FATTY ACID SYNTHESIS FROM PROPIONATE

IN ASCARIS LUMBRICOIDES

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CHAPTER I

INTRODUCTION

The parasitic nematode, Ascaris lumbricoides suum, can be found in the small intestines of a large number of the world's swine. This worm lies, unattached to the gut wall, usually in the anterior half of the small intestine. Here, it is confronted with an environment which is very favorable to its survival. The worm is presented with an abundant supply of food, the temperature varies only a small amount, and the pH is just on the basic side of neutrality. The ionic environment is hypertonic to the worm, but this seems to present no particular problems. The atmosphere is a highly variable one depending upon the state of digestion which is occurring at a given time. The oxygen tension is almost invariably low (0.08-3.62% of the total gases, 69), while the percentages of CO_2, N_2, H_2, and CH_4 can be quite high (CO_2, 2.16-79.89%; N_2, 2.02-78.86%; H_2, 4.25-39.56%; CH_4, 0.2-28.29%, 69). The atmosphere has very few adverse effects on the animal because it thrives in this environment and is very prolific. It has been estimated that the female ascarid lays upwards of 200,000 eggs per day. The eggs consist of 7.5% protein, 16.6% carbohydrate, and 17.5% lipid (24). They have a thick coating which consists of an outer protein layer, a thick chitinous layer, and a thin inner membrane composed of lipid-like glycosides known as ascarosides (24). A high rate of metabolism is essential for such tremendous egg production. Undoubtedly, there is a rapid turnover of genetic material (DNA and RNA), protein, carbohydrate, and fat in the reproductive tissue. The unusual aspect of such rapid metabolic activity is that the parasite lives in an environment in which the

oxygen tension is generally as low as 0.08 percent. This rules out most of the biological oxidation-reduction reactions which involve oxygen as a terminal electron acceptor. One might ask how the animal has been able to survive and flourish in such an environment. The answer lies, of course, in the fact that the parasite has undergone many modifications in its metabolic pathways. Some of these modifications will be described in the following paragraphs.

Little is known about the metabolism of proteins in Ascaris. Transaminations must undoubtedly occur, because alanine is synthesized from pyruvate and ammonia by ovarian tissue of the worm (67). When starved Ascaris were fed amino acids, the protein content of the perienteric fluid was found to increase(72). The most prevalent nitrogenous waste product excreted by Ascaris is ammonia (71% of the total nitrogen excreted; 92). This would suggest a method of deamination. A small amount of urea is also excreted which would support the existence of an ornithine cycle (92). Whether or not amino acids can be used as an energy source is not known. The tricarboxylic acid cycle is not functional in Ascaris (70). Amino acids which are known to feed into the TCA cycle via transamination in mammalian tissue must be broken down in some other manner in Ascaris. Pollack (67) states that alanine-α-keto acid transaminases may be more important in Ascaris than glutamic-α-ketoglutaric transaminase systems.

Carbohydrate metabolism in the worm is somewhat different than that found in mammalian species. In higher animals, sugars are the main source of energy and this is also true for Ascaris (24). Glucose is broken down by the Embden-Meyerhof sequence, i.e., glycolysis (71, 12, 15). However, the end-product of glycolysis is not lactate. Instead it is one of several volatile fatty acids. After pyruvate is formed there are three possible pathways for further metabolism: (a) it can be converted to amino acids, (b) it can be decarboxylated to acetate or acetyl CoA, or (c) it can be carboxylated to form oxalacetate.

When oxalacetate is formed, it is converted to succinate via malate and fumarate. Succinate is then decarboxylated to yield CO_2 and propionate (12, 73, 74). Thus the animal can produce two propionic acid molecules from the catabolism of one molecule of glucose.

Propionic acid is not the only acid produced in the tissues of Ascaris as the result of carbohydrate catabolism. The following is a list of the most prevalent volatile fatty acids found in Ascaris tissues and the concentration of each: formate, 1.15%; acetate, 29.2%; propionate, 7.8%; 2-methyl-butyrate, 18.35%; and 2-methyl-valerate, 43.00% (12, 14, 19, 77, 5). A mechanism for the formation of the two branched-chain volatile fatty acids has been elucidated by Saz and Weil (75, 76). In the case of 2-methylbutyrate, the carboxyl carbon of acetate condenses with the alpha-carbon of propionate. The resulting keto acid is reduced via a pyridine nucleotide, dehyrated, and reduced again. The mechanism of formation of 2-methylvalerate entails condensation of 2 moles of propionate in the same manner as in 2-methylbutyrate.

The utility in forming volatile fatty acids as excretion products of carbohydrate metabolism can be seen in the fact that their synthesis requires reduced cofactors (75, 76). When oxygen is not present as a terminal electron acceptor, other compounds must be present to oxidize reduced cofactors so they can be used again. Syntheses of volatile fatty acids serve this purpose. The conversion of one mole of glucose to 2 moles of pyruvate requires the presence of 2 moles of NAD. This process generates 2 moles of NADH + H^+. The formation of propionate from pyruvate requires 4 moles of NADH + H^+. Further condensation reactions of acetate and propionate also require 2 moles of reduced pyridine nucleotides. Therefore, reduced cofactors arising from various biological oxidations (amino acid oxidation, purine and pyrimidine metabolism) are regenerated by the formation of volatile fatty acids which are then excreted.

A high energy phosphate bond is formed in the conversion of fumarate to succinate. Oxygen is not needed in this phosphorylation (47, 79).

It was pointed out earlier that a complete TCA cycle is not functional in adult Ascaris. This cycle can be demonstrated in the larvae during their development in an aerobic environment (24). Most of the reactions associated with the cycle are lost in the last stages of the larval development as it becomes an adult. Only the steps from succinate to fumarate to malate to oxalacetate to pyruvate are retained. This portion of the cycle is responsible for the formation of propionate and the oxidation of reduced cofactors. Further, attempts to describe cytochrome c and cytochrome oxidase in the mitochondria of adult Ascaris have not been successful (13, 17). Electron transport and oxidative phosphorylation as it is known in the mammalian system is apparently of little value to Ascaris.

Very little work has been done in the field of lipid metabolism of Ascaris. Von Brand (10) could find little change in the lipids of Ascaris after starvation for 5 days. Beames and coworkers (8) found no change in the lipid content of Ascaris which had been starved under atmospheres of 95% N_2-5% CO_2, 100% N_2, air or 95% air-5% CO_2. In fact, the concentration of lipid present in the starved worms was slightly higher than the controls. Jacobsen (42) found that the mitochondria of the muscle tissue of Ascaris had the ability, as indicated by oxidation of the dye 2,6-dichlorophenolindophenol, to oxidize propionate, butyrate, 2-methylbutyrate, and 2-methylvalerate. Palmitate was not oxidized to any extent by the system. The primary products of oxidation were acetate and formate. Beames et al. (7) have shown that acetate labeled with carbon-14 was incorporated into fatty acids by cell free, supernatants of Ascaris muscle and ovary-oviduct tissue. The pathway of this incorporation appeared to be the malonyl CoA pathway which is normally found in the cytoplasmic fraction of the cell. The main fatty acids synthesized by the ovary-oviduct system were 16-and 18-carbon

fatty acids. Beames (5) found that fatty acids with 18-carbons comprise approximately 70% of the total non-volatile fatty acids present in the worm. It is feasible then, that the main products of fatty acid synthesis would be 16-and 18-carbon acids. Beames (5) also showed that branched-chain, odd-numbered fatty acids were present as 5-6% of the total non-volatile fatty acids. A pathway for the synthesis of these acids in Ascaris has not been investigated to date.

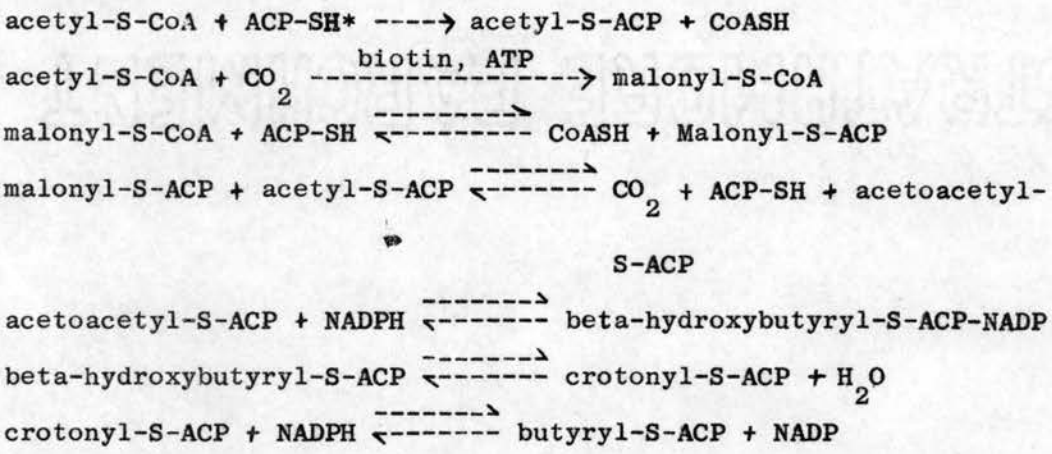
It is evident that the lipid metabolism of Ascaris needs much more study before the overall metabolic picture of this parasite can be understood. Are the branched-chain acids synthesized by the same systems which builds the normal even-numbered fatty acids? Is the synthesis of these acids an elongation of the odd-numbered, branched-chain, volatile fatty acids which are the end-products of carbohydrate metabolism? If this is the case, is acetate the preferred substrate and are the branched acids just "accidentally" elongated? This study was designed to try to answer these questions.

CHAPTER II

LITERATURE REVIEW

Fatty Acid Synthesis

Within the past few years the mechanism of de novo fatty acid synthesis has been elucidated to a great extent (89, 11, 54, 32, 91, 56). It proceeds in the following steps:



The butyryl-S-ACP then reacts with another molecule of malonyl-S-ACP which forms a beta-keto acid and the cycle repeats itself. The chain is usually elongated to 16 carbons, and the acid is then released from the acyl carrier protein. The de novo pathway of fatty acid synthesis is found within the soluble or cytoplasmic fraction of the cell (86, 18, 1, 2). Each of the above reactions has been demonstrated in soluble extracts of Escherichia coli (3). The enzymes necessary for this conversion are easily fractionated from E. coli, but mammalian fatty acid synthetase resists this fractionation (91). The acyl carrier

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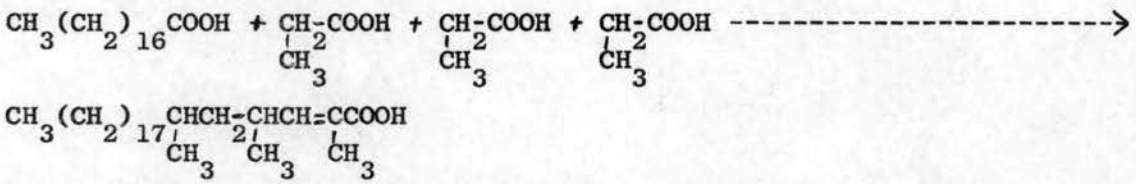
*ACP-SH - Acyl Carrier Protein.

protein which is involved in these series of reactions appears to take the place of CoA. It was first described in soluble extracts of E. coli by Goldman, Alberts, and Vagelos, (32). It is a heat-stable protein with 86-88 amino acid residues and has as the prosthetic group, 4'-phosphopantetheine (56). Although 4'-phosphopantetheine is also the main prosthetic group of CoA, the enzymes of fatty acid synthesis metabolize acyl CoA derivatives very poorly or not at all (56). Thus, the protein itself imparts some specificity on the reactions. Synthesis proceeds after the acyl fraction of acyl CoA is transferred to acyl carrier protein by a series of specific acylases (3). The 4'-phosphopantetheine is bound to the protein by a phosphodiester linkage with the amino acid serine (68, 57). The amino acid sequence around the serine has been determined as; Gly-Ala-Asp-Ser-Leu (58). Further amino acid analyses of ACP are being conducted at the present time (58). Attempts to isolate an acyl carrier protein from the mammalian fatty acid synthetase have not been possible because of the homogeneity of the enzyme complex, but Wakil (93) has demonstrated the presence of 4'-phosphopantetheine in preparations of highly-purified pigeon liver fatty acid synthetase. He has suggested that the ACP exists in a complex with fatty acid synthetase in mammalian tissues (93).

In 1960, it was demonstrated (90) that the mitochondria could incorporate acetyl-1-C^{14}-CoA into fatty acids. Harlan and Wakil (35, 36) were then able to demonstrate three distinct pathways in the mitochondria for the uptake of acetyl-1-C^{14}-CoA into fatty acids. The first pathway was similar to the de novo pathway and was dependent upon biotin for the synthesis. The second pathway did not require biotin and appeared to be a reversal of the beta-oxidation system, i.e., a simple add-on system. This elongation system could add one or more two-carbon units to existing fatty acyl CoA's. The third pathway was one in which oleic acid was produced and could be elongated to C-20 and C-22 polyunsaturated acids. Thus, the mitochondria can serve the purpose of elongating and desaturating fatty acids which are formed in the soluble,

cytoplasmic fraction of the cell. Phospholipids of mitochondria in mammals have large numbers of unsaturated acids (55). Phospholipids have also been implicated in membrane transport phenomena (39). Therefore, synthesis of fatty acids by the mitochondria could serve the purpose of providing fatty acids for structural or transport functions (36).

