Methods.

Values are the average of three determinations ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

suggesting that PCB might probably be influencing a reaction step that is common to the formation of all these lipid fractions.

The major radioactive products under the incubation conditions in this system are the glycerides. It can be seen that their formation was significantly inhibited, 41 and 37% at 0.86 mM and 0.43 mM PCB respectively (Tables 4a and 4b). The total glyceride fraction is composed of mono-, di- and triglycerides, thus it was deemed necessary to determine which of these fraction(s) was being inhibited by PCB. Therefore an aliquot of the total extracted radioactive lipids was separated in a different solvent system as described under the methods section to separate the individual glycerides. The results are shown in Tables 5a and 5b. Both tables show that PCB inhibited the formation of all three glyceride fractions, but the major effect was on the triglyceride fraction which was inhibited 76 and 54% at 0.86 mM and 0.43 mM PCB respectively.

Since triglycerides were inhibited to a much greater extent than all the fractions examined (compare Tables 4a and 4b with 5a and 5b) it will be interesting to speculate that there is probably an additional inhibitory effect of PCB on the enzyme that catalyzes the last reaction in triglyceride synthesis, namely diglyceride acyltransferase, since the formation of diglyceride was not inhibited to the same extent. The results of the effect of PCB on this enzyme will be discussed in a later section.

Table 5a. *In vitro* effect of PCB on [^{14}C]glycerol-3 phosphate incorporation into individual glycerides.*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
	Control	PCB	Control	PCB
Monoglycerides	683 ± 58	508 ± 57	45 ± 4	59 ± 7
Diglycerides	308 ± 44	222 ± 16	20 ± 3	26 ± 2
Triglycerides	542 ± 69	132 ± 19	35 ± 5	15 ± 2

*The concentration of PCB was 8.6×10^{-4} M. Homogenates were prepared and glycerides determined as described under Materials and Methods.

Values are the average of three determinations ± stnadard deviation.

Table 5b. *In vitro* effect of PCB on [^{14}C]glycerol-3 phosphate incorporation into individual glycerides by rat liver.*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
	Control	PCB	Control	PCB
Monoglycerides	767 ± 75	460 ± 97	34 ± 5	35 ± 10
Diglycerides	319 ± 44	223 ± 14	14 ± 3	17 ± 2
Triglycerides	539 ± 86	246 ± 34	24 ± 5	19 ± 4

*The concentration of PCB was 4.3×10^{-4} M. The same assay conditions were employed as for Table 5a.

Values are the mean ± standard deviation.

Effect of HCB on Microsomal sn-Glycerol-3 Phosphate Acyltransferase Activity

The rationale for using a commercial PCB preparation in this study stemmed from the fact that these commercial PCB preparations are the ones that are used industrially and thus find their way into the environment. Hence, any studies on the environmental impact of PCBs must include these commerical preparations also. However, since these commercial PCB preparations are mixtures of different isomers and the results described so far indicate that at least Aroclor 1254 does alter lipid metabolism it was deemed necessary to test whether a purified isomer also elicits the same kind of response. Therefore, the effect of 2,4,5,2',4',5'-hexachlorobiphenyl (HCB), in vitro, on phospholipid and glyceride biosynthesis was investigated in microsomes and liver homogenate. This isomer was chosen due to the fact that it is one of the major components of Aroclor 1254 and because of its persistance in the liver. In addition, this particular isomer is available in 99% pure form.

Table 6 summarizes the results of the effect of HCB in vitro on microsomal sn-glycerol-3 phosphate acyltransferase activity. It can be seen that the incorporation of substrate into the major radioactive product, lysophosphatidate was significantly inhibited (76%) at 0.55 mM HCB. This value is very close to that obtained with PCB at comparable concentrations. Lysolecithin was also significantly decreased by HCB (77%) whereas the other fractions were not significantly affected. These results imply that the HCB elicits the same kind of reponse as PCB in vitro on the acyltransferase activity and

Table 6. Activity of microsomal sn-glycerol-3 phosphate acyltransferase in the absence and presence of 2,4,5,2',4',5'-hexachlorobiphenyl (HCB) in vitro.*

	CPM/mg Protein		% of Total CPM	
Compound	Control	HCB	Control	HCB
Lysolecithin	1850 ± 290	433 ± 63	16 ± 2	12 ± 2
Lysophosphatidate	9147 ± 1653	2203 ± 400	78 ± 14	64 ± 12
PC + PE	463 ± 70	524 ± 77	4 ± 0.6	15 ± 2
Glycerides	273 ± 67	307 ± 47	2 ± 1.0	9 ± 1

*The concentration of HCB was 5.5×10^{-4} M. sn-Glycerol-3 phosphate acyltransferase activity was determined as described under Materials and Methods.

Values are the average of three determinations ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

that the effect seen with PCB could be partly attributed to the presence of this isomer in the mixture.

Inhibition of the Incorporation of [U-^{14}C]Glycerol-3 Phosphate Into Liver Lipids by HCB in vitro

The results obtained when the capacity of a cell-free homogenate to incorporate radioactivity into phospholipids and glycerides in the presence of HCB was determined are summarized in Table 7. As can be seen, there was a general decline in the radioactivity incorporated into all lipid fractions in the presence of HCB. There was also a concomitant alteration in the distribution pattern of the total radioactivity in contrast to the results observed with PCB. This suggests that in the cell-free homogenate, the presence of other isomers in the PCB might be influencing the overall results obtained.

Table 7. Incorporation of [U-^{14}C]glycerol-3 phosphate into phospholipids and glycerides by rat liver in the presence of 2,4,5,2',4',5'-hexachlorobiphenyl (HCB) in vitro.*

	CPM/mg DNA		% of Total CPM	
Compound	Control	HCB	Control	HCB
Lysolecithin	788 ± 75	138 ± 25	6 ± 1	2 ± 0.4
Phosphatidate	6738 ± 456	1288 ± 500	49 ± 3	22 ± 9
PC + PE	1263 ± 63	756 ± 188	9 ± 0.5	13 ± 3
Glycerides	5069 ± 313	3681 ± 319	37 ± 2	63 ± 5

*The concentration of HCB was 5.5×10^{-4} M. Phosphatides and glycerides were determined and homogenates prepared as described under Materials and Methods.

Values are the average of three determinations ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Thus, in general, both PCB and HCB decreased the synthesis of phospholipids from ^{14}C-glycerol-3 phosphate. Glyceride synthesis was slightly increased in the microsomes by both PCB and HCB but decreased in the cell-free homogenate. The general decrease in phospholipid synthesis by all three cell fractions used was directly correlated with a decrease in the activity of microsomal sn-glycerol-3 phosphate acyltransferase. The reaction catalyzed by this enzyme has been proposed as a possible regulatory step in hepatic lipogenesis by Fallon and Kemp (67), Tzur et al. (68), Howard and Lowenstein (69) and Bortz and Lynen (70).

In vivo Effect of PCB on Phospholipid and Glyceride Biosynthesis

In order to fully assess the mode of action of PCBs on phospholipid and glyceride synthesis it was necessary to determine their *in vivo* effects on the biosynthetic process. Comparison of the *in vivo* data with one obtained *in vitro* will indicate whether the observed direct effects on the enzymes are manifested in the intact system. In addition, having data of both types will enable one to properly assess the significance of the observed responses to PCBs. Polychlorinated biphenyls are also primarily metabolized in the animal system to hydroxylated (phenolic) derivatives (12-20). Phenols are uncouplers of oxidative phosphorylation, thus their presence in the system could influence the biosynthesis of phospholipids and glycerides since this process requires energy in the form of ATP. For the *in vivo* experiments, rats were fed, *ad libitum*, control and PCB (0.1% w/w) diet for 30 days. At the end of the treatment period the rats were sacrificed, subcellular fractions of the liver prepared and incubated with radioactive substrates as indicated under Materials and Methods.

Synthesis and Distribution of Radioactivity in Microsomal Lipids

The *in vivo* effect of PCB on the incorporation of radioactivity into phospholipids and glycerides by microsomal sn-glycerol-3 phosphate acyltransferase was determined and the results are summarized in Table 8, experiments 1, 2 and 3 and Table 9, experiments 1 and 2. Table 8

Table 8. Experiment 1: The distribution of radioactivity in phospholipids and glycerides of microsomes after in vivo treatment of rats with PCB.*

	CPM/μg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	8615 ± 1230	3422 ± 761	95 ± 14	95 ± 21
Monoglycerides	47 ± 10	32 ± 8	0.5 ± 0.1	1 ± 0.2
Diglycerides	203 ± 53	71 ± 17	2 ± 0.5	2 ± 0.5
Triglycerides	66 ± 13	12 ± 1.1	0.7 ± 0.1	0.4 ± 0.06

*Microsomes were prepared and incorporation of radioactivity determined as indicated under Materials and Methods.

Values are the average of three determinations ± standard deviation. [U-^{14}C]glycerol-3 phosphate was the substrate.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

Table 8: Experiment 2: In vivo effect of PCB on the distribution of radioactivity in individual phospholipids and total glycerides by microsomes.*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
Compound	Control	PCB	Control	PCB
Lysolecithin	4,123 ± 746	2,550 ± 375	3 ± 0.5	2 ± 0.3
Lysophosphatidate	148,746 ± 11,808	74,290 ± 11,127	90 ± 7	87 ± 13
PC + PE	1,354 ± 389	1,245 ± 573	1.5 ± 0.3	2.4 ± 0.9
Glycerides	6,262 ± 300	4,054 ± 772	4 ± 0.2	5 ± 1

*Microsomes were prepared and incorporation of radioactivity determined as indicated under Materials and Methods.

Values are means ± standard deviation. [U-^{14}C]glycerol-3 phosphate was the substrate. PC, phosphatidyl choline: PE, phosphatidyl ethanolamine.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

Table 8. Experiment 3: The distribution of ^{14}C-palmitoyl-CoA radioactivity in phospholipids and glycerides of microsomes after *in vivo* treatment of rats with PCB.*

	CPM/μg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	17,706 ± 2,738	9,305 ± 309	35 ± 5	26 ± 1
Monoglycerides	199 ± 19	112 ± 14	0.4 ± 0.05	0.3 ± 0.03
Diglycerides	27,833 ± 4,181	24,573 ± 2,201	54 ± 8	70 ± 6
Triglycerides	1,427 ± 238	396 ± 32	3 ± 0.5	1 ± 0.1

*Microsomes were prepared and incorporation of radioactivity determined as indicated under Materials and Methods.

Values are means ± standard deviation.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

experiments 1 and 2 show the effect on glycerol-3 phosphate incorporation into phospholipids and glycerides after the *in vivo* treatment of rats with PCB. In Table 8, experiment 1, the data were analyzed as total phospholipids and individual glycerides. Over 90 percent of the total radioactivity was incorporated into the phospholipids both under control and treated conditions. There was, however, about 60% decline in the radioactivity incorporated into the phospholipids after the PCB treatment. In Table 8, experiment 2, the results were analyzed for individual phospholipids and total glycerides. It can be seen that lysophosphatidate, the direct product of sn-glycerol-3 phosphate acyltransferase reaction constituted 90 and 87% of the total radioactivity under control and treated conditions, respectively. This fraction was inhibited 50% after the PCB treatment. In general, there was a decline in the radioactivity incorporated into all the lipid fractions after

the PCB treatment, both in Table 8, experiments 1 and 2.

Table 8, experiment 3, shows the effect on ^{14}C-palmitoyl-CoA incorporation into lipids by the same microsomal preparation whose results are shown in Table 8, experiments 1 and 2. It can be seen that the major products obtained with this substrate were the phospholipids and diglycerides both under control and treated conditions. The total phospholipids declined 47%, diglycerides declined 33%, while triglycerides declined 72% after the PCB treatment. Thus, the results obtained with palmitoyl-CoA are in agreement with those obtained with glycerol-3 phosphate, in that PCB treatment resulted in a general decline in the radioactivity incorporated into all lipid fractions.

Table 9, experiments 1 and 2, are a repeat of the experimental results described in Table 8, i.e. rats were again treated with PCB for 30 days, microsomal preparations were made and incorporation of ^{14}C-glycerol-3 phosphate into lipids determined. In Table 9, experiment 1, it can be seen that the total phospholipids, constituting 92 and 86% of the total radioactivity under control and treated conditions, respectively, decreased 41% after PCB treatment. Slight increases were noted in the monoglycerides and triglycerides fraction although these fractions consituted only a minor portion of the total products. In Table 9, experiment 2, the results were analyzed for individual phospholipids and total glycerides. It is again clear from this table that a general decline in the radioactivity incorporated into all lipid fractions occurred after the PCB treatment. Again, the major product was lysophosphatidate under both control and experimental conditions and this decreased 58% after the PCB treatment. The

Table 9. Experiment 1: The distribution of ^{14}C-glycerol-3 phosphate radioactivity in phospholipids and glycerides of microsomes after *in vivo* treatment of rats with PCB.*

	CPM/ug DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	6,817 ± 1,065	3,992 ± 317	94 ± 14	86 ± 7
Monoglycerides	184 ± 19	267 ± 41	2 ± 0.3	6 ± 1
Diglycerides	334 ± 30	216 ± 17	4 ± 0.4	5 ± 0.4
Triglycerides	99 ± 14	150 ± 17	1 ± 0.2	3 ± 0.4
Observed Total Count	9,000 ± 2,911	5,864 ± 1,000		

*Microsomes were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are the means ± standard deviation.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

Table 9. Experiment 2: The distribution of radioactivity in individual phospholipids and total glycerides of microsomes after *in vivo* treatment of rats with PCB.*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
Compound	Control	PCB	Control	PCB
Lysolecithin	2,720 ± 220	1,450 ± 117	2 ± 0.2	2 ± 0.2
Lysophos-phatidate	102,640 ± 6,140	42,960 ± 3,400	86 ± 5	77 ± 6
PC + PE	4,080 ± 420	2,400 ± 243	3 ± 0.4	6 ± 1
Glycerides	8,440 ± 1,220	5,300 ± 500	7 ± 1	13 ± 1

*Microsomes were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are means ± standard deviation. ^{14}C-glycerol-3 phosphate was the substrate. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

radioactivity incorporated into the other minor products also decreased; for instance, lysolecithin declined 47%, whereas PE and PC declined 41%.

When this experiment was repeated a third time, similar results were obtained as those described for Tables 8 and 9, i.e. the incorportaion of ^{14}C-glycerol-3 phospahte into total phospholipids decreased 46% and the glycerides also decreased slightly (15%) after PCB treatment. The apparent differences in the radioactivity incorporated into the products in different experiments stems from the fact that large variations in the activity of the liver preparations were observed, thus accounting for these differences.

Distribution of Radioactivity in Mitochondrial Lipids

The capacity of the mitochondria to synthesize phospholipids and glycerides from [U-^{14}C]glycerol-3 phosphate after PCB treatment of rats for 30 days was also determined. These results are summarized in Table 10, experiments 1 and 2. It can be seen in each of these experiments that a general increase in the radioactivity incorporated into all major lipid fractions occurred after PCB treatment. In experiment 1 (Table 10) PCB increased the incorporation of radioactivity into phospholipids (14-fold) and monoglycerides (89%). In addition, there was a concomitant alteration in the distribution of total radioactivity, such that most appeared in the phospholipids (58.5%) and lesser amounts in monoglycerides (32%). In Table 10, experiment 2, PCB *in vivo* increased the incorporation of [U-^{14}C] glycerol-3 phosphate radioactivity into phospholipids (35%),

Table 10. Experiment 1: In vivo effect of PCB on the distribution of ^{14}C-glycerol-3 phosphate radioactivity in phospholipids and glycerides of mitochondria.*

	CPM/µg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	138†	3257 ± 221	16.0	58.8
Monoglycerides	595 ± 114	1764 ± 86	69.8	31.9
Diglycerides	8 ± 2	230 ± 36	1.0	4.2
Triglycerides	16 ± 2	192 ± 31	1.9	3.5

*Mitochondria were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are the average of three determinations ± standard deviation. Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

†Value is a mean of three pooled bands.

