# BIOSYNTHESIS OF GLYCEROLIPIDS BY THE EPITHELIAL CELLS OF *ASCARIS* MID-GUT

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# CHAPTER I

# INTRODUCTION

## Fat Absorption by the Mammalian Intestine

Great progress in understanding the mechanism of fat absorption by the mammalian intestine has been made in the past fifteen years. The mechanism can be described in summary as follows: (1) The major hydrolytic products which are produced in the intestinal lumen by the action of pancreatic lipase and bile salts are fatty acids and monoglycerides (Isselbacher, 1965; Shapiro, 1967). (2) The uptake of micellar monoglycerides and fatty acids into the intestinal mucosa is followed by re-esterification into triglycerides and this occurs by two major metabolic pathways (Isselbacher, 1965; Hüscher, 1963; Clark and Hüscher, 1961; Senior, 1964; Benson and Rampone, 1966). (3) One of these is by direct esterification of monoglycerides and the other involves the esterification of L-α-glycerophosphate with fatty acyl CoA.

### Intraluminal Micelle Formation

Once dietary fats get into the stomach they are converted to a coarse emulsion. The chief role of the stomach seems to be to liberate fats and phospholipids from protein by proteolytic digestion, to churn the mixture into an emulsion, and squirt the emulsified fats into the duodenum where they may be mixed with bile and pancreatic juice (Senior,

1964). In the small intestine the triglycerides are hydrolyzed by the pancreatic lipase, which has been shown to preferentially catalyze the breakage of the primary ($\alpha$, $\alpha'$) glyceride ester bonds (Borgström, 1964a; Hofmann and Borgström, 1963). Products of the cleavage are free fatty acids, 1,2-diglyceride, 2-monoglyceride, and slowly, free glycerol. The 2-monoglyceride will be hydrolyzed either directly or after isomerization to the $\alpha$-monoglyceride (Hofmann, 1963). Pancreatic lipolysis, therefore, is responsible for two products (Monoglycerides and fatty acids) which interact with bile salts to form mixed micelles (Hofmann, 1962).

Bile salts, which occur almost exclusively conjugated through a peptide bond with glycine or taurine, have most of the properties of normal anionic detergents. Compared to normal detergents bile salt solutions have a low capacity for the solubilization of non-polar substances but a high solubility for polar substances such as long-chain monoglycerides and fatty acids. Mixed micelles do have a much higher capacity to solubilize non-polar substances than do the bile solutions alone (Borgström, 1964b). Micelle formation occurs when bile salts are present at greater than critical concentrations (Hofmann, 1961). It was shown that by using bile salts in varying concentrations and incubation these with monoglyceride or fatty acid for 96 hours at 38°C, above certain critical concentrations of conjugated bile salts these lipid fractions could be solubilized; unconjugated bile salts were ineffective in inducing solubilization (Hofmann, 1961).

The exact mode of the penetration of bile salt micelles into or through the microvilli of the gut epithelial cells is still unknown. Bile salt micelles may serve as a means of transportation for the

monoglycerides and fatty acids from the emulsified triglyceride phase to the brush border (Borgström, 1964 a and b). Conjugated bile salts which are so important in forming micelles are not absorbed across the mucosa with the fatty acids and monoglyceride, instead they remain in the lumen and eventually are absorbed in the distal small bowel, and then re-circulate via liver and bile (Senior, 1964).

Johnston and Borgström (1964) reported that the uptake of micellar $C^{14}$ labelled monoglyceride by hamster intestine in vitro was not prevented by prior boiling of the tissue nor by including 2, 4-dinitrophenol or phlorizin in the medium. Therefore, the uptake of micellar monoglycerides and fatty acids by intestinal mucosa is apparently a non-energy dependent process. The concept of pinocytotic vesicles formation has been described as a possible mechanism for lipids to gain entry into the intestinal mucosa (Senior, 1964), but still there is a controversy of ideas.

Intracellular Synthesis of Glycerides in the Small Intestinal Mucosa

The pathways of synthesis of glyceride lipids was first described by Kornberg and Price (1953 a and b). They demonstrated that the activation of long chain fatty acid and phosphatidic acids may be intermediates in the biosynthesis of phospholipids (1953 a). At the same time, Kennedy (1953), in a study of the incorporation of $P_i^{32}$ into the phospholipids of a particulate enzyme preparation from rat liver, showed that L-α-glycerophosphate is an obligate intermediate in the process. Weiss, Smith and Kennedy (1956) isolated an enzyme from chicken liver which rapidly dephosphorylate phosphatidic acids with the production of orthophosphate and D-1,2-diglyceride. The D-1,2-diglyceride produced by

the action of this enzyme may then react with cytidine diphosphate choline to form lecithin (Weiss et al., 1956) or with long chain fatty ester of CoA to form triglyceride (Weiss et al., 1960).

Investigators demonstrated that rat liver homogenates contain two enzyme systems, a microsomal and a mitochondrial one, which catalyze the incorporation of labeled palmitic acid into neutral lipids. (Tietz and Shapiro, 1956; Stein, Tietz and Shapiro, 1957; Stein and Shapiro, 1957). The microsomal fraction, requires the addition of L-α-glycerophosphate, Coenzyme A, $Mg^{++}$, and ATP for maximal activity (Stein and Shapiro, 1958). The second system found in the mitochondrial fraction, requires the addition of ATP, and a small amount of "native supernatant". The addition of boiled "native supernatant" to the active system resulted in an increased rate of palmitic acid incorporation (Stein, Tietz and Shapiro, 1957). Unfortunately, the "native supernatant" was a mitochondrial-free supernatant and it was thus not clear whether the stimulation was due to the microsomal fraction or particulate-free supernatant. Exploring further the observation of Shapiro and co-worker, Hübscher and co-workers (1964) were able to demonstrate that the biosynthesis of glycerides in both mitochondrial and microsomal fractions of liver tissue was stimulated by adding either fresh or boiled particulate-free supernatant to a microsomal preparation from the intestinal mucosa of the cat. It was found to greatly stimulate the incorporation of labeled palmitic acid into triglycerides. Maximum activity was observed in the system containing fresh and boiled particulate-free supernatant. It appears that a heat labile factor in the supernatant as well as heat stable factors are required for maximum incorporation of palmitic acid into triglycerides. A similar, but lower stimulation was

observed when the particulate-free supernatant was added to a mitochondrial preparation (Hübscher, et al., 1964). The possible functions of the factors from the particulate-free supernatant in triglyceride biosynthesis are not yet fully understood.

The formation of triglycerides and phospholipids from α-glycerophosphate and long chain fatty acids can be outlined as follows:

$$\text{Palmitic acid} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{++}} \text{Palmityl CoA} + \text{AMP} + PP_i \quad (1)$$

$$\text{Palmityl CoA} + \text{L-}\alpha\text{-Glycerophosphate} \longrightarrow \text{L-}\alpha\text{-Phosphatidic acid} + \text{CoA} \quad (2)$$

$$\text{L-}\alpha\text{-Phosphatidic acid} \longrightarrow \text{D-}\alpha,\beta\text{-Diglyceride} + \text{Phosphate} \quad (3)$$

$$\text{D-}\alpha,\beta\text{-Diglyceride} + \text{Palmityl CoA} \longrightarrow \text{Triglyceride} + \text{CoA} \quad (4)$$

$$\text{D-}\alpha,\beta\text{-Diglyceride} + \text{CDC*} \longrightarrow \text{Lecithin} + \text{CMP} \quad (4)$$

*CDC - Cytidine-diphosphate-choline

Johnston (1959), used everted segments of golden hamster intestine to study $C^{14}$ palmitic acid uptake in vitro. He showed that after incubation under aerobic condition, approximately 5% of the original activity, initially placed on the mucosal side, was found in the serosal compartment. Ninety per cent or more of the activity on the serosal side was in the form of glyceride. The activity in the serosal solution and the intestinal wall was found predominantly as triglyceride and free fatty acids.

By using homogenates of rat and human intestinal mucosa, a very active system has been demonstrated which is capable of incorporating long-chain fatty acids into neutral lipids (Dawson and Isselbacher, 1959 and 1960). This system was dependent upon ATP, Coenzyme A, and magnesium, and was more active in the jejunum than in the ileum or colon. The addition of potassium fluoride and Tween "80"

(polyoxyethylene sorbitan monooleate) to the homogenate increased the total incorporation (Dawson and Isselbacher, 1960). Stimulation of palmitate incorporation by Tween "80" could be explained partially by the inhibitory effect of Tween "80" on a lipase present in the homogenate (Dawson and Isselbacher, 1960). Stimulation of palmitate incorporation by fluoride would most likely be due either to the inhibition of an ATPase or of a lipase (Weinstein, 1935). Experiments by Dawson and Isselbacher (1960) showed that this might be due to the latter effect, and the addition of both Tween "80" and potassium fluoride gave maximum inhibition of the lipase activity.

Demonstration that the incorporation of free fatty acids into glycerides by homogenates of human- and rat-gut mucosa is dependent on CoA, ATP and $Mg^{++}$ suggest that the initial step in the process might be activation of the fatty acids to acylCoA theoester. Biosynthesis of glycerides in the epithelial cells of the intestine might have the same mechanism that occurs in the liver. Senior and Isselbacher (1960) was able to demonstrate a long-chain fatty acid thiokinase in the rat-gut mucosa. The enzyme was more active in the microsomes than in the mitochondria. At the same time Clark and Hübscher (1960 and 1961) had carried out a series of experiments to study the biosynthesis of glycerides using mitochondrial preparations of the intestinal mucosa of rabbit small intestine. They showed a definite requirement of CoA, ATP, magnesium chloride for incorporation. α-monstearin, α,β-dipalmitin and α-glycerophosphate all stimulated the incorporation of labelled fatty acid into glyderide though α-glycerophosphate was able to produce a greater degree of stimulation. Similar studies were carried out using mitochondrial preparations from rat intestine, but there it was found that

α-glycerophosphate did not stimulate the incorporation of labelled palmitate to any great extent (Clark and Hübscher, 1960). Using mitochondria of the rabbit small intestine, α-glycerophosphate could not be replaced by β-glycerophosphate or glycerol (Clark and Hübscher, 1961). The latter is obviously not converted to α-glycerophosphate due to lack of glycerol kinase. However later works by Clark and Hübscher (1962) and Haesseler, et al. (1963) demonstrated that a glycerokinase exists in the cytoplasm of the intestinal mucosa which leads to the formation of α-glycerophosphate from glycerol and ATP.

The above results indicate a pathway similar to that suggested by Stein, Tietz and Shapiro for the synthesis of triglycerides in rat liver where α-glycerophosphate and phosphatidic acid are precursor and intermediate respectively.

The appreciable incorporation found with monoglyceride would suggest an additional pathway (Clark and Hübscher, 1960 and 1961). The direct acylation of monoglyceride in mucosal homogenates has been confirmed by Senior and Isselbacher (1962). Their studies of the microsomal fraction derived from isolated rat intestinal epithelial cells have indicated the presence of an enzyme system, referred to as monoglyceride acylase, which catalyzes the direct condensation of palmityl Coenzyme A and monoglyceride to diglyceride as follows:

$$\text{Monoglyceride} + \text{Palmityl Coenzyme A} \longrightarrow \text{Diglyceride} + \text{Coenzyme A}$$

The enzyme system was found to be heat labile, and the highest specific activity was present in the microsomal fraction of the mucosal cells. The reaction did not require ATP, nor were phosphorylated intermediates detected (Senior and Isselbacher, 1962). An active monoglyceride lipase

and a fatty acid thiokinase have been shown to have their highest activity in the microsomal fraction of rat gut mucosa (Senior and Isselbacher, 1960; Senior and Isselbacher, 1962).

It was shown that in mucosal homogenates of rat intestine that β-monoglyceride was preferred to α-monoglyceride as a substrate (Ailhaud, Samuel and Densnuelle, 1963). The monoglyceride acylase, therefore, expresses a preference for the outer positions on the monoglyceride substrates.

The pathways of glycerides synthesis in the intestinal mucosa are illustrated in Figure 1.