Odd-numbered and branched-chain fatty acids exist as minor components of the lipids of animals (40). On the other hand, the major fatty acids present in some bacteria are odd-numbered and branched-chain acids (66, 45). As would be expected, the majority of the metabolic studies of these acids have been carried out with bacteria. The possibilities of branched-chain fatty acid syntheses are very numerous. One pathway which has been studied extensively is the so-called "propionic acid mechanism". In 1951, Polgar and Robinson (66) hypothesized that mycolipenic acid (2,4,6-trimethyltetracos-2-enoic acid) was synthesized by a reaction of 1 mole of stearic acid and 3 moles of propionic acid in the following manner:



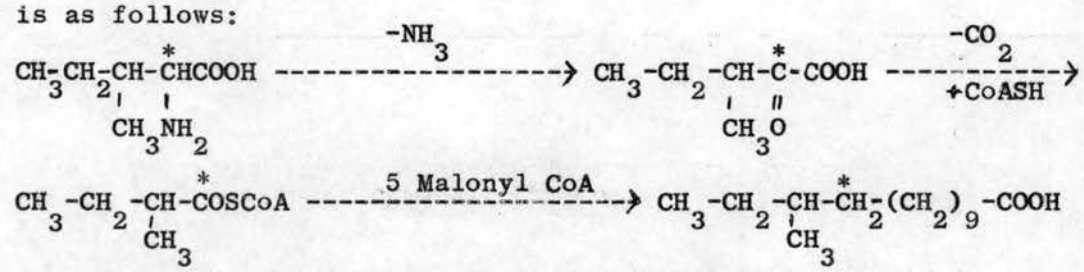
Lederer (48) was able to show that the condensation of "several moles of propionic acid" resulted in the formation of the branched chain of erythronolide, the macrocycliclactone moiety of the antibiotic, erythromycin. Kanada and Corcoran (44) have demonstrated that the active form of propionate in these reactions is methylmalonic acid, probably as the CoA derivative. This derivative corresponds to the active form of acetate, malonyl CoA. Gastambide-Odier et al. (29) have shown that mycocerosic acid is synthesized by a condensation of a normal 20-carbon acid in much the same manner as that hypothesized for mycolipenic acid by Polgar and Robinson (66). An example of this type of synthesis in animals has been described by Noble and co-workers (62). The preen

gland of the goose primarily secretes 2,4,5,8-tetramethyldecanoic acid. Noble et al. (62) have found that this acid is synthesized from one mole of acetate and four moles of propionate. Saz and Weil (75,76) have shown in Ascaris muscle that α-methylbutyric and α-methylvaleric acids are formed by condensations of acetate and propionate.

Another method for the synthesis of methyl-branched acids is C-methylation with methionine. In 1956, Karlsson (45) stated that tuberculostearic acid (10-methylstearic acid) "may be synthesized from oleic acid by methylation at the double bond." This thesis was proven in 1962 by the work of Lennarz, Scheuerbrandt, and Bloch (50) on Mycobacterium phlei. Their work showed that stearic acid was first desaturated to form oleic acid which was then methylated with methionine. A similar structure and mode of synthesis is seen with the cyclopropane fatty acid first identified from the fatty acids of Lactobacillus arabinosis, lactobacillic acid (37). The structure of this acid was later shown to be cis-11,12-methyleneoctadecanoic. Liu and Hofmann (52) demonstrated that this acid was synthesized from methionine and cis-octadec-11-enoic acid by methylation at the double bond (38). O'Leary (1962) and Zalkin and Law (95, 96) later found that the methyl group donor in this reaction was S-adenosylmethionine. Other works (48) have shown that C-methylation with S-adenosylmethionine is responsible for a variety of bacterial branched-chin fatty acids. No system of this type, however, has been described in animal systems. In a recent communication, Chung (16) reported that mitochondria from cat liver are unable to metabolize the cyclopropane ring of cis-9,10-methylenehexadecanoic acid. Therefore, the metabolism of these compounds in mammals seems rather questionable.

The third method used to form branched fatty acids is one in which Lennarz (49) has described for Micrococcus lysodeikticus. This method entails the deamination and oxidative decarboxylation of branched-chain amino acids to form branched-chain volatile fatty acids which are then elongated via malonyl CoA to form long-chain, methyl-branched fatty

acids. In the case of isoleucine the sequence as taken from Lennarz (49) is as follows:



In a highly-purified enzyme system from rat adipose tissue, Horning et al. (40) have been able to synthesize odd-numbered, iso, and anteiso 15- and 17-carbon fatty acids from malonyl-2-C^{14}-CoA and the corresponding odd-numbered or branched-chain volatile fatty acyl CoA. They suggested that the low level of these odd-numbered and branched non-volatile fatty acids in vivo was caused by the lack of initial substrates. They also postulated that the branched volatile acids could arise from branched amino acids.

Mevalonic acid is a well-known precursor of steroids in animal tissues (9). According to Lederer (48), phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) can be synthesized from mevalonate in plants. Mize et al. (61) have studied the metabolism of phytanic acid in rat liver homogenates by adding the alcohol (phytol) to the homogenate. Radioactive carbon dioxide was produced by liver slices when phytanate-U-C^{14} was incubated with them, but the rate was less than 1% of that found with stearic acid. Therefore, the methyl branches appear to inhibit metabolism by animal enzymes. Whether mevalonate can be a precursor to branched fatty acids in animals is not known.

Gastambide-Odier and Lederer (28) have found that corynomycolic acid ($C_{32}H_{64}O_3$) can be formed by Corynebacterium diphtheriae by the condensation of the carboxyl group of one mole of palmitic acid with the alpha-carbon of another mole of palmitic acid. Thus, the fatty acid formed has a branch 16 carbons long.

In summary, there are many possibilities of synthesis of odd-numbered and branched-chain fatty acids. They can be made from propionic acid (66, 44, 62, 29), methylation of double bonds by S-adeno-

sylmethionine (50, 52, 63, 95, 96), elongation of odd-numbered and branched-chain volatile fatty acids, or deamination and elongation of amino acids or some other source (40, 49). According to Lederer (48) mevalonate can be incorporated into branched-chain fatty acids of plants. Condensation mechanism involving 2 moles of long chain fatty acids also results in the formation of branched acids (28).

The question now arises, which, if any, of the preceding mechanisms is utilized by tissues of Ascaris to build odd-numbered and branched-chain non-volatile fatty acids? The mechanism of Horning et al. is certainly a possibility, because the initial substrates are present in abundant quantities. Beames et al. (6, 7) have shown that the muscle and reproductive tissues appear to utilize the malonyl CoA pathway for the incorporation of acetate-1-C^{14} into fatty acids. However, the level of odd-numbered and branched-chain fatty acids does not correspond to the high level of the odd-numbered and branched-chain precursors (5). Thus, there may be less synthesis via the malonyl CoA than would be expected.

Lipids of Ascaris

The lipids of Ascaris are a unique group of compounds. The volatile fatty acids, which are actually fermentation products, are the most readily observed because of their characteristic odor. These acids are unique within themselves because of their chemical structure. Nowhere in the animal kingdom can there be found a greater assortment of acids excreted in such abundance. Bacteria have long been known to form such compounds (81, 49) and indeed the ruminant animal probably derives the greater portion of its energy from the volatile fatty acids formed by the ruminal bacteria. We do not know if the volatile fatty acids excreted by Ascaris are absorbed and utilized by the hog.

In Ascaris, many of these acids are esterified (27) to a group of fatty alcohols which were discovered by Flury (26) and Faure-Fremiet

(25) and named by these workers as ascaryl alcohols. These lipid-like substances are found in all the tissues of Ascaris, but are most plentiful in the reproductive system (20,22). Fouquey and co-workers (27) determined the structure of these alcohols and found them to be long-chain hydrocarbons (29-45 carbons) with a minimum of one and maximum of three hydroxyl groups. They were also able to identify a hexose which was bound in glycosidic linkage with the ascaryl alcohol thus forming an ascaroside. This hexose, 3,6-dideoxyaldohexose, was designated as ascarylose. Jezyk and Fairbairn (43) have proposed a mechanism of synthesis of the ascaryl alcohols in which 2 moles of fatty acyl CoA condense in a carboxyl-alpha-carbon fashion with subsequent decarboxylation and reduction to form the straight carbon chain with one hydroxyl group. The mechanism of the placement of other hydroxyl groups is unknown, although it is possible that the carbon chain could be desaturated and water added to the double bond. Ascarylose is formed from C-14 glucose (43) in the same manner as that described in bacteria by Matsuhashi et al. (60). Fairbairn and Passey (21) found that ascarosides accounted for 77% of the unsaponifiable lipid present in the vitelline membrane of the egg. The membrane is semipermeable; thus, the ascarosides may function in forming an envelope around the egg which will allow certain materials to pass through and retard the flow of other compounds. The function of the ascarosides in the muscle and cuticle is not known.

Flury (26) was the first to identify phospholipids in Ascaris. Rogers and Lazarus (71) were able to demonstrate the presence of cephalins and lecithins in the reproductive tissue of the worm. Fairbairn (20, 22) found that phospholipids comprised 8.3% of the total lipids in the reproductive tissue and 38% of the total in muscle tissue. Fairbairn (22) also noted that plasmalogens constituted about 30% of phospholipid in muscle tissue. Beames (4) has studied the phospholipids of the muscle, cuticle, and reproductive systems extensively. In the muscle tissue he found that phosphatidyl choline and its corresponding plasmalogen comprised 70% of the total phospholipid while

the remainder was primarily phosphatidyl ethanolamine. In the cuticle ethanolamine phospholipids accounted for 60% and choline phospholipids were present as 42% of the total. In the reproductive system the ratio was choline, 56%, and ethanolamine, 38%. Beames (4) also found that the concentrations of plamalogens were high in the phospholipids. He established values of 38%, 29% and 20% of the total phospholipide in the muscle, cuticle, and reproductive system, respectively. Beames (4) found that the C-18 fatty acids and aldehydes comprised 60-80% of the total acids and aldehydes in the phospholipids and plasmalogens of Ascaris. This is, of course, in contrast to the mammalian system in which the 16-carbon acid predominates (33). Beames (4) established that the unsaturated 18-carbon acids were present in greater quantities (40-56%) than the saturated 18-carbon acids (5-25%). This finding is rather unique because an animal which lives in an essentially anaerobic environment has problems in oxidizing reduced cofactors. Therefore, the purpose of desaturation of fatty acids in Ascaris appears to be rather nebulous. The presence of large amounts of plasmalogens in the tissues of Ascaris is another unique occurrence. The worm must have some type of enzyme which will reduce fatty acids to their respective aldehydes, although this has not been demonstrated in the tissues of Ascaris. Phospholipids have been thought of as structural components of cells for some time (9). The presence of larger amounts of phospholipids in the muscle tissue than in the reproductive tissue may be an indication of this role of phospholipids in Ascaris. However, the half-life of phospholipid molecules has not been determined, so statements on the function of phosphatides in the tissues of Ascaris cannot be made at the present time.

Fairbairn (20, 22) has studied the triglyceride content of Ascaris tissues. In the muscle and integument he found that triglycerides composed approximately 47% of the total lipid. In the reproductive system, 63% of the total lipid was present as triglyceride. Beames (5) established the presence of some 22 fatty acids ranging from 12-20 carbons

in the neutral lipids of Ascaris. The 18-carbon fraction accounted for 72% of the total, while only 12-15% was the 16-carbon acid, palmitic acid. Sixty-one percent of the total was unsaturated 18-carbon acids (18:1, 18:2, 18:3). These were approximately the same values found in the fatty acids of the phospholipid fraction (4). The triglycerides in higher animals serve the purpose of energy storage. Beta-oxidation of the fatty acids yields acetyl CoA which is then shuttled into the tricarboxylic acid cycle. As was mentioned previously, the TCA cycle appears to be non-existent in the tissues of Ascaris. Besides, acetate is an excretion product of the nematode. Von Brand (10) and Beames et al. (8) found no change in the total lipid of Ascaris which had been starved up to 5 to 6 days. Therefore, it is doubted that the worm can use glycerides as a source of energy. One possibility is, however, that the lipids are synthesized and stored for energy reserves for the eggs (24). Passey and Fairbairn (64) have demonstrated a net conversion of lipid to carbohydrate during embryonation of Ascaris eggs. Costello and Brown (17) have shown that the unembryonated eggs of Ascaris contain the enzymes of the TCA cycle, so it is plausible that these cleidoic eggs are furnished with all the food necessary for development from the lipid in the egg.

Sterols account for 1.7% of the total unsaponifiale lipids in Ascaris (20). Twenty-five percent of the sterols were saturated. Fairbairn and Jones (23) identified cholesterol as the main component of the sterols. The presence of this compound in Ascaris is not clear. In mammalian species cholesterol serves a structural function as well as being a precursor for hormones, bile acids, and other compounds, but the need of the latter compounds in Ascaris has not been demonstrated. This leads one into a discussion of assimilation or synthesis. Up to now the discussion of the lipids of Ascaris has assumed that each lipid was synthesized by the worm for some specific purpose. However, there is some disagreement upon the amount of lipids and other compounds which are actually synthesized by intestinal parasites. They

live in an environment in which starvation is usually no threat to their existence, so a large portion of their foodstuffs may be assimilated and incorporated unchanged into their tissues. Indeed, Fairbairn (personal communication) found that the fatty acid composition of the contents of the hog intestine is very much like that of Ascaris. Certainly, in Ascaris the process of natural selection has chosen an animal, which by the very nature of its metabolism, is able to survive in an environment without oxygen. The TCA cycle is no longer present in the adult, cytochrome c and cytochrome oxidase are also conspicously absent. The answer to the question of assimilation or synthesis and to what degree it exists in Ascaris remains unanswered at the present time. This research problem was designed to aid in finding the correct answer.