Table 10. Experiment 2: In vivo effect of PCB on the distribution of ^{14}C-glycerol-3 phosphate radioactivity in phospholipids and glycerides of mitochondria.*

	CPM/µg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	3,152 ± 364	8,000 ± 825	33 ± 3	22 ± 2
Monoglycerides	3,546 ± 364	12,400 ± 6,250	37 ± 4	34 ± 2
Diglycerides	2,470 ± 1,030	15,900 ± 1,775	26 ± 11	43 ± 4
Triglycerides	515 ± 152	375 ± 75	5 ± 2	1 ± 0.3

*Mitochondria were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are means ± standard deviation.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

monoglycerides (53%) and diglycerides (74%). The percent distribution of total radioactivity in phospholipids and monoglycerides was not significantly changed after PCB treatment, however, the percent radioactivity in the diglycerides was higher than that of the controls (49 and 19%) respectively.

It should be mentioned that the experimental results reported in Table 10 were obtained at different times using different sets of rats, but from the same suppliers. This could probably account for the different distribution patterns of radioactivity observed in the two experiments, since all other experimental conditions were controlled to the same extent. When this experiment was repeated a third time, similar results to those described in Table 10, experiments 1 and 2, were obtained. The radioactivity incorporated into total phospholipids increased (60%) and the glycerides increased (75%) after PCB treatment.

When ^{14}C-palmitic acid was incubated with the mitochondrial preparations, total phospholipid synthesis increased (76%), monoglycerides increased 1.6-fold and diglycerides also increased 35% after PCB treatment (Table 11). Triglyceride radioactivity, however, decreased (70%). There was no significant change in the distribution of the total radioactivity.

Effect on the Distribution of Radioactivity in Lipids of Liver Homogenate

In addition to determining the *in vivo* mode of action of PCB on the capacity of the mitochondria and microsomes to incorporated labelled

Table 11. In vivo effect of PCB on the distribution of ^{14}C-palmitic acid radioactivity in phospholipids and glycerides of mitochrondia.*

	CPM/µg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	738 ± 47	2,040 ± 114	0.6	0.8
Monoglycerides	177 ± 19	737 ± 71	0.2	0.3
Diglycerides	111,480 ± 1,473	236,526 ± 6,282	95.9	97.3
Triglycerides	1,799 ± 359	855 ± 57	1.6	0.4

*Mitochondria were prepared and incorporation of radioactivity determined as indicated under Materials and Methods.

Values are means ± standard deviation.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

substrates into phospholipids and glycerides, a cell-free homogenate (contains both microsomes and mitochondria) was also used. These results are summarized in Table 12, experiments 1, 2 and 3 and Table 13.

Table 12 summarizes the results obtained when [U-^{14}C]glycerol-3 phosphate was used as the labelled substrate in three different experiments. As can be seen in each of these experiments, there was consistently an increase in the radioactivity incorporated into all lipid fractions after PCB treatment. After the treatment, phospholipids increased 93, 53 and 62% in experiments 1 through 3, respectively, while triglycerides increased 91, 88 and 70%, respectively, in the same order. The relative distribution pattern of the total radioactivity among the various lipid fractions was also altered after the PCB treatment. Thus, whereas 35% of the total radioactivity was incorporated into monoglycerides under control conditions, this fraction only

Table 12. Experiment 1: In vivo effect of PCB on the distribution of radioactivity in phospholipids and glycerides of rat liver.*

	CPM/µg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	155†	3886 ± 341	15	24 ± 2
Monoglycerides	363 ± 80	2109 ± 213	35 ± 8	13 ± 1
Diglycerides	191 ± 97	4376 ± 399	19 ± 10	28 ± 3
Triglycerides	293 ± 65	5508 ± 509	28 ± 7	35 ± 3

*Homogenates were prepared and incorporation of radioactivity determined as indicated under Materials and Mathods.
Values are means ± standard deviation. ^{14}C-glycerol-3 phosphate was the substrate.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

†Value is mean of three pooled bands.

Table 12. Experiment 2: In vivo effect of PCB on the distribution of radioactivity in phospholipids and glycerides of rat liver.*

	CPM/mg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	12,542 ± 375	28,818 ± 1,500	29 ± 1	15 ± 1
Monoglycerides	11,375 ± 1,083	39,500 ± 5,818	18 ± 3	22 ± 3
Diglycerides	7,875 ± 1,167	39,500 ± 5,818	18 ± 3	22 ± 3
Triglycerides	6,792 ± 958	61,455 ± 8,364	16 ± 2	33 ± 4
Observed Total Count	42,626 ± 5,125	182,408 ± 12,542		

*Homogenates were prepared and incorporation of radioactivity determined as described under Materials and Methods.
Values are means ± standard deviation. ^{14}C-glycerol-3 phosphate was the substrate.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

Table 12. Experiment 3: In vivo effect of PCB on the distribution of radioactivity in phospholipids and glycerides of rat liver.*

	CPM/µg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	1,377 ± 217	5,673 ± 1,068	37 ± 6	33 ± 6
Monoglycerides	933 ± 57	4,958 ± 184	25 ± 2	29 ± 1
Diglycerides	757 ± 70	2,853 ± 247	20 ± 2	17 ± 1
Triglycerides	660 ± 50	3,532 ± 384	18 ± 1	19 ± 2
Observed Total Count	3,100 ± 700	20,474 ± 5,947		

*Homogenates were prepared and incorporation of radioactivity determined as indicated under Materials and Methods.
Values are means ± standard deviation. ^{14}C-glycerol-3 phosphate was the substrate.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

represented 13 percent after treatment. The distribution of radioactivity in phospholipids, diglycerides and triglycerides was not significantly changed (Table 12, experiment 1). In experiment 2 (Table 12) the percent distribution pattern of the total radioactivity among the various lipid fractions was such that more radioactivity was incorporated into the triglycerides (33, compared to 16%) and less into the total phospholipids (15 compared to 29%) after PCB treatment. The other fractions were not significantly changed. No major difference in the distribution pattern of the total radioactivity was observed in the third experiment. Thus, consistently, after PCB treatment the homogenates, like the mitochondria always had a higher capacity to synthesize phospholipids and glycerides from [U-^{14}C] glycerol-3 phosphate.

Table 13 shows the results of ^{14}C-palmitic acid incorporation into lipids by control and treated homogenates. The most striking observation in this experiment was the finding that whereas diglycerides are the major products under the control conditions (93.6%), this is shifted into triglycerides (94.7%) after PCB treatment. The observation that [U-^{14}C]glycerol-3 phosphate and ^{14}C-palmitic acid were differently incorporated into the lipid fractions even when these substrates were assayed in the same liver preparations suggests they are compartmented.

Table 13. *In vivo* effect of PCB on the distribution of ^{14}C-palmitic acid radioactivity in phospholipids and glycerides of liver.*

	CPM/μg DNA		% of Total CPM	
Compound	Control	PCB	Control	PCB
Phospholipids	1,369+	3,617 ± 474	1.4	2.2
Monoglycerides	693 ± 47	659 ± 99	0.7	0.2
Diglycerides	89,862 ± 4,238	1,276 ± 74	93.6	0.8
Triglycerides	1,238 ± 432	154,086 ± 2,630	1.3	94.7

*Homogenates were prepared and incroporation of radioactivity determined as indicated under Materials and Methods.

Values are means ± standard deviation.
Rats were fed control or 0.1% (w/w) PCB diet for 30 days.

+Value is a mean of three pooled bands.

Synthesis and Distribution of Radioactivity in Lipids Upon Mixing Liver Cell Fractions from Control and PCB-treated Rats

In order to more carefully analyze the *in vivo* data obtained using the three subcellular fractions, experiments were carried out in which each subcellular fraction from control and treated rats was mixed in fixed ratios, based upon their initial protein concentration and incubated with radioactive substrates. As will be discussed in a later section, with the exception of sn-glycerol-3 phosphate acyltransferase, the activities of other enzymes in the pathway were not significantly altered by PCB. Thus, if the overall results obtained *in vivo* was due to a direct effect of PCB on the activity of sn-glycerol-3 phosphate acyltransferase alone, then one should expect an additive effect when enzyme preparations from control and treated rats are mixed in fixed

ratios based on their intial activities. If, however, other factors are indirectly influencing the overall results, then such an additive effect would not be observed.

In Table 14 the results obtained from such an experiment using microsomes from control and PCB treated rats are summarized. The microsomes were mixed in the ratio 1:1 (based on initial protein concentration) and incubated with [U-^{14}C]glycerol-3 phosphate. It can be seen that the radioactivity incorporated into the total lipids as well as that incorporated into the major product was higher than would be expected from half the sum of the initial activities (expected values in parentheses). Mixing of the two microsomes, however, did not change the overall distribution of the total radioactivity. The results suggest that the PCB treated microsomes have a synergistic effect when added to the control microsomes, although when assayed separately they always had lower activity than the control microsomes.

Tables 15 and 16 summarize the results upon mixing homogenates from control and PCB treated rats. In Table 15 the homogenates were mixed in the ratio 1:1 based on initial protein, and in Table 16 the ratio was 1:3 (control:PCB). It can be seen from these two tables that the radioactivity incorporated both into the total lipids and the major products were not additive when the two preparations were mixed. The values obtained were in general lower than expected (expected values in parentheses). This was true whether the preparations were mixed in the ratio 1:1 or 1:3 (control:PCB). The results rather suggest that there was some factor in the PCB treated homogenate and this was diluted out upon the addition of the control homogenate so that its full stimulatory effect is no longer apparent. The

Table 14. Incorporation of [U-^{14}C]glycerol-3 phosphate radioactivity into lipids by a mixture of microsomes from control and PCB treated rats.*

Compound	Control CPM	PCB CPM	Mixture† CPM
Phospholipids	5385 ± 842	2635 ± 209	5150 ± 500 (4010)
Monoglycerides	146 ± 15	176 ± 27	150 ± 17
Diglycerides	264 ± 24	143 ± 11	139 ± 39
Triglycerides	78 ± 1	99 ± 11	39 ± 5
Observed Total Count	7110 ± 2300	3870 ± 660	7760 ± 1080 (5490)

*Microsomes were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are the mean of three determinations ± standard deviation.

†Homogenates were mixed 1:1 based on protein. Values in parentheses are the expected values.

Table 15. Incorporation of [U-^{14}C]glycerol-3 phosphate radioactivity into lipids by a mixture of homogenates from control and PCB treated rats.

Compound	Control CPM	PCB CPM	Mixture† CPM
Lysolecithin	26 ± 1	41 ± 16	36 ± 5
Phosphatidic Acid	321 ± 53	866 ± 196	503 ± 58 (593)
PC + PE	170 ± 8	145 ± 38	176 ± 21
Glycerides	483 ± 45	1788 ± 579	740 ± 86 (1135)
Observed Total Count	930 ± 210	3890 ± 1130	1610 ± 150 (2410)

*Homogenates were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are mean ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

†Homogenates were mixed 1:1 based on protein. Values in parentheses are the expected values.

Table 16. Incorporation of [U-^{14}C]glycerol-3 phosphate radioactivity into lipids by a mixture of cell-free homogenates from control and PCB treated rats.*

Compound	Control CPM	PCB CPM	Mixture† CPM
Lysolecithin	26 ± 1	41 ± 16	28 ± 5
Phosphatidic Acid	321 ± 53	866 ± 196	623 ± 159 (721)
PC + PE	170 ± 8	135 ± 38	167 ± 39
Glycerides	483 ± 45	1788 ± 579	991 ± 226 (1461)
Observed Total Count	930 ± 210	3890 ± 1130	2520 ± 500 (3150)

*Homogenates were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are means ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

†Homogenates were mixed in the ratio 1:3 (control:PCB) based on protein. Values in parentheses are the expected values.

distribution pattern of the total radioactivity was such that in general the pattern seen after mixing the two homogenates more closely resembles that under the control conditions rather than that under treated conditions (Tables 15 and 16).

When mitochondria were used, similar results were obtained as those described for the homogenate, i.e. control, 260 ±50; PCB, 1060 ±270; mixture 1:1, 560 ±130 counts per minute. Thus, from these results there is no clear evidence that the overall *in vivo* results are due to a direct effect of PCB on sn-glycerol-3 phosphate acyltransferase activity. It rather suggests that other indirect factors might be contributing to the overall response seen *in vivo*.

Addition of PCB *in vitro* to Subcellular Fractions from PCB Treated Rats on Incorporation of Radioactivity Into Lipids

In order to determine whether the activity of the liver subcellular fractions prepared from PCB treated rats could be influenced by the *in vitro* addition of PCB, microsomes and homogenates, each separately were incubated with substrate and a known amount of PCB. The amount of PCB added to each incubation has been shown previously to effectively inhibit synthesis of phospholipids and glycerides from sn-glycerol-3 phosphate. Addition of 0.46 mM PCB to microsomes prepared from PCB treated rats resulted in a decline in the total radioactivity (treated, 1810 ±410; treated + 0.46 mM PCB, 980 ±380 counts per minute), the percent decline being 46.

The results of such an experiment using the homogenate are summarized in Table 17. It can be seen that the same PCB treated

Table 17. The effect of added PCB, in vitro, to a homogenate from PCB treated rats on the incorporation of radioactivity into phospholipids and total glycerides.*

	PCB Treated		PCB Treated + 0.62 mM PCB	
Compound	CPM/mg DNA	% CPM	CPM/mg DNA	% CPM
Lysolecithin	212 ± 5	1	121 ± 5	3 ± 0.4
Phosphatidic Acid	3,732 ± 347	45 ± 4	1,658 ± 26	38 ± 0.6
PC + PE	326 ± 37	3	258 ± 58	6 ± 1.4
Glycerides	4,200 ± 300	50 ± 4	2,300 ± 279	53 ± 6.4
Observed Total Count	13,737± 1,211		6,105 ± 3,263	

*Homogenates were prepared and incorporation of radioactivity determined as described under Materials and Methods.
Values are means ± standard deviation. ^{14}C-glycerol-3 phosphate was the substrate. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

homogenate that was about four times more active than control homogenate now showed a lower incorporation of radioactivity when 0.62 mM PCB was added in vitro. The inhibition of total radioactivity incorporated was 56%, which is within the same range as normally observed with homogenate in vitro.

Levels of Glycerol-3 Phosphate in Liver Homogenates and Mitochondria of Control and PCB Treated Rats

In order to explore the possibility that the reason one sees a higher incorporation of radioactivity into lipids after PCB treatment is due to differences in pool sizes of glycerol-3 phosphate, the levels of this substrate in the liver homogenate and mitochondria were determined. These results are shown in Table 18. As can be seen, there

Table 18. Concentration of glycerol-3 phosphate in homogenate and mitochondria of livers from control and PCB treated rats.*

	Homogenate Glycerol-3 Phosphate (nMoles/g wet liver)		Mitochondria Glycerol-3 Phosphate (nMoles/g wet liver)
	Experiment I	Experiment II	
Control	536 ± 150	580 ± 9.8	169 ± 7.6
Treated	590 ± 150	573 ± 38	164 ± 15.3

*Homogenate and mitochondria were prepared and the amount of glycerol-3 phosphate determined as described under Materials and Methods.

Values are means of two determinations ± standard deviation.

was no difference in the pool sizes of glycerol-3 phosphate either in the homogenate or mitochondria. Thus, dilution of the specific activity of [U-^{14}C]glycerol-3 phosphate in the control preparations could not be the reason for their lower activity than those from PCB treated rats.

Levels of PCB Residues in Whole Liver and Subcellular Fractions After 30 Days on 0.1% (w/w) PCB Diet

Since the activity of a homogenate preparation from PCB treated rats was inhibited upon the addition of PCB *in vitro*, the question arose as to whether any PCBs accumulate in the livers at all after the 30-day treatment. Therefore the levels of PCB in the whole liver, mitochondria and microsomes after the 30-day PCB treatment was determined. These results are shown in Table 19. The results show that there was indeed a significant accumulation of PCBs in the whole liver, and in concentrations that effectively inhibit incorporation of

Table 19. PCB residue levels in whole liver and subfractions after 30 day treatment.*

	Whole Liver Range (PPM)	Mitochondria Range (PPM)	Microsomes Range (PPM)
Control	0.089 - 0.326		
Treated	246 - 450	60 - 87	5.5 - 6.4

*PCB residues were determined with a Hewlett-Packard 5700A series chromatograph with a Ni^{63} electron capture detector as described under Materials and Methods.