Transport and Distribution of Absorbed Glycerides

After absorption from the small intestine, the long-chain and short-chain fatty acids of dietary origin enter the circulation by different routes and in different forms. Major proportions of the fatty acids with less than 12 carbon atoms are transported directly to the liver via the portal vein. The fatty acids with 12 or more carbons are converted to triglyceride and secreted in the lymph in the form of very low density lipoproteins called chylomicrons. Chylomicrons contain small amounts of phospholipids and cholesterol in addition to the protein moiety and triglyceride (Isselbacher, 1965). Evidence has been presented to show that intestinal mucosa has the potential for making lipoprotein (Isselbacher, 1965). Almost all the cholesterol and phospholipids absorbed from the gastrointestinal tract enter the lymphatic system as chylomicrons as well as small amounts of phospholipids that are continually synthesized by the intestinal mucosa (Guyton, 1965). Chylomicrons are then transported up the thoraic duct and are emptied into the

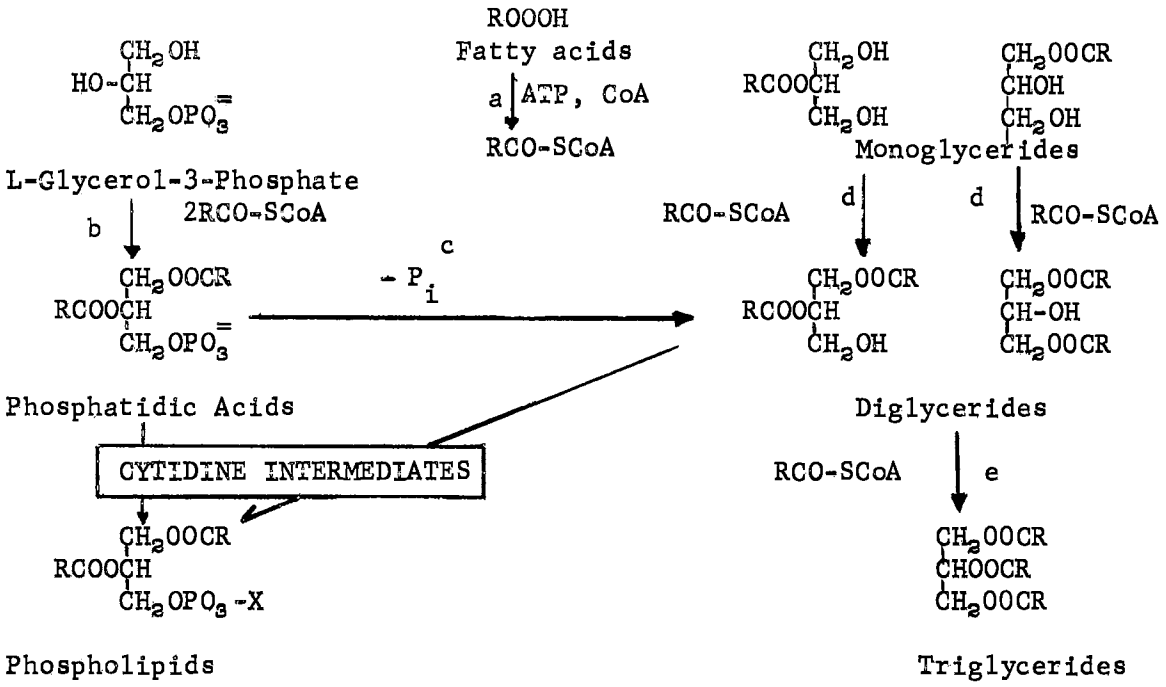



Figure 1. Pathways for the Biosynthesis of Glycerides in the Intestinal Mucosa. Enzymes involved are a, (fatty) acid: CoA ligase (AMP), b, acyl CoA:L-glycerol-3-phosphate acyl transferase, c, L-α-phosphatidate phosphohydrolase, d, acylCoA:monoglyceride acyl transferase, e, acyl CoA:diglyceride acyl transferase. (Enzymes d and e have not been isolated and purified, nor officially named as yet.)

venous blood at the juncture of the jugular and subclavian veins (Guyton, 1966).

The fat of the chylomicrons is removed from the blood in two different ways. The triglycerides portion of the chylomicrons is probably hydrolyzed into glycerol and fatty acids by an enzyme (lipoprotein lipase) in the blood. The fatty acids are transported in combination with serum albumin, which plays a major role in transport of unesterified fatty acids, to the various cells of the body (Guyton, 1966). The fatty acid can there be utilized as energy source, or they can be stored in adipose tissue as triglycerides (Guyton, 1966). Chylomicrons may also be removed from the blood by transport through the capillary wall into liver cells, which then either use the lipids of the chylomicrons for energy or convert them into other lipid substances (Guyton, 1966).

The scheme of absorption of fats in the mammalian intestine is illustrated in Figure 2.

## Absorption in *Ascaris* Intestine

In *Ascaris*, and all the common-known nematodes, the intestine is divided into three regions, the anterior (ventricular) region, the mid-region and the posterior (pre-rectal) region. The intestinal wall consists of a single layer of epithelial cells, and a basement membrane (basal lamella) covering the mucosal surface of the intestinal wall (Lee, 1965). Each intestinal cell is delimited from the others by a distinct membrane (Kessel, *et al.*, 1961). The electron microscope reveals that a multitude of protoplasmic projections in the form of microvilli (bacillary layer), comprise the lumen surface of the cell (Kessel *et al.*, 1961) The inner core of each microvillus extends for a short

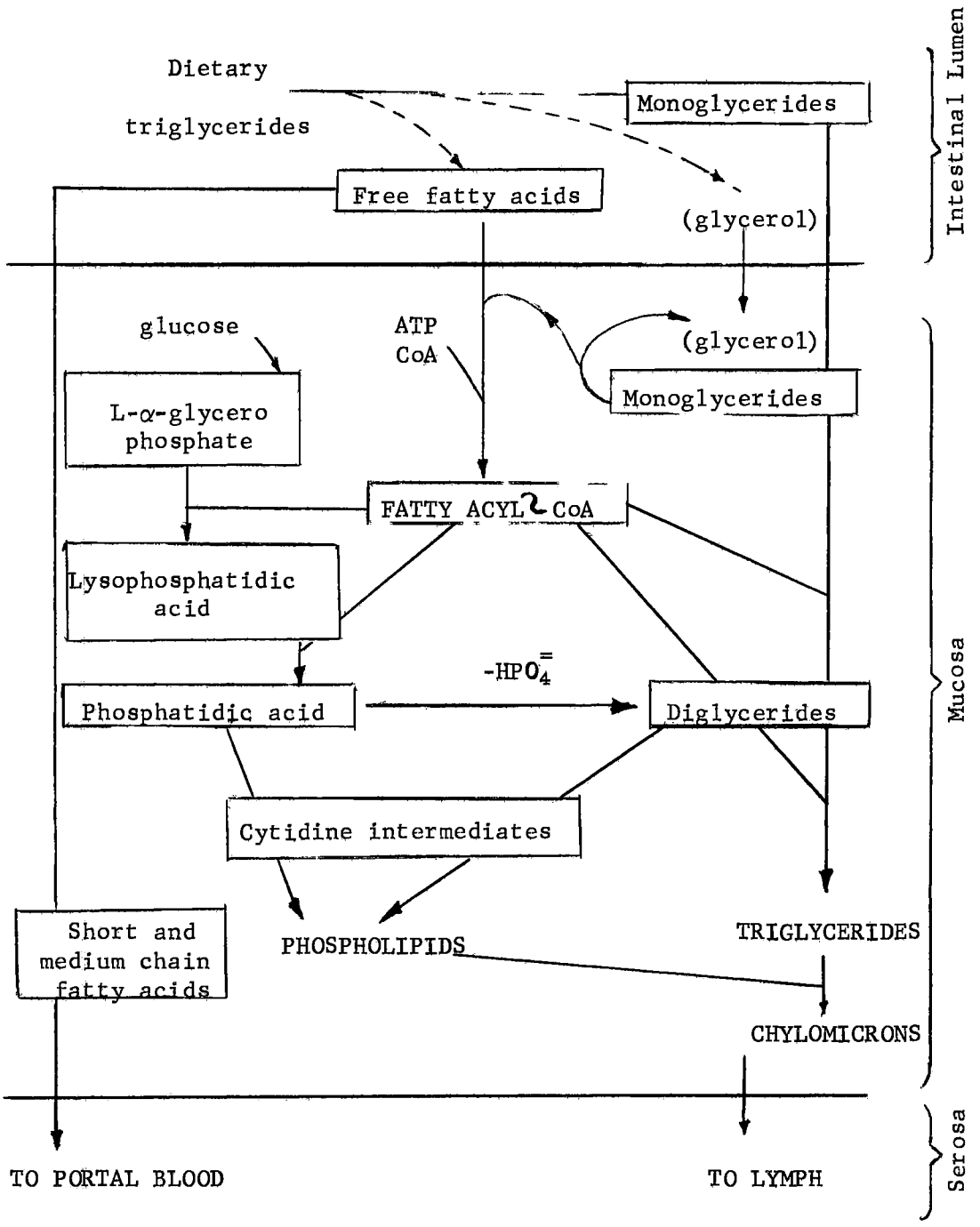



Figure 2. Pathways of Lipid Synthesis in the Intestinal Epithelial Cell, from Absorption of the Products of Digestion to Production of Chylomicron Lipids.

distance into the apical cytoplasm in the form of roolets which are continuous with a filamentous system forming the terminal web; specializtions (desmosomes) of the plasma membrane are located in the apical region of these cells. The endoplasmic reticulum is present throughout the cell as tubules which sometimes expanded into cristernae. Mitochondria are numerous and pleomorphic, with some occuring in the form of dense beaded strands. In the less dense mitochondria, the cristae are distinct. (Kessel et al., 1961). Cytochemical tests, indicate that lipid droplets appear in highest concentration in the central portion of the intestinal cell (Kessel et al., 1961).

Rogers (1940, 1941) established the presence of amylase, lipase, esterase and protease in Ascaris intestine. He demonstrated esterase and lipase activity in the gut extracts. The rate of activity of both enzymes was enhanced by sodium glycocholate, but sodium taurocholate retarded fat digestion. Also both enzymes were activated by sodium bicarbonate.

Carpenter (1952) has studied the hydrolytic enzymes of Ascaris gut in some detail. She confirmed Rogers' discovery of amylase, lipase and esterase. She found, in general, that these enzymes were most concentrated in the foregut, this is the region of the gut which contains the greatest number of secretory type cell. The relative activity of the hydrolytic enzyme is -- peptidases>proteases>maltase>lipase>amylase. On the basis of histological and chemical analysis it is thought that the foregut is mainly secretory in function and the mid-gut and posterior region is mainly absorption (Carpenter, 1952).

Very little is known about the absorption of food material by Ascaris. Most evidence indicates that absorption takes place through

the intestine and not through the cuticle (Lee, 1965). Feeding experiments have shown that Ascaris can absorb several simple sugars, such as glucose, fructose, sorbose, sucrose or maltose to produce glycogen in the tissue (Lee, 1965; Fairbairn, 1957). Fischer (personal communication) has developed a technique which he used to study amino acid and sugar transport in the intestine of Ascaris. Under aerobic condition, he found that the system can transport these compounds only for a limited period of time.

The absorption of lipids by Ascaris gut in vitro was first investigated by King (1966). He used an evert gut sac preparation to study the transport of palmitic 1-$C^{14}$ acid and showed that if an energy source was added to the system the movement of palmitic acid across the intestinal wall was enhanced. Apparently, this process functioned best under anaerobic conditions. The distribution of radioactivity in the lipids of Ascaris gut was determined. 66.7 per cent of the radioactivity was present as the free fatty acid, approximately 22.5 per cent was present as triglycerides, and only 1.5 per cent was found in the phospholipids.

The ability of the intact gut to incorporate palmitic acid into glycerides suggests that Ascaris acquires fatty acids by mechanisms similar to those described in the vertebrate small intestine. There might be an enzyme system in the cells of Ascaris gut that esterify long-chain fatty acids into triglycerides and then move them into the hemolymph and subsequently to other parts of the body. This paper reports a series of experiments which have been carried out to determine the distribution and characteristics of glyceride biosynthesis in the cells of Ascaris mid-gut.

# CHAPTER II

# MATERIALS AND METHODS

## Chemicals

Ammonium palmitate was prepared following the procedure described by Kornberg and Pricer (1953a). Palmitic acid-1-$C^{14}$ (obtained from Nuclear-Chicago, Des Plaines, Illinois) was dissolved in an alkaline solution of 50% ethanol and washed three times with petroleum ether to remove lipid impurities. The alcoholic solution was acidified and the fatty acid was extracted with petroleum ether. The petroleum ether was evaporated with a stream of nitrogen. Water was added to the fatty acid residue, and the solution was adjusted to pH 9.0 with ammonium hydroxide. The mixture was heated in a steam bath until it was clear.