CHAPTER III

MATERIALS AND METHODS

Chemicals

Adenosine triphosphate (ATP), Coenzyme A (CoA), nicotinamide adenine dinucleotide, reduced form (NADH), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) were obtained from Sigma Chemical Company, St. Louis. Sodium propionate-1-C^{14} was purchased from Baird-Atomic, Chicago. Sodium propionate-2-C^{14}, sodium propionate-3-C^{14}, and malonic acid-2-C^{14} were bought from New England Nuclear, Boston. Sodium malonate-1-C^{14} and sodium malonate-2-C^{14} were were obtained from Nuclear-Chicago, Des Plaines, Illinois. Propionyl CoA was synthesized by the method of Simon and Shemin (79). For this synthesis, sodium propionate was converted to the acid and dried according to the method of Gerhke and Lamkin (30). Malonyl CoA was synthesized by the method of Trams and Brady (82) with the modification that the basic CoA solution was allowed to react with monomalonylthiophenol for only 15 minutes instead of the prescribed 3 hours. No decrease was noted in the percent yield when this was done. The concentration of the acyl CoA derivatives was measured by the hydroxymate procedure developed by Lipmann and Tuttle (51). The purity of the radioactive CoA derivatives was tested by chromatographing the hydroxymate of the derivative in the system described by Vagelos (83). Total protein on all samples of enzymes was determined by the method of Lowry and workers (53).

Intact Tissue Incubation

Adult female Ascaris lumbricoides suum were obtained from a local slaughterhouse and transported to the laboratory in a salt solution (42, 8) maintained at 37°C. The ovary-oviduct tissue was dissected out and placed in salt solution at 0-2°C until used. Five-tenths millimoles of sodium propionate-1-C^{14} (5.5×10^7 cpm) were incubated with 2.5 g. of ovary-oviduct tissue suspended in 5 ml of salt solution. The incubation was carried out for 2 hours at 37° in a shaking water bath.

Extraction of Lipids and Fatty Acids

The incubation was stopped by the addition of 5 volumes of chloroform: methanol (2:1, v/v). The flask was shaken vigorously for 3 hours, and the precipitated tissue was removed by filtration. The filtration. The filtrate was washed with 10 volumes of tap water for 3 hours, and the bulk of the water was removed with a vacuum aspirator. The remainder of the water was frozen out by placing the beaker in a deep-freeze at -25° for 3 to 4 hours. The solution was filtered in the cold, and the chloroform which contained the lipids was collected. The solution was dried further over anhydrous sodium sulfate, filtered, and the chloroform was removed in vacuo at 40°. The lipid residue was dissolved in 10 ml of chloroform, and aliquots were taken for counting. Aliquots were also taken for thin-layer chromatographic analysis.

Fatty acids were isolated in the following manner. An aliquot of the chlorofrom solution was evaporated, and the residue was suspended in 20 ml of 10% KOH in methanol plus 5 ml of water. The suspension was saponified by refluxing for 6 to 8 hours. After saponification, 50 ml of water were added to the solution and the aqueous layer was extracted 3 times with equal volumes of ether. The ether extracts were combined and washed 3 times with equal volumes of water. The aqueous fractions were combined and acidified to pH 1 by the drop-wise addition of 10 N H_2SO_4. The acidified solution was extracted 3 times with equal volumes

of ether. The ether washes were combined, dried over sodium sulfate, and concentrated *in vacuo* at 45°. In this manner, the fatty acids were effectively separated from unsaponifiable lipids in the original extract.

Enzyme Preparation

The ovary-oviduct tissue was homogenized in 2 volumes (w/v) of cold potassium phosphate buffer (0.05 M, pH 7.5) with a Potter-Elvejhem tissue grinder equipped with a teflon pestle. The resultant homogenate was centrifuged in the cold for 30 minutes at 12,000 x g (International Centrifuge, Model HR-1) to remove cell debris nuclei, and mitochondria. The supernatant (S_{12}) was then spun at 100,000 x g (Beckman Ultra-centrifuge, Spinco Model L 2) for one hour to remove microsomes. The supernatant (S_{100}) was used in some instances as the enzyme source. Further purification was accomplished by ammonium sulfate fractionation of the S_{100} supernatant. The protein which precipitated between 25-40% saturation of ammonium sulfate was shown to have the greatest activity in incorporating propionate-C^{14} into fatty acids. This precipitate could be stored for at least 1 month at 0-2°C without appreciable loss of activity.

Incubations

Most of the cofactors were dissolved in potassium phosphate buffer (0.05 M, pH 7.5), and the pH was carefully adjusted to 7.5 for all acidic cofactors immediately before use. After all cofactors and substrates were added to a screw-cap culture tube, the experiment was started by the addition of S_{100} supernatant or protein from the 25-40% ammonium sulfate-protein precipitate of S_{100}. Individual reaction tubes were flushed with nitrogen for 15-20 seconds, capped and allowed to incubate for a set period of time at 37.5° in a shaking water bath. The total volume of the reactants was either 2.5 or 3 ml.

Extraction of Fatty Acids

After the incubation was completed, the reaction was stopped by the addition of 3 ml 10% KOH in methanol. Saponification was accomplished by allowing the capped tubes to heat overnight at 60° or for one hour at 100°. The methanol was removed by evaporation with a stream of nitrogen in a water bath maintained at 60°. The tubes were then cooled to 0° in an ice bath and the pH was adjusted to 1 by carefully adding 0.5 ml of cold, concentrated HCl to each tube. Five milliters of pentane were added to each tube and they were vigorously shaken on a Vortex mixer. Organic and aqueous phases were separated by centrifugation, and the pentane layer containing the fatty acids was removed with a Pasteur pipette and saved. The procedure was repeated 2 more times, and the combined washes were evaporated to dryness under a stream of nitrogen in a water bath maintained at 45°. Fatty acid residue was dissolved in 5 ml of pentane and washed once with 3 ml of 1 N HCl. Centrifugation separated the two phases, and the pentane was transferred to another tube with a Pasteur pippette. The pentane was evaporated under a stream of nitrogen at 45°, and the temperature of the water bath was then increased to 60°. The tubes were allowed to stand at this temperature with nitrogen blowing through them for 10 minutes. This procedure insured the removal of the volatile fatty acids. The non-volatile fatty acids were dissolved in 0.5 ml of hexane, and an aliquot was taken for counting with a Packard Tri-Carb Liquid Scintillation Spectrometer. Tests with carbon-14 labeled palmitic acid indicated that this extraction procedure removed 90 to 100% of the fatty acids present in the aqueous phase.

Thin-Layer Chromatographic Analysis

This was carried out on 20 x 20 cm plates which were coated with a thin layer of Silica Gel G. Before use the silica gel was activated

by heating for 1 hour at 110°. The developing solvent was usually hexane:ether:acetic acid (60:40:1, v/v). In order to attain better separation, the plates were allowed to develop twice in this solvent system. The spots were visualized with either iodine vapors or charring with sulfuric acid. Antimony trichloride was used to detect sterols and sterol esters (59). Phospholipids were identified by the ammonium molybdate-perchloric spray developed by Wagner and workers (85). Hydroxylamine-ferric chloride (59) was used to detect fatty acid esters and glycerides. Additional identification was made by running standard compounds concomitantly with the unknowns. The spots were eluted from the thin-layer plate by scraping the gel off the plate and extracting it with chloroform:methanol (2:1, v/v) or hexane.

Preparation of Acids for Analysis

The methyl esters of the fatty acids were synthesized by reacting freshly distilled diazomethane with an ethereal solution of the fatty acids. Unsaturated fatty acids were reduced to their saturated forms with platinum oxide and hydrogen (34).

Gas Chromatographic Analysis

A Barber-Coleman Model 5000 Gas Chromatograph equipped with a column temperature programmer and an ionization detector were used for the analysis of the methyl esters. Six foot glass columns packed with either ethylene glycol succinate (EGS) coating Gas Chrom CLP (100-120 mesh) or SE-30 coating Gas Chrom Q (80-100 mesh, Applied Science Laboratories, State College, Pennsylvania) were utilized to separate the methyl esters. The EGS column packing was 14.5% liquid and 85.5% solid, while the SE-30 column packing was 3% liquid and 97% solid. The effluent fatty esters were collected on glass cartridges filled with anthracene. A Packard Fraction Collector was used to facilitate collection. The radioactivity of the individual methyl esters was

determined by placing the cartriduges directly into the Packard Tri-Carb. Anthracene acted as the fluorescent compound. Tests with standard methyl palmitate-C^{14} revealed that 60 to 90% of the radioactivity injected into the gas chromatograph could be recovered in this manner. With the EGS column, the column temperature was 185°, Flash-heater temperature was 260°, and the detector temperature was 220°. When the SE-30 column was used the column temperature was programmed between 175-215° with a temperature rise of 1.5° C/minute. The flash-heater and the detector temperatures were the same as with the EGS column.

CHAPTER IV

RESULTS

Intact Tissue Incubation

Incubation of intact ovary-oviduct tissue with sodium propionate-1-C^{14} resulted in the uptake of radioactivity into the lipids of the tissue. The various classes of lipids were separated by thin-layer chromatography, and the percentage of carbon-14 that was incorporated into each class can be seen in Figure 1. Phospholipids, which do not migrate in this solvent system, contained very small amounts of radioactivity. A spot which migrated the same as a standard monoglyceride (#2) contained 7% of the total radioactivity. Spot #4, which may have been an ascaryl alcohol (Jezyk, personal communication), exhibited 16.4% of the total carbon-14 that was recovered. Spot #6 routinely gave a positive test with antimony trichloride, which is specific for sterols and sterol esters. This spot contained 10.1% of the total radioactivity. King (46) has demonstrated that an ascaroside ester also migrates with these sterols and is very difficult to separate from them. It is possible that the radioactivity in this spot was in the ascaroside ester. Spot #7 contained nearly one-fifth of the total isotope. It was not identified but it may be an ascaroside ester. Free fatty acids and possibly ascaroside esters were found in spot #10. A significant percentage of the carbon-14 (16.2%) was located in this spot. The tryglyceride spot (#11) contained 15.5% of the radioactivity. It was the most predominant spot on the chromatogram. Spot #12 was usually very faint but 11.7% of the total radioactivity was present in this area. Sterol esters, hydrocarbons, and fatty acid esters were

Spot Number	Percentage of Total C^{14}	Tentative Identification
		Sterol ester
		Hydrocarbon
12	11.7	Fatty ester
11	15.5	Triglyceride
		Free Fatty Acid
10	16.2	Ascaroside ester?
9	1.7	Ascaroside ester?
8	1.3	Ascaroside ester?
		Diglyceride?
7	18.6	Ascaroside ester?
		Cholesterol
6	10.1	Ascaroside ester?
		Ascaroside ester?
5	1.7	Unknown
4	16.4	Ascaryl Alcohol?
3	1.4	Unknown
2	7.0	Monoglyceride
1	1.9	Phospholipid

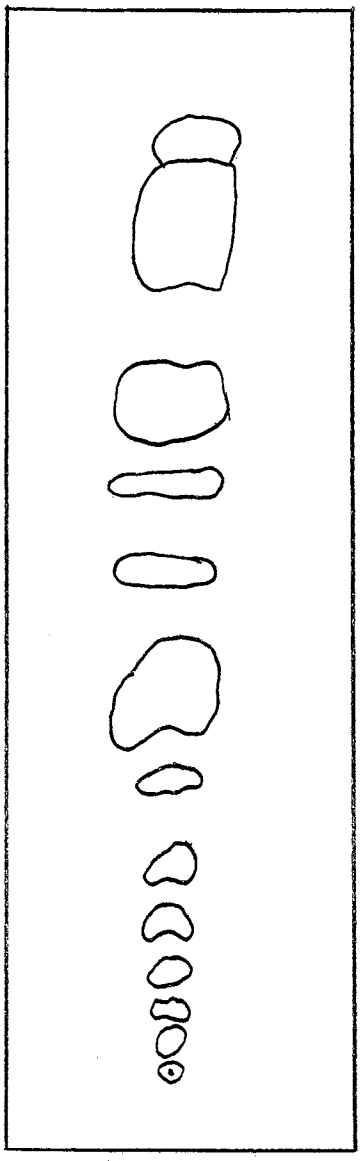


Figure 1. Thin-Layer Chromatogram of the Lipids of Ovary-Oviduct Tissue Incubated with Sodium Propionate-1-C^{14}. The solvent was hexane:ether: acetic acid (60:40:1, v/v). The chromatogram was developed two times and the spots were visualized with iodine vapors.

tentatively identified as the main components of this spot. It can be seen in these data that the presence of ascaryl alcohols and ascaroside esters make the task of separating the lipid classes of Ascaris very difficult. However, since the main interest was the labeled material in the free fatty acids and triglycerides, these spots were studied extensively. Triglyceride and fatty acid spots were eluted from the gel and saponified. The resultant soaps were acidified, and extracted from the aqueous solution with pentane. The fatty acids were methylated, reduced, and analyzed by gas chromatography. The distribution of the radioactivity in the different fatty acids can be seen in Table I. Since preliminary studies revealed that only trace quantities of radioactivity were found in the unsaturated fatty acids, only the saturated forms will be reported here. The greatest percentage of the recovered label was in the 16-carbon fatty acid. Very small amounts of activity were shown to be in the odd-numbered acids. The same sample was analyzed on the EGS column and comparable results were obtained. Saz and Vidrine (74) have shown that succinate could be converted to propionate by the muscle tissue of Ascaris. It is possible that the reverse reaction occurs and the propionate is converted into acetate and then incorporated into fatty acids. If the pathway described by Saz and Vidrine (74) is utilized, then the radioactivity from propionate-1-C^{14} would be lost as CO_2. Further, in studies by Beames and co-workers (7) of acetate incorporation into intact tissue, radioactivity was more evenly distributed in the even-numbered fatty acids than is observed here with propionate.