Values are determinations from three animals.

radioactivity when tested in vitro. The amounts accumulating in the mitochondria were moderate while that in the microsomes was very low. Thus, in spite of the fact that there is some PCB accumulating in the liver, one still sees a higher activity when homogenate and mitochondria were used. The question naturally to ask then is where are the PCBs located in these organelles with respect to the enzyme system in the intact animal? No attempt has been made to answer this question in the present work, but it is something that is worthy of consideration for future purposes.

Determination of the Activities of Other Key Enzymes in the Presence of PCB

In order to ascertain whether other key enzymes in the pathway of phospholipid and glyceride biosynthesis could be influenced by PCB and thus contribute to the overall results obtained, changes in the activities of some other key enzymes in the absence and presence of PCB were determined.

Glycerol Kinase Activity

The activity of glycerol kinase was measured as described in the Methods section. It can be seen in Table 20 that PCB at both concentrations used had no effect on the activity of this enzyme *in vitro*. It should be noted that the higher PCB concentration used is about three times as high as the concentration at which sn-glycerol-3 phosphate acyltransferase was inhibited.

Hydrolysis of Phosphatidate by Microsomal Phosphatidate Phosphatase

The reaction catalyzed by phosphatidate phosphatase is of importance in the pathway since this reaction can regulate the amount of phospholipids and glycerides formed. Therefore, changes in the activity of this enzyme can be important in the regulation of the overall process. Thus, the determination of the mode of action of PCB on this enzyme was deemed important. Figures 10a, 10b and 11 illustrate the changes in the activity of this enzyme in the presence of PCB under *in vitro* and *in vivo* conditions, respectively. As can be seen, PCB had no effect *in vitro* on the initial hydrolysis of phosphatidate by phosphatidate phosphatase, but after a prolonged incubation there was a small increase (22 ±2%) in the activity of this enzyme under *in vitro* conditions. After *in vivo* treatment of rats for 30 days, the activity of this enzyme slightly decreased (18%) after prolonged incubation.

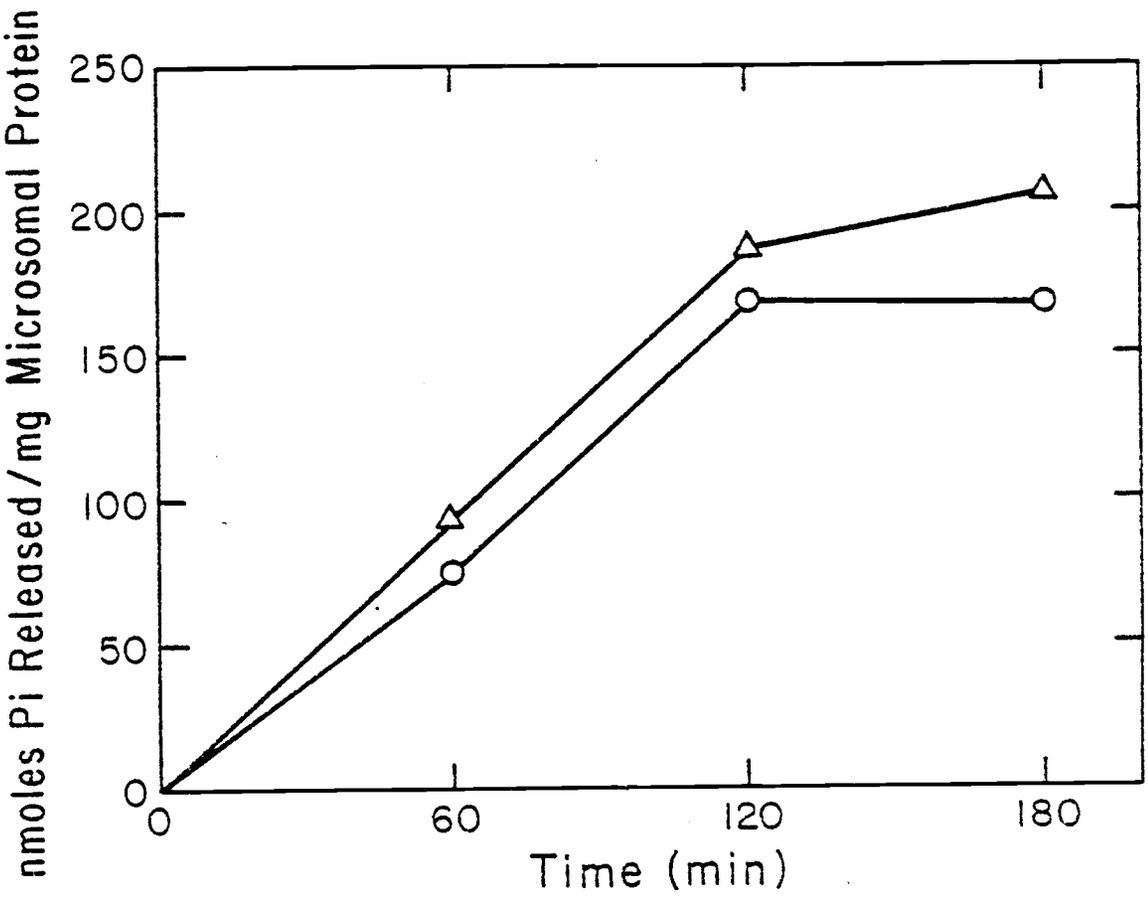

Figure 10a. Hydrolysis of phosphatidate by microsomal phosphatidate phosphatase in the absence and presence of PCB in vitro. Enzyme activity was measured as described under Materials and Methods. (O) control; (Δ) PCB, 1.2 mM.

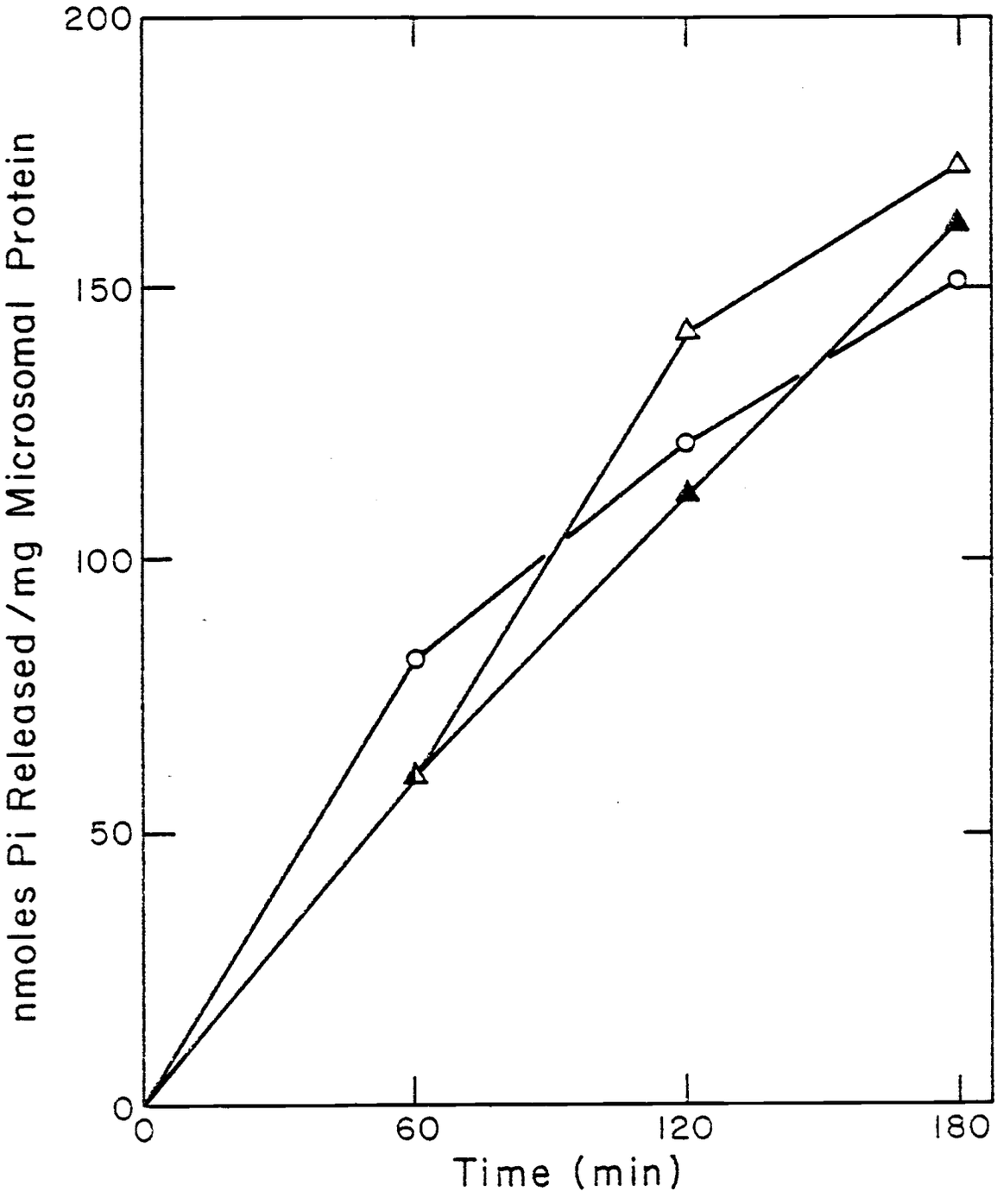

Figure 10b. In vitro effect of lower concentrations of PCB on microsomal phosphatidate phosphatase activity. (O) controls; (Δ) PCB, 0.47 mM; (▲) PCB, 0.18 mM.

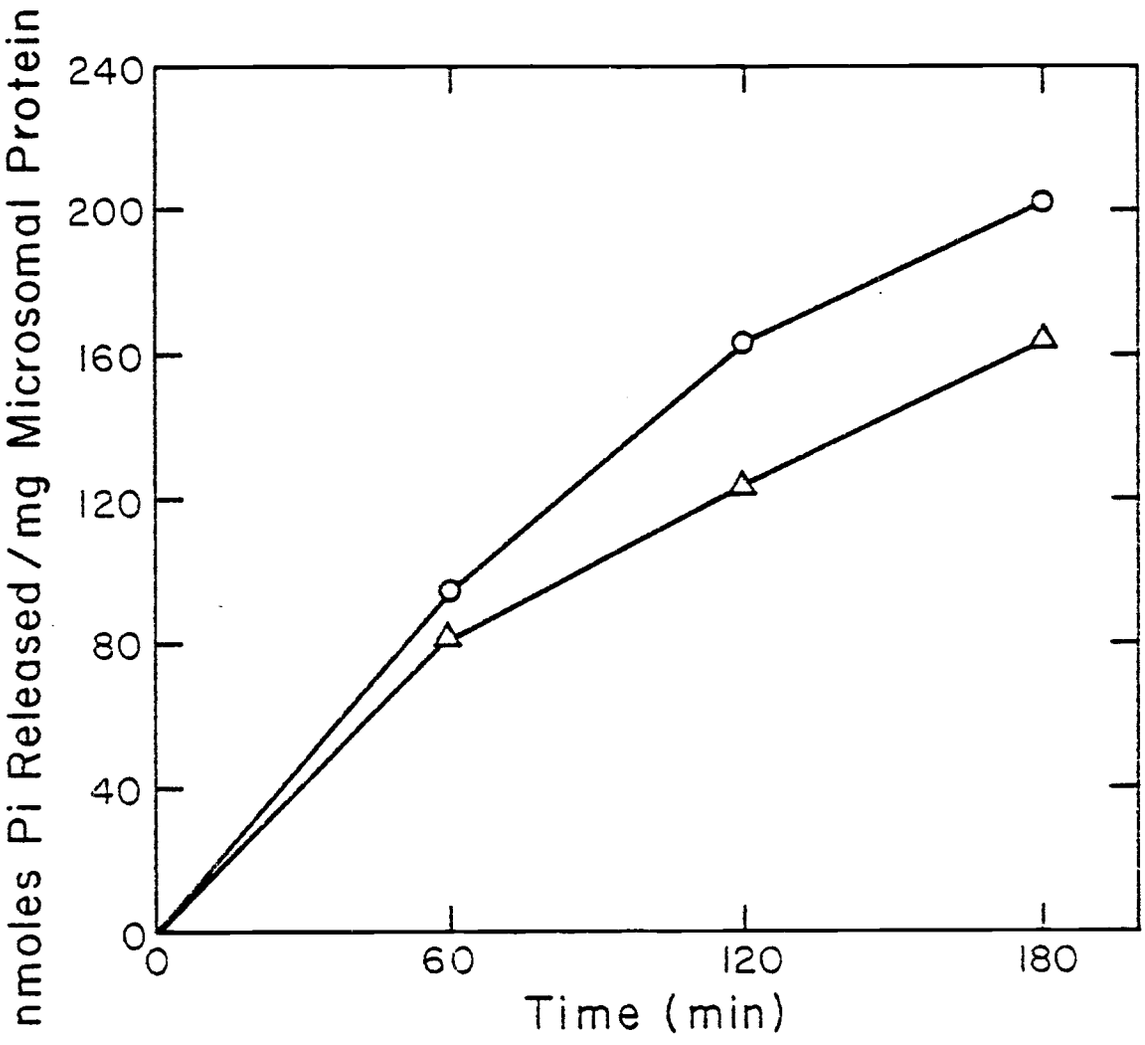

Figure 11. Hydrolysis of phosphatidate by microsomal phosphatidate phosphatase after in vivo treatment of rats with PCB for 30 days. Assay conditions are described under Materials and Methods. (O) control, (Δ) PCB.

Table 20. *In vitro* effect of PCB on glycerol kinase activity from rat liver.*

	Concentration mM	Glycerol-3 Phosphate CPM
Control		9000 ± 962
PCB	0.75	8087 ± 1075
PCB	1.5	9125 ± 1213

*Glycerol kinase was assayed by the method of Robinson and Newsholme as modified by Adams *et al*. (58). Homogenates were prepared as described under Methods and Materials.

Values are average of three determinations ± standard deviation.

Diglyceride Acyltransferase

The activity of microsomal diglyceride acyltransferase (acyl-CoA: 1,2-diglyceride O-acyltransferase EC 2.3.1.d) in the absence and presence of PCB *in vitro* was also determined. These results are summarized in Table 21. It can be seen that there was no significant difference in the radioactivity incorporated into the total lipids or that incorporated into triglycerides at both PCB concentrations used.

At 1.5 mM PCB, a 41% decline in the formation of free fatty acids which probably were formed by triglyceride lipase action on the synthesized triglycerides or by hydrolysis of the labelled palmitoyl-CoA was observed. It should be noted that the total free fatty acids formed represents only 12 and 7% of the total radioactive substrate added to the incubation medium under control and experimental conditions, respecively. Thus, even if this free fatty acid was being formed by hydrolysis of some of the substrate, there was still at least 88 to 93%

Table 21. Activity of microsomal diglyceride acyltransferase in the presence of PCB *in vitro*.*

Compound	Control CPM	0.6 mM PCB CPM	1.5 mM PCB CPM
Total Lipids	91,906 ± 4,014	99,028 ± 3,711 (+8)†	75,088 ± 4,808 (-18)
Triglycerides	54,548 ± 2,500	63,076 ± 2,300 (+16)	51,938 ± 3,167 (-5)
Free Fatty Acids	34,722 ± 1,514	34,645 ± 1,411 (-0.2)	20,431 ± 1,641 (-41)

*Microsomes were prepared and diglyceride acyltransferase activity determined as described under Materials and Methods.

Values are the average of three determinations ± standard deviation. ^{14}C-palmitoyl-CoA was the substrate.

†Values in parentheses represent percent difference from control values.

of the total substrate available for utilization by diglyceride acyltransferase. Thus, the interpretation of the overall results cannot be influenced to any significant degree by the formation of the free fatty acids.

Phosphorylcholine-Glyceride Transferase

Since under our incubation conditions there was no significant radioactivity getting into the membranous phospholipids such as phosphatidyl choline, phosphatidyl ethanolamine, etc., it was not readily possible to determine whether the synthesis of any of these phospholipids was being influenced by PCB or not. Thus, the mode of action of PCB *in vitro* on the synthesis of phosphatidyl choline, one of the major membranous phospholipids was determined by measuring the activity

Table 22. In vitro effect of PCB on microsomal phosphorylcholine-glyceride transferase activity.*

Compound	Control CPM	0.62 mM PCB CPM	1.5 mM PCB CPM
Lysolecithin	262 ± 22	290 ± 59	210 ± 24
Phosphatidyl Choline	7437 ± 401	6599 ± 899 (-11)†	5200 ± 380 (-30)
Others	186 ± 13	70 ± 8	113 ± 26
Observed Total Count	9970 ± 97	8340 ± 1190 (-16)	6490 ± 380 (-35)

*Microsomes were prepared and phosphorylcholine-glyceride transferase activity determined as described under Materials and Methods section. Values are the average of three determinations ± standard deviation. CDP-choline-[methyl-^{14}C] was the substrate.