Monoglycerides or diglycerides were prepared as suggested by Senior and Isselbacher (1962). α-monpalmitin (obtained from Sigma Chemical Co., St. Louis, ‹99% purity) or dipalmitin (approx. 80% purity) was dissolved in a small amount of diethyl ether and homogenized gently in 5% or 10% bovine albumin solution (3 μmoles of substrate per ml of bovine albumin). The ether was evaporated at 35-40 °C under vacuum, leaving a clear, stable dispersion of monoglycerides or diglycerides.

D-L-β-glycerophosphate (0.1% D-L-α-Isomer), D-L-α-glycerophosphate, D-fructose-1-6-diphosphate ‹99% purity), adenosine 5' triphosphate (ATP), Coenzyme A (CoA), were obtained from Sigma Chemical Co., St.

Louis, Mo. Tween "80" (polyoxyethylene sorbitan mono-oleate) and glutathione monosodium (GSH) were obtained from Nutrition Biochemical Co., Cleveland, Ohio.

S-Palmityl-1-$C^{14}$-CoA was purchased from New England Nuclear Corp., Boston, Mass. S-Palmityl-CoA (free acid 90% purity) was obtained from Sigma Chemical Company, St. Louis, Mo. Both were suspended in acetate buffer (pH 6.0) and made up to the concentration needed.

Standard phosphatidic acid was prepared from rat liver following the procedure described by Hübscher and Clark (1960).

Magnesium chloride , potassium fluoride and glycerin (95% purity) were purchased from Mallinckrodt Chemical Works, St. Louis, Mo.

All solvents used for chromatography were either spectrophotometric grade or glass distilled.

## Tissue Preparation

Adult female Ascaris lumbricoides suum was collected from Wilson & Co., Inc., Oklahoma City, Oklahoma, and transported to the laboratory in a saline holding solution (Jacobsen, 1965) maintained at 32°-40°C.

Worms were dissected longitudinally and the mid-gut was removed and placed in a petri dish containing ice cold saline. Worms were never held for more than six hours.

## Intact Tissue Incubation

The mid-gut was dissected longitudinally and the ribbon was washed in ice cold Ascaris saline. 1.25 μmole ($7.0 \times 10^5$ d.p.m.) of ammonium palmitate-1-$C^{14}$ was incubated with 0.75 gm of gut tissue in 5.0 ml of 0.043 M glucose in potassium phosphate buffer (0.05 M, pH7.5). It was

incubated under 99.9% $N_2$ for two hours at 37°C in a metabolic shaker. After two hours, each gut strip was rinsed three times in saline. Then 5 volumes of chloroform:methanol (2:1,v/v) was added, and allowed to sit overnight. The tissue was removed by filtration. The filtrate (lipid extract) was put in a small beaker (50 ml) which was then put in a large beaker (600 ml). Tap water was added on the top of the chloroform-methanol extract until the small beaker was submerged and half of the large beaker was filled. After 4 hours the bulk of water was removed, and the last trace of water in the small beaker was removed by freezing. The solution was filtered in the cold, and the lipid extract was allowed to come to room temperature. The solution was dried over anhydrous sodium sulfate, filtered, and the solvent was removed *in vacuo*. The lipid residue was dissolved in 2.0 ml of chloroform and saved for thin layer chromatography.

## Tissue Extract Incubation

### Enzyme Preparation

When sufficient gut tissue was collected it was blotted dry on filter paper and weighed. The tissue was homogenized in 4 volumes (w/v) of cold potassium phosphate buffer (0.05M, pH 7.5) using a Potter-Elvejhem tissue grinder equipped with a teflon pestle. Cellular debris was removed by centrifuging at 900 x g (International Centrifuge, Model 1 HR-1) for 20 minutes at 0°C. The supernatant ($S_1$) was centrifuged at 12,000 x g for 30 minutes to remove mitochondria. The pellet was resuspended in potassium phosphate buffer and re-centrifuged at 12,000 x g for 30 minutes. The supernatant ($S_{12}$) was centrifuged at 100,000 x g

(Beckman Ultracentrifuge Spinco Model L 2) for one hour to remove microsomes. Both mitochondria and microsomes were suspended in one volume (w/v) of cold potassium phosphate buffer (pH 7.5), and used immediately as the enzyme source for individual experiments.

## Incubation

Cofactors were dissolved in potassium phosphate buffer (0.05 M pH 7.5). The experimental reaction mixtures were placed in screw cap culture tubes, adjusted to a volume of 2.0-3.0 ml by the addition of the appropriate enzyme source, flushed with nitrogen for 20-30 seconds, capped and incubated for 2 hours at 37°C with constant shaking in a water bath.

Protein concentration was determined according to Lowry *et al.* (1951).

## Extraction of Lipids

The reaction was stopped by the addition of an equal volume of 1 N HCl and 10 ml of ethanol:heptane:diethyl ether (1:1:1,v/v) was added together with 3.0 mg of carrier lipids (mixture of standard tripalmitin, dipalmitin, monopalmitin and lipid extract from *Ascaris* mid-gut). The tubes were shaken vigorously with a Vortex mixer, and centrifuged. The upper layer containing the lipid was removed with a pasteur pipette and saved. The extraction procedure was repeated twice. The extracts were combined and evaporated to dryness with a stream of nitrogen in a water bath maintained at 45°C. the residue was dissolved in 1-2 ml of chloroform and saved for thin-layer chromatography.

## Thin-Layer Chromatography

### Separation of Neutral Lipids

Glass plates (20 cm x 20 cm) were coated with a slurry of 30 grams of Silica Gel G (obtained from Brinkmann Instruments Inc., Westbury, L.I., N.Y.) in 60 ml of an 0.02% aqueous 2', 7'-dichlorofluorescein (DCF) solution (pH 7.0-7.5) to give a layer of 250 $\mu$ thick (Brown and Johnston, 1962). The plates were activated at 110°C for 1 hour. The developing solvent was hexane:diethyl ether: acetic acid (60:40:1, v/v) and each plate was developed twice in a saturated chromatography chamber lined with filtered paper. Spots were visualized under ultraviolet light and identification was made by reference to standards run simultaneously. All the neutral lipids migrated from the origin while the phospholipids remained at the origin. The various neutral lipis spots were scraped from the plate with the aid of a spatula, and the silica gel was transferred to a scintillation vial. Ten ml of a naphthalene-dioxane scintillation fluid (Bray, 1960) was then added to the vial. The samples were analyzed for radioactivity in a Tri-Carb Scintillation Spectrometer.

### Separation of Phospholipids

Thin-layer plates were prepared in the same manner as above except the dye was omitted from the 60 ml of water. A solvent mixture of chloroform: methanol: water (65:25:4, v/v) was used to separate the phospholipids. The spots were visualized by placing the plates in iodine vapors. Total radioactivity in each phospholipid was determined as described above for the neutral lipids.

### Identification of Phospholipid Spots

Iodine vapors were used as a general detecting agent for lipids (Mangold, 1961). Phospholipids were detected by ammonium-molybdate-perchloric acid spray (Wagner et al., 1961). Plasmalogens (aldehyde and ketone) were detected by dinitrophenylhydrazine spray (Reisema, 1954). Ninhydrin (Mangold, 1961) for phospholipids contained free amino acid. The modified dragendorff reagent (Wagner et al., 1961) for phosphatidyl choline, and the modified malachite green reagent was for lysocompounds (Ansell and Hawthorne, 1964).

## Gas Chromatography Analysis

### Preparation of Acids for Analysis

Fatty acid methyl esters were prepared by reacting the fatty acids with diazomethane in ether. In all instances the ethereal solution of diazomethane was freshly distilled. Following methylation the ether was evaporated and the residue was dissilved in hexane (chromatoquality reagent).

### Gas Chromatography

A Barber-Coleman Model 5000 Gas Chromatograph equipped with a β-ionization detector was used for the identification of the methyl esters. A six foot glass column packed with 14.5% ethylene glycol succinate (EGS) coating Gas Chrom CLP (100-120 mesh, Applied Science Laboratories, State College, Pennsylvania) was prepared to separate the methyl esters. The injector temperature was 215°C, the detector temperature was 210°C, and the column temperature was 185°C. The flow rate

was 20 p. s. i. Fatty acid esters were trapped in vials containing anthracene crystals as they were eluted from the column. Radioactivity of the individual methyl ester was determined by placing the anthracene vials in a scintillation counter.

## Acid Hydrolysis of Plasmalogens

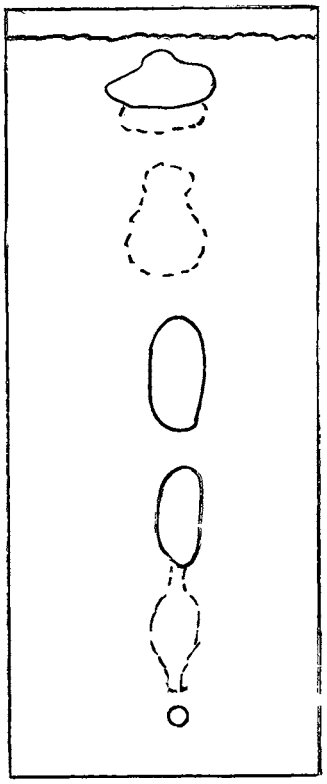
The procedure was followed as described by Gray (1958). Lipid residue was dissolved in 2 ml of 90% acetic acid and incubated at 38°C for 18 hours. After incubation the solution was cooled, neutralized with sodium hydroxide and extracted with an equal volume of chloroform: methanol (2:1, v/v). The phases were separated by centrifuge and the upper aqueous methanol layer was removed. The chloroform layer was washed twice with 0.2 volume of 0.5 M NaCl and dried over anhydrous $Na_2SO_4$. The solvent was removed under reduced pressure and the residuce was dissolved in 2.0 ml of chloroform and ready for thin-layer or gas chromatography.

# CHAPTER III

## RESULTS

### Identification of Lipids of *Ascaris* Gut

Total lipids of *Ascaris* mid-gut were isolated for analysis by thin layer chromatography employing techniques worked out earlier in this laboratory by King (1966). The neutral lipids were examined in detail by King (1966), but no effort was made to separate and identify the phospholipids. Phosphatidic acid is one of the intermediates in the synthesis of glycerides in many tissues. With this in mind a series of determinations were carried out to determine the phospholipids of *Ascaris* mid-gut. The results of this analysis are shown in Figure 3. Spot number 1 which had the same Rf as standard neutral lipids, and hydrocarbons was a very rapid staining, distinct spot when placed in iodine vapors. It gave a negative test with ammonium-molybdate-perchloric acid, but reacted quite readily with 2,4-dinitrophenylhydrazine and hydroxylamine ferric chloride spray. Fatty acid esters, hydrocarbons, and fatty aldyhydes were identified as the main components of this spot. Spot number 1(a) did not react with ammonium-molybdate-perchloric acid. It had the same Rf as standard sterol. Spot number 2 always appeared very faint. It gave a positive test for ammonium-molybdate-perchloric reagent and 2,4-dinitrophenylhydrazine. When standard labelled palmitic acid was run along with the gut lipid, almost all of the radioactivity was recovered in this spot. Spot number 2 had the same Rf as isolated



| Spot Number | Tentative Identification |
|---|---|
| 1 | Neutral lipids<br>Hydrocarbon<br>Aldehyde (?) |
| 1(a) | Sterol |
| 2 | Free fatty acids<br>Phosphatidic acid<br>Aldehyde (?) |
| 3 | Cephalin (serine & ethanolamine)<br>Choline plasmalogen |
| 4 | Lecithin<br>Choline plasmalogen |
| 5 | Lysolecithin<br>Lysophosphatidic acid |
| 6 | Lipoprotein (?) |

Figure 3. Thin-Layer Chromatogram of the Ascaris Gut Lipids. Solvent system was chloroform:methanol:water (65:25:4, v/v). Spots were visualized with iodine vapor.

phosphatidic acid (from rat liver). The spot apparently contains free fatty acids, phosphatidic acid, and fatty aldehydes. Spot number 3 gave a positive test for phospholipid when sprayed with ammonium-molybdate-perchloric reagent. Also it reacted instantly with ninhydrin reagent to give a positive test for cephalin. It gave a positive test when sprayed with 2,4-dinitrophenylhydrazine and it reacted slightly with dragendorff spray. This spot migrated the same as standard cephalin. Spot number 4 gave a positive test with dragendorff reagent, ammonium-molybdate-perchloric acid and 2,4-dinitrophenylhydrazine. It had the same Rf value as standard lecithin. Spot number 5 appeared faintly when visualized under iodine vapor. It gave a positive test for phosphate when sprayed with ammonium-molybdate-perchloric reagent. It reacted quite slowly with malachite green reagent, which indicates the presence of lyso-compounds. Further determinations were made by scraping off the sliica gel corresponding to the lyso-compounds and eluted with chloroform and methanol (2:1, v/v). The lipid material eluted from the silica gel was then concentrated and rechromatographed. A distinct spot was detected. It gave a slight positive test for choline. Lysolecithin and lysophosphatidic acid are tentatively identified as the main components of this spot. Spot number 6 was not identified but it may be lipoprotein, since it did not migrate off the origin.