Tissue-Extract Incubation With Propionate-1-C^{14}

With intact tissue incubations, the lack of label in the odd-numbered fatty acids, and the significant amount of isotope in the 16-carbon acid pointed out the need for a more clearly defineable system. Thus, homogenization and differential centrifugation of the ovary-

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN THE FATTY ACIDS FROM INTACT OVARY-OVIDUCT TISSUE INCUBATED WITH PROPIONATE-1-C^{14}

Fatty Acid Methyl Ester	Percentage of Total Recovered Radioactivity
12:0	0.86
13:0	0.65
14:0	2.45
15:0	0.92
16:0	80.30
17:0	0.46
18:0	1.84
19:0	0.11
20:0	1.07
22:0	2.40
24:0	1.08
Unknown	6.88

oviduct tissue was carried out in an attempt to isolate such a system. Beames and workers (6) have purified a system from Ascaris ovary-oviduct tissue that incorporates acetate into fatty acids. Table II depicts the distribution of radioactivity when the different subcellular fractions were incubated with propionate-1-C^{14}. The crude homogenate incorporated propionate into fatty acids, but the mitochondria demonstrated very limited ability in this respect. Beames et al. (6, 7) also showed this to be the case with regard to acetate incorporation by muscle and ovary-oviduct tissue of Ascaris. The presence of microsomes inhibited the incorporation of propionate because S_{100} incorporated more radioactivity into fatty acids than did S_{12}. The protein from S_{100} which precipitated between 25-40% saturation of ammonium sulfate was very active in promoting uptake of propionate into fatty acids. This fraction also incorporated acetate into fatty acids (6). This protein precipitate was used for all subsequent experiments.

Notably, variation can be observed in sets of numbers which essentially represent the same type of experiment. When female ascarids were harvested at the slaughter house, they were taken from a great variety of hosts. Thus, the status of the host with regard to nutrition and health was highly variable. The conditions for growth of the worms were highly variable also. For this reason, it is difficult, if not impossible, to compare data from one set of experiments to the other. However, care was taken to complete a given set of experiments on the same batch of protein and in this manner meaningful data could be obtained.

In order to make certain that an enzyme-catalyzed reaction was occurring, protein concentration, substrate concentration, and time sequence experiments were run with the 25-40% protein precipitate and propionate-1-C^{14}. The results of these experiments are illustrated by Figures 2, 3, and 4. The concentration of protein from the S_{100} protein precipitate played a significant role in the amount of incorporation of propionate into fatty acids. Figure 2 depicts this role.

TABLE II

SUBCELLULAR SITE OF PROPIONATE INCORPORATION INTO FATTY ACIDS

Each tube contained the following additions: source of enzyme; sodium propionate-1-C^{14} (30 µmoles, 4.5 x 10^6 cpm); ATP (40 µmoles); CoA (0.05 µmoles); NADPH (0.025 µmoles); NADH (0.025 µmoles); $MnCl_2$ (0.03 µmoles); $KHCO_3$ (10 µmoles); disodium malonate (30 µmoles) potassium phosphate buffer (50 µmoles, pH 7.5). The tubes were flushed with nitrogen and incubated for 2 hours at 37°. The values represent millimicromoles propionate converted to fatty acids/mg protein/2 hrs.

Homogenate	Mitochondria	S_{12}	S_{100}	S_{100} (protein precipitate)
4.74	0.05	3.83	4.75	9.12
5.67	0.18	4.98	6.04	13.68

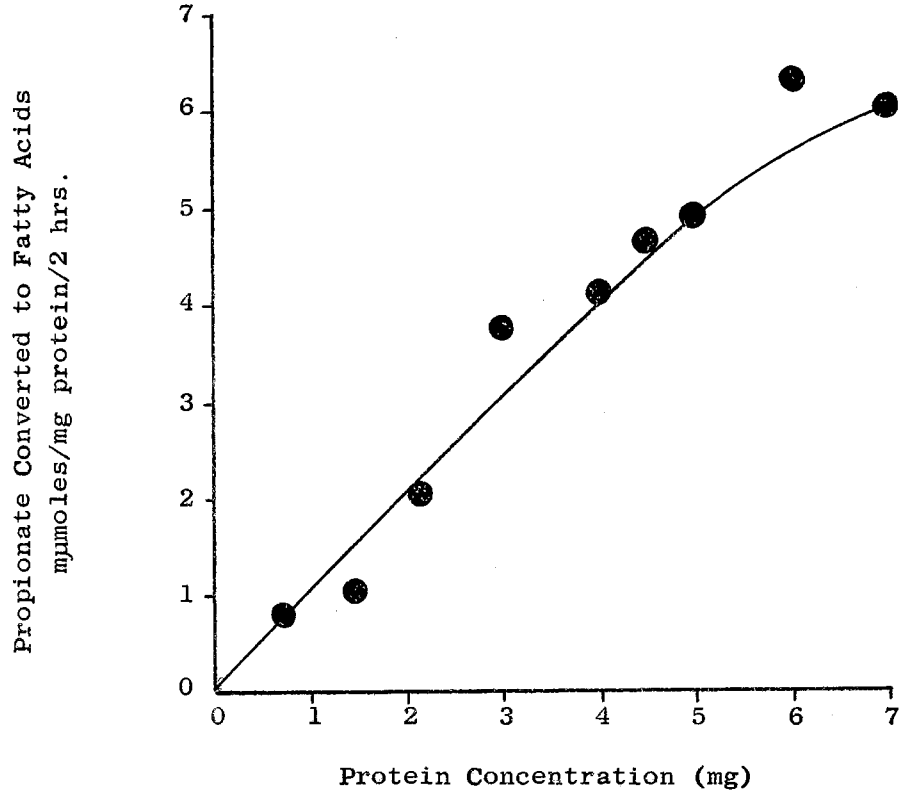


Figure 2. The Influence of Protein Concentration on the Uptake of Propionate into Fatty Acids. Each tube contained the following additions: sodium propionate-1-C^{14} (40 μmoles, 4.6 x 10^6 cpm); CoA (0.04 μmoles); ATP (40 μmoles); NADPH (0.25 μmoles); $MnCl_2$ (0.01 μmoles); disodium malonate (30 μmoles); potassium phosphate buffer (50 μmoles, pH 7.5); 25-40% ammonium sulfate-protein precipitate (as indicated). Tubes were incubated for 2 hours at 37° under nitrogen.

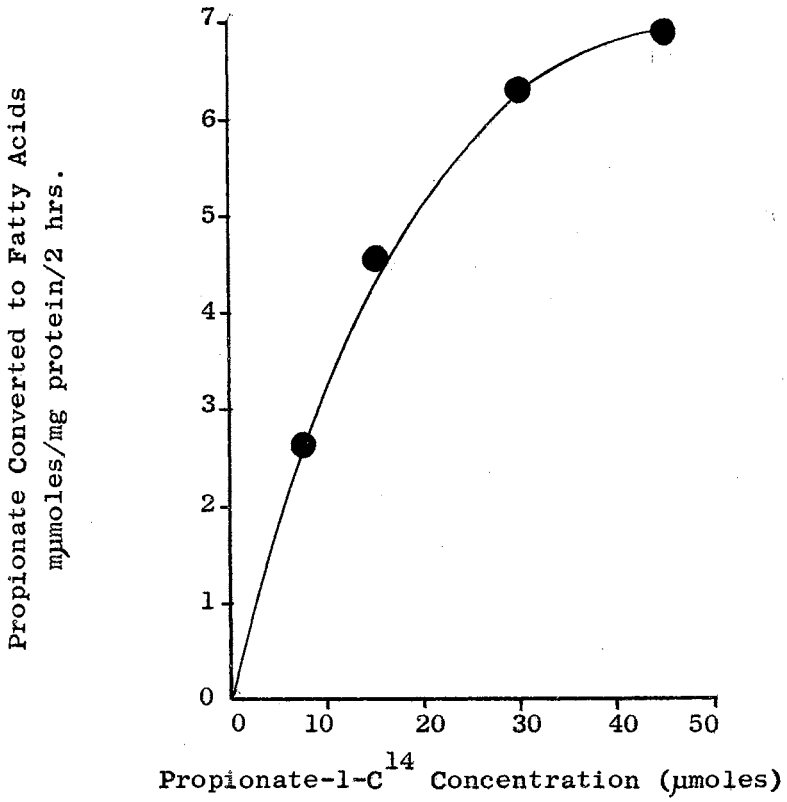


Figure 3. The Influence of Propionate Concentration on Fatty Acid Synthesis. The tubes contained the same additions as Figure 2 with the exception that the propionate-1-C^{14} was varied. 3 mg of protein was added to each tube.

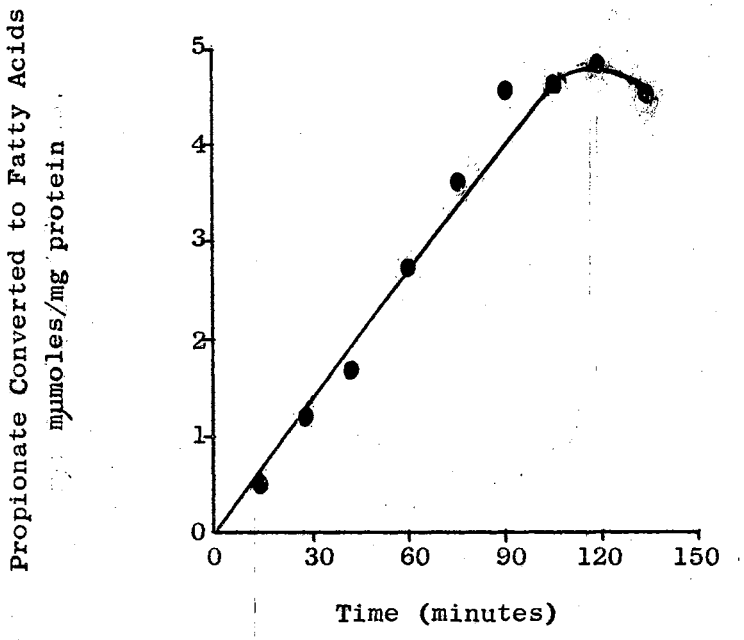


Figure 4. The Effect of Time on the Synthesis of Fatty Acids From Propionate. The tubes contained the same additions as Figure 2. Three milligrams of protein was added to each tube.

All subsequent incubations were first order with respect to enzyme concentration. Figure 3 depicts the optimum substrat concentration experiments. The incorporation plateaus at a level of 30 to 40 μmoles of propionate. Forty μmoles of propionate were then used in all the following experiments. The effect of time on the synthesis of fatty acids from propionate can be seen in Figure 4. The response was linear up to a period of 90 minutes and then leveled off. All subsequent incubations were run for at least an hour and a half.

One method of establishing the metabolic pathway of a compound is to simply add it to the tissue or enzyme preparation and after a period of time, analyze the products. Another method is to add cofactors which are theoretically essential for metabolism of the compound, together with the enzyme preparation. If these cofactors stimulate the metabolism of the compound, then a particular metabolic pathway can be theorized. Various cofactor requirements have been elucidated for the synthesis of fatty acids from acetate (84, 31, 87, 88, 89, 6, 7). These are as follows: ATP, CoA, and magnesium ions for the formation of the acetyl CoA derivative; ATP, biotin, manganese ions and CO_2 for the subsequent reduction reactions. The cofactor requirements for the uptake of propionate into fatty acids by the system isolated from Ascaris are depicted in Table III. There was an absolute requirement for ATP in the system. Omission of it from the incubation medium resulted in almost complete cessation of fatty acid synthesis. CoA was likewise required. When NADPH was left out of the medium, a decrease was noted in the incorporation of propionate into fatty acids. As will be seen later, NADH could replace NADPH, but the rate of fatty acid synthesis was not as high. $KHCO_3$ was also needed for optimum synthesis, although it's effects were not as dramatic as those of malonate. The requirement for malonate in the system tended to indicate that the malonyl CoA pathway of fatty acid synthesis may have been instrumental in aiding the incorporation of propionate into the fatty acids. Beames et al. (7) could not demonstrate a clear-cut need for malonate in a system

TABLE III

COFACTOR REQUIREMENTS

The tubes contained the same additions as described in Table II with the exceptions that NADH was left out and the source of enzyme was 2.5 mg of the ammonium sulfate precipitate of S_{100}.

Omissions	Additions	Percent of Control Synthesis
ATP		0.0
CoA		16.8
NADPH		22.7
$KHCO_3$		79.0
Malonate		22.4
$MnCl_2$		22.6
$MgCl_2$		103.2
Biotin		93.7
Biotin	0.5 mg avidin	0.3
None	0.5 mg avidin + 10 μg biotin	95.0
None	0.5 mg avidin + 20 μg biotin	103.1

from Ascaris muscle which incorporated acetate into fatty acids, but Beames et al. (6) demonstrated that malonate was required for optimum uptake of acetate into the fatty acids of Ascaris ovary-oviduct tissue. Manganese chloride stimulated uptake of propionate into fatty acids, but magnesium chloride had no effect. A need for biotin could not be demonstrated by simply omitting it from the medium. Wakil et al. (88) found that avidin could be utilized to implicate biotin in a reaction. The presence of avidin inhibited incorporation in the system from Ascaris. This inhibition could be reversed by the addition of exogenous biotin. These data indicate that some type of carboxylation reaction is occurring, but the nature of the reaction is unknown.

In most of the fatty acid synthesizing systems studied thus far, the primary pyridine nucleotide required for acetate incorporation into fatty acids has been NADPH (85, 2, 50, 41). NADPH is also required for fatty acid synthesis from acetate in Ascaris ovary-oviduct tissue (6). Studies with propionate incorporation into fatty acids by the present system revealed about the same data (cf. Table IV). Although there was statistically insignificant variation, a trend was demonstrated that indicated that NADPH was the preferred cofactor. This trend was not manifiested in Ascaris muscle tissue (7). Neither NADPH nor NADH would stimulate the uptake of acetate as well as the two combined. An explanation for this phenomenon is lacking at the present.