†Values in parentheses represent percent inhibition from controls.

of phosphorylcholine-glyceride transferase, the last enzyme in the pathway of this phospholipid synthesis.

The activity of the enzyme was tested at comparable PCB concentrations that were found to inhibit sn-glycerol-3 phosphate acyltransferase in vitro. These results are summarized in Table 22. It can be seen that a 16 and 35% decline in the total radioactivity occurred at 0.62 mM and 1.5 mM PCB, respectively. Correspondingly, the radioactivity incorporated into phosphatidyl choline was decreased 11 and 30%. The results also indicate that the inhibition of both the total lipids as well as phosphatidyl choline is proportional to the concentration of PCB used.

Kinetics of sn-Glycerol-3 Phosphate Acyltransferase Reaction

Since among all the enzymes tested sn-glycerol-3 phosphate acyltransferase appeared to be the most sensitive to the presence of PCB in the system both under *in vitro* and *in vivo* conditions, it was of interest to determine how PCB affects the kinetics of the reaction catalyzed by this enzyme. The microsomal preparation was incubated with [U-^{14}C] glycerol-3 phosphate in the absence and presence of PCB, reaction stopped at predetermined times and radioactive products isolated and determined. Figure 12 illustrates the results of this experiment. At the PCB concentration used (0.62 mM) there was a dramatic decline in the radioactivity of the total lipids as well as in the major product, lysophosphatidate over the entire 60 minute incubation period studied.

Lineweaver-Burk Reciprocal Plot of sn-Glycerol-3 Phosphate Acyltransferase Activity

In order to determine the nature of the inhibition of sn-glycerol-3 phosphate acyltransferase by PCB, the Lineweaver-Burk reciprocal plot of the enzyme activity was determined. This is illustrated in Figure 13. It can be seen that the inhibition is a noncompetitive type suggesting that the PCB binds to the enzyme at a site that is different from its active site. The Km of the enzyme for sn-glycerol-3 phosphate was 7.4 mM and the inhibitor constant, Ki was 6.5 mM for PCB.

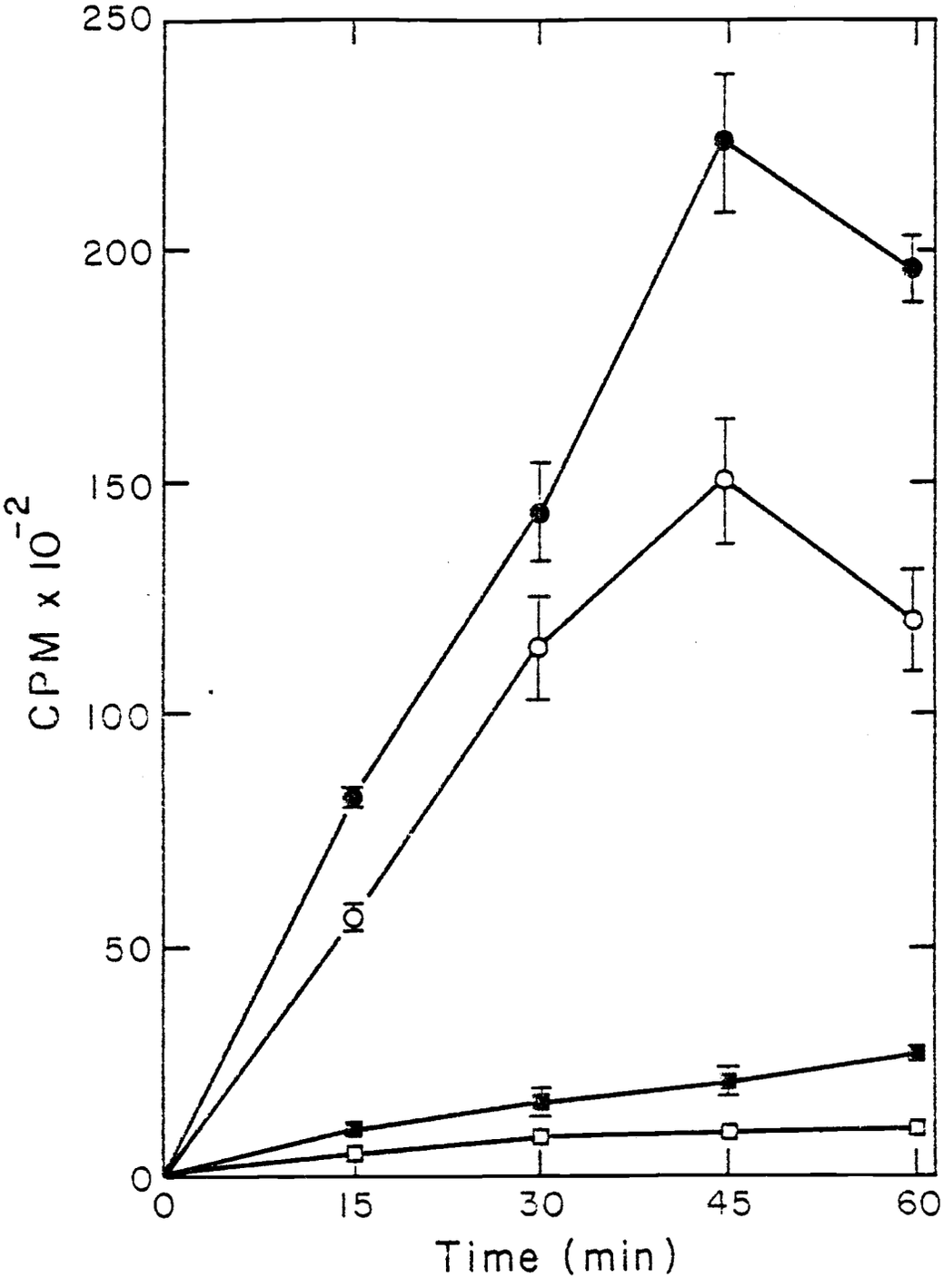

Figure 12. Kinetics of sn-glycerol-3 phosphate acyltransferase reaction in the absence and presence of PCB in vitro. Enzyme activity was measured as described under Materials and Methods. (O) control, total lipid, (O) control, lysophosphatidate, (■) 0.62 mM PCB, total lipid, (□) 0.62 mM PCB, lysophosphatidate.

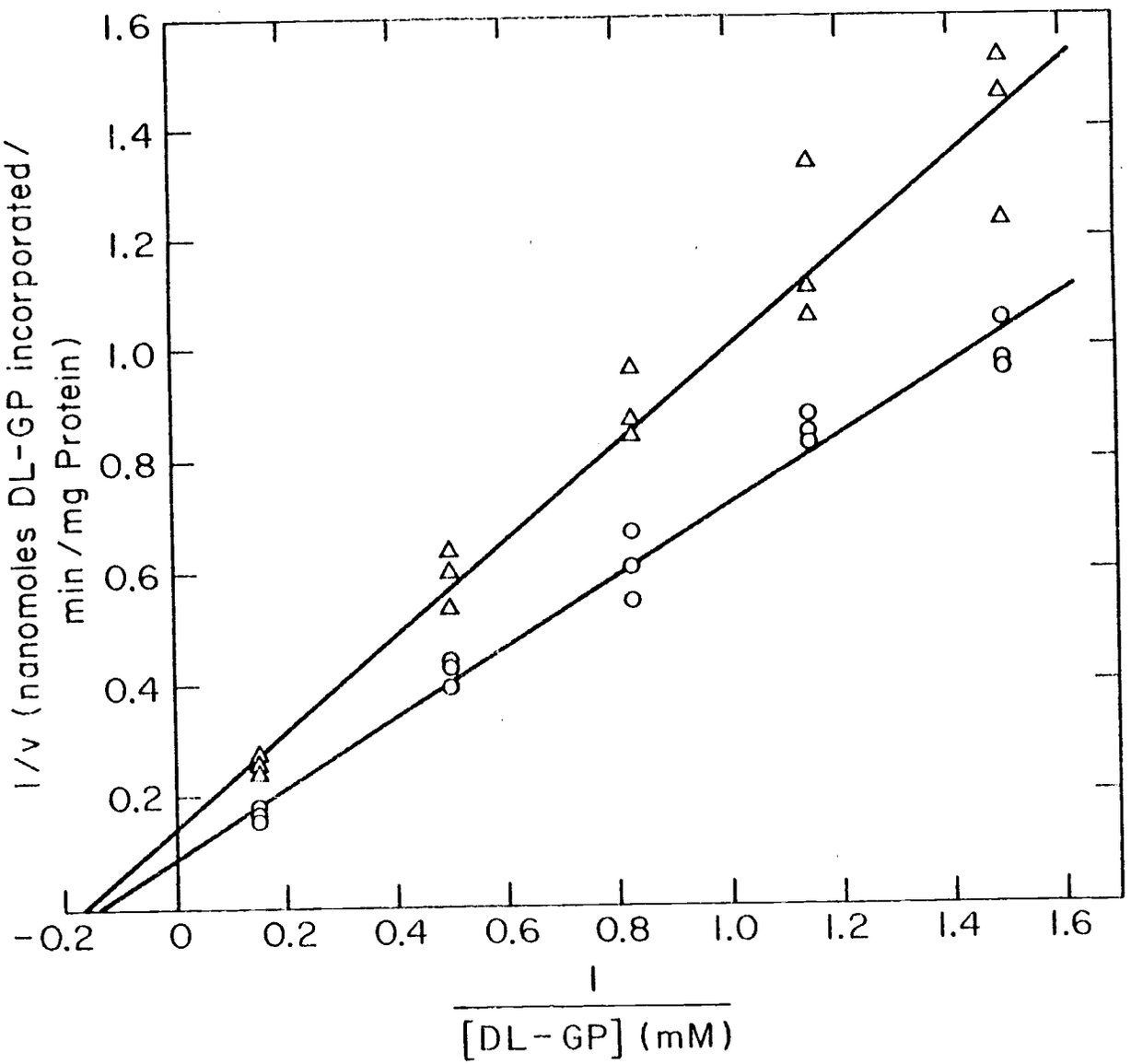

Figure 13. Lineweaver-Burk reciprocal plot of sn-glycerol-3 phosphate acyltransferase activity in the presence of PCB in vitro. (O) control, (Δ) PCB. 9.2×10^{-3} M

The Mode of Action of Chlorinated Biphenylols on Phospholipid and Glyceride Biosynthesis

Polychlorinated biphenyls are primarily metabolized to hydroxylated derivatives (12-20) and since the effect of these metabolites on phospholipid and glyceride biosynthesis has not been reported in the literature, it was of interest to determine whether these compounds also influence the biosynthesis of the above lipids. In addition, information obtained from experiments with these metabolites will indicate whether they contributed to the in vivo results seen with PCB when mitochondria and homogenates were used.

Two chlorinated biphenylols were used in this study, namely, 4'-chloro-4-biphenylol and 2',3',4',5,5'-pentachloro-2-biphenylol. The former was chosen as a model compound while the latter was more likely to be a product formed from Aroclor 1254 metabolism. All three subcellular fractions, namely microsomes, mitochondria and homogenates were employed in these experiments.

sn-Glycerol-3 Phosphate Acyltransferase and Microsomal Lipids

The effects of the above chlorinated biphenylols on the in vitro incorporation of [U-^{14}C]glycerol-3 phosphate into lipids by microsomal sn-glycerol-3 phosphate acyltransferase are summarized in Tables 23 and 24. Table 23 shows the results of [U-^{14}C]glycerol-3 phosphate incorporation into microsomal lipids in the presence of 4'-chloro-4-biphenylol (CB-OH). It can be seen that at 0.65 mM there occurred a 53% decline in the radioactivity incorporated into the total lipids.

Table 23. In vitro effect of 4'-chloro-4-biphenylol (CB-OH) on the incorporation of radioactivity into phospholipids and glycerides of microsomes.*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
Compound	Control	CB-OH	Control	CB-OH
Lysolecithin	520 ± 110	400 ± 67	1 ± 0.2	2 ± 0.3
Lysophosphatidate	38,160 ± 4,450	10,250 ± 783	85 ± 10	47 ± 4
PC + PE	1,230 ± 140	1,200 ± 83	3 ± 0.3	6 ± 0.4
Glycerides	4,240 ± 260	9,550 ± 1,217	9 ± 0.6	44 ± 6
Observed Total Count	65,700 ± 4,700	31,000 ± 9,442		

*Microsomes were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are means ± standard deviation. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine. The conentration of CB-OH was 0.65 mM.

Table 24. In vitro effect of 2',3',4',5,5'-pentachloro-2-biphenylol (PCB-OH) on the incorporation of radioactivity into phospholipids and glycerides of microsomes.*

	CPM/mg Protein		% of Total CPM	
Compound	Control	PCB-OH	Control	PCB-OH
Lysolecithin	540 ± 65	98 ± 3	3 ± 0.4	2 ± 0.1
Lysophosphatidate	13,418 ± 1,480	2,798 ± 510	85 ± 9	61 ± 11
PC + PE	320 ± 93	220 ± 38	2 ± 0.6	5 ± 0.8
Glycerides	660 ± 33	1,005 ± 123	4 ± 0.2	22 ± 3
Observed Total Count	20,252 ± 3,475	4,900 ± 1,050		

*Microsomes were prepared and incorporation of radioactivity determined as indicated under Materials and Methods.

Values are means ± standard deviation. ^{14}C-glycerol-3 phosphate was the substrate. The concentration of PCB-OH was 0.35 mM. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

In addition, lysophosphatidate radioactivity was decreased 73% whereas the total glycerides were increased 56 percent. The CB-OH also altered the distribution of the total radioactivity in such a way that lysophosphatidate decreased while the glyceride percentage increased.

In Table 24, the effect of the pentachlorobiphenylol is summarized. At 0.35 mM the radioactivity incorporated into the total lipids declined 76%. Correspondingly, there was a 79% decline in the radioactivity of the major product, lysophosphatidate, whereas glycerides increased 34%. There was also a change in the distribution of the total radioactivity such that lysophosphatidate decreased while the glycerides increased.

Table 25 shows the results obtained when ^{14}C-palmitoyl-CoA was used as the labelled substrate. In this particular experiment, considerable hydrolysis of the labelled substrate into free fatty acids was observed. Thus, only a very small percentage of the label was found in lysophosphatidate and the other lipids. In spite of this problem, it is still evident that at 0.35 mM pentachlorobiphenylol, there was 94% inhibition of lysophosphatidate formation from this substrate, while phosphatidyl choline was inhibited 75%. In contrast, lysolecithin although constituting only a small fraction of the total radioactivity, was stimulated 53%.

Since the free fatty acid formed represents about 12% of the total radioactivity added to the incubation medium under control conditions and much less under experimental conditions, there was still at least 88% of the labelled substrate available for sn-glycerol-3 phosphate acyltransferase reaction. Disregarding the free fatty acid

Table 25. Incorporation of radioactivity into phospholipids and glycerides of microsomes in the presence of 2',3',4',5,5'-pentachloro-2-biphenylol (PCB-OH) in vitro.*

	CPM/mg Protein		% of Total CPM	
Compound	Control	PCB-OH	Control	PCB-OH
Lysolecithin	190 ± 47	403 ± 78	0.2 ± 0.05	1 ± 0.3
Lysophosphatidate	19,884 ± 797	1,178 ± 288	17 ± 1	4 ± 1
PC	2,744 ± 447	678 ± 147	2 ± 0.4	2 ± 0.5
Free Fatty Acids + PE?†	83,409 ± 4,475	18,190 ± 4,184	73 ± 4	63 ± 15
Glycerides	7,588 ± 1,813	7,172 ± 1,653	7 ± 2	25 ± 6

*Microsomes were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are means ± standard deviation. ^{14}C-palmitoyl-CoA was the substrate. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

†Over 90% of the counts in this band were shown to be in the free fatty acids when an aliquot of the samples were separated in a different solvent system. The rest could be PE or something else.

band, one can see that the percent radioactivity of the glycerides was increased while that of lysophosphatidate decreased. It is of interest to know that the formation of the free fatty acids was considerably inhibited (83%) by the pentachlorobiphenylol.