## Intact Gut Tissue Incubation

A series of experiments were carried out in which strips of intact gut were incubated with ammonium palmitate-1-$C^{14}$. The result of these experiments provided information which one could compare with data from experiments with cell-free systems. The distribution of neutral lipids

is shown in Figure 4. Most of the radioactivity was distributed between triglycerides and phospholipids. Triglycerides (spot number 2) had 34.2 per cent of the total radioactivity, and the phospholipids (spot number 7) had 41.1 per cent of the total radioactivity. Free fatty acids had only 5.8 per cent of the total radioactivity.

Aliquots of the total lipid was utilized for phospholipid analysis. These results are presented in Figure 5. Most of the radioactivity was found in spot number 4, which consists of phosphatidyl choline (lecithin) and choline plasmalogen. Since over three-fourths of the total radioactivity in the polar lipids was in this area, further analysis was carried out to determine the distribution of radioactivity in the two components. The spot was eluted from the plate with chloroform: methanol (2:1, v/v), and taken to dryness. The residue was subject to acid hydrolysis for 18 hours. If the lipid residue contained plasmalogens, the ether bond would be cleaved and the fatty aldehyde would be released (Gray, 1958). Other lipids remained intact. The hydrolysate was spotted on a thin-layer plate and run in chloroform:methanlo:water (65:25:4, v/v). Phosphatidyl choline, which is not subject to acid hydrolysis, had 73.9 per cent of the total radioactivity. The spot corresponding to lyso-compounds had 11.5 per cent of the total radioactivity. 14.7 per cent of the activity was in the spot identified as fatty aldehydes. The amount of radioactivity found in the lysocompounds and fatty aldehyde fractions were most probably the hydrolysate of plasmalogen. From the above data, it was concluded that of the 40 per cent of the total radioactivity recovered in spot number 4 (lecithin and choline plasmalogen), three-fourth was incorporated into phosphatidyl choline, and one-fourth was incorporated into choline plasmalogen.

| Spot Number | Percentage of Total $C^{14}$ | Tentative Identification |
|---|---|---|
| 1 | 3.6 | Sterol ester |
| 2 | 34.2 | Triglyceride |
| 3 | 4.2 | Ascaroside Ester |
| 4 | 5.8 | Free fatty acids |
| 5 | 2.6 | Unknown |
| 6 | 8.5 | Ascaroside Ester ?<br>Diglyceride (?)<br>Cholesterol |
| 7 | 41.1 | Phospholipids |

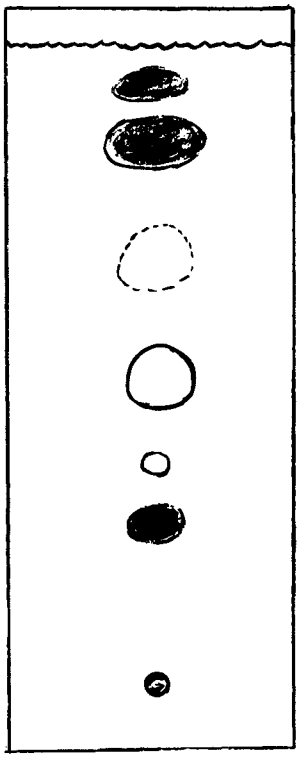


Figure 4. Thin-Layer Chromatogram of the Neutral Lipids of Ascaris Gut After Incubation with Ammonium Palmitate-1-$C^{14}$. The solvent was hexane:ether:acetic acid (60:40:1, v/v). The chromatogram was developed twice and the spots were visualized with iodine vapor.

| Spot Number | Percentage of Total $C^{14}$ | Tentative Identification |
|---|---|---|
| | | Neutral Lipids<br>Hydrocarbon<br>Aldehyde (?) |
| 1 | 47.5 | Sterol |
| 2 | 3.3 | Free fatty acids<br>Phosphatidic acid<br>Aldehyde (?) |
| 3 | 4.3 | Cephalin<br>(serine & ethanolamine)<br>Choline plasmalogen |
| 4 | 40.0 | Lecithin<br>Choline plasmalogen |
| 5 | 3.3 | Lysolecithin<br>Lysophosphatidic acid |
| 6 | 1.6 | Lipoprotein (?) |

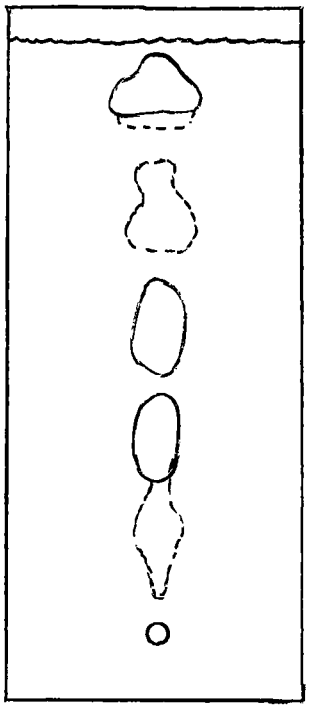


Figure 5. Thin-Layer Chromatogram of the Phospholipids of Ascaris Gut After Incubation with Ammonium Palmitate-1-$C^{14}$. The solvent was chloroform:methanol:water (65:25:4, v/v). The spots were visualized with iodine vapor.

## Formation of Labeled Glycerides from α-Glycerophosphate and Palmitate-1-$C^{14}$

### Palmitic Acid Incorporation by Cell-Free Supernatant

Determinations were made on the effects of pH, protein concentrations and time on the incorporation of palmitate into glycerolipids. The results of these determinations are presented in Figures 6, 7 and 8. Supernatant ($S_1$) which excluded cell debris and nuclei was used as the enzyme sources. As seen in Figure 6, palmitate esterification increased in proportion to the protein concentration until the protein concentration reached two milligrams. Above this point reduction of the incorporation occured. Figure 7 shows the effect of time on the synthesis of glycerolipids. The response was linear for a period of 120 minutes, and then leveled off. All subsequent incubations were run for two hours. Figure 8 shows the influence of pH on the rate of incorporation and the optimum pH was found to be 7.5.

### Possibility of Bacterial Contamination

Although the mid-gut tissue for the above experiments was washed carefully, individual guts were not split along their length prior to homogenizing. It was possible that bacteria present in the lumen of the gut were responsible for synthesizing part or all of the glycerolipids. So a series of experiments were designed in which mid-guts were split longitudinally and washed repeatedly in Ascaris saline before they were homogenized. Table I shows the comparison between palmitate incorporation of the supernatant ($S_1$) from the split gut tissue and washed intact

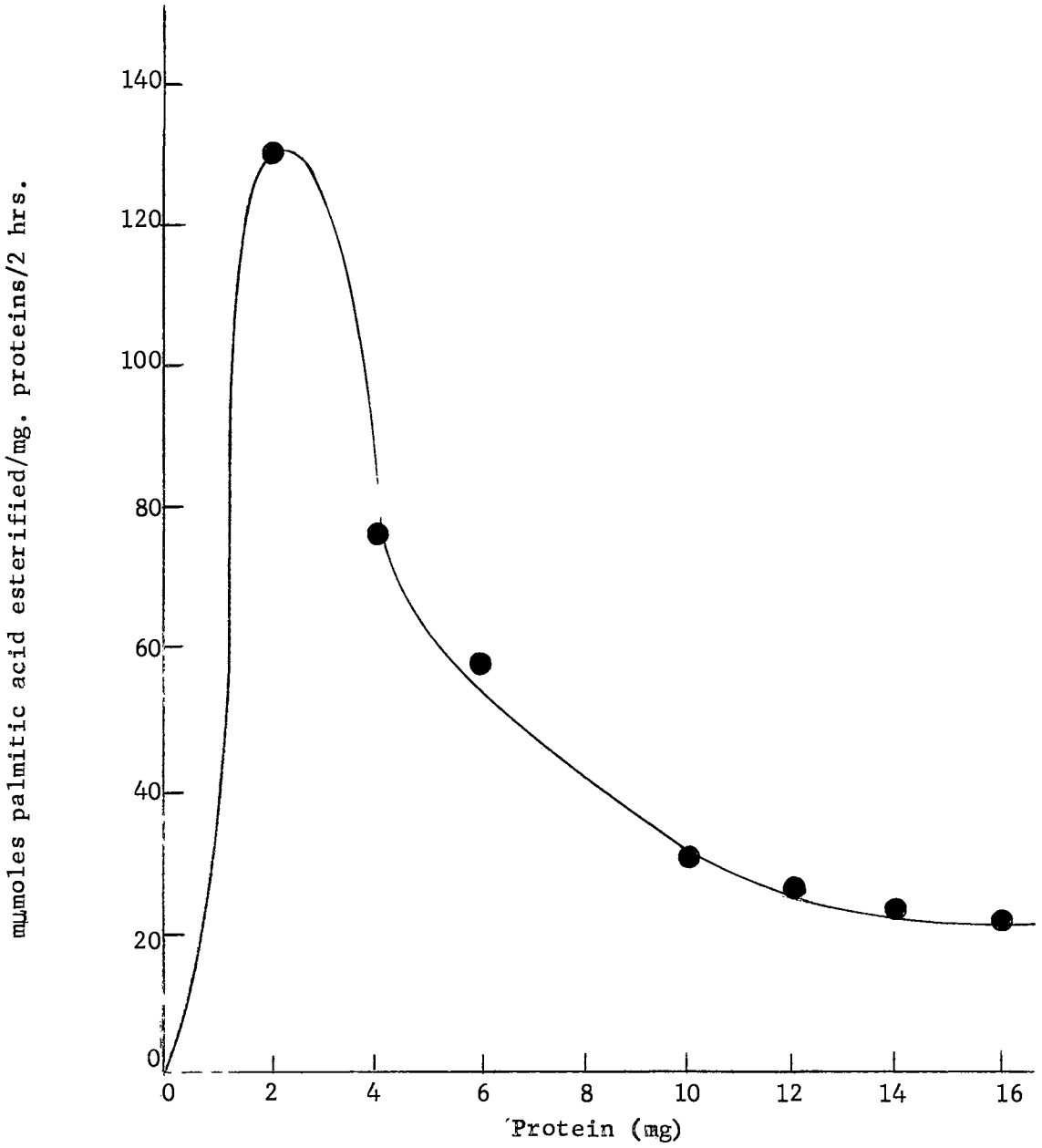



Figure 6. The Effect of Protein Concentration ($S_1$ fraction) on the Synthesis of Lipids. The incubation medium contained: 30 μmoles ATP; 0.5 μmoles CoA; 10 μmoles $MgCl_2$; 25 μmoles KF; 25 μmoles glutathione; 10 μmoles α-glycerophosphate; 0.5 μmoles ($C^{14}$) Ammonoim palmitate (283,862 d.p.m.); 0.1 ml. 10% Tween "80"; 0.05 M potassium phosphate buffer (pH 7.5) to make up to a final volume of 3.0 ml. Incubated under 99.9% $N_2$ at 37°C for two hours.