Figures 5, 6, 7, 8, 9, and 10 depict optimum cofactor concentration curves for the incorporation of propionate-1-C^{14} into fatty acids by the S_{100} ammonium sulfate-protein precipitate of Ascaris ovary-oviduct tissue. In Figure 5, it can be seen that concentrations of Coenzyme A in the system greater than 0.1 μmoles caused an inhibition in the uptake of propionate into fatty acids. An explanation for the suppression of synthesis is lacking. Figure 6 illustrates the influence of manganese ions on the incorporation of propionate into fatty acids. Metal ion is definitely required as its presence stimulate synthesis some 30-fold when 30-35 μmoles are added to the incubation

TABLE IV

PYRIDINE NUCLEOTIDE REQUIREMENTS

Control tubes contained the same cofactors as in Table II. The values indicate the percent of control synthesis, i.e., +NADPH, +NADH.

Experiment Number	-NADPH +NADH	+NADPH -NADH	-NADPH -NADH
1	94.5	78.2	56.4
2	62.9	150.0	8.1
3	90.3	152.0	61.8
4	97.5	158.2	80.6
5	80.8	101.6	27.9
6	41.9	110.0	0.3
7	94.1	133.3	5.0
8	97.8	87.9	52.9
Average	82.5	121.4	36.7

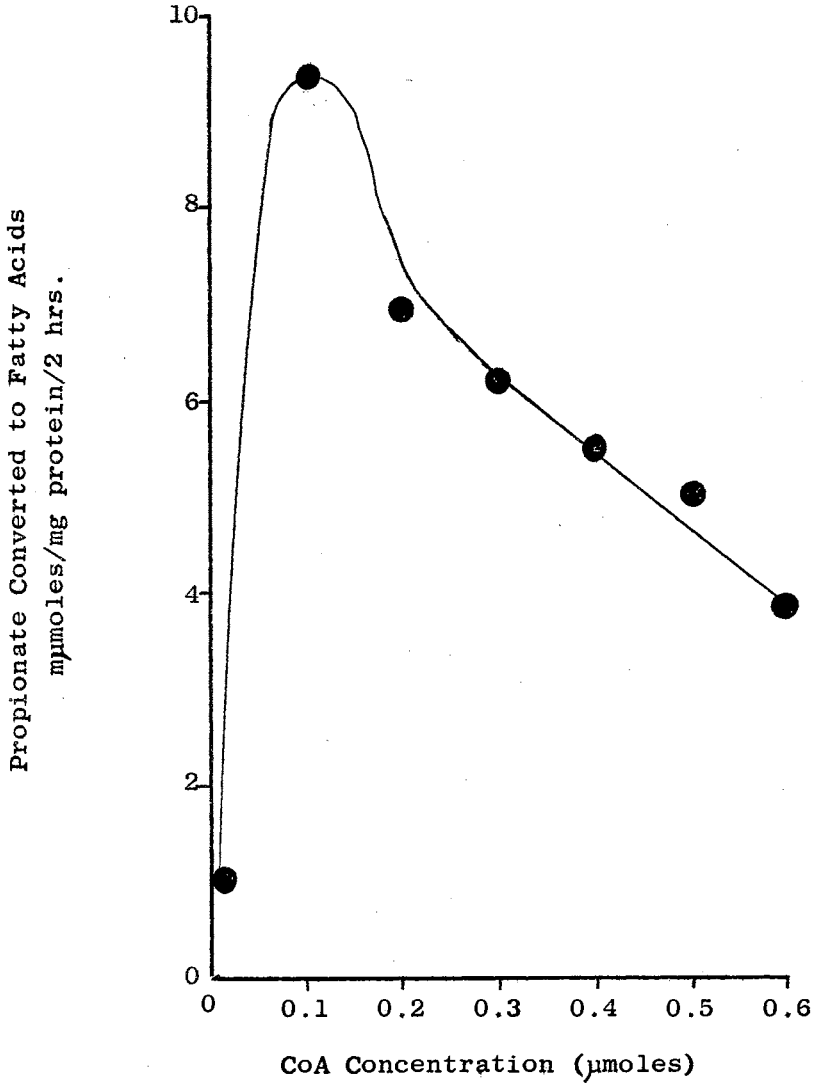


Figure 5. The Effect of Coenzyme A Concentration on the Synthesis of Fatty Acids From Propionate. Each tube contained the following additions: sodium propionate-1-C^{14} (40 μmoles, 5.5 x 10^6 cpm); ATP (20 μmoles); CoA (as indicated); NADPH (2.5 μmoles); $KHCO_3$ (2.5 μmoles); malonate (30 μmoles); $MnCl_2$ (7.5 μmoles); biotin (10 μg); potassium phosphate buffer (50 μmoles, pH 7.5). The enzyme source was 2 mg of protein precipitate of S_{100}. The incubation was run under nitrogen for 2 hours at 37°.

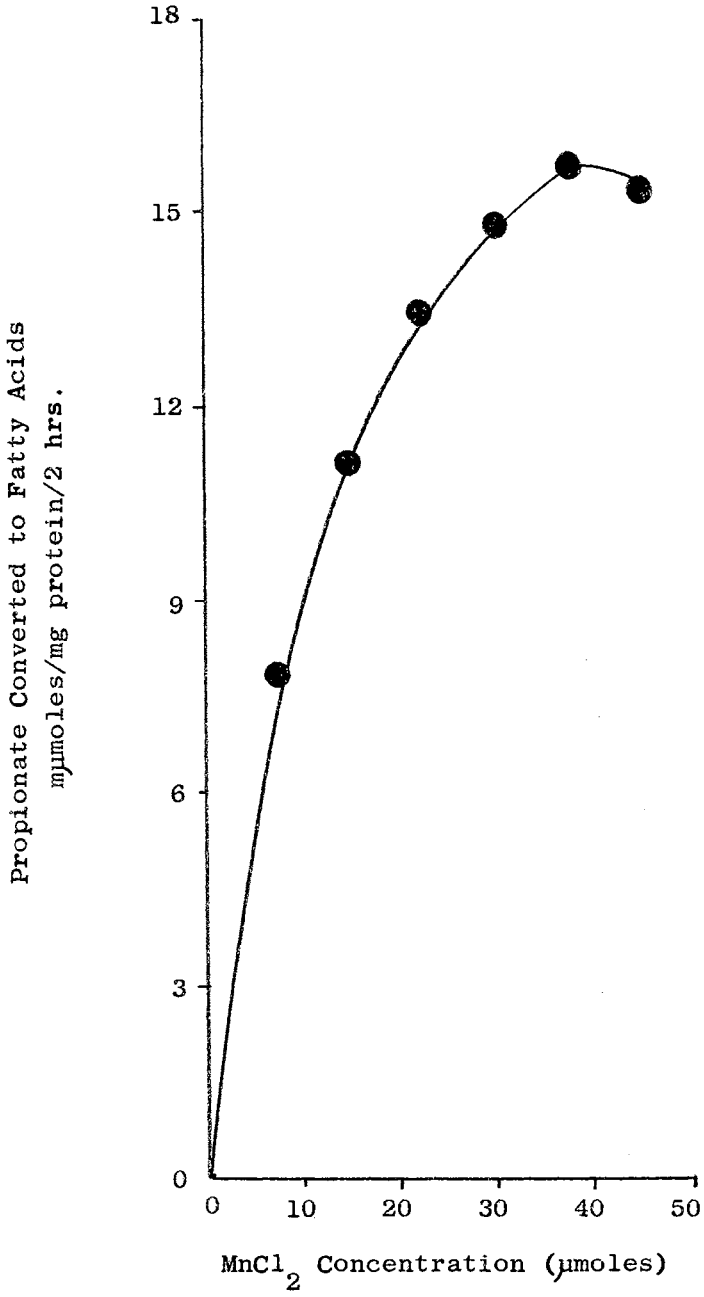


Figure 6. The Effect of Manganese Chloride on the Uptake of Propionate Into Fatty Acids. Tubes contained the same cofactors as in Figure 7 with the exception that $MnCl_2$ was added as indicated and CoA was in a concentration of 0.1 μmoles.

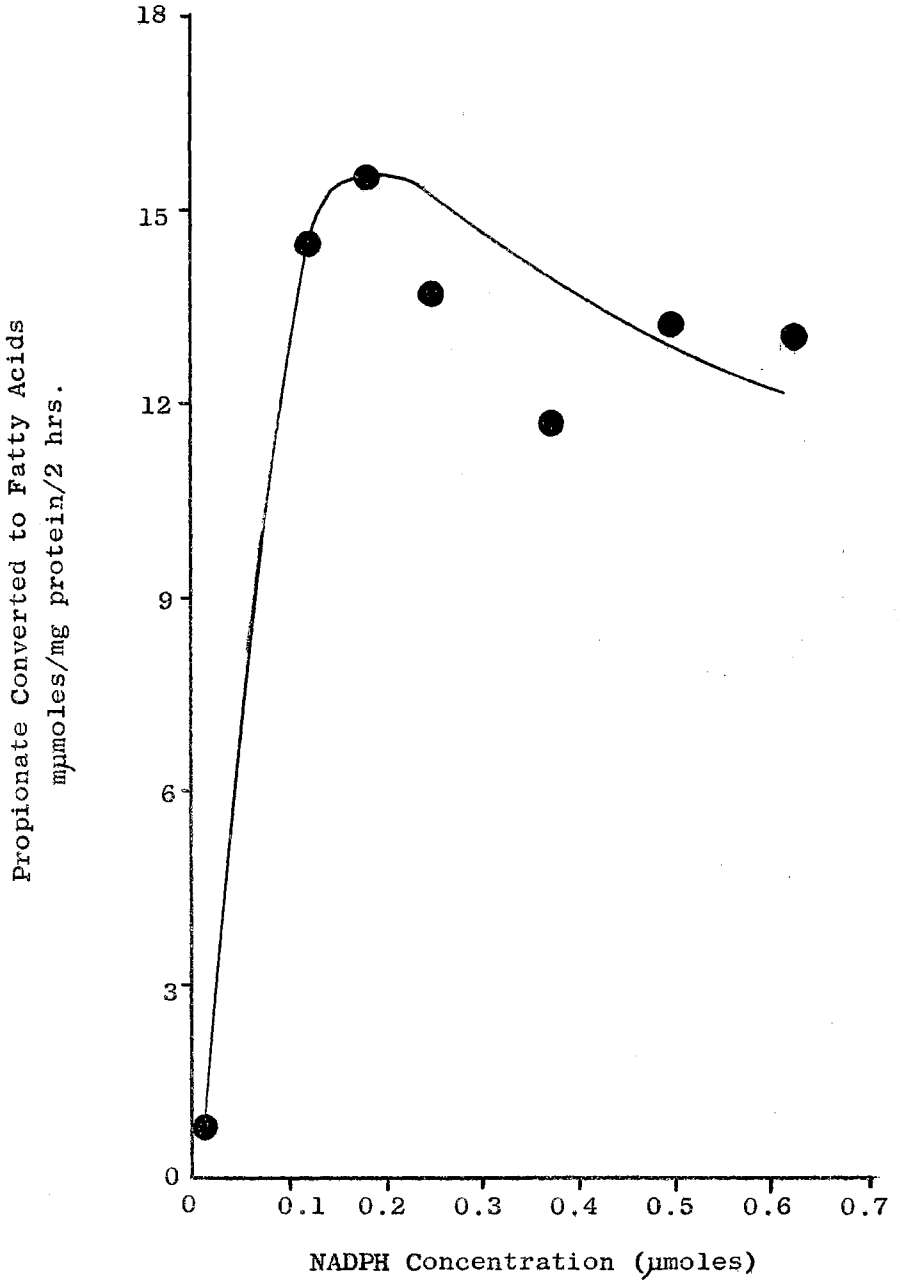


Figure 7. The Effect of NADPH Concentration on the Synthesis of Fatty Acids From Propionate. Tubes contained the same additions as Figure 5 with the exception of NADPH, CoA (0.1 μmoles), and $MnCl_2$ (50 μmoles).