Synthesis and Distribution of Radioactivity in Mitochondrial Phospholipids and Glycerides

Mitochondria were incubated in vitro with [U-^{14}C]glycerol-3 phosphate in the absence and presence of 2',3',4',5,5'-pentachloro-2-biphenylol (PCB-OH) and the synthesis and distribution of the radioactivity of labelled substrate in the various lipid fractions was

Table 26. In vitro inhibition of [U-^{14}C]glycerol-3 phosphate incorporation into mitochondrial lipids by 2',3',4',5,5'-pentachloro-2-biphenylol (PCB-OH).*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
Compound	Control	PCB-OH	Control	PCB-OH
Lyso PC	21 ± 3	9†	1 ± 0.1	3
Phosphatidic Acid	626 ± 123	111	25 ± 5	38
PC + PE	18 ± 0	21	1 ± 0	1
Glycerides	1,869 ± 233	152	74 ± 9	52
Observed Total Count	3,718 ± 436	318		

*Mitochondria were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are the average of three determinations ± standard deviation. The concentration of PCB-OH was 0.47 mM. ^{14}C-glycerol-3 phosphate was the substrate. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

†Due to the small radioactivity incorporated, the three samples were pooled together and chromatographed. The values shown, therefore, are the average of three pooled samples.

determined as described in the Methods section. These results are summarized in Table 26. A large decline in the radioactivity incorporated into the total lipids as well as the major products was observed in the presence of the pentachlorobiphenylol. Changes in the distribution of the total radioactivity was also observed, in that more of the total radioactivity was incorporated into the glycerides under the control conditions than under experimental conditions.

Incorporation of Radioactivity into Phospholipids and Glycerides by Rat Liver

In addition to microsomes and mitochondria, rat liver cell-free homogenates were also used to study the mode of action of chlorinated biphenylols on the biosynthetic process. Cell-free homogenates were prepared and incorporation of radioactivity into lipids determined with or without the addition of 2',3',4',5,5'-pentachloro-2-biphenylol. These results are shown in Table 27. It can be seen that the radioactivity incorporated into total lipids, phosphatidic acid and glycerides, in vitro, decreased 51, 55 and 41%, respectively, at 0.47 mM pentachlorobiphenylol. The other lipid fractions were much less affected. There was no major change in the distribution of the total radioactivity.

Lineweaver-Burk Reciprocal Plot of sn-Glycerol-3 Phosphate Acyltransferase Activity

In order to determine the mode of inhibition of sn-glycerol-3 phosphate acyltransferase activity by 2',3',4',5,5'-pentachloro-2-biphenylol as well as to compare the nature of inhibition to that of the parent PCBs, the Lineweaver-Burk reciprocal plot of the enzyme activity was determined. This result is illustrated in Figure 14. The inhibition apparently is a noncompetitive type as is the case with the parent PCBs.

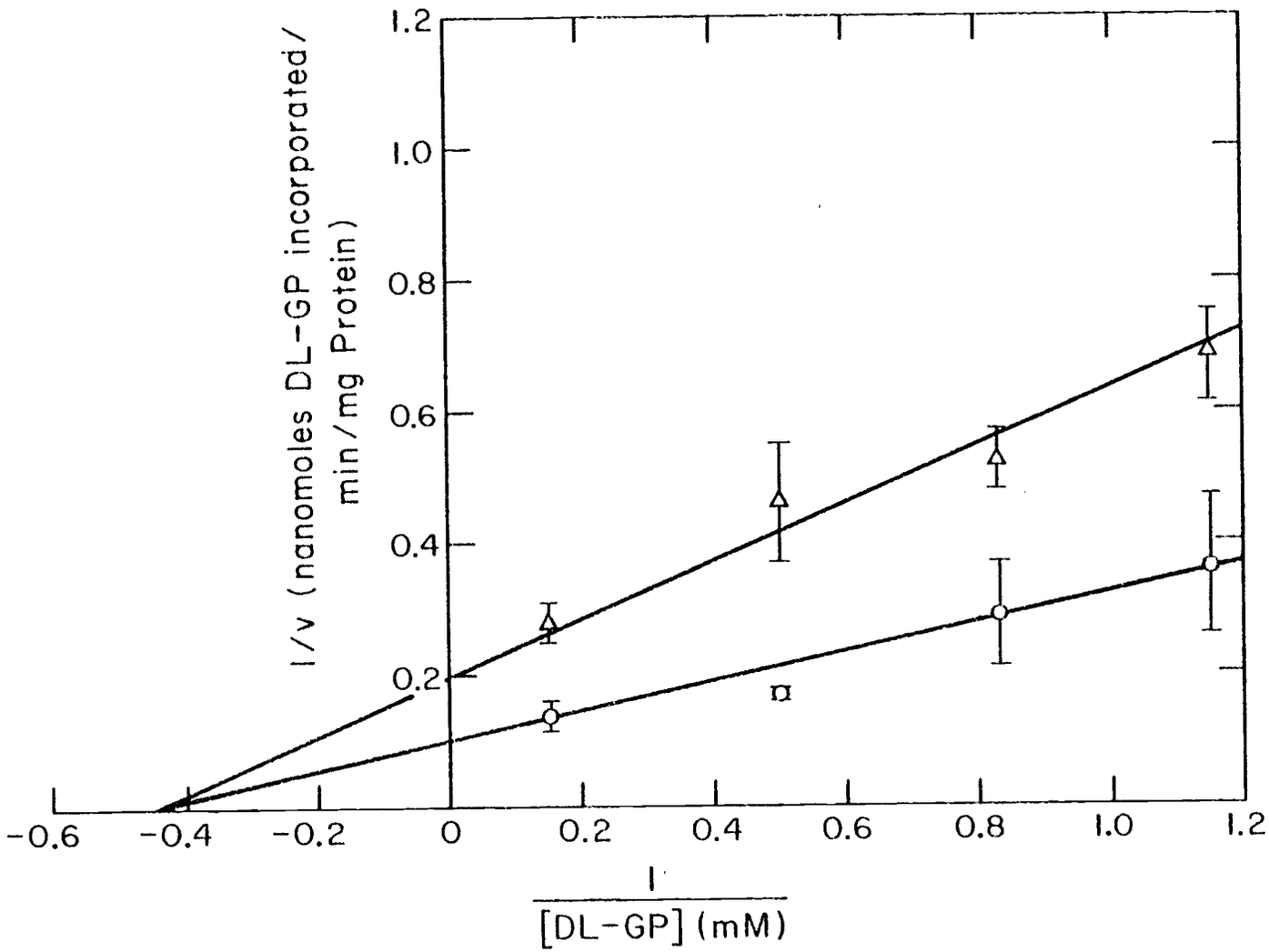

Figure 14. Lineweaver-Burk reciprocal plot of sn-glycerol-3 phosphate acyltransferase activity in the presence of 2',3',4',5,5'-pentachloro-2-biphenylol (PCB-OH). (0) control, (Δ) PCB-OH 2.3 x 10^{-4} M.

Table 27. In vitro inhibition of [U-^{14}C]glycerol-3 phosphate incorporation into liver lipids by 2',3',4',5,5'-pentachloro-2-biphenylol (PCB-OH).*

	CPM/μmole Lipid Phosphorus		% of Total CPM	
Compound	Control	PCB-OH	Control	PCB -OH
Lysolecithin	71 ± 5	59 ± 5	1 ± 0.07	1 ± 0.08
Phosphatidate	5,486 ± 552	2,361 ± 282	57 ± 6	50 ± 6
PC + PE	290 ± 14	250 ± 11	3 ± 0.01	5 ± 0.01
Glycerides	3,698 ± 202	2,095 ± 248	39 ± 2	44 ± 5
Observed Total Count	14,476 ± 2,262	6,727 ± 1,455		

*Homogenates were prepared and incorporation of radioactivity determined as described under Materials and Methods.

Values are means ± standard deviation. The concentration of PCB-OH was 0.47 mM. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Effect on Microsomal Phosphatidate Phosphatase

The in vitro effect of 4'-chloro-4-biphenylol (CB-OH) and 2',3',4',5,5'-pentachloro-2-biphenylol (PCB-OH) on microsomal phosphatidate phosphatase activity are illustrated in Figures 15 and 16. Figure 15 shows that at 0.65 mM, the CB-OH had no effect on the kinetics of hydrolysis of phosphatidate by phosphatidate phosphatase. At 0.12 mM PCB-OH, there was no effect on the enzyme activity, but at 0.35 mM there was a small (25%) increase in the rate of hydrolysis.

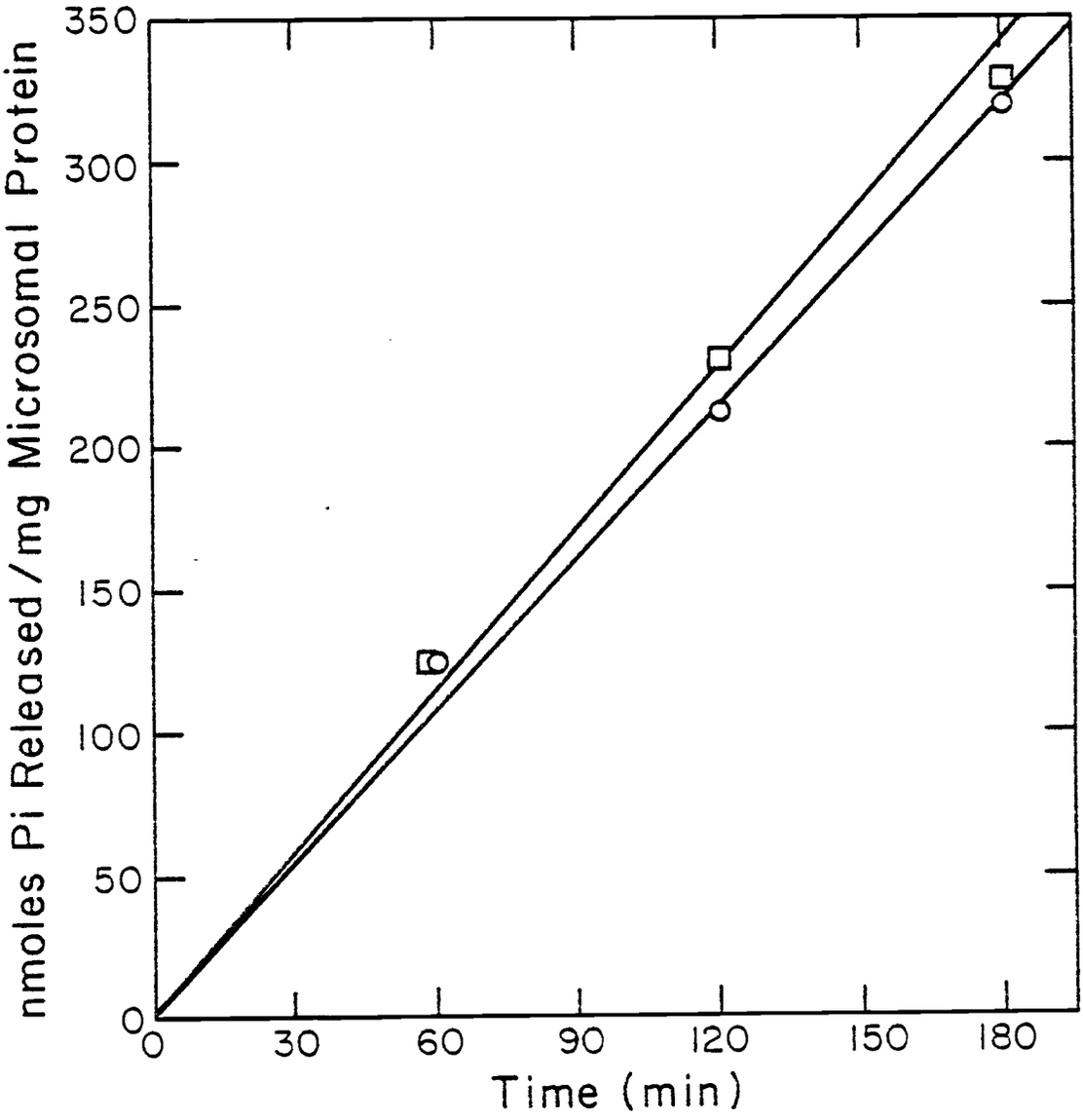

Figure 15. Hydrolysis of phosphatidate by microsomal phosphatidate phosphatase in the absence and presence of 4'-chloro-4-biphenylol (CB-OH) in vitro. (O) control; (□) CB-OH, 0.65 mM.

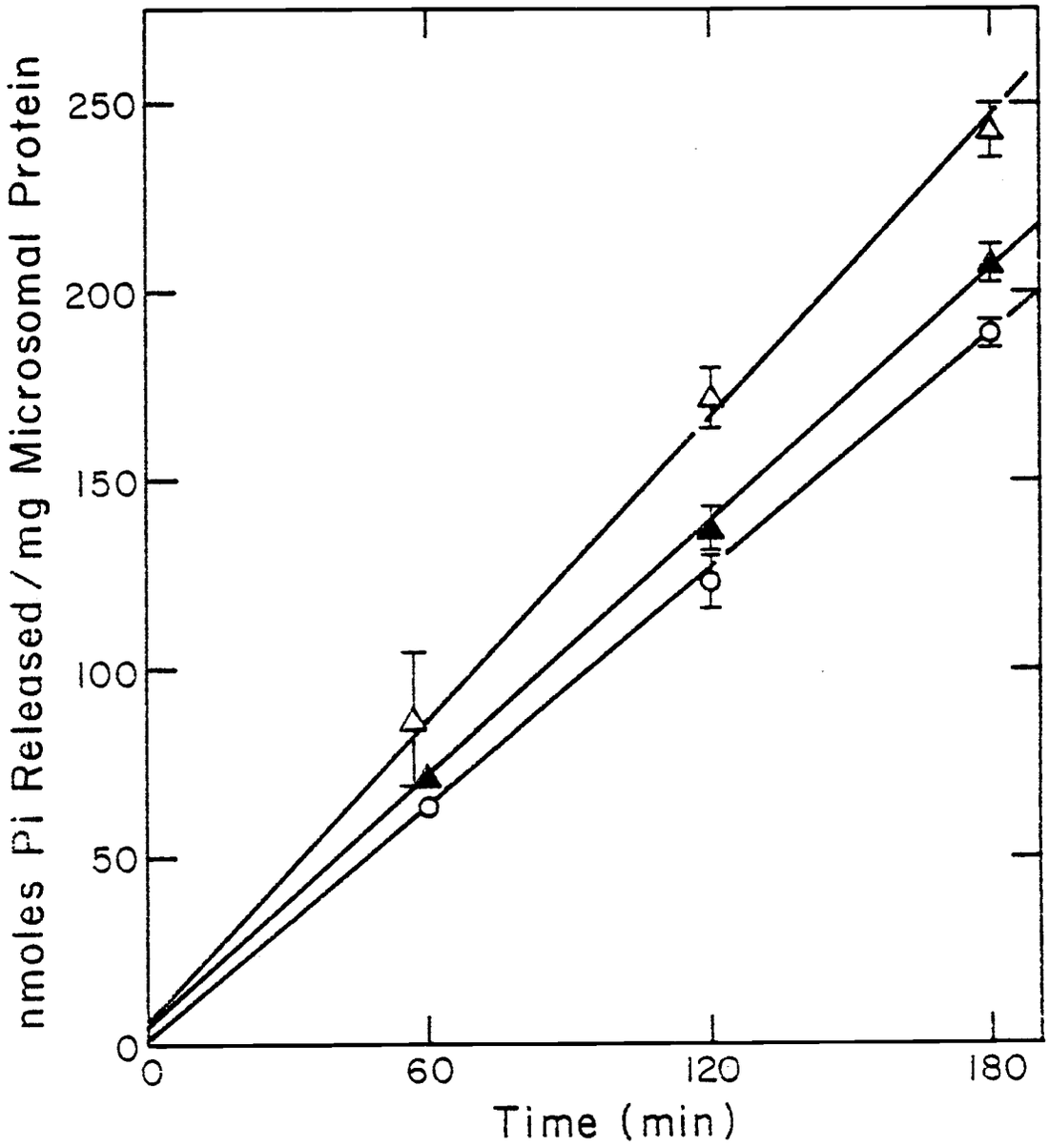

Figure 16. In vitro effect of 2',3',4',5,5'-pentachloro-2-biphenylol (PCB-OH) on microsomal phosphatidate phosphatase activity. The enzyme activity was determined as indicated in the Materials and Methods section. (O) control; (▲) 0.12 mM PCB-OH; (Δ) 0.35 mM PCB-OH.