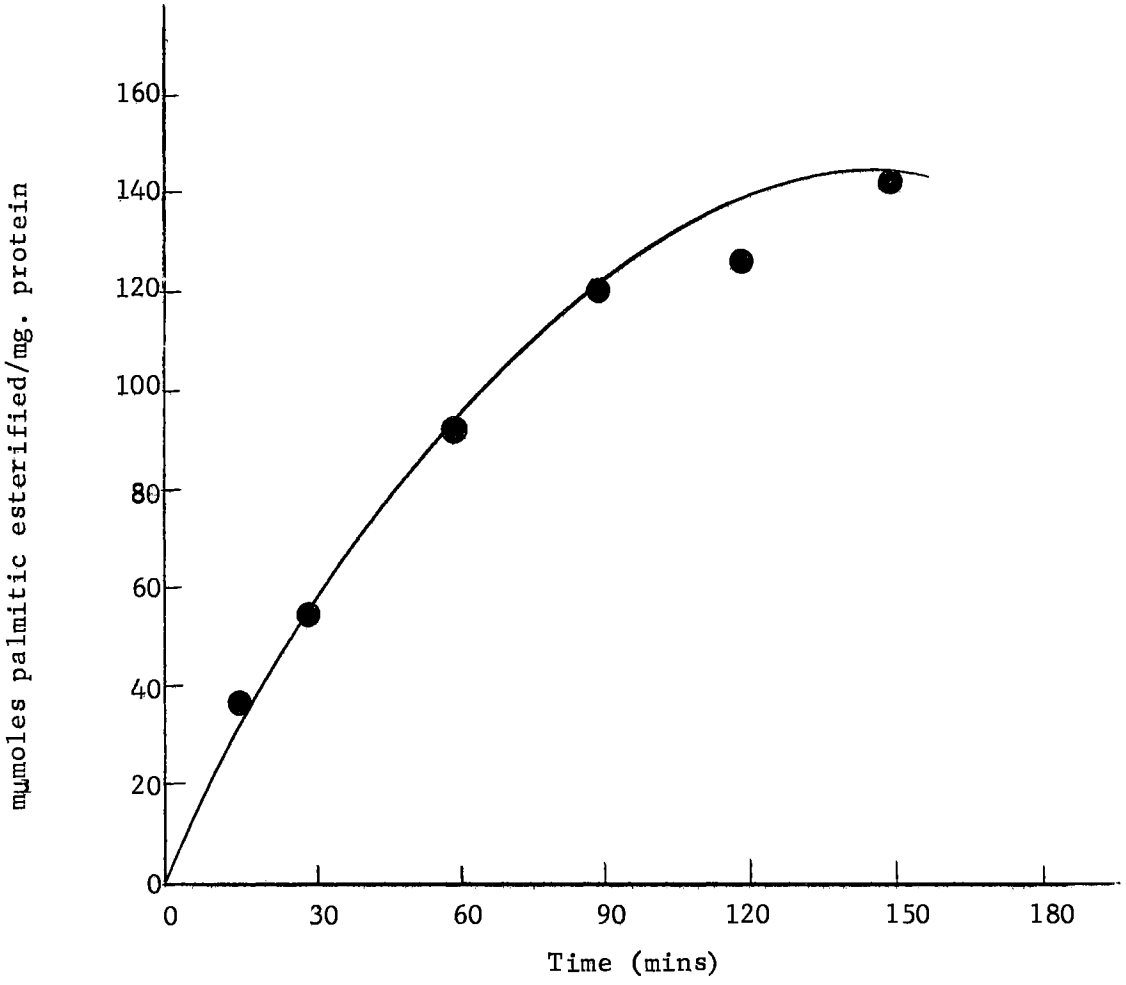



Figure 7. The Effect of Time on the Synthesis of Lipids. The incubation medium contained: 30 μmoles ATP; 10 μmoles $MgCl_2$; 0.5 μmoles CoA; 25 μmoles KF; 25 μmoles glutathione; 10 μmoles α-glycerophosphate; 0.5 μmoles ($C^{14}$) Ammonium palmitate (283,862 d.p.m.); 0.1 ml. 10% Tween "80"; 0.05 M potassium phosphate buffer (pH 7.5) to make up to a final volume of 2.0 ml. The enzyme source was 2 mg. of protein from $S_1$ fraction. Incubated under 99.9% $N_2$ at 37°C.

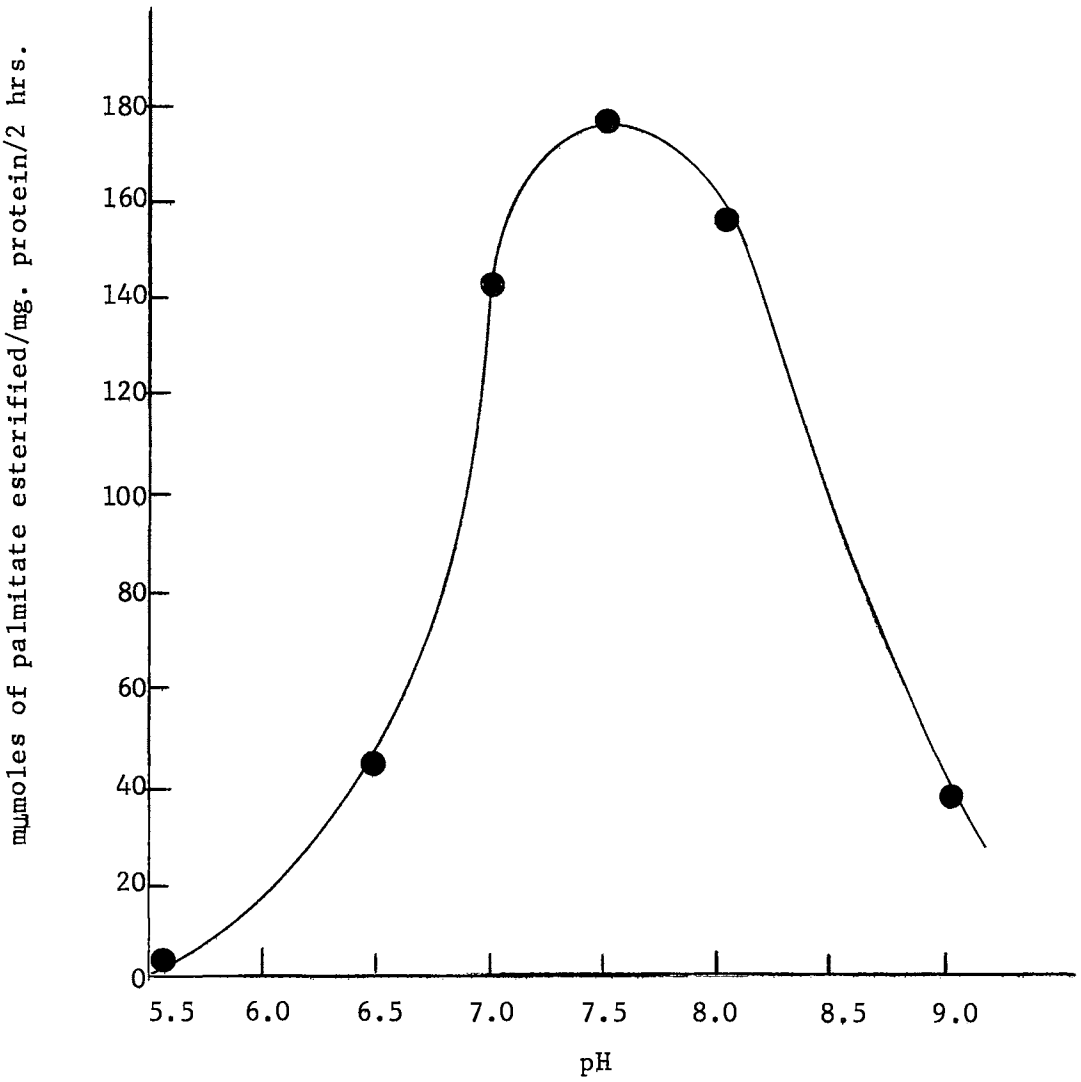



Figure 8. The Effect of pH on the Synthesis of Lipids. The gut supernatant was suspended in 0.25 M sucrose. The incubation medium contained: 30 μmoles ATP; 10 μ-moles $MgCl_2$; 0.5 μmoles CoA; 25 μmoles KF; 25 μmoles glutathione; 10 μmoles α-glycerophosphate; 0.5 μ-moles ($C^{14}$) ammonium palmitate (283,862 d.p.m.); 0.1 ml. 10% Tween "80"; 0.05M potassium phosphate buffer of the appropriate pH was added to make up to a final volume of 3.0 ml. The enzyme source was 2 mg of protein from $S_1$ fraction. Incubated under 99.9% $N_2$ at 37°C for 2 hours.

TABLE I

COMPARISON OF LIPID SYNTHESIS BY THE SUPERNATANT ($S_1$) OF GUT TISSUE ISOLATED TWO DIFFERENT WAYS

| System | mμmoles palmitate-1-$C^{14}$ esterified/mg protein/2hrs | | | |
|---|---|---|---|---|
| | Glycerides | | | |
| | Tri- | Di- | Mono- | Phospholipids |
| Supernatant A* ($S_1$) | 1.50 (4) | 18.12 (3) | 2.17 (4) | 80.30 (4) |
| Supernatant B** ($S_1$) | 1.14 (3) | 14.70 (3) | 1.73 (3) | 82.57 (3) |

A* Gut was not split but the intact gut was washed.

B** Gut split longitudinally and washed.

The incubation medium contained: 30 μmoles ATP; 0.5 μmoles CoA; 10 μmoles $MgCl_2$; 25 μmoles KF; 25 μmoles glutathione; 10 μmoles α-glycerophosphate; 0.5 μmole ($C^{14}$) ammonium palmitate (508,000 d.p.m.); 0.1 ml 10% Tween "80"; 0.05 M potassium phosphate buffer (pH 7.5) to make a final volume of 2.0 ml; 1.0-1.5 mg of protein: Gas phase 99.9% $N_2$; temperature 37°C. Incubation time was 2 hours.

( ) indicate the number of determinations.

mid-gut. As can be seen, no significant difference in incorporation of palmitate was found.

Subcellular Site of Palmitic Acid Incorporation in Ascaris Gut

A series of experiments were designed to determine the subcellular site(s) of glycerides and phospholipids synthesis in the cells of Ascaris mid-gut. A number of subcellular fractions were prepared and incubated in a medium containing α-glycerophosphate. The results of these experiments are presented in Table II. The various neutral lipids were separated by thin-layer chromatography and a zero time control was run simultaneously with each experimental determination. Possible contamination of glycerides with labeled palmitic acid was checked (this is discussed later) by running the control along with the experimental samples. The amount of $C^{14}$ incorporation reported in Table II and further experiments was determined by subtracting the radioactivity of the zero time control from the experimental samples.

Mitochondrial and microsomal fractions stimulate incorporation of ammonium palmitate-1-$C^{14}$ into glycerolipids. In fact, the mitochondrial fraction incorporates one-fifth more $C^{14}$ per unit time than does the microsomal fraction. The amount of palmitate-1-$C^{14}$ incorporated into phospholipids in both subcellular fractions was quite large. This would be expected when α-glycerophosphate is present to serve as the acceptor in the esterification of a fatty acid. In the mitochondrial fraction, 92.7 per cent of the total radioactivity was found in the phospholipids, and in the microsomal fraction, 88.4 per cent of the total radioactivity was in the phospholipids. This suggests that in Ascaris gut tissue the synthesis of phospholipids occurs to a much greater degree than the

TABLE II

PALMITIC ACID INCORPORATION INTO α-GLYCEROPHOSPHATE
BY SUBCELLULAR FRACTIONS OF ASCARIS GUT

| System | mμmoles palmitate-1-$C^{14}$ esterified/ mg protein/ 2 hrs incubation | | | | |
|---|---|---|---|---|---|
| | Glycerides | | | | |
| | Tri- | Di- | Mono- | Phospholipids | Total incorporation |
| Supernatant ($S_1$) | 1.50 ± 0.7(4) | 18.12 ± 7.9(3) | 2.17 ± 0.8(4) | 80.30 ± 20.7(4) | 111.26 |
| Mitochondria | 5.47 ± 2.0(5) | 21.00 ± 5.5(5) | 10.55 ± 5.1(7) | 468.00 ± 48.0(7) | 505.02 |
| Soluble fraction | 1.55 ± 2.0(5) | 8.29 ± 5.7(5) | 0.66 ± 0.5(5) | 1.99 ± 2.5(5) | 12.49 |
| Microsomes | 5.52 ± 2.4(7) | 30.10 ±11.0(6) | 15.90 ±11.6(5) | 392.80 ± 61.0(3) | 444.32 |

The incubation medium contained: 30 μmoles ATP; 0.5 μmole CoA; 10 μmoles $MgCl_2$; 25 μmoles KF; 25 μmoles glutathione; 10 μmoles α-glycerophosphate; 0.5 μmole ($C^{14}$) ammonium palmitate (508,000 d.p.m); 0.1 ml 10% Tween "80"; 0.05 M potassium phosphate buffer (pH 7.5) to make a final volume of 2.0 ml; 0.4-1.5 mg of protein. Gas phase 99.9% $N_2$; temperature 37°C. Incubation time was 2 hours.

( ) indicate the number of determinations.
± indicate standard deviation of the mean.

synthesis of triglycerides. When distribution of radioactivity in the neutral lipids was determined, the diglycerides had the highest activity. The monoglyceride fraction had less radioactivity than the diglycerides, but more than the triglycerides.