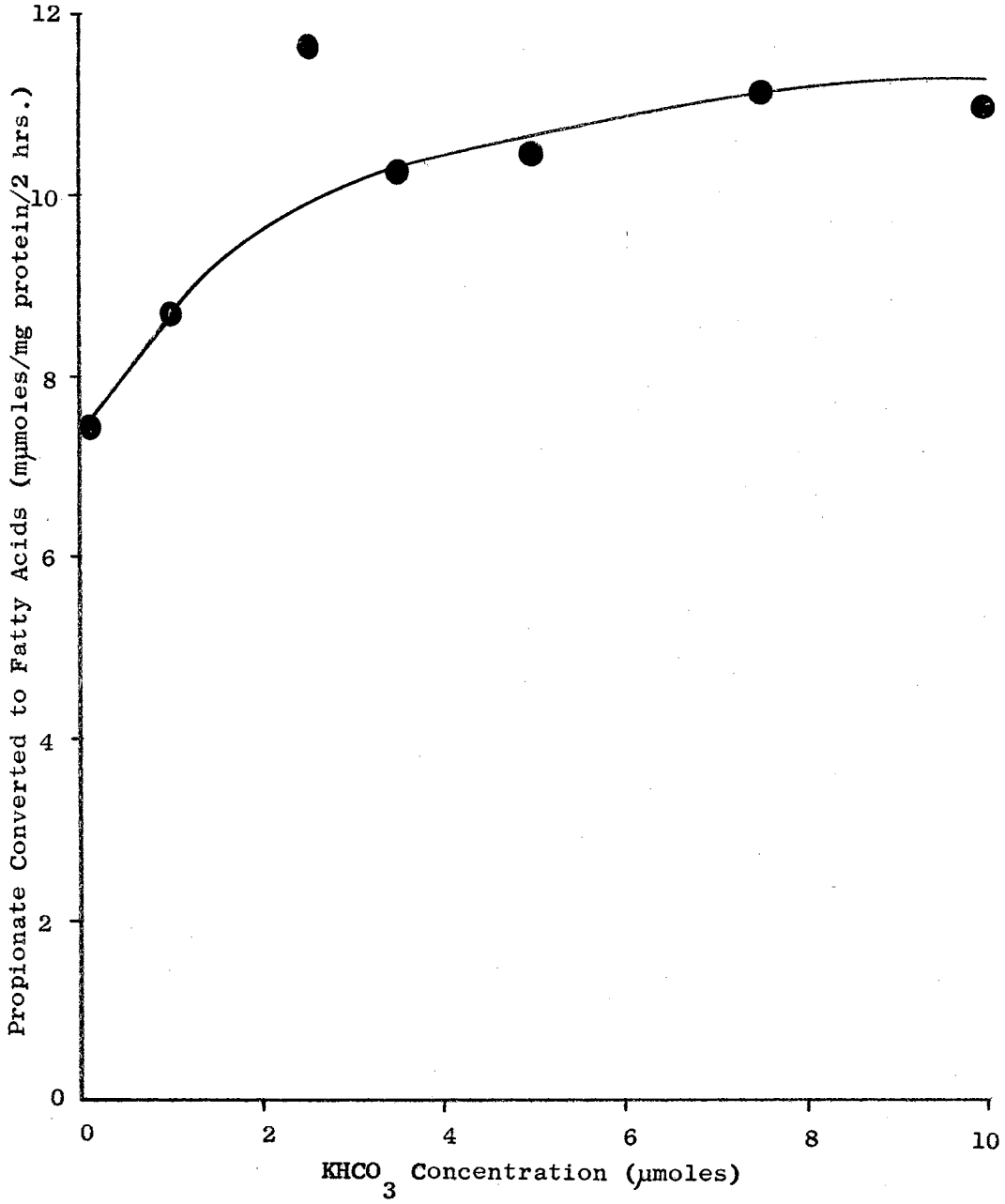


Figure 8. The Effect of Bicarbonate on the Synthesis of Fatty Acids. Tubes contained the same additions as Figure 5 with the following exceptions: CoA (0.1 μmoles); $MnCl_2$ (50 μmoles); NADPH (2.5 μmoles); $KHCO_3$ (as indicated).

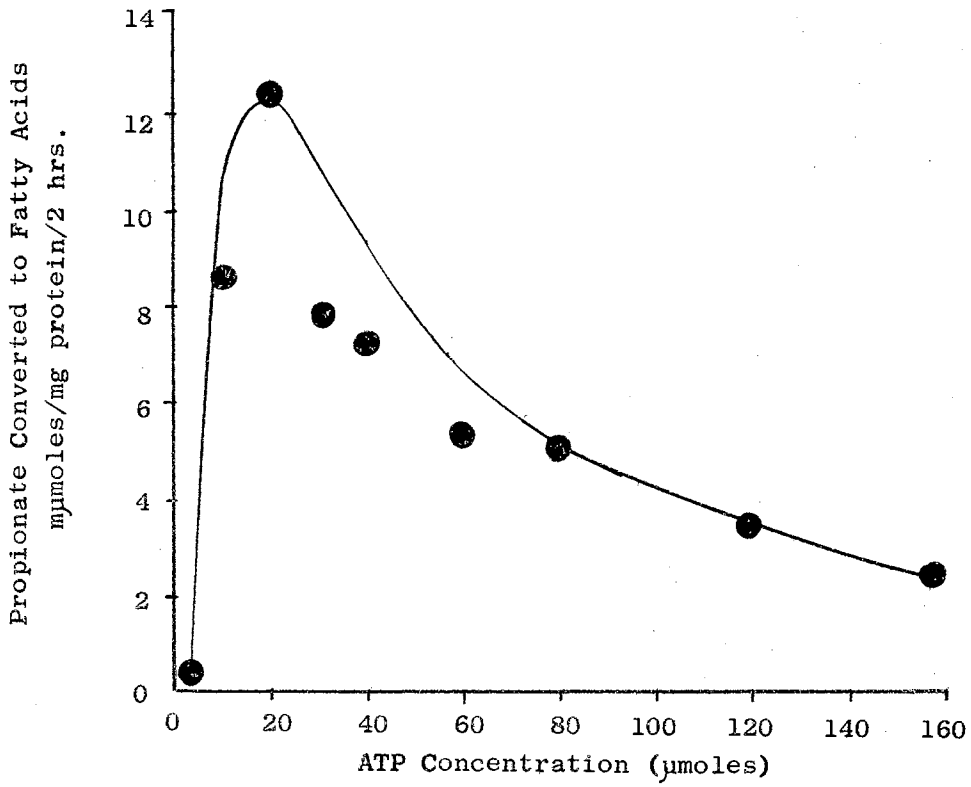


Figure 9. The Effect of ATP on the Synthesis of Fatty Acids From Propionate. Tubes contained the same additions as Figure 5 with the following exceptions: CoA (0.1 μmoles); $MnCl_2$ (50 μmoles); $KHCO_3$ (2.5 μmoles); ATP (as indicated).

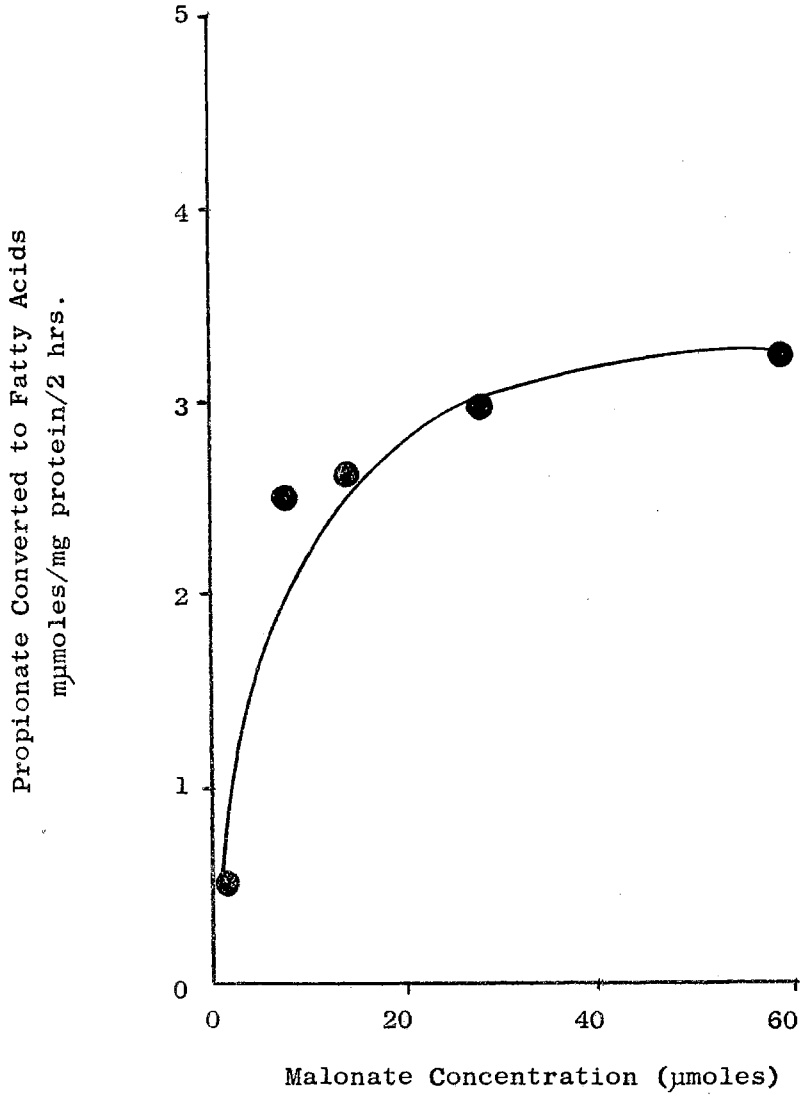
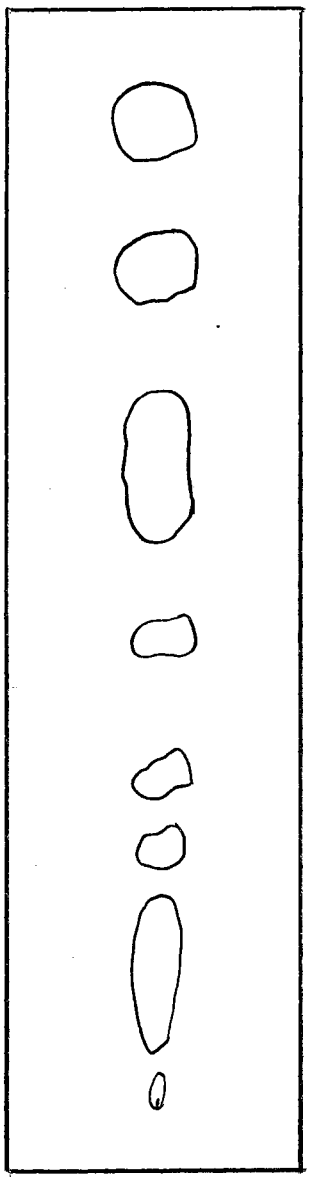


Figure 10. The Effect of Malonate Concentration on the Synthesis of Fatty Acids From Propionate. The conditions of incubation were the same as Figure 5 with the following exceptions: CoA (0.1 μmoles); $MnCl_2$ (50 μmoles); $KHCO_3$ (2.5 μmoles); ATP (20 μmoles); malonate (as indicated).

medium. The optimum concentration of NADPH in the system is approximately 0.2 μmoles (Figure 7). Levels higher than this do not elicit greater synthesis. Bicarbonate is needed in fatty acid synthesizing systems which use acetate as the precursor. The CO_2 is attached to acetyl CoA thus forming malonyl CoA. In essence, the CO_2 activates the α-carbon of acetate so that it can react with another mole of acyl CoA (87). In the present system, acetate was not present; thus, the requirement for an exogenous source of CO_2 is questionable. This point is made stronger if one observes Figure 8. Although bicarbonate did stimulate synthesis, the stimulation was not very powerful. There is a possibility that malonate added to the medium was decarboxylated and thus a source of endogenous CO_2 was generated by the system. Further experiments on the need for bicarbonate were not run. Beames and workers (7) found that the addition of ATP in large quantities to a fatty acid synthesizing system from Ascaris muscle elicited a drastic change in the pH of the system. These workers then showed that the change in pH was brought about by endogenous adenosine triphosphatase activity in the muscle tissue. Thus, when large quantities of ATP were added to the system, the terminal phosphate groups on the ATP were hydrolyzed, which in turn brought about a decrease in the pH. This same phenomenon appeared to have occurred with respect to ATP stimulation of propionate uptake into fatty acids by the system from Ascaris ovary-oviduct tissue. This effect was not as pronounced as was found in the muscle tissue. Figure 9 illustrates the effect of increasing the concentration of ATP on the synthesis of fatty acids from propionate. When ATP was added at levels greater than 20 μmoles, a depression of synthesis occurred. An experiment was run to test for ATPase activity and the pH was noted to change up to 0.2 pH units during a 2 hour incubation. Thus, it appears that the ovary-oviduct tissue contains a small amount of ATPase activity. Malonate readily stimulated the uptake of propionate into fatty acids (Figure 10). The incorporation reached a maximum when malonate was at a concentration of

30 μmoles.

The radioactive lipid residue extracted from the system in which sodium propionate-1-C^{14} served as the initial substrate was chromatographed on the thin-layer system as described previously. A typical chromatogram is present in Figure 11. The distribution of radioactivity remained the same each time the lipids were chromatographed. Spot #6 usually contained 30-40% of the total radioactivity and spot #8 retained 40-50% of the C^{14}. The R_f value of spot 6 corresponded to that of free fatty acids, while spot 8 migrated with sterol esters, hydrocarbons, and fatty esters. Since the possibility arose that spot 8 might contain fatty acids as their ester, spots 6, 7 and 8 were combined, eluted from the silica gel and saponified for 24 hours. Hydrolysis for this length of time insured saponification of sterol esters. The fatty acids were extracted from the acidified aqueous soltuion as described previously. They were then analyzed by gas chromatography. The results of the gas chromatographic analyses are presented in Table V. Again, a large portion of the radioactivity was found in the 16-carbon acid with very little, if any, label in the odd-numbered acids. A large number of counts also emerged in an area which corresponded to a branched-chain 25-carbon fatty acid ester. No peak was distinguishable on the chromatograph tracing but a definite peak of radioactivity was obtained. The absence of any radioactivity in the odd-numbered fatty acids again emphasized that propionate was either degraded or converted into a different form before incorporation. If it was being degraded to acetate by the method described by Saz and Vidrine (74), then succinate should be one of the intermediates. Various concentrations of succinate were tested with the system, but they failed to either stimulate or inhibit the incorporation of propionate-1-C^{14} into fatty acids (Figure 12). From these data, it would appear that propionate is not being converted into acetate by this method.