DISCUSSION

The work described in this thesis demonstrates that changes in the activity of sn-glycerol-3 phosphate acyltransferase is sufficient to account for most of the observed differences seen in phospholipid and glyceride biosynthesis in the presence of PCBs or their metabolites. A model is proposed in this discussion to account for all the observed results. The model also will support our conclusion that a decrease in the activity of sn-glycerol-3 phosphate acyltransferase could account for the general decline in phospholipid and glyceride biosynthesis as has been reported in the literature and confirmed for the most part by this work. In addition, the model will also explain the overall results obtained with the metabolites of PCBs.

In vivo and *in vitro* Modifications of Phospholipid and Glyceride Biosynthesis by PCB

In general, the *in vitro* synthesis of phospholipids from sn-glycerol-3 phosphate by microsomes, mitochondria and cell-free homogenates was decreased in the presence of PCB. In microsomes PCB decreased the synthesis of phospholipids from sn-glycerol-3 phosphate whereas glycerides, although constituting only a small fraction of the total radioactivity, was actually increased. Both phospholipid and glyceride synthesis from sn-glycerol-3 phosphate by mitochondria decreased in the presence of PCB *in vitro*. There was also a concomitant alteration in the distribution of radioactivity such that less appeared in phosphatidate and more in the glycerides. When homogenates were used similar inhibitory effects of PCB on the *in vitro*

synthesis of phospholipids and glycerides was observed. In contrast to the microsomes and mitochondria, however, there was no significant alteration in the distribution of the total radioactivity.

When the total glyceride fraction obtained with the cell-free homogenate was analyzed for the individual glycerides, the synthesis of triglycerides was found to be most sensitive to the presence of PCB. At the same PCB concentration, the percent decline in triglyceride formation *in vitro* was much higher than that of phosphatidate, which is the direct intermediate in triglyceride and phospholipid synthesis.

The activity of microsomal sn-glycerol-3 phosphate acyltransferase was inhibited by PCB *in vitro*. The inhibition was noncompetitive suggesting that the PCB binds to a site on the enzyme other than the active site. When 2,4,5,2',4',5'-hexachlorobiphenyl (HCB) was used it elicited the same kind of response by inhibiting the activity of sn-glycerol-3 phosphate acyltransferase *in vitro*. This decrease in the enzyme activity was accompanied by a decline in the incorporation of glycerol-3 phosphate radioactivity into phospholipids and glycerides with changes in the distribution of total radioactivity.

The hexachlorobiphenyl is known to persist in the liver with only very small amounts metabolized. Thus, the observed *in vitro* results are at least in part due to unaltered PCBs. It should be mentioned that at comparable concentrations, the PCB was slightly more potent in inhibiting the sn-glycerol-3 phosphate acyltransferase activity than the HCB, suggesting that probably other isomers in the commercial PCB preparation might be contributing to the overall

inhibition by PCB. Holub et al. (44) have also reported the in vitro inhibition of sn-glycerol-3 phosphate acyltransferase by 2,4,2',4'-tetrachlorobiphenyl, a constituent of Aroclor 1254.

Garthoff et al. (11) have reported alterations in mitochondrial function of rats pretreated with Aroclor 1254 for three weeks. The PCB treatment resulted in an increased succinate oxidation by the mitochondria. The results presented here demonstrate that PCB in vitro alters the capacity of the mitochondria to synthesize phospholipids, compounds that are integral part of the mitochondrial membrane. Since many of the mitochondrial enzymes, especially those in the inner membrane are closely associated with the membrane structure and are dependent on the integrity of the membrane for activity, changes in the phospholipid composition or content of the mitochondrial membrane could result in a malfunction of this vital organelle.

It is important to note that we observed a decline in the radioactivity incorporated into lipid fractions as a result of the inhibition of sn-glycerol-3 phosphate acyltransferase activity by PCB. This observation is consistent with the idea that the reaction catalyzed by this enzyme controls the flux of substrate through the pathway as one might expect. The only exception to the above observation was the increase in the glyceride fraction when microsomes were used. Our proposed model described later in this discussion could, however, account for this difference.

After in vivo treatment of rats for 30 days with PCB, there was a general increase in the incorporation of [U-^{14}C]glycerol-3 phosphate into phospholipids and glycerides by mitochondria and homogenates.

This phenomenon was consistently observed after three consecutive experiments. In contrast, with the exception of one initial experiment, microsomes consistently exhibited a decline in the incorporation of radioactivity into phospholipids and glycerides. This was true whether [U-^{14}C]glycerol-3 phosphate or ^{14}C-palmitoyl-CoA was used. It should be remembered that the incubation conditions in the microsomes allows one to specifically limit the major part of the reaction to that catalyzed by sn-glycerol-3 phosphate acyltransferase. This will imply that the inhibition of incorporation of radioactivity seen in microsomes under *in vitro* or *in vivo* conditions should in part be due to a direct impairment of the activity of this enzyme. Since in most of the experiments described here, microsomes, mitochondria and homogenates were usually prepared by differential centrifugation of homogenized pooled livers from three to five rats, the different *in vivo* results obtained with microsomes from those with mitochondria and homogenates should be inherent in the liver subcellular fractions themselves and not due to differences inherent in individual rats used.

It is noteworthy that under the *in vivo* conditions, homogenates always showed very high incorporation of both [U-^{14}C]glycerol-3 phosphate and ^{14}C-palmitic acid into triglycerides. Elevations in the serum triglyceride levels in rabbits poisoned with PCB for 11 days has been reported by Ito *et al*. (41). Hirayama (40) reported that in PCB poisoned humans elevated serum triglyceride levels were among one of the first metabolic disorders observed. Litterst *et al*. (8) also reported high triglyceride levels in rats fed Aroclor 1254 in the diet for four weeks.

The data obtained under *in vitro* and *in vivo* conditions when homogenates and mitochondria were used appear to be opposing but if one considers the possibility that after the *in vivo* treatment of rats with PCB, some product(s) was formed that could cause an increase in the activity of sn-glycerol-3 phosphate acyltransferase or in its content, then the results obtained under the two conditions should not be surprising. In an attempt to test this possibility, experiments were performed in which microsomes, mitochondria and homogenates each separately from control and PCB treated rats were mixed in fixed ratios based on their initial protein concentration and incubated with radioactive substrates.

The rationale behind such an experiment is that if the overall results obtained *in vivo* were due to a direct effect of PCB (or product) on the activity of sn-glycerol-3 phosphate acyltransferase, then one should expect an additive effect when enzyme preparations from control and treated rats were mixed in fixed ratios based on their initial activities. However, such an additive effect would not be observed if other unknown factors were indirectly influencing the lipid synthesis. The results obtained in these experiments were not equal to the sum of the two individual activities as would be expected. For example, when microsomes were mixed in the ratio 1:1, the incorporation of radioactivity into the total lipids as well as the major product was higher than would be expected from the sum of the two activities. When homogenates were mixed either in the ratio 1:1 or 1:3 (control:PCB), the radioactivity incorporated into the total lipids as well as the major product was somewhat lower than the sum of the

two activities. The results rather appeared as though there was a factor (PCB?) in the treated preparations which was diluted out upon mixing so that it was no longer stimulatory. In addition, the PCB present in the treated preparations (from our GC analysis) could inhibit the activity of the control preparation upon mixing the two. These two factors taken together could account for the lower incorporation of radioactivity observed upon mixing the two preparations. This observation was found to be true for the mitochondria also, although the difference was not as significant as observed with the cell-free homogenate system.

In order to determine whether the activity of the liver preparations from PCB treated rats could be influenced by exogenously added PCB, the activities of microsomes and cell-free homogenates were determined in the presence of PCB in vitro. The activities of both preparations declined when the PCB was added. This phenomenon was observed despite our finding that there was already high accumulation of PCB in the livers of rats fed the PCB diet. This is a further indication that the results seen in vivo with mitochondria and cell-free homogenates might not be due to a simple direct effect of PCB per se.

Since intermediary metabolism is a highly integrated process, the interpretation of the in vivo data should be guarded with care since indirect effects of other reactions might also come into play. The rats in the experiments were not pair-fed and thus slightly different amounts of food were consumed by control rats and treated rats. However, analysis of the average food consumption did not reveal any

statistically significant difference between the two groups and therefore cannot account for the overall *in vivo* results.

Perhaps further experiments might be useful in explaining the different *in vivo* response as observed in the mitochondria and the cell-free homogenate. For example, if the mitochondria responded to the PCB treatment by making more glycerol-3 phosphate acyltransferase while the total mitochondrial protein remains unchanged, then it could account for the results. Thus, if one could show that there is more mitochondrial glycerol-3 phosphate acyltransferase formed after the PCB treatment, then it could be a valid reason for the increased activity of the mitochondrial fraction. In addition, no *in vivo* dose-related response experiments were performed in this project. Thus, the PCB concentration at which the mitochondrial system begins to show an increased activity is not known. Therefore, such an experiment might also lend a further insight to the observed results.

It is clearly noticeable in some of the results that there are variations in the radioactivity incorporated into the lipid fractions when one particular experiment was repeated using different animals a number of times. The reason for this variation stems from the problem mentioned earlier that the liver preparations exhibited varying degrees of activity from one preparation to another. Despite these differences, however, there was a high degree of consistency in the results with respect to the effect of PCB on the biosynthetic process under each particular experimental condition when the same preparations were employed.

In the *in vivo* experiments when microsomes were incubated with

either [U-^{14}C]glycerol-3 phosphate or ^{14}C-palmitoyl-CoA different distribution patterns of radioactivity in lipids were observed. Thus, whereas 85 to 95% of the radioactivity of [U-^{14}C]glycerol-3 phosphate was incorporated into total phospholipids, the radioactivity of ^{14}C-palmitoyl-CoA was evenly distributed between phospholipids and glycerides. The reasons for this appear to be two-fold. In the first place, the Km of sn-glycerol-3 phosphate acyltransferase for palmitoyl-CoA is about ten times lower than for sn-glycerol-3 phosphate as reported by Fallon and Lamb (57). Therefore, more palmitoyl-CoA is usually incorporated into the lipids than sn-glycerol-3 phosphate. Secondly, although lysophosphatidate acyltransferase, the second enzyme in the pathway (see Figure 5) was operating at a very low rate under the incubation conditions (74), its activity has been reported by Lamb and Fallon (67) to be about 20 times that of sn-glycerol-3 phosphate acyltransferase. Therefore, whenever there was a build-up of radioactivity in lysophosphatidate, one would expect lysophosphatidate acyltransferase to become active and convert the lysophosphatidate to phosphatidate. This phosphatidate could then be hydrolyzed to diglycerides by microsomal phosphatidate phosphatase. It should be mentioned that under the incubation conditions, diglycerides were the only major glycerides found. The same results were observed by Lamb and Fallon (74) whose microsomal preparation and incubation conditions were identical to the ones used in this work.

Response of Other Enzymes to the Presence of PCB *in vitro*

The response of some other key enzymes in the pathway of phospholipid and glyceride biosynthesis to the presence of PCB *in vitro* was studied in order to determine whether changes in the activities of these enzymes could contribute to the overall observed results. The activity of glycerol kinase was not affected by PCB *in vitro*, even at concentrations that were about three times as high as that used for the inhibition of sn-glycerol-3 phosphate acyltransferase.

The activity of microsomal phosphatidate phosphatase, an enzyme that occupies a branching point in the pathway, was found under *in vitro* conditions to be unaffected during the initial stages of catalysis. After prolonged incubation, however, there was a slight increase in the activity. This latter observation would imply that there was probably a biochemical transformation of the parent PCB into a metabolite by microsomal mixed-function oxidase system and this metabolite was responsible for the slight increase in the activity of the phosphatidate phosphatase at longer periods of incubation. This interpretation is supported by the finding that, *in vitro*, the activity of the phosphatidate phosphatase was slightly stimulated (25%) by 2',3',4',5,5'-pentachloro-2-biphenylol. After *in vivo* treatment of rats for 30 days, phosphatidate phosphatase was initially unaffected followed by a slight decrease in the activity after prolonged incubation. Lamb and Fallon (67) have suggested that phosphatidate phosphatase is the rate limiting enzyme in the biosynthesis of triglycerides *in vitro*. However, the changes observed in the activity of this enzyme

were small in comparison to the overall effects of PCB on phospholipid and glyceride synthesis. Thus, the changes in activity of this enzyme cannot account for the results. Furthermore, the fact that one still saw a large decline in triglyceride synthesis *in vitro* despite a small increase in the phosphatase activity *in vitro* lends further support to the argument that changes in the activity of this enzyme cannot account for the overall results.

The possibility that diglyceride acyltransferase, the last enzyme in the pathway of triglyceride synthesis might be inhibited by PCB *in vitro* and thus account for the observed large decline in triglyceride formation was also found not to be true. At comparable concentrations of PCB, there was very little, if any, effect on diglyceride acyltransferase. Thus, it is probable that the reason for the large decline in triglyceride formation *in vitro* might be due to increased breakdown of this compound by triglyceride lipases which would be present in the liver preparations used. An alternative explanation is that the radioactivity incorporated into phosphatidate, the direct intermediate for triglyceride synthesis, is usually low in the presence of PCB and thus could ultimately affect the radioactivity incorporated into the final product.

The activity of microsomal phosphorylcholine-glyceride transferase, the last enzyme in the biosynthesis of phosphatidyl choline was inhibited (30%) at 1.5 mM PCB *in vitro*. At 0.62 mM the inhibition was only 16%. It should, however, be pointed out that since the reaction catalyzed by this enzyme has been reported by Bjornstad and Bremer (75), Kanoh and Ohno (76, 77) and Sundler *et al*. (78) to be freely

reversible, the inhibition could have been much higher than observed, as no attempt was made to block the backward reaction under the assay conditions employed. Ishidate and Nakazawa (7) reported that after PCB treatment of rats for three days, the in vivo incorporation of (^{32}P)orthophosphate and (^{14}C)choline into choline-containing phospholipids declined, whereas the radioactivity incorporated into phosphatidyl ethanolamine and other phospholipids was unaffected. Based on these results they (7) concluded that PCB or its metabolies cause a severe inhibition of hepatic phospholipid synthesis at the level of CDP-choline formation either at the reaction catalyzed by choline kinase to form phosphorylcholine and/or that catalyzed by phosphocholine cytidyltransferase to form CDP-choline. In addition, based on their findings that the incorporation of (^{32}P)orthophosphate and (Me-^{14}C) choline into phosphatidyl choline and sphingomyelin was decreased to the same extent, whereas (^{3}H)glycerol incorporation was unaffected one hour after injection, they (7) concluded that the reaction catalyzed by phosphorylcholine-glyceride transferase was not affected. Contrary to the conclusions drawn by these authors, the results presented here do demonstrate that a direct test of phosphorylcholine-glyceride transferase found it to be inhibited by PCB in vitro.

It should be noted that under the in vivo conditions, several factors could account for the inhibition of choline kinase or phosphocholine cytidyltransferase. For example, if there is an inhibition of the activity of phosphorylcholine-glyceride transferase, the enzyme that converts CDP-choline to phosphatidyl choline, then there will be a build-up in the level of CDP-choline. This in turn will result in a

reduced activity of phosphocholine cytidyltransferase, the enzyme that forms CDP-choline from phosphocholine and CTP (inhibition by product accumulation). This will be especially true since the reaction catalyzed by phosphocholine cytidyltransferase has not been shown to be reversible. A decrease in the activity of phosphocholine cytidyltransferase could then account for the accumulation of phosphorylcholine reported in a subsequent experiment by Ishidate et al. (79). Alternatively, stimulation of choline kinase activity after PCB treatment with no effect on phosphocholine cytidyltransferase could also account for phosphorylcholine accumulation under in vivo conditions. It must also be mentioned that an additional possibility to the arguments presented above could equally be true. For example, a decline in the activity of phosphocholine cytidyltransferase will result in low levels of CDP-choline and thus account for the decline in activity of phosphorylcholine-glyceride transferase. Despite this alternative explanation, our results do suggest that the decline in activity of phosphorylcholine-glyceride transferase could in part account for the decreased synthesis of microsomal phosphatidyl choline in the presence of PCB.