Cofactor Requirements

The cofactor requirements for the uptake of palmitate-1-$C^{14}$ into glycerolipids was determined in both the mitochondrial and microsomal systems. Table III presents the results of these studies. An absolute requirement of ATP and CoA was shown for both systems. This suggests the presence of a fatty acyl CoA synthetase in Ascaris gut tissue which will form fatty acyl CoA derivatives from fatty acids in the presence of ATP, CoA and magnesium ions. An enzyme of this type has been described by Senior and Isselbacher (1960) in rat-gut mucosa, and by Stein and Shapiro (1957 and 1958) in rat liver. The magnesium requirement for incorporation in both the mitochondrial and the microsomal systems was less important than ATP or CoA. Ommission of KF or GSH in the incubation did not greatly affect the uptake of palmitic acid in the mitochondrial system. As a matter of fact, ommission of KF and GSH in the microsomal preparation resulted in a slight increase in the rate of palmitate incorporation. ATPase activity may be more active in the mitochondria of Ascaris gut tissue. The limited effect of reduced glutathione (GSH) on palmitate incorporation by the microsomal fraction suggests that microsomes have the ability to reduce Coenzyme A. Similar results have been reported in mammalian system (Clark and Hübscher, 1960).

TABLE III

COFACTOR REQUIREMENTS FOR INCORPORATION OF PALMITATE-1-$C^{14}$ INTO α-GLYCEROPHOSPHATE BY SUBCELLULAR FRACTIONS OF ASCARIS GUT

| System | Percent of total radioactivity in complete system | |
|---|---|---|
| | Mitochondria | Microsomes |
| Complete* | 100 | 100 |
| Minus ATP | 0.30 | 0.72 |
| Minus CoA | 1.70 | 1.13 |
| Minus $Mg^{++}$ | 11.40 | 19.40 |
| Minus KF | 86.07 | 102.20 |
| Minus GSH | 65.62 | 102.10 |

*The incubation medium contained the same additions as described in Table II. The enzyme source was 0.4-0.6 mg of protein.

## The Biosynthesis of Glycerolipids from Substrates Related to α-Glycerophosphate

Table IV summarizes experiments in which an attempt was made to replace α-glycerophosphate with metabolically related compounds. In both enzyme preparations, it is evident that neither glycerol, β-glycerophosphate nor fructose-1, 6-diphosphate was effective in replacing α-glycerophosphate to synthesize glycerolipids. Next to α-glycerophosphate, glycerol was the most effective substrate to stimulate the incorporation of palmitate to glycerolipids. The rate of incorporation of palmitate was considerably lower when β-glycerophosphate was substituted for α-glycerophosphate. The stock β-glycerophosphate contains 0.1 per cent impurity of D-L-α isomer. In all probability, if an absolutely pure β-glycerophosphate had been used, the rate of incorporation of palmitate would have been insignificant. The utilization of fructose 1, 6-diphosphate as the substrate for lipid synthesis is doubtful, since both the mitochondria and microsomes are not likely to have any of the glycolytic enzymes. The incorporation of palmitate with fructose 1, 6-diphosphate may be partly due to the impurities (about 1%) presented in the stock fructose 1, 6-diphosphate.

## The Distribution of Radioactivity in the Phospholipids Synthesized by Mitochondria and Microsomes

Since a large amount of radioactivity was incorporated into phospholipids when particulate-fractions were incubated with α-glycerophosphate, further investigations were needed to determine the distribution of radioactivity among these lipids. The total lipid residue was applied to a thin layer plate with the aid of a mechanical streaker.

TABLE IV

THE BIOSYNTHESIS OF GLYCEROLIPIDS FROM SUBSTRATES RELATED TO α-GLYCEROPHOSPHATE

| System | Addition (10 μmoles) | mμmoles of palmitate-1-$C^{14}$ esterified/mg protein/2hrs |
|---|---|---|
| A. Mitochondria | None | 6.67 |
| | L-α-glycerophosphate | 505.02 |
| | L-β-glycerophosphate | 268.97 |
| | glycerol | 403.05 |
| | fructose-1, 6-diphosphate | 177.25 |
| B. Microsomes | None | 9.42 |
| | L-α-glycerophosphate | 444.32 |
| | L-β-glycerophosphate | 110.90 |
| | glycerol | 226.92 |
| | fructose-1, 6-diphosphate | 172.32 |

The incubation medium contained the same additions as described in Table II except the substrate.

After the plate was developed in the neutral lipid solvent, the phospholipids were eluted from the origin of the chromatogram with chloroform: methanol (2:1, v/v). The phospholipids were concentrated and re-chromatographed along with carrier lipid (gut tissue lipid) in the phospholipid solvent. Figure 9 shows the distribution of radioactivity in the phospholipids of the mitochondrial and microsomal fractions. Most of the radioactivity was discovered in spot number 4 (lecithin and choline plasmalogen) and spot number 5 (lysolecithin and lysophosphatidic acid). With the lipids from the microsomal incubations, some 78 per cent of the total radioactivity was present in lysolecithin and lysophosphatidic acid. About 10 per cent of the total radioactivity was recovered in the spot tentatively identified as a mixture of phosphatidyl choline and choline plasmalogen. Approximately 7 per cent of the total radioactivity remained at the origin, which was tentatively identified as lipoprotein. Neutral lipids had little radioactivity since most of the neutral lipids and fatty acids were removed from phospholipids. Phosphatidic acid had 2.7 per cent of the total radioactivity. Cephalin and choline plasmalogen contained only about 1.1 per cent of the total radioactivity. The distribution of radioactivity in the phospholipids from the mitochondrial fraction is similar to the distribution with the microsomal fraction. Lysocompounds (spot number 5) contained most of the radioactivity (approximately 72%). Lipoprotein had about 16 per cent of the total radioactivity. The radioactivity recovered in phosphatidic acid was relatively low.

Further experiments were carried out to determine whether the amount of radioactivity found in spot number 4 was phosphatidyl choline or choline plasmalogen. This spot corresponding to phosphatidyl choline

| Spots Number | Average per of Total $C^{14}$ in Systems | | Tentative Identification |
|---|---|---|---|
| | Microsomes | Mitochondria | |
| 1 | 1.52 ± 0.53 (2) | 0.90 ± 0.02 (2) | Neutral lipids<br>Hydrocarbon<br>Aldehyde (?)<br>Sterol |
| 2 | 2.73 ± 0.26 (2) | 1.79 ± 0.35 (2) | Free fatty acids<br>Phosphatidic acid<br>Aldehyde (?) |
| 3 | 1.08 ± 1.00 (2) | 0.91 ± 0.07 (2) | Cephalin<br>(serine & ethanolamine)<br>Choline plasmalogen |
| 4 | 10.03 ± 5.00 (2) | 6.81 ± 0.16 (2) | Lecithin<br>Choline plasmalogen |
| 5 | 78.24 ± 19.00 (2) | 72.81 ± 1.04 (2) | Lysolecithin<br>Lysophosphatidic acid |
| 6 | 7.27 ± 3.90 (2) | 16.61 ± 0.64 (2) | Lipoprotein (?) |

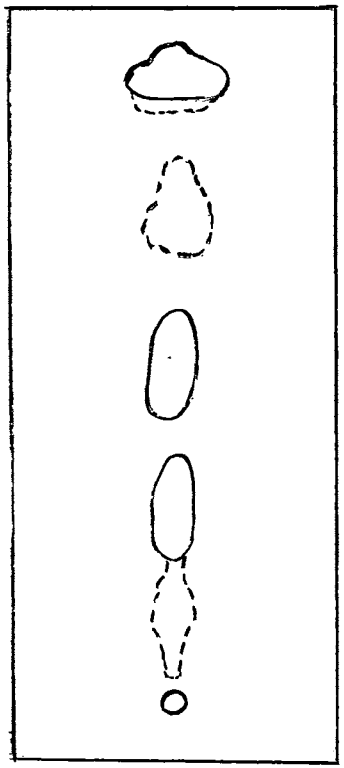


Figure 9. Thin-Layer Chromatogram of the Phospholipids Formed by Subcellular Fractions of Ascaris Gut Incubated with α-Glycerophosphate. Solvent system was chloroform:methanol:water (65:25:4, v/v).
( ) indicate the number of determinations
± indicate standard deviation of the mean

and choline plasmalogen was eluted from the silica gel and subject to acid hydrolysis as described previously. If the compound is plasmalogen, lysocompounds would be the end product. However, if it is lecithin, it will not be subject to acid hydrolysis. Both control and experimental samples were then re-chromatographed and developed in the phospholipid solvent system. The results are summarized in Figure 10. Approximately 92 per cent of the total radioactivity was recovered in the fatty acid and phosphatidic acid fractions, whereas in the control sample, some 91 per cent of the total radioactivity was found in the same spot. Further identification was obtained by developing the same samples in a different solvent system of hexane:diethyl ether:acetic acid (60:40:1, v/v), and the spot corresponding to free fatty acid was eluted from the plate for further analysis. The fatty acids were methylated with diazomethane and analyzed by gas chromatographic analysis. The results are presented in Table V. Methyl palmitate contained 94.9 per cent of the total radioactivity eluted from the column. Small amounts of radioactivity were distributed among other long-chained fatty acids.

Two possibilities to explain the above results occur to the investigation. First, it is possible that the bulk of radioactivity found in phospholipids is lysophosphatidic acid, which is unstable enough to break down when developed on Silica gel G. The amount of radioactivity found in the spot corresponding to phosphatidyl choline and choline plasmalogen (Figure 9) in both mitochindrial and microsomal could be the tailing of lysophosphatidic acid, since as soon as this spot was eluted and rechromatographed (Figure 10) it broke down completely to free fatty acids. Second, it is possible that the large amount of

(I)

| Spots Number | Percentage of Total $C^{14}$ Before Hydrolysis | After Hydrolysis | Tentative Identification |
|---|---|---|---|
| 1 | 5.7 | 4.9 | Neutral lipids<br>Hydrocarbon;aldehyde(?)<br>Sterol |
| 2 | 91.4 | 91.9 | Fatty acid;aldehyde(?)<br>Phosphatidic acid |
| 3 | 1.0 | 1.1 | Cephalin<br>Choline Plasmalogen |
| 4 | 1.2 | 1.4 | Lecithin<br>Choline Plasmalogen |
| 5 | 0.1 | 0.3 | Lysolecithin<br>Lysophosphatidic acid |
| 6 | 0.4 | 0.0 | Lipoprotein |

(II)

| | | | |
|---|---|---|---|
| 1 | 4.3 | 3.6 | Triglyceride |
| 2 | 95.0 | 93.8 | Free fatty acids |
| 3 | 0.5 | 1.5 | Diglycerides |
| | 0.0 | 0.4 | |
| 4 | 0.0 | 0.0 | Monoglycerides |
| 5 | 0.0 | 0.4 | Phospholipids |

Figure 10. Analysis of Lecithin and Choline Plasmalogen Synthesized by the Mitochondrial Fraction of Ascaris Gut When Incubated with α-Glycerophosphate. Solvent systems are (I) chloroform:methanol:water (65:25:4, v/v). (II) hexane:ether:acetic acid (60:40:1, v/v).

TABLE V

DISTRIBUTION OF RADIOACTIVITY IN FATTY ACIDS FROM LECITHIN AND CHOLINE PLASMALOGEN FORMED BY THE MITOCHONDRIA OF *ASCARIS* GUT

Unk = Unknown

| Fatty Acid Methyl Ester | Percentage of Total Recovered Radioactivity |
|---|---|
| 12:0 and less | 0.42 |
| 14:0 | 0.42 |
| 15 Unk | 1.30 |
| 16:0 | 94.90 |
| Unk | 1.20 |
| 18:0 | 0.00 |
| 19:0 and more | 1.80 |

Conditions: See Chapter II, *Materials and Methods*.

radioactivity placed on the origin after developing the plate in neutral lipid solvent is not phospholipid. It could be due to the presence of some labelled palmitic acid attached to the non-labelled carrier phospholipids (gut lipid) by van der Waal force.

Experiments were carried out to test the second possibility. Palmitic-1-$C^{14}$ acid was added to a lipid misture containing total lipid extracted from Ascaris mid-gut plus commercial standards of tri-, di and monoglyceride. The glycerides were added to assure adequate quantities for their detection in iodine vapor. This mixture was placed on a thin layer plate and developed in the neutral lipid solvent. The triglyceride, free fatty acid, diglyceride, monoglyderide and phospholipids (origin) spots were eluted and counted for radioactivity. From an average of nine experiments, about 0.3 per cent of the total radioactivity was found in triglyceride, 97.0 per cent in free fatty acid, 1.7 per cent in diglyceride, 0.2 per cent in monoglyceride and 0.6 per cent in phospholipids. As an additional check on the separation procedure, radioactive phospholipid synthesized by the mitochondrial fraction was isolated by thin-layer chromatography and mixed with neutral lipids and palmitic acid. The mixture was chromatographed in neutral lipid solvent. About 7.8 per cent of the total radioactivity was recovered in the neutral lipids and free fatty acid. Further, palmitic acid-1-$C^{14}$ was mixed with non-labelled phospholipids (isolated from Ascaris gut tissue), and chromatographed in the phospholipid solvent system. Distribution was as follows: 1.7 per cent of the total radioactivity was found in neutral lipids (spot number 1), 94.9 per cent was recovered in the area containing fatty acid and phosphatidic acid (spot number 2), 0.2 per cent was in cephalin and choline plasmalogen (spot number 3),

0.3 per cent was in lecithin and choline plasmalogen (spot number 4), 2.4 per cent was in the lyso-compounds (spot number 5), and no radioactivity was detected in the origin.