Spot Number	Percentage of Total C^{14}	Tentative Identification
8	40.88	Sterol ester Hydrocarbon? Fatty esters?
7	4.14	Triglyceride
6	35.48	Free Fatty Acid - Ascaroside ester?
5	0.03	Ascaroside ester?
4	4.28	Cholesterol Ascaroside ester?
3	9.47	Ascaroside ester?
2	4.44	Monoglyceride Ascaryl Alcohol?
1	1.32	Phospholipids

Figure 11. Thin-Layer Chromatogram of the Lipids of S_{100} 25-40% Ammonium Sulfate-Protein Precipitate Incubated With Propionate-1-C^{14}. The chromatogram was allowed to develop two times in hexane:ether:acetic acid (60:40:1, v/v). The spots were visualized with iodine vapors.

TABLE V

RADIOACTIVITY INCORPORATED INTO THE FATTY ACIDS FROM PROPIONATE-1-C^{14} BY THE PROTEIN PRECIPITATE OF S_{100}

Fatty Acid Methyl Ester	Percent of Total Recovered Radioactivity
12:0	4.55
13:0	0.00
14:0	2.70
15:0	0.51
16:0	33.10
17:0	0.00
18:0	6.25
19:0	0.02
20:0	5.13
22:0	7.72
Unknown	3.60
24:0	8.95
Unknown	27.39

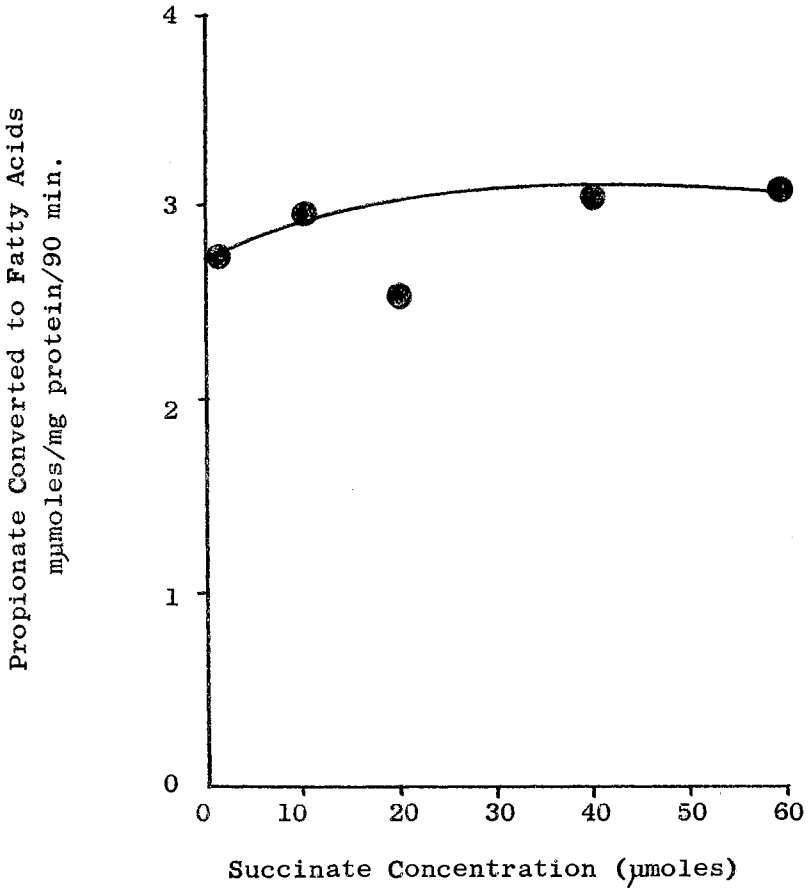


Figure 12. The Effect of Succinate on the Synthesis of Fatty Acids From Propionate. Tubes contained the following additions: disodium succinate (as indicated); S_{100} protein precipitate (3 mg); sodium propionate-1-C^{14} (40 μmoles, 5.5 x 10^6 cpm); ATP (20 μmoles); CoA (0.1 μmoles); NADPH (0.25 μmoles); $MnCl_2$ (30 μmoles); malonate (30 μmoles); potassium phosphate buffer (50 μmoles, pH 7.5). The tubes were incubated for 90 minutes at 37° under nitrogen.

Tissue Extract Incubation With Malonate-C^{14}

Abraham and workers (1) and Dils and Popjak (18) demonstrated that malonate stimulated the uptake of acetate into fatty acids by rat mammary gland supernatants. The stimulation in some cases was very high (40-50 fold, 18). Beames and workers (6, 7) found that malonate stimulated the synthesis of fatty acids from acetate by the protein precipitate from Ascaris ovary-oviduct tissue, but they could not demonstrate a clear-cut stimulation of synthesis in the muscle tissue. Malonate stimulated synthesis of fatty acids from propionate in the present system (cf. Figure 10). This indicated that malonate or some portion of the malonate molecule was being incorporated into the fatty acids. Dils and Popjak (18) found that malonate was not incorporated into fatty acids synthesized from acetate by a high-speed supernatant of lactating rat mammary gland tissue. This would be expected in a system in which acetate can be convered to malonyl CoA. These workers concluded that malonate stimulated their system in a manner other than being incorporated. However, in the present system, acetate was omitted and it its place was propionate, which is not as easily converted into malonyl CoA. A source of malonate or malonyl CoA would then be required to elongate the propionate. These results dictated the use of labeled malonate to establish whether or not it was being incorporated into fatty acids when propionate was present. Malonate-1-C^{14} and malonate-2-C^{14} were tested. It is reasonable to assume that if malonate is decarboxylated to acetate which would then be converted to acetyl CoA and malonyl CoA, then the fatty acids synthesized with malonate-1-C^{14} would contain half as much isotope as those synthesized from malonate-2-C^{14}. This was not the case. Malonate-1-C^{14} was incorporated as effeciently as the species with the label in the 2-position (cf. Figure 13). It should be noted here that the malonate was taken up into fatty acids 5 to 6 times faster than propionate which may indicate its role in the elongation mechanism. Further experiments were

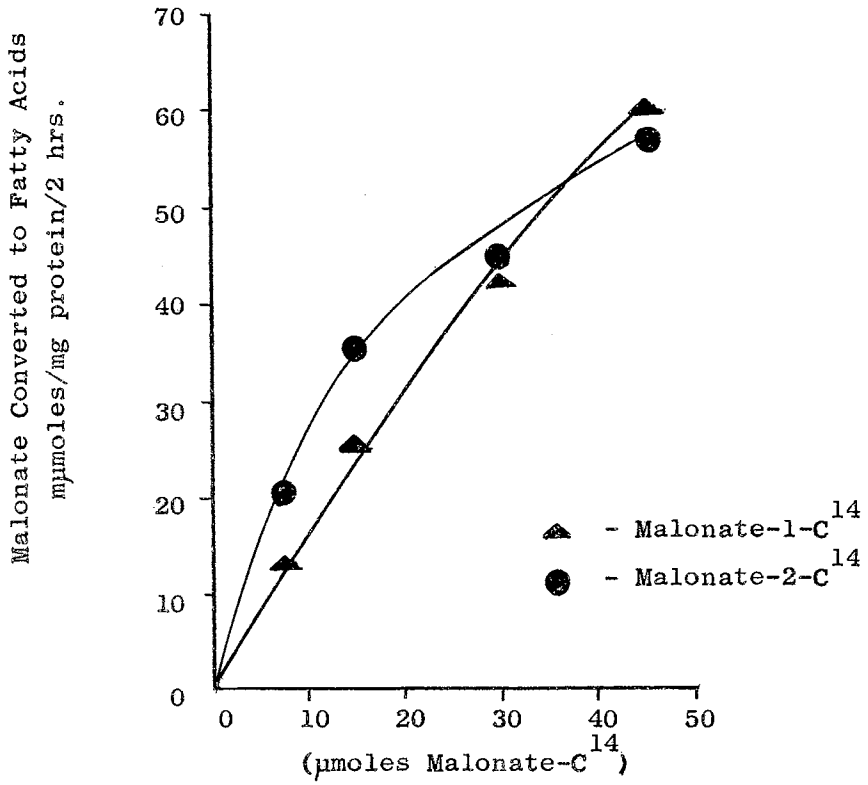


Figure 13. The Incorporation of Malonate-C^{14} into Fatty Acids. Each tube contained the following additions: sodium propionate (40 μmoles); ATP (20 μmoles); CoA (0.1 μmoles); $MnCl_2$ (30 μmoles); NADPH (0.25 μmoles); $KHCO_3$ (2.4 μmoles); biotin (10 μg); potassium phosphate buffer (50 μmoles, pH 7.5); sodium malonate-1- or -2-C^{14} (as indicated, 1.5 x 10^6 cpm/10 μmoles); S_{100} protein precipitate (2 mg protein). The tubes were flushed with nitrogen and incubated for 2 hours at 37°.

run with two labeled species of malonate. If a carboxylation reaction was occurring, then biotin would be required. Table VI depicts experiments in which the need for biotin was demonstrated. The addition of avidin to the system suppressed incorporation by some 90%. The uptake of both species of labeled malonate was likewise inhibited. Addition of exogenous biotin stimulated the incorporation of both species. Malonate may in some manner be converted to acetyl CoA which in turn can be converted to malonyl CoA and thus be incorporated into fatty acids. The nature of the conversion of malonate to acetyl CoA is not known. A species of the bacterium, Pseudomonas, has the ability to convert malonic semialdehyde to acetyl CoA and CO_2 (94). In order for Ascaris ovary-oviduct tissue to complete this feat, malonate would have to be reduced to the semialdehyde. This mechanism was not tested in the present system. If malonate is decarboxylated to yeild CO_2, then addition of CO_2 to the medium should decrease the labeling of the fatty acids synthesized from malonate-1-C^{14}. Figure 14 shows an experiment in which increasing concentrations of $KHCO_3$ were added to separate systems containing malonate-1- or-2-C^{14}. Bicarbonate stimulated the uptake of malonate-1-C^{14} into fatty acids while it had no effect on the incorporation of malonate-2-C^{14}. This does not necessarily rule out the possibility that malonate is decarboxylated. Perhaps the CO_2 arising from malonate remains attached to the coenzyme and is then fixed back into acetyl CoA to form malonyl CoA. However, CO_2 would be lost when malonyl CoA reacts with the acyl CoA acceptor. An experiment in which unlabeled acetate was added to systems containing malonate-1- or -2-C^{14} resulted inalmost complete cessation of uptake of radioactivity from either species. It appears then, that malonate may be converted to acetate, but the pathway of the reaction is unknown at the present.

TABLE VI

EFFECT OF BIOTIN AND AVIDIN ON THE UPTAKE OF MALONATE-1-C^{14} AND MALONATE-2-C^{14} INTO FATTY ACIDS

Each tube contained the following additions; malonate-1- or -2-C^{14} (30 µmoles, 1.5 x 10^6 cpm); 2.5 mg S_{100} protein precipitate; ATP (20 µmoles); CoA (0.2 µmoles); NADPH (0.25 µmoles); $MnCl_2$ (30 µmoles); $KHCO_3$ (10 µmoles); biotin (10 µg); potassium phosphate buffer (50 µmoles, pH 7.5). The tubes were flushed with nitrogen and incubated for 2 hours at 37°. The values represent mµmoles/mg protein/2 hrs.

Substrate	System	Malonate Converted to Fatty Acids
Malonate-1-C^{14}	Complete	126.10
	" + Avidin	9.03
	" + Avidin + 10 µg Biotin	27.00
	" + Avidin + 20 µg Biotin	106.03
Malonate-2-C^{14}	Complete	118.75
	" + Avidin	9.53
	" + Avidin + 10 µg Biotin	29.53
	" + Avidin + 20 µg Biotin	98.30

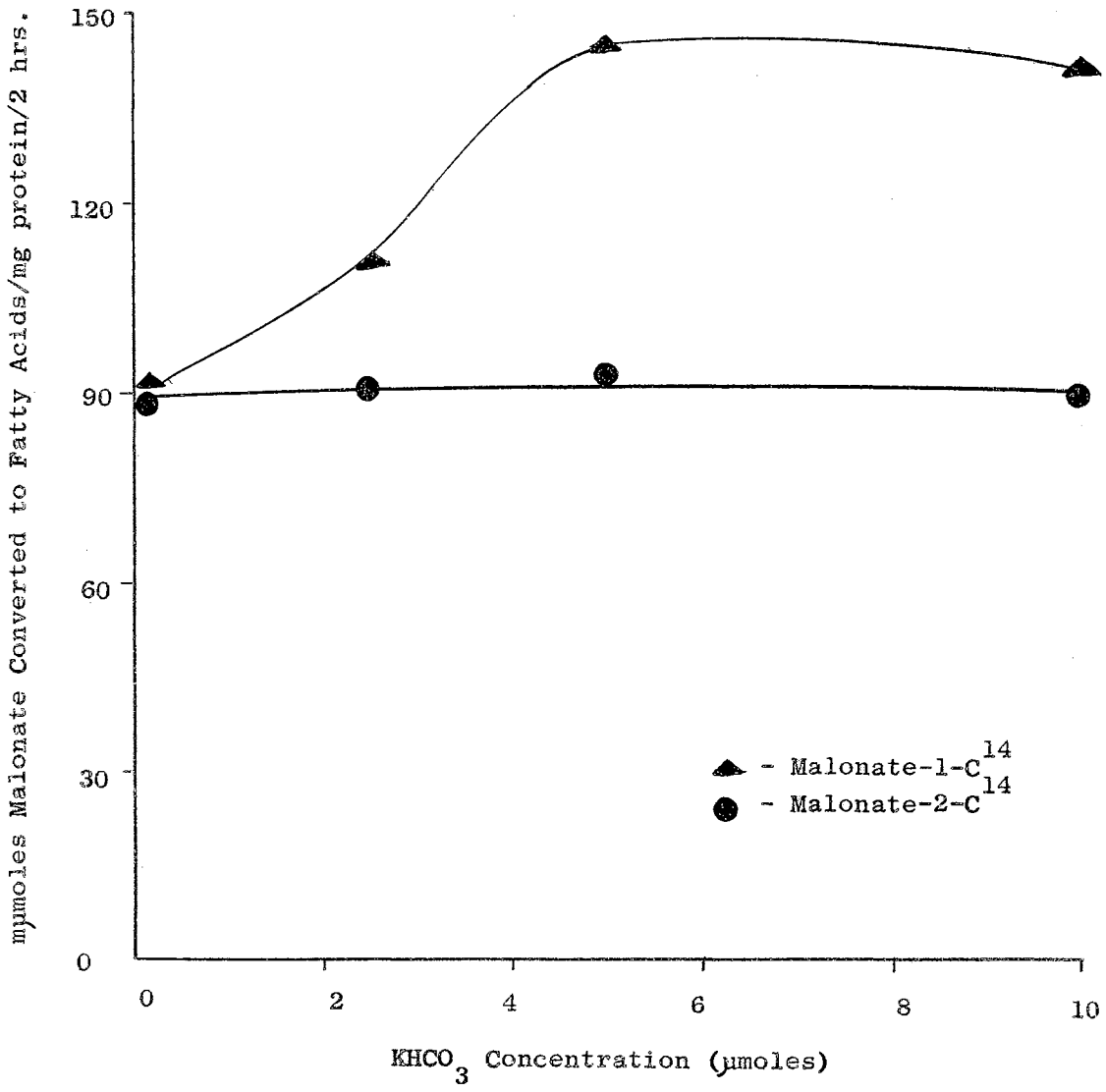


Figure 14. The Effect of Bicarbonate on the Uptake of Malonate-C^{14} into Fatty Acids. Tubes contained the same additions as Figure 13 with the following exceptions: malonate-1- or -2-C^{14} (30 μmoles, 1.5 x 10^6 cpm); $KHCO_3$ as indicated.