Mode of Action of Chlorinated Biphenylols on Phospholipid and Glyceride Biosynthesis in vitro

Polychlorinated biphenyls are primarily metabolized in animal systems into hydroxy (phenolic) derivatives. The effect of these PCB metabolites on phospholipid and glyceride biosynthesis has not been reported, thus it was of interest to determine whether these metabolites

also interfere with lipid metabolism in animals and secondly whether they contribute to the observed results seen with PCBs *in vivo*. As previously reported (54, 55) changes in sn-glycerol-3 phosphate acyltransferase activity appeared to account for most of the observed effects of PCB on phospholipid and glyceride biosynthesis. Therefore, the effect of PCB metabolites on the activity of this enzyme *in vitro* was first determined.

Both 4'-chloro-4-biphenylol and 2',3',4',5,5'-pentachloro-2-biphenylol were found to severely inhibit the incorporation of [U-^{14}C] glycerol-3 phosphate into lipids by microsomal sn-glycerol-3 phosphate acyltransferase at concentrations of 0.65 mM and 0.35 mM respectively. The pentachlorobiphenylol was without effect at 0.07 mM but was completely inhibitory at 0.7 mM (results not shown). Both chlorinated biphenylols inhibited the formation of lysophosphatidate from sn-glycerol-3 phosphate but with increases in glyceride formation. When mitochondria and cell-free homogenates were used, the pentachlorobiphenylol similarly inhibited the synthesis of phospholipids from [U-^{14}C]glycerol-3 phosphate. There was also decreases in glyceride formation in contrast to observed results in the microsomes. At comparable concentrations, the pentachlorobiphenylol was more potent in inhibiting sn-glycerol-3 phosphate acyltransferase activity than the parent PCBs.

On the premise that the increased glyceride synthesis observed in the microsomes could be a result of a stimulatory effect on phosphatidate phosphatase activity, the activity of this enzyme was tested *in vitro*. The 4'-chloro-4-biphenylol was found to have no significant

effect on the enzyme, although at the same concentration it inhibited sn-glycerol-3 phosphate acyltransferase activity 73%. At 0.12 mM the pentachlorobiphenylol had no significant effect on phosphatidate hydrolysis by phosphatidate phosphatase, but at 0.35 mM there was a slight (25%) stimulation of this enzyme indicating that this stimulation might in part account for the increased glyceride synthesis from sn-glycerol-3 phosphate at 0.35 mM pentachlorobiphenylol.

Our results are for the most part in agreement with those reported in the literature. For example, Ishidate and Nakazawa (7) observed both decreased and increased synthesis of phospholipids in PCB-treated liver microsomes. The decreases occurred at early periods and the increases after longer duration of incubation. Hinton et al. (9) also reported decreases in microsomal phospholipid and triglyceride synthesis in rats injected with Aroclor 1254 for three or six days. The incorporation of radioactivity into phospholipids and triglycerides by liver homogenates was also found to decrease after the PCB treatment (9). This latter observation, however, does not agree with our in vivo results when mitochondria and the cell-free homogenates were employed. Perhaps the reason for the differences could either be due to the longer duration of PCB pretreatment employed in our experiments or differences in the experimental conditions. Hinton et al. (9) determined the in vivo effect of PCB on lipid synthesis by injection of substrates directly into the rats while our determinations were performed on isolated systems after the PCB treatment. Holub et al. (44) did not observe any effect of 2,4,2',4'-tetrachlorobiphenyl on lipid synthesizing enzymes, three days after the administration of the

tetrachlorobiphenyl. However, the activities of some of the enzymes were inhibited and some stimulated with the tetrachlorobiphenyl was tested *in vitro*. Among the enzymes inhibited was sn-glycerol-3 phosphate acyltransferase, which has been shown in this work to be inhibited both under *in vitro* and *in vivo* conditions.

Thus the general findings in this project indicate that commercial PCB preparations as well as purified isomers severely inhibit the activity of sn-glycerol-3 phosphate acyltransferase both *in vivo* and *in vitro*. The hydroxy metabolites of PCB were also found to severely inhibit the activity of this enzyme *in vitro* whereas phosphatidate phosphatase was only slightly affected. The decrease in the activity of sn-glycerol-3 phosphate acyltransferase was accompanied by a general decline in the *in vitro* synthesis of phospholipids and glycerides by microsomes, mitochondria and cell-free homogenates. Phospholipid and glyceride synthesis by microsomes also declined after the *in vivo* treatment of rats with PCB whereas mitochondria and cell-free homogenates showed apparent increases in the synthesis of lipids *in vivo*. With the exception of phosphorylcholine-glyceride transferase, PCB was found to have no significant effect on any of the other enzymes tested. A decrease in the activity of sn-glycerol-3 phosphate acyltransferase in the presence of PCB could account for the general decline in phospholipid and glyceride synthesis seen under both *in vitro* and *in vivo* conditions.

This conclusion is arrived at by a careful systematic analysis of all the data obtained under *in vitro* conditions together with the *in vivo* results obtained with microsomes. Taking the microsomal system

as an example we have proposed the following model to account for all the results obtained with this system. It is observed that when glycerol-3 phosphate in incubated with the microsomal system at pH 6.5, the major reaction is limited to the formation of lysophosphatidate with only small amounts of glycerides formed. Thus, the reaction:

$$\text{Glycerol-3P} + \text{Acyl-CoA} \xrightarrow{K_1} \text{lysophosphatidate} \xrightarrow{K_2} \text{Glycerides}$$

proceeds in such a way that $K_1 >> K_2$. In this model, K_1 and K_2 indicate the relative amounts of radioactivity incorporated into lysophosphatidate and glycerides, respectively and do not refer to the actual rates of reaction. Likewise, $K_{1(PCB)}$ and $K_{2(PCB)}$ are the corresponding notations when PCB is present in the system. When PCB is added to the microsomal system <u>in vitro</u>, the major reaction product is still lysophosphatidate so that in the presence of PCB, we still have $K_{1(PCB)} >> K_{2(PCB)}$. However, the formation of the lysophosphatidate decreases and the glycerides increase when PCB is present so that $K_1 > K_{1(PCB)}$ and $K_2 < K_{2(PCB)}$.

If one assumes that lysophosphatidate formation, that is, the sn-glycerol-3 phosphate acyltransferase reaction is the limiting one in the pathway, then the action of PCB was to uncouple this reaction from the other reactions in the pathway, along with a decrease in phosphorylcholine-glyceride transferase activity. This would result in a decrease in the regulation of the process leading to an increased activity of other enzymes, resulting in more glyceride formation. Under <u>in vivo</u> conditions, the same mechanism was operative in that lysophosphatidate was still the major product both in the absence and presence of PCB. However, under the <u>in vivo</u> conditions, the PCB

treatment resulted in a decrease in both lysophosphatidate and glyceride formation. The reason for the decreased glyceride formation was due to the fact that although the acyltransferase reaction was being perturbed by PCB, this perturbation was not sufficient enough (presumably because of lower PCB concentration in the system) to uncouple the reaction from the others so as to give rise to stimulation of other reactions. But since the substrate for glyceride synthesis was decreased by PCB and the formation of this substrate is the limiting step in the pathway, we see a decrease in glyceride synthesis.

The results obtained in vitro when the mitochondria and cell-free homogenates were used could be explained on the same basis as described for the microsomal system. In both of these systems when the sn-glycerol-3 phosphate acyltransferase reaction was allowed to proceed under optimal conditions (pH 7.5), lysophosphatidate no longer accumulated; instead we formed phosphatidate and the glycerides, the latter being the major product. Addition of PCB to each of these systems in vitro did not change the reaction pattern in that glycerides were still formed in larger amounts than phosphatidate. Here again there was decreased formation of phosphatidate and the glycerides. Since the acyltransferase reaction was not inhibited to such an extent as to uncouple it from the other reactions, regulatory control of the other reactions was not observed. The reason for the general decrease in the formation of both phosphatidate and glycerides was simply due to the inhibition of the acyltransferase reaction which controls the flux of substrate through the pathway.

A careful examination of all the data obtained using the chlorinated

biphenylols will indicate that most of these results can also be accounted for by the changes in activity of sn-glycerol-3 phosphate acyltransferase. This is not surprising since both the PCB and the metabolites inhibited the activity of this enzyme in a similar manner, i.e. noncompetitively.

Previous reports (7, 9) have indicated that PCBs in vivo caused a general decrease in the synthesis of microsomal phospholipids and triglycerides. However, the particular enzymatic reaction(s) responsible for this decreased lipid synthesis have not been identified. It is proposed here that the inhibition of sn-glycerol-3 phosphate acyltransferase activity by PCBs and their metabolites could account for most of these observations.

Under in vitro conditions, mitochondria and cell-free homogenates showed decreased incorporation of radioactivity into lipids in the presence of PCB. After in vivo treatment of rats with PCB, however, both systems showed increases in lipid synthesis. The different results obtained under the two experimental conditions could be due to several reasons, as explained below. It should first of all be emphasized that under the in vivo conditions, microsomes for the most part showed decreased activity whereas the mitochondrial activity increased. When the two systems were present together as was the case with the cell-free homogenate, the mitochondrial response was dominant. Thus, the homogenate activity under the in vivo conditions should be mainly due to the mitochondrial response. In the first place it is probable that the physicochemical state of the PCB entering the mitochondria upon feeding might not be the same as the PCB

added exogenously. Gas chromatographic analysis indicated that there was some PCB present in the mitochondria. PCB reaching the liver from the gut was most likely associated with the chylomicrons or lipoproteins and transported to the liver cells and subsequently to the mitochondria where it becomes associated with other hydrophobic proteins.

Another explanation and probably the most plausible one is that the PCB treatment might result in the alterations of the mitochondrial integrity. Carter and Cameron (10) and Garthoff et al. (11) have reported changes in the mitochondrial structure and integrity of rats pretreated with PCB. It is possible that when the mitochondrial structure is altered the organelles leak so that when exogenous substrates are added they readily traverse the membrane and become more available to the enzymes of interest. Since the mitochondrial structure is not altered in the liver cells of untreated rats, the added substrate does not traverse the membrane to the same extent. Hence, less substrate is made available and we see less incorporation of radioactivity under these conditions. The above explanation will also account for why the cell-free homogenates also reflect the mitochondrial response in vivo. Since the cell-free system contains both microsomes and mitochondria, but the microsomal enzyme was inhibited by PCB, less of the added substrate was used leaving more substrate available to the mitochondria. This explanation seems to be the most plausible one, but other possibilities cannot be excluded. For example, the presence of other minor metabolites such as the sulfate, glucuronide and sulfonyl derivatives have been reported to be formed from PCB metabolism in the liver. No reports have been made as to how

these minor metabolites affect the biosynthesis of phospholipids and glycerides. A simplified summary of the overall results is shown in Tables 28 and 29.

Table 28. The effect of PCB, HCB and PCB-OH under *in vitro* or *in vivo* conditions on the incorporation of ^{14}C radioactivity from sn-glycerol-3 phosphate into phospholipids and glycerides.

		In vitro			In vivo		
		Microsomes	Mitochondria	Homogenate	Microsomes	Mitochondria	Homogenate
PCB	Phospholipids	-	-	-	-	++	++
	Glycerides	+	-	-	-	++	++
HCB	Phospholipids	-	ND	-			
	Glycerides	+	ND	-			
PCB-OH	Phospholipids	-	-	-			
	Glycerides	+	-	-			

PCB, Aroclor 1254, a polychlorinated biphenyl mixture; HCB, 2,4,5,2',5',5'-hexachlorobiphenyl; PCH-OH, 2',3',4',5,5'-pentachloro-2-biphenylol
ND, not determined
+, stimulation
-, inhibition of incorporation of radioactivity into lipids
in vivo, rats fed 0.1% (w/w) PCB diet for 30 days
in vitro, see Tables 2, 3, 4, 6, 24 for examples of concentrations of toxicants used

Table 29. Effect of PCB and PCB-OH on the activities of other enzymes in the pathway of phospholipid and glyceride synthesis.

		Phosphatidate Phosphatase	Glycerol Kinase	Diglyceride Acyltransferase	Phosphorylcholine-glyceride transferase
PCB	*in vitro*	+ (small)	no effect	no effect	-
	in vivo	- (small)	ND	ND	ND
PCB-OH	*in vitro*	+ (small)	ND	ND	ND

PCB, polychlorinated biphenyl; PCB-OH, 2',3',4',5,5'-pentachloro-2-biphenylol; ND, not determined; +, stimulation; -, inhibition of enzyme activity.

SUMMARY

This project was undertaken in an attempt to determine the mode of action of polychlorinated biphenyls and their related metabolites on phospholipid and glyceride biosynthesis. The problem was approached in several ways. Firstly, the effect of PCB on phsopholipid and glyceride biosynthesis was investigated in different subcellular fractions of the rat liver in order to compare their similarities and differences. Secondly, a purified PCB isomer was used so as to compare its mode of action on the biosynthetic process with that of the commercial preparation, Aroclor 1254. In addition, results obtained after *in vivo* pretreatment of rats with PCB were compared with those obtained under *in vitro* conditions in order to properly assess the manner in which PCBs affect these biosynthetic processes. Finally, PCBs are primarily metabolized into phenolic derivatives in animals. The effects of these PCB metabolites on phospholipid and glyceride biosynthesis, to our knowledge, have not been reported in the literature. Therefore, this project also determined the mode of action of these PCB metabolites on phospholipid and glyceride biosynthesis.

The results indicate that PCBs or their metabolites cause a severe inhibition of phospholipid and glyceride biosynthesis at the level of the reaction catalyzed by sn-glycerol-3 phosphate acyltransferase. The inhibition of the activity of this enzyme *in vitro* was accompanied by a general decline in the incorporation of radioactivity into phospholipids by microsomes, mitochondria and cell-free homogenates. Glyceride synthesis was decreased in mitochondria and homogenates while it increased in the microsomes. Under *in vivo* conditions,

mitochondria and homogenates showed an apparent increase in the synthesis of phospholipids and glycerides whereas there was a decline in the incorporation when microsomes were used. The inhibition of the acyltransferase activity was noncompetitive suggesting a binding of the PCB to a site other than the active site of the enzyme. When a purified isomer, 2,4,5,2',4',5'-hexachlorobiphenyl was used, it elicited the same kind of response. It inhibited the activity of sn-glycerol-3 phosphate acyltransferase, which caused a general decrease in the synthesis of phospholipids and glycerides *in vitro*.

The activities of glycerol kinase and diglyceride acyltransferase were not affected by PCB *in vitro*. Microsomal phosphatidate phosphatase activity was not affected during the initial stages of catalysis, but after prolonged incubation, there was a slight increase or decrease in the activity under *in vitro* and *in vivo* conditions, respectively. The activity of microsomal phosphorylcholine-glyceride transferase was inhibited by PCB at 1.5 mM.

Both 4'-chloro-4-biphenylol and 2',3',4',5,5'-pentachloro-2-biphenylol severely inhibited the activity of microsomal sn-glycerol-3 phosphate acyltransferase accompanied by large decreases in the *in vitro* synthesis of phospholipids and glycerides by mitochondria and cell-free homogenates. With the exception of the *in vivo* response of the mitochondria and cell-free homogenates to the PCB treatment, the overall results are consistent with the conclusion that decreases in the activity of sn-glycerol-3 phosphate acyltransferase both *in vivo* (in microsomes) and *in vitro* in all the three systems could account for the decreased synthesis of phospholipids and glycerides in the

presence of PCB. In addition the inhibition of phosphorylcholine-glyceride transferase activity by PCB could account for the specific decrease in the synthesis of phosphatidyl choline as reported by Ishidate and Nakazawa.

BIBLIOGRAPHY

1. Widmark, G. 1967. Pesticide Residues. Possible Interference by Polychlorinated Biphenyls. J. Ass. Off. Anal. Chemists 50:1069.

2. Hutzinger, O., Safe, S., and Zitko, V. 1974. The Chemistry of Polychlorinated Biphenyls. p. 93. CRC Press, Cleveland, Ohio.