The above experiments indicate that the method is quite satisfactory for separating phospholipids from palmitic acid. As noted in the first experiment, when labelled palmitic acid was developed in the neutral lipid solvent, only about 0.6 per cent of the total radioactivity of the labelled palmitic acid remained in the origin where it could contaminate the phospholipids. The results of the second experiment indicated that some phospholipids might eventually break down on the plate. It was noted in the third experiment that if palmitic-1-$C^{14}$ was spotted on plate and developed in phospholipid solvent system, the bulk of the radioactivity was located in free fatty acid, and only trace amount of radioactivity were found elsewhere. In contrast to this, the distribution of radioactivity in Figure 9 was found mostly in lysocompounds, and not in the spot corresponding to free fatty acid. These results indicate that the phospholipid was not contaminated with radioactive palmitic acid. Hence the second possibility is ruled out.

## Formation of Labelled Glycerides from α-Monoglyceride and Palmitate-1-$C^{14}$

### Subcellular Site of Palmitic Acid Incorporation into Ascaris Gut

Direct esterification of monoglycerides by Ascaris gut tissue was also investigated. Experiments were carried out to determine the site(s) of the greatest glyceride synthesis by various subcellular fractions. The results are shown in Table VI. Both the mitochondria and microsomes possess the ability to form di- and triglycerides from

TABLE VI

PALMITIC ACID INCORPORATION INTO MONOGLYCERIDES BY SUBCELLULAR FRACTIONS OF ASCARIS GUT

| System | mμmoles palmitate-1-$C^{14}$ esterified/mg protein/2 hrs incubation | | | | |
|---|---|---|---|---|---|
| | Glycerides | | | | |
| | Tri- | Di- | Mono- | Phospholipids | Total incorporation |
| Supernatant ($S_1$) | 3.97 (8) | 1.66 (6) | 0.02 (8) | 1.27 (7) | 6.82 |
| Mitochondria | 7.50 (9) | 4.01 (9) | 0.29(10) | 0.54(10) | 12.34 |
| Soluble fraction | 0.33 (8) | 1.04 (8) | 0.35 (8) | 0.31 (7) | 2.03 |
| Microsomes | 7.17(10) | 3.26(10) | 0.41 (8) | 1.33 (9) | 12.17 |

The incubation medium contained 30 μmoles ATP; 0.5 μmoles CoA; 10 μmoles $MgCl_2$; 25 μmoles KF; 25 μmoles glutathione; 3 μmoles α-monopalmitin in 10% bovine albumin; 0.5 μmoles ($C^{14}$) ammonium palmitate (508,000 d.p.m.); 0.1 ml of 10% Tween "80"; 0.05M potassium phosphate buffer (pH 7.5) to make up to a final volume of 2.5 ml; 0.4-1.5 mg of protein. Incubated under 99.9% $N_2$ at 37°C for two hours.

( ) indicate the number of determinations.

α-monoglycerides and palmitic acid. The highest concentration of radioactivity was found in triglycerides in both the subcellular preparations. Diglycerides had only half as much radioactivity as triglycerides and only trace amounts of radioactivity were recovered from phospholipids.

In this experiment only 3 μmoles of α-monopalmitin was used, while in the first experiment 10 μmoles of α-glycerophosphate was added to the medium. This difference should have little influence on the uptake of palmitic acid. The reason for using only 3 μmoles of α-monopalmitin is due to the solubility of this substance in bovine albumin. The amount of α-glycerophosphate used in the last experiment was far in excess of the palmitate (0.5 μmole) in the medium. Since each triglyceride is formed by using one mole of glycerol and 3 moles of fatty acid, the 3 μmoles of α-monopalmitin used in this experiment is also in excess.

Cofactor Requirements

The cofactor requirements of both mitochondrial and microsomal fractions were also determined with monopalmitin as the acceptor substance in the medium. The results are presented in Table VII. ATP, CoA and magnesium ions were essential cofactors. Ommission of either of them from the reaction mixture reduced the incorporation of palmitic acid into complex lipids by 80-90 per cent. The requirements of KF and reduced glutathione (GSH) were not as essential as the above cofactors, but they still affected the rate of uptake of palmitate-1-$C^{14}$ into glycerolipids.

TABLE VII

COFACTOR REQUIREMENTS FOR INCORPORATION OF PALMITATE-1-$C^{14}$ INTO MONOGLYCERIDES BY SUBCELLULAR FRACTIONS OF *ASCARIS* GUT

| System | Percent of total radioactivity in complete system | |
|---|---|---|
| | Mitochondria | Microsomes |
| Complete* | 100 | 100 |
| Minus ATP | 13.05 | 9.45 |
| Minus CoA | 7.62 | 9.37 |
| Minus $Mg^{++}$ | 21.63 | 10.76 |
| Minus KF | 76.10 | 61.00 |
| Minus GSH | 69.37 | 30.90 |

*The incubation medium contained the same additions as described in Table VI. The enzyme source was 0.4-0.6 mg of protein.

## Tissue Extract Incubated with Palmityl-1-$C^{14}$-CoA

Beames and coworkers (1967) demonstrated that there is an ATPase present in Ascaris muscle. It seemed possible that the absences of significant phosphatidic acid and triglycerides synthesis were due to ATPase interferring with the synthesis of acyl CoA derivatives. By supplying the system with palmityl-CoA it would be possible to avoid any interference from an ATPase. Palmityl-1-$C^{14}$-CoA was suspended in acetate buffer (pH 6.0) and made up to a final concentration of 0.1 μmole/0.15 ml. Incubation was carried out as described previously. As seen in Tables VIII and IX, when palmityl-1-$C^{14}$-CoA was incubated with α-glycerophosphate, the total amount of radioactivity recovered in glycerolipids was quite low. The rate of incorporation was one-tenth of the rate observed (Table II) when palmitic acid was incubated with α-glycerophosphate. Experiments to determine the direct acylation of monoglycerides in the presence of palmityl-1-$C^{14}$-CoA were also performed. Here too, total incorporation was lower than when palmitic acid was incubated with monoglyceride (Table VI). It is interesting to note that the amount of radioactivity recovered in the phospholipids was some ten times higher than it was with palmitic acid and monoglycerides. Further experiments were carried out using diglycerides as substrate. The amount of incorporation is quite similar to those with monoglyceride.

From the above results, it is quite obvious that activation of fatty acid in the presence of ATP, $Mg^{++}$ and CoA is needed to stimulate the incorporation of palmitic acid to higher glycerides. The presence of free palmityl-CoA does not stimulate the metabolic systems which are responsible for the synthesis of glycerolipids in Ascaris mid-gut.

TABLE VIII

INCORPORATION OF PALMITYL-1-$C^{14}$-CoA INTO COMPLEX LIPIDS BY THE MICROSOMAL FRACTION OF ASCARIS GUT TISSUE

| | mμmoles palmitate-1-$C^{14}$ esterified/mg protein/2 hrs | | | |
|---|---|---|---|---|
| | Glycerides | | | |
| Substrate | Tri- | Di- | Mono- | Phospholipids |
| α-glycerophosphate (10 μmoles) | 0.29(3) | 3.61(3) | 1.16(3) | 47.06(3) |
| α-Monopalmitin* (3 μmoles) | 0.28(2) | 2.76(2) | 0.29(2) | 8.39(2) |
| Dipalmitin* (3 μmoles) | 0.29(2) | 2.65(2) | 0.53(2) | 7.12(2) |

Each tube contained the following additions: 10 μmoles KF; 0.1 ml of 10% Tween "80", 25 μmoles of GSH; 0.1 μmoles of palmityl-1-$C^{14}$-CoA ($3.6 \times 10^5$ d.p.m.); 0.05 M potassium phosphate buffer (pH 7.5) to make up a final volume of 2.0 ml. 0.5-0.9 mg of protein. Incubated under 99.9% $N_2$ at 37°C for 2 hours.

( ) indicate the number of determinations.

* indicate the substrate is suspended in 5% bovine albumin to a final concentration of 3 μmoles per ml.

TABLE IX

INCORPORATION OF PALMITYL-1-$C^{14}$-CoA INTO COMPLEX LIPIDS BY THE MITOCHONDRIAL FRACTION OF *ASCARIS* GUT TISSUE

| | mμmoles palmitate-1-$C^{14}$ esterified/mg protein/2 hrs | | | |
|---|---|---|---|---|
| | Glycerides | | | |
| Substrate | Tri- | Di- | Mono- | Phospholipids |
| α-glycerophosphate (10 μmoles) | 0.59(3) | 2.95(3) | 0.88(3) | 35.22(3) |
| α-Monopalmitin* (3 μmoles) | 0.34(2) | 4.56(2) | 0.27(2) | 9.44(2) |
| Dipalmitin* (3 μmoles) | 0.56(2) | 3.23(2) | 0.09(2) | 8.12(2) |

Conditions as described in Table VIII.

( ) indicate the number of determinations.

* indicate the substrate is suspended in 5% bovine albumin to a final concentration of 3 μmoles per ml.

# CHAPTER IV

## DISCUSSION

The mid-gut tissue of Ascaris lumbricoides suum possesses the ability to incorporate fatty acids into glycerolipids. When palmitate-1-$C^{14}$ was incubated with intact gut strips, most of the radioactivity recovered from the gut lipid was in triglycerides and phospholipids. Triglycerides had approximately 34 per cent of the radioactivity and phospholipids had 41 per cent of the radioactivity. Most of the radioactivity in the phospholipids was distributed between phosphatidyl choline and choline plasmalogen. Phosphatidyl choline had three times more radioactivity than choline plasmalogen. No reports have yet been made concerning the lipid composition of Ascaris gut. Determinations on the lipids distribution of the reproductive tissues, body wall and cuticle have been reported by Beames (1964 and 1965) and Fairbairn (1955 and 1956). In general, the total lipids from these tissues are quite evenly distributed among neutral and phospholipids. Triglyceride is the predominate one in neutral lipids. Choline phospholipids and plasmalogens account for half of the total nitrogen-containing phospholipids, and plasmalogens account for some 40-60 of the nitrogen-containing phospholipids (Beames, 1964). Therefore, it does not seem unusual for the intact Ascaris gut to incorporate palmitate into these lipids.

Efforts were made to elucidate the mechanism of synthesis of glycerides in Ascaris gut epiethelial cells. Two pathways have been

described in mammalian intestinal mucosa. The first of these, termed the α-glycerophosphate pathway, was described by Dawson and Isselbacher (1960), and later confirmed by Clark and Hübscher (1960 and 1961). The second pathway, termed the monoglyceride pathway, was suggested by Clark and Hübscher (1960 and 1961), Senior and Isselbacher (1961). Both the mitochondria and microsomes of the mammalian intestinal mucosa were reported by these workers to be solely responsible for esterification of fatty acids into glycerides. With this in mind, Ascaris gut tissue was homogenized and the cell-free supernatant (mitochondria + microsomes + soluble fraction) was isolated to run some preliminary tests. The enzyme system in Ascaris gut cells has its optimum pH at 7.5 and the time course for maximum incorporation is 2 hours.

The sites for the esterification of palmitate-1-$C^{14}$ via the α-glycerophosphate was determined. Mitochondrial and microsomal fractions of the Ascaris gut cells have the highest specific activity in catalyzing the reactions (Table II). Enzymes in the mitochondria are most active in catalyzing phospholipids biosynthesis, while the microsomal fraction has higher ability to synthesize glycerides. About 88-93 per cent of the radioactivity was found in the phospholipids. When cell-free supernatant (mitochondria + microsomes + soluble fraction) was incubated with α-glycerophosphate, no specific stimulatory effect in the synthesis of triglycerides has been observed. Evidences have been presented by Hüdscher et al. (1964) and Johnston et al. (1967) that the addition of supernatant (soluble fraction) to the enzyme systems stimulated the incorporation of fatty acids into triglycerides. The major role of supernatant fraction is to provide the enzyme L-α- phosphatidate phosphohydrolase (Johnston et al., 1967). It seems from the present results,

the supernatant (soluble fraction) of Ascaris gut cells does not behave like in mammalian system to stimulate triglyceride synthesis.

The labeled neutral lipids formed by both of these two subcellular preparations were a mixture of mono-, di- and triglycerides, and most likely represent a balance between incorporation of palmitate-1-$C^{14}$ into higher glycerides and lipolysis. No attempts were made to measure the lipase activity intracellularly. However, lipase activity in the lumen of Ascaris gut had been measured by Carpenter in 1952. Lipase activity was found to be relatively low as compared with other hydrolytic enzymes in the lumen, and the activity is especially low towards the mid-gut section. In the present study, Tween "80" and potassium fluoride were added to suppress most of the lipase activity. From the results presented in Table I, an intraluminal lipase activity was also demonstrated. When individual guts were not split along their length and washed out all the intestinal contents, the amount of di- and monoglycerides formed were a little more when compared with washed gut. In the subcellular fractions, microsomes probably seemed to have highest lipase activity. The evidence is based on the higher amount of di- and monoglycerides formed (Table II). Greatest specific lipase activity was also found in the microsomal fraction of rat intestinal mucosa (Playoust and Isselbacher, 1964).

The large amount of phospholipid formed via the α-glycerophosphate pathway was subject to further identification by means of thin-layer chromatography techniques. Most of the radioactivity was found in the fraction tentatively identified as lysophosphatidic acid (Figure 9). Trace amounts of radioactivity were incorporated into phosphatidic acid. It is possible that some cofactors or enzymes which are present in the

intact gut are inactivated when the gut tissue is broken down. These cofactors or enzymes might be responsible for converting lysophosphatidic acid to phosphatidic acid. The actual requirements and mechanism are still unknown. The questionable amount of radioactivity in the phosphatidic acid suggests that there is a highly active phosphohydrolase, associated with both subcellular fractions of Ascaris gut, hydrolyzes phosphatidic acid into diglyceride as soon as it is formed. For this reason, the added supernatant (soluble fraction) did not have a stimulatory effect on triglyceride synthesis as described earlier.

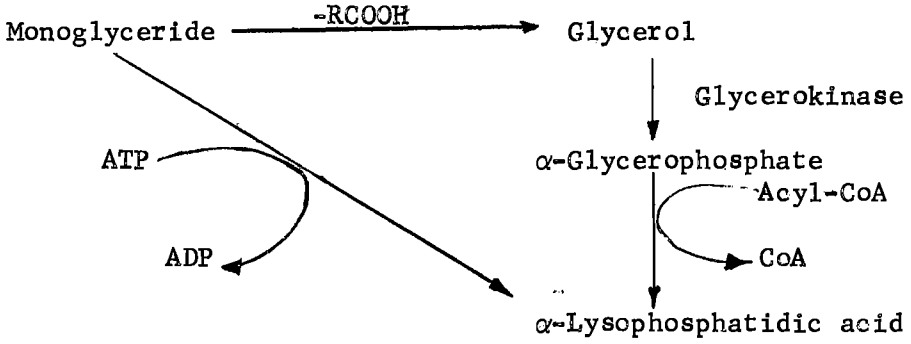
Cofactor requirements by these two subcellular preparations in Ascaris gut demonstrated that the synthesis of glycerides via α-glycerophosphate are similar to that described for mammalian systems. An absolute requirement of ATP, CoA and $Mg^{++}$ suggests the presence of a fatty acyl-CoA synthetase in Ascaris gut. The system activates the free fatty acids to fatty acyl-CoA thiolesters in the presence of ATP, CoA and $Mg^{++}$. Glutathione and potassium fluoride did not significantly affect the uptake of palmitate-1-$C^{14}$ in the microsomal fraction. It is possible that the microsomes can reduce the Coenzyme A which had not been reduced before addition. On the other hand, glutathione and potassium fluoride were definitely needed by the mitochondria for more incorporation.

The ability of both the mitochondrial and microsomal fractions to utilize other substitutes were also observed. All the substitutes used are either precursors or related substrates of α-glycerophosphate. Glycerol was the most effective substrate besides α-glycerophosphate to stimulate the incorporation of palmitate into glycerolipids. This indicates that a glycerokinase might be present in these enzyme

preparations and convert glycerol to α-glycerophosphate. β-glycerophosphate and fructose-1, 6-diphosphate could also stimulate the uptake of palmitate, however, the utilization of these two substrates is doubtful. Most likely the observed stimulation was due to the impurities present in the reagents.

Evidence present in Table V indicates that palmitic acid is not altered in chain length before it is incorporated into triglycerides.

The incorporation found with added monoglyceride (Table VI) suggests an additional pathway, that is, the direct esterification of monoglyceride to diglyceride. Monoglyceride might either be hydrolyzed to glycerol and then phosphorylated to α-glycerophosphate, or acted on by a kinase to lysophosphatidic acid. The amount of phospholipid formed is insignificant however, when compared with glycerides. For this reason, the direct esterification appears to be the principal route for triglycerides synthesis. Mitochondria and microsomes have almost identical activity with respect to triglycerides synthesis. The dependence of this activity upon ATP, CoA and $Mg^{++}$ suggests that the fatty acid is converted to acyl-CoA before it is esterified. This is quite similar to the system described in mammalian intestinal mucosa.



The failure of palmityl-CoA to replace palmitic acid plus its activation system in both the acylation of α-glycerophosphate and monoglyceride is surprising. Similar results are reported by Johnston et al. (1967), with the incubation of palmityl-CoA with the microsomal fraction of hamsteral intestinal mucosa. Palmityl-CoA has been demonstrated by Srere (1965) to have an inhibitory effect on citrate-condensing enzyme probably due to its detergent properties.

A flow-chart is presented in Figure 11, which summarized all the possible biochemical reactions involved in synthesis of triglycerides and phospholipids in Ascaris gut cells. No pathways or mechanisms are worked out for the phospholipid or plasmalogens synthesis. It might involve cytidine intermediates as described by Weiss et al. (1956) in chicken liver. All the lipids synthesized in the gut are eventually hydrolyzed into fatty acids in the hemolymph (personal communication with Sullivan). This may be due to a highly active lipase in the hemolymph.

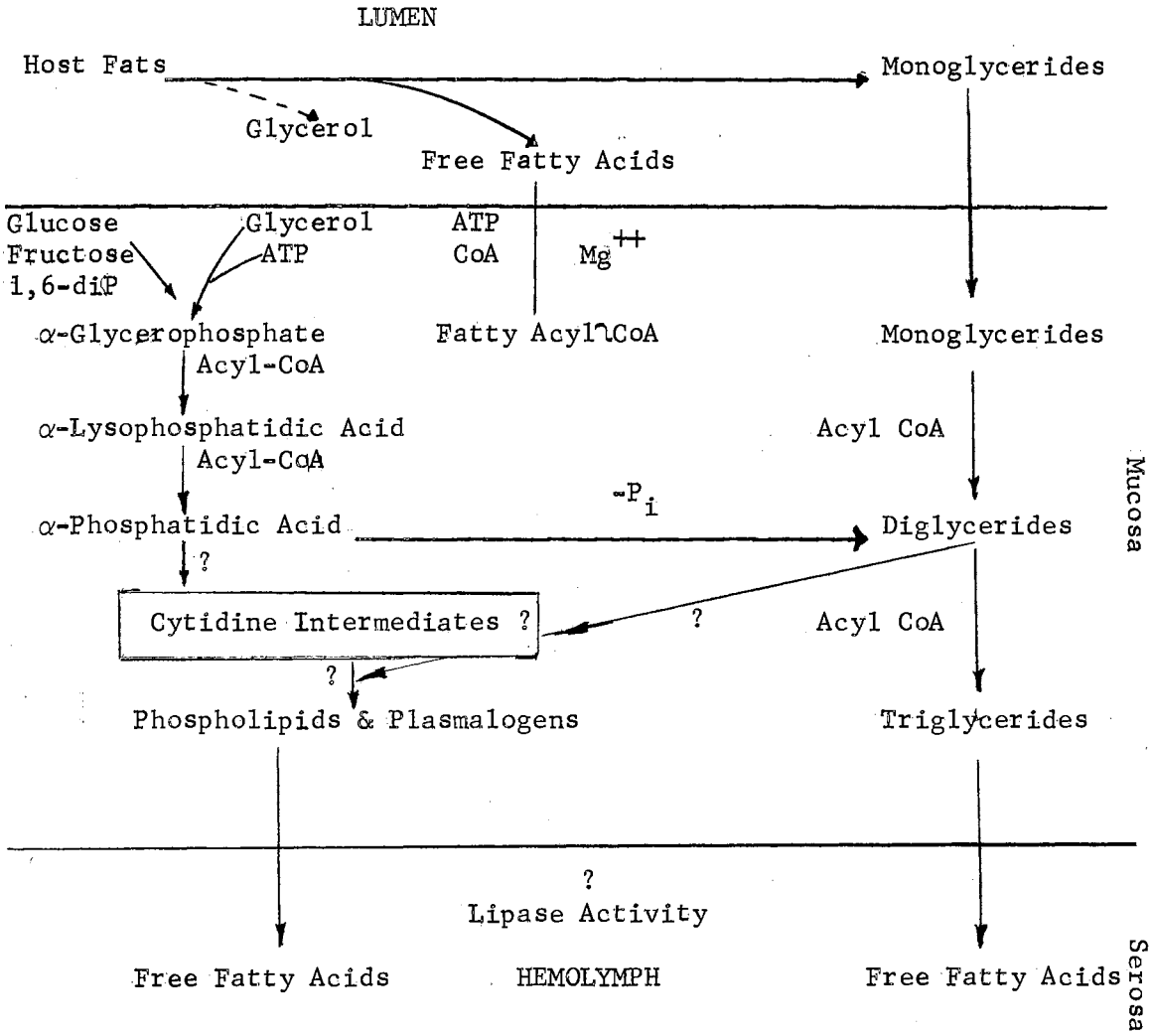



Figure 11. Proposed Pathways of Lipid Synthesis in the Cells of Ascaris Gut

# CHAPTER V

## SUMMARY AND CONCLUSIONS

The present experiments are designed to determine the pathways of glyceride synthesis in Ascaris gut tissue. Two pathways have been described for mammalian intestinal mucosa. The first one is called the α-glycerophosphate pathway, and the second one the monoglyceride pathway. By studies of both the intact gut and the subcellular fractions of Ascaris gut tissue, the following conclusions are reached.

1. When palmitate-1-$C^{14}$ was incubated with intact gut cells, most of the radioactivity was incorporated into the triglycerides, phosphatidyl choline, and choline plasmalogens.

2. Mitochondria and microsomes have the highest activity for the esterification of palmitate-1-$C^{14}$ via the α-glycerophosphate pathway. This incorporation was dependent on several cofactors. ATP, CoA, and magnesium ions were absolutely required by both subcellular preparations. Potassium fluoride, and glutathione were needed for maximum esterification by mitochondria, but had little effect with the microsomes. Incorporation was found among tri-, di-, and monoglycerides, which accounted for 7-12 per cent of the total incorporation for both the subcellular fractions of Ascaris gut tissue. The other 88-93 per cent of the radioactivity was found in phospholipids. Most of the radioactivity found in the phospholipid fraction was recovered in lysophosphatidic acid. Trace amount of radioactivity was incorporated into phosphatidic

acid.

3. Mitochondria and microsomes both possess the ability to incorporate palmitate-1-$C^{14}$ into glycerides via the monoglyceride pathway. Radioactivity was located mainly in the mono-, di-, and triglycerides. Little radioactivity was found in the phospholipids. The amount of neutral lipid synthesis via this pathway was less than the amount via the α-glycerophosphate pathway. ATP, CoA, magnesium ions, potassium fluoride, and glutathione were required by both subcellular preparations for optimal incorporation.

4. The activation of fatty acid into acyl-CoA could not be replaced by added commercial acyl-CoA. When palmityl-1-$C^{14}$ Coenzyme A was added to both mitochondrial and microsomal fractions of Ascaris gut tissue, only limited incorporation of palmitic acid into glycerolipids was observed.

Further studies are needed to determine these pathways in more detail. The tremendous amount of lysophosphatidic acid synthesis by the subcellular fractions via the α-glycerophosphate pathway indicates this step is limited by the absence of some unknown cofactor(s) or by the inactivation of some enzyme when the gut tissue is broken down. Experiments involving the determinations of ATPase and lipase in these subcellular fractions of Ascaris gut epithelial cells are also desired.

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