3. Risebrough, R. W. et al. 1968. Polychlorinated Biphenyls in the Global Ecosystem. Nature 220:1098-1102.

4. Peakall, D. B., and Lincer, J. L. 1970. Polychlorinated Biphenyls: Another Long-life Widespread Chemical in the Environment. Bioscience 20:958-968.

5. Risebrough, R. W., and deLappe, B. 1972. Polychlorinated Biphenyls in Ecosystems. Environ. Health Perspect. 1:39-45.

6. Price, H. A., and Welch, R. L. 1972. Occurrence of Polychlorinated Biphenyls in Humans. Environ. Health Perspect. 1:73-78.

7. Ishidate, K., and Nakazawa, Y. 1976. Effect of Polychlorinated Biphenyls (PCBs) Administration on Phospholipid Biosynthesis in Rat Liver. Biochem. Pharmacol. 25:1255-1260.

8. Litterst, C. L. et al. 1972. Effect of Polychlorinated Biphenyls on Hepatic Microsomal Enzymes in the Rat. Toxicol. Appl. Pharmacol. 23:112-122.

9. Hinton, D. E., Glaumann, H., and Trump, B. F. 1978. Studies on the Cellular Toxicity of Polychlorinated Biphenyls. Virchows Archiv. B Cell Pathol. 27:279-306.

10. Carter, J. W., and Cameron, I. L. 1977. Sublethal Effects of a Pure Polychlorinated Biphenyl on Mice. Exp. Mol. Pathol. 26:228-250.

11. Garthoff, L. H. et al. 1977. Biochemical and Cytogenetic Effects in Rats Caused by Short-term Ingestion of Aroclor 1254 or Firemaster BP6. J. Toxicol. Environ. Health 3:769-796.

12. Yoshimura, H. et al. 1971. Studies on the Tissue Distribution and the Urinary and Fecal Excretion of ^{3}H-Kanechlor (Chlorobiphenyls) in Rats. Fukuoka Acta Medica 62:12-19.

13. Hutzinger, O. et al. 1972. Polychlorinated Biphenyls: Metabolic Behavior of Pure Isomers in Pigeons, Rats and Brook Trout. Science 178:312-314.

14. Block, W. D., and Cornish, H. H. 1959. Metabolism of Biphenyl and 4-chlorobiphenyl in the Rabbit. J. Biol. Chem. 234:3301-3302.

15. Safe, S., Hutzinger, O., and Jones, D. 1975. The Mechanism of Chlorobiphenyl Metabolism. J. Ag. Fd. Chem. 23:851-853.

16. Wyndham, C., and Safe, S. 1978. In vitro Metabolism of 4-chlorobiphenyl by Control and Induced Rat Liver Microsomes. Biochemistry 17:208-215.

17. Gardner, A. M. et al. 1973. Polychlorinated Biphenyls: Hydroxylated Urinary Metabolites of 2,5,2',5'-Tetrachlorobiphenyl Identified in Rabbits. Biochem. Biophys. Res. Commun. 55:1377-1384.

18. Goto, M. et al. 1974. Metabolism of 2,3-dichlorobiphenyl-^{14}C and 2,4,6-trichlorobiphenyl-^{14}C in the Rat. Chemosphere 3:227-232.

19. Goto, M. et al. 1974. Metabolism of 2,3,5,6-tetrachlorobiphenyl-^{14}C and 2,3,4,5,6-pentachlorobiphenyl- ^{14}C in the Rat. Chemosphere 3:233-238.

20. Jensen, S., and Sundstrom, G. 1974. Metabolic Hydroxylation of a Chlorobiphenyl Containing Only Isolated Unsubstituted Positions - 2,4,5,2',4',5'-hexachlorobiphenyl. Nature 251:219-220.

21. Data of Monsanto. 1970. Cited in the FDA Status Report on the Chemistry and Toxicology of Polychlorinated Biphenyls, PCBs or Aroclors.

22. Grant, D. L., and Phillips, W. E. J. 1974. The Effect of Age and Sex on the Toxicity of Aroclor 1254, a Polychlorinated Biphenyl, in the Rat. Bull. Environ. Contam. Toxicol. 12:145-152.

23. Miller, J. W. 1944. Pathologic Changes in Animals Exposed to a Commercial Chlorinated Diphenyl. U. S. Publ. Health Rep. 59:1085-1093.

24. Tucker, R. K., and Crabtree, D. G. 1970. Handbook of Toxicity of Pesticides to Wildlife. U. S. Dept. of Interior, Bureau of Sports Fisheries and Wildlife Resources Publication 84. Washington, D. C.

25. Righter, H. F., Booth, N. H., and Teske, R. H. 1976. Acute Effects of Aroclor 1254 on the Feline Cardiovascular System. Environ. Res. 11:353-358.

26. Baker, F. D. et al. 1977. Toxicity and Persistence of Low-Level PCB in Adult Wistar Rats, Fetuses and Young. Arch. Environ. Contam. Toxicol. 5:143-156.

27. Kikuchi, M., and Masuda, Y. 1976. The Pathology of Yusho. In: PCB Poisoning and Pollution, ed. K. Higuchi. New York, Academic Press. pp. 69-86.

28. Allen, J. R., Carstens, L. A., and Barsotta, D. A. 1974. Residual Effects of Short-term Low-level Exposure of Nonhuman Primates to Polychlorinated Biphenyls. Toxicol. Appl. Pharmacol. 30: 440-451.

29. Itokawa, Y. et al. 1975. Effect of Simultaneous Administration of Polychlorinated Biphenyls and Aklylbenzene Sulfonic Acid Salts in Rats. Arch. Environ. Contam. Toxicol. 3:115-131.

30. Mehlman, M. A. et al. 1975. The Effects of a Polychlorinated Biphenyl Mixture (Aroclor 1254) on Liver Gluconeogenic Enzymes of Normal and Alloxan-Diabetic Rats. Toxicology 5:89-95.

31. Kuratsume, M. et al. 1972. Epidemiologic Study on Yusho, a Poisoning Caused by Ingestion of Rice Oil Contaminated with a Commercial Brand of Polychlorinated Biphenyls. Environ. Health Perspect. 1:119-128.

32. Nishizumi, M. 1970. Light and Electron Microscopic Study of Chlorobiphenyl Poisoning. Arch. Environ. Health 21:620-632.

33. Vos, G. J., and Notemboom-Ram, E. 1972. Comparative Toxicity Study of 2,4,5,2',4',5'-hexachlorobiphenyl and Polychlorinated Biphenyl Mixture in Rabbits. Toxicol. Appl. Pharmacol. 23:563-578.

34. Villeneuve, D. C. et al. 1971. Effects of PCB Administration on Microsomal Enzyme Activity in Pregnant Rabbits. Bull. Environ. Contam. Toxicol. 6:120-128.

35. Shimada, T., and Ugawa, M. 1978. Induction of Liver Drug Metabolism by Polychlorinated Biphenyls Whose Gas Chromatographic Profile Having Much in Common With That in Human Milk. Bull. Environ. Contam. Toxicol. 19:198-205.

36. Ecobichon, D. J., Hansell, M. M. and Safe, S. 1977. Halogen Substituents at the 4- and 4'- Positions of Biphenyl: Influence on Hepatic Function in the Rat. Toxicol. Appl. Pharmacol. 42:359-366.

37. Goldstein, J. A. et al. 1975. A Comparative Study of Two Polychlorinated Biphenyl Mixtures (Aroclors 1242 and 1016) Containing 42% Chlorine on Induction of Hepatic Porphyria and Drug Metabolizing Enzymes. Toxicol. Appl. Pharmacol. 32:461-473.

38. Hansell, M. M. et al. 1977. The Relationship Between Retention of Pure Chlorobiphenyl Congeners and Hepatic Function in the Rat. Exp. Mol. Pathol. 26:75-81.

39. Schmoldt, A., Herzberg, W., and Benthe, H. F. 1977. On the Inhibition of Microsomal Drug Metabolism by Polychlorinated Biphenyls and Related Phenolic Components. Chem.-Biol. Interactions 16:191-200.

40. Hirayama, C. 1976. Clinical Aspects of PCB Poisoning. In: PCB Poisoning and Pollution. ed. H. Higuchi. New York, Academic Press, pp. 87-104.

41. Ito, Y., Uzawa, H., and Notomi, A. 1971. Lipid Composition of the Rabbit Liver Poisoned with Chlorobiphenyls. Fukuoka-Igaku Zosshi 62:48-50.

42. Spalding, J. W., Ford, E., and Lane, D. 1976. Effect of 4-chlorobiphenyl on Substrate Transport and Phospholipid Metabolism in Mouse L5178Y Lymphoma Cells. Biochem. Pharmacol. 25:2051-2058.

43. Nagai, J., Kaoru, H., and Yae, Y. 1972. The Influence of the Oral Administration of PCB on Lipid Metabolism of Rat. 1. Skin Lipid. Fukuoka Acta Medica 63:367-373.

44. Holub, B. J., Piekarski, J., and Nilsson, K. 1975. Effect of a PCB (2,4,2',4'-tetrachlorobiphenyl) on Lipid Synthesizing Enzymes in Rat Liver Microsomes. Bull. Environ. Contam. Toxicol. 11:415-421.

45. Park, D. V. 1968. The Biochemistry of Foreign Compounds. New York, Pergamon Press, Vol. V, 269 pp.

46. Grant, D. L., Phillips, W. E. J., and Villeneuve, D. C. 1971. Metabolism of a Polychlorinated Biphenyl (Aroclor 1254) Mixture in the Rat. Bull. Environ. Contam. Toxicol. 6:102-112.

47. Schulte, E., and Acker, L. 1974. Identifizierung Und Metabolisierbarkeit von Polychlorierten Biphenylen. Naturwissenschaften 61:79-80.

48. Jerina, D. M., and Dally, J. W. 1974. Arene Oxides: A New Aspect of Drug Metabolism. Science 185:573-582.

49. Hutzinger, O. et al. 1974. Identification of Metabolic Dechlorination of a Highly Chlorinated Biphenyl in Rabbit. Nature 252: 698-699.

50. Goto, M., Hattori, M., and Sugiura, K. 1975. Metabolism of Pentachloro and Hexachlorobiphenyls in the Rat. Chemosphere 4:177-180.

51. Tulp, M. Th. M., Bruggeman, W. A., and Hutzinger, O. 1977. Reductive Dechlorination of Chlorobiphenylols by Rats. Experientia 33: 1134-1136.

52. Mizutani, T. 1978. Identification of Sulfur-containing Metabolites of 2,4,2',4'-tetrachlorobiphenyl in Mice. Bull. Environ. Contam. Toxicol. 20:219-226.

53. Hesse, S., Mezger, M., and Wolff, T. 1978. Activation of [^{14}C]-chlorobiphenyls to Protein Binding Metabolites by Rat Liver Microsomes. Chem.-Biol. Interactions 20:353-365.

54. Dzogbefia, V. P., and Gamble, W. 1978. Alterations in Phsopholipid and Acylglycerol Biosynthesis by Polychlorinated Biphenyls. Fed. Proc. 37:1836 (Abstract).

55. Dzogbefia, V. P., Kling, D., and Gamble, W. 1978. Polychlorinated Biphenyls: In vivo and in vitro Modifications of Phospholipid and Glyceride Biosynthesis. J. Environ. Path. Toxicol. 1: 841-856.

56. Dzogbefia, V. P., and Gamble, W. 1979. In vitro Effects of Chlorinated Biphenylols on the Metabolism of Phospholipids and Glycerides. Fed. Proc. 38:236 (Abstract).

57. Fallon, H. J., and Lamb, R. G. 1968. Acylation of sn-glycerol-3-phosphate by Cell Fractions of Rat Liver. J. Lipid Res. 9:652-660.

58. Adams, L. L., Webb, W. W., and Fallon, H. G. 1971. Inhibition of Hepatic Triglyceride Formation by Clofibrate. J. Clin: Invest. 50:2339-2345.

59. Folch, J., Lees, M., and Sloane-Stanley, G. H. 1957. A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues. J. Biol. Chem. 226:496-509.

60. Parker, F., and Peterson, N. F. 1965. Quantitative Analysis of Phospholipids and Phospholipid Fatty Acids from Silica Gel Thin-layer Chromatograms. J. Lipid Res. 6:455-460.

61. Freeman, C. P., and West, D. 1966. Complete Separation of Lipid Classes on a Single Thin-layer Plate. J. Lipid Res. 7:324-327.

62. Raheja, R. K. et al. 1973. New Colorimetric Method for the Quantitative Estimation of Phospholipids Without Acid Digestion. J. Lipid Res. 14:695-697.

63. Lowry, O. H. et al. 1951. Protein Measurement With Folin Phenol Reagent. J. Biol. Chem. 193:265-275.

64. Burton, K. 1968. Determination of DNA Concentration With Diphenylamine. In: Methods in Enzymology. eds. L. Grossman and K. Moldave. New York, Academic Press, Vol. XII, Part B. pp. 163-166.

65. Robinson, J., and Newsholme, E. A. 1969. Some Properties of Hepatic Glycerol Kinase and Their Relation to the Control of Glycerol Utilization. Biochem. J. 112:455-464.

66. Newsholme, E. A., Robinson, J., and Taylor, K. 1967. A Radiochemical Enzymatic Activity Assay for Glycerol Kinase and Hexokinase. Biochim. Biophys. Acta 132:338-346.

67. Lamb, R. G., and Fallon, H. J. 1974. Glycerolipid Formation from sn-Glycerol-3 Phosphate by Rat Liver Cell Fractions: The Role of Phosphatidate Phosphohydrolase. Biochim. Biophys. Acta 348:166-178.

68. Young, D. L., and Lynen, F. 1969. Enzymatic Regulation of 3-sn-phosphatidyl Choline and Triacylglycerol Synthesis in States of Altered Lipid Metabolism. J. Biol. Chem. 244:377-383.

69. Gerhard, M., and Lang, G. 1974. L-(-)-Glycerol-3 Phosphate. In: Methods of Enzymatic Analysis, ed. H. H. Bergmeyer. New York, Academic Press, Vol. III, pp. 1415-1418.

70. Fallon, H. J., and Kemp, E. L. 1968. Effects of Diet on Hepatic Triglyceride Synthesis. J. Clin. Invest. 47:712-719.

71. Tzur, R., Tal, E., and Shapiro, B. 1964. α-Glycerol Phosphate as Regulatory Factor in Fatty Acid Esterification. Biochim. Biophys. Acta. 84:18-23.

72. Howard, C. F., Jr., and Lowenstein, J. M. 1965. The Effect of Glycerol-3 Phosphate on Fatty Acid Synthesis. J. Biol. Chem. 240:4170-4175.

73. Bortz, W. M., and Lynen, F. 1963. Elevation of Long-chain Acyl CoA Derivatives in Livers of Fasted Rats. Biochem. Z 339:77.

74. Lamb, R. G., and Fallon, H. J. 1970. The Formation of Monoacylglycerophosphate from sn-glycerol-3 Phosphate by a Rat Liver Particulate Preparation. J. Biol. Chem. 245:3075-3083.

75. Bjornstad, P., and Bremer, J. 1966. In vivo Studies on Pathways for the Biosynthesis of Lecithin in the Rat. J. Lipid Res. 7:38-45.

76. Kanoh, H., and Ohno, K. 1973. Utilization of Endogenous Phospholipids by the Back-reaction of CDP-choline (Ethanolamine): 1,2-Diglyceride Choline (Ethanolamine)-Phosphotransferase in Rat Liver Microsomes. Biochim. Biophys. Acta 306:203-217.

77. Kanoh, H., and Ohno, K. 1973. Studies on 1,2-Diglycerides Formed from Endogenous Lecithins by the Back-reaction of Rat Liver Microsomal CDP-Choline: 1,2-Diacylglycerol Choline-Phosphotransferase. Biochim. Biophys. Acta 326:17-25.

78. Sundler, R., Åkesson, B., and Nilsson, Å. 1974. Sources of Diacylglycerols for Phospholipid Synthesis in Rat Liver. Biochim. Biophys. Acta 337:248-254.

79. Ishidate, K., Yoshida, M., and Nakazawa, Y. 1978. Effect of Typical Inducers of Microsomal Drug-Metabolizing Enzymes on Phospholipid Metabolism in Rat Liver. Biochem. Pharmacol. 27:2595-2603.