Tissue Extract Incubation With Acyl CoA Derivative

Horning and workers (40) isolated a system from rat adipose tissue that would form odd-numbered acids when propionyl CoA and malonyl-2-C^{14} CoA were added as the substrates. When CoA derivatives are used, the need for ATP, CoA, manganous ion, bicarbonate, and biotin is removed. Only CoA derivatives and NADPH are needed to elicit fatty acid synthesis. In some cases, the presence of the CoA moiet on the substrate may retard degradtion of the substrate molecule. With these obvious advantages in mind, propionyl CoA and malonyl-2-C^{14}CoA were synthesized and were tested with the rat adipose tissue system described by Horning and workers (40). Over 80% of the radioactivity was found in the 15- and 17-carbon fatty acids. These were approximately the same values that these workers reported. The CoA derivatives were then run with the system isolated from Ascaris ovary-oviduct tissue. There was essentially no label in the odd-numbered acids, but there was radioactivity in the 16-carbon acid and in the high molecular weight unknown that was seen in Table V. Also an extra peak of radioactivity was found in the area of a branched-chain 27-carbon fatty acid. The isotope present in the 16-carbon acid actually came as no surprise. There was a possibility that the system contained a malonyl CoA decarboxylase which would convert malonyl-2-C^{14} CoA into acetyl-2-C^{14} CoA which in turn would be elongated by malonyl-2-C^{14} CoA onto a 16-carbon acid. If this was occurring, the propionyl CoA would not be incorporated. To insure that propionyl CoA was being incorporated, three labeled species of it were synthesized; propionyl-1-, -2-, and -3-C^{14} CoA. Unlabeled malonyl CoA, propionyl-C^{14} CoA, and NADPH were then tested with the system from Ascaris. Table VII shows the cofactor requirements for the system. Malonyl CoA and NADPH were definite requirements for the uptake of labeled propionyl CoA into fatty acids. Very little synthesis occurred when they were omitted from the medium. Mercaptoethanol, which was added to keep the sulhydryl groups of the

TABLE VII

COFACTOR REQUIREMENTS FOR THE SYNTHESIS OF FATTY ACIDS FROM PROPIONYL-1-C^{14} CoA

Control tubes contained the following additions: propionyl-1-C^{14} CoA (0.15 µmoles, 30,000 cpm); malonyl CoA (0.12 µmoles); NADPH (0.25 µmoles); mercaptoethanol (0.5 µmoles); potassium phosphate buffer (100 µmoles, pH 7.5); 2.6 mg of protein from the S_{100} precipitate. The tubes were flushed with nitrogen and incubated for 1 hour at 37°.

Omissions	CPM/mg Protein/hr.
None	2800
Malonyl CoA	385
NADPH	289
Mercaptoethanol	2699

enzyme reduced, effected the incorporation only slightly.

All three labeled species of propionyl CoA were then used with the system from Ascaris. Gas chromatographic analysis of fatty acids synthesized by the system are depicted in Table VIII. In each case, there was a small amount of radioactivity in the 16-carbon acid. The odd-numbered acids contained small but significant amounts of label (3-4% of the total radioactivity). The majority of the radioactivity was recovered in the high molecular weight unknowns. Seventy to eighty percent of the total recovered radioactivity was present in these two compounds. Although radioactivity appeared in definite areas, no peaks were visible on the tracing and positive identification by gas chromatographic means was not possible. There appeared to be no significant difference in the labeling patterns, hence, the degradation of proionate to acetate was doubtful.

Since radioactivity was found in the 16-carbon acid, the possibility of contamination of the propionyl-C^{14}CoA by acetyl-C^{14}CoA was investigated. The hydroxymante of the propionyl-C^{14}CoA was made and chromatographed in the system described by Vagelos (83), but only one peak of radioactivity was found. This peak migrated where standard propionyl hydroxymate was found. This sytem separated acetyl hydroxymate (R_f 6.0) from propionyl hydroxymate (R_f 7.2). Thus, it was concluded that the radioactivity must come from the propionate.

Since the greatest percentage of the C^{14} was found in the high molecular weight compounds, these were studied more extensively in an attempt to identify them. The effluent from the gas chromatograph containing these compounds was captured on glass beads (60-80 mesh). The compounds were eluted with hexane and analyzed by thin-layer chromatography. In this regard, use of microscope slides coated with Silica Gel G proved very effective. They developed very fast and good separation was attained. The high molecular weight unknowns migrated in two areas (Figure 15). Spot #1 had the same R_f value as cholesterol, but it was negative to the antimony trichloride test, and tests with stand-

TABLE VIII

DISTRIBUTION OF RADIOACTIVITY IN FATTY ACIDS SYNTHESIZED FROM THREE LABELED SPECIES OF PROPIONYL-C-14 COA

Fatty Acid Methyl Ester	Percent of Total Recovered Radioactivity		
	-1-C^{14}	-2-C^{14}	-3-C^{14}
12:0	0.54	0.06	0.56
13:0	0.40	0.20	1.59
14:0	0.90	0.00	0.33
15:0	1.63	0.40	1.28
16:0	7.04	6.40	8.61
17:0	1.64	1.00	1.80
18:0	1.40	0.00	0.77
19:0	0.21	0.34	0.00
20:0	0.90	0.80	1.47
Unknown	2.10	0.80	1.47
22:0	0.77	1.49	1.19
Unknown	2.77	1.79	1.35
24:0	2.55	1.16	5.00
Unknown	24.12	27.89	25.05
Unknown	6.92	9.79	2.88
Unknown	46.79	49.41	44.87

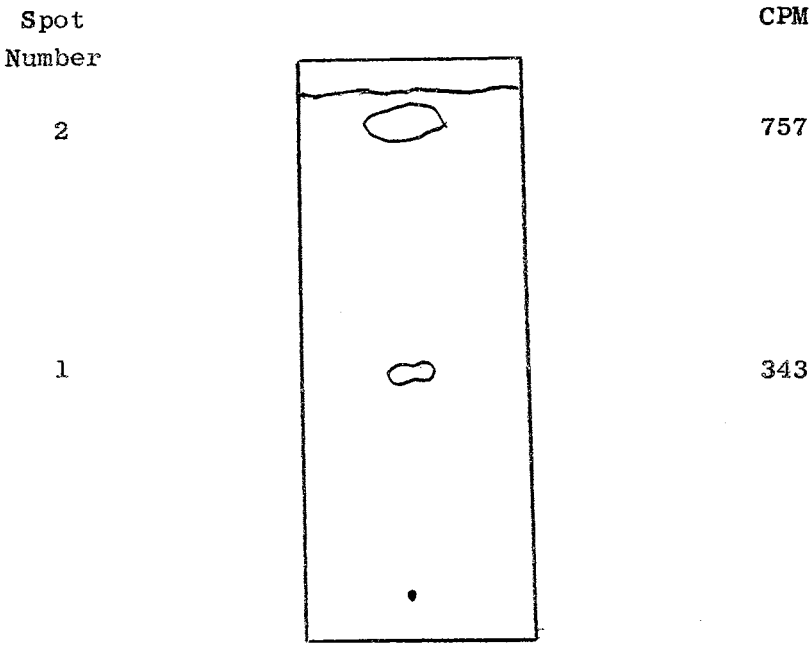


Figure 15. Thin-Layer Chromatogram of High Molecular Weight Unknowns Synthesized by Ovary-Oviduct Tissue Extract From Propionyl-C^{14} CoA. The solvent was hexane:ether:acetic acid: (60:40:1, v/v). The chromatogram was allowed to develop two times. The spots were visualized with iodine vapors.

ards indicated that very minute amounts of cholesterol would give a positive test. Cholesterol was run on the gas chromatograph and the retention time was much longer than that for the unknown compound. There is a possibility that it is an ascaryl alcohol or an ascaroside ester, but this is not known at the present. Spot #2 migrated with sterol ester, hydrocarbons, and fatty esters. Saponification for 24 hours did not alter the compound, because it migrated on TLC in the same manner. The spot was not positive for sterols with antimony trichloride. Since the unknowns were not altered by saponification, it was surmised that they could be separated from the fatty acids by washing the basic aqueous solution with hexane. This proved to be the case; thus, they appeared to be unsaponifiable. Methylation with diazomethane did not alter their migration on TLC or GLC, and decarboxylation by the method of Phares (65) produced no detectable radioactivity. Therefore, it was concluded that the compounds were not fatty acids. Their identity is still unknown.

CHAPTER V

DISCUSSION

The ovary-oviduct tissue of Ascaris lumbricoides suum incorporated propionate into fatty acids. This incorporation occurred in a cell-free system as well as in intact tissue. When fatty acids from intact tissue incubations were analyzed by gas chromatography, seventy to eighty percent of the recovered radioactivity was present in the 16-carbon acid, palmitic acid. This data indicated that the ovary-oviduct tissue might metabolize propionate in a manner which was different from that found in muscle tissue (74, 75, 76). Since the present study was begun, Saz and Lescure (78) reported that ovary-oviduct tissue of Ascaris does not incorporate propionate into α-methyl butyric or α-methyl valeric acids as does the muscle tissue of the parasite. These workers also found that propionate was esterified into the tissues in such a manner that saponification and steam distillation could remove it. They concluded from their work that the muscle tissue produced propionate and it was transported to the reproductive tissue via the hemolymph. In the reproductive tissue propionate is esterified into compound which are incorporated into the egg. Thus, when the egg begins it's development in an aerobic environment, propionate could be used as an energy source. The work of Saz and Lescure (78) has shown that metabolism of propionate by the muscle and reproductive tissues is not the same. In the muscle tissue, propionate is a waste product, while in the reproductive tissue, it is incorporated into the tissue lipids.

The presence of radioactivity in an even-numbered acid from an odd-numbered precursor was very surprising. Propionate appeared to be metabolized before it was incorporated into fatty acids, but the mecha-

nism of this pathway is unknown. The fact that the fatty acids from Ascaris tissues contain only minute quantities of odd-numbered fatty acids (5) indicate that the odd-numbered volatile acids are not incorporated into non-volatile fatty acids or they are methabolized before incorporation. Since there was some doubt that the tissue fatty acids were products of the worm, then studies on the incorporation of propionate by a more discrete system seemed feasible. Therefore, the tissues were homogenized and fractionated in order to obtain a more discrete system which might incorporate propionate *in toto* into fatty acids. The system which was isolated, the 25-40% ammonium sulfate-protein precipitate of the S_{100} fraction, had already been shown to be the subcellular site of acetate incorporation into fatty acids (6). This fraction also incorporated radioactivity into fatty acids from propionate more efficiently than any other portion of the cell. Thus, it was used as the source of enzymes.

Cofactor requirements of the cell-free system indicated that a *de novo* pathway of propionate uptake might be present. Malonate was an absolute requirement for incorporation of propionate which implicated the malonyl CoA pathway. Studies with malonate-1- and -2-C^{14} incorporation into fatty acids demonstrated that malonate was incorporated at a rate which was 5 to 6 times faster than that of propionate, thus implicating it in some type of elongation mechanism. The reason both species were incorporated at approximately the same rate is unknown at the present. This data certainly did not fit into that known about the malonyl CoA pathway. Studies with avidin and biotin showed that a carboxylation reaction was necessary for either species of malonate to be incorporated into fatty acids. The presence of carbon dioxide stimulated the incorporation of malonate-1-C^{14}, but did not effect the incorporation of malonate-2-C^{14}. These data implied that all three carbon atoms of malonate were incorporated into fatty acids. A pathway to explain these data is not known at the present.

Horning and workers (40) reported that propionyl CoA was incorporated into the fatty acids of rat adipose tissue as rapidly as acetyl CoA. Although radioactivity from propionyl-C^{14}CoA was readily incorporated into fatty acids by the protein precipitate of Ascaris ovary-oviduct tissue, it proceeded at a rate which was 5 times slower than acetyl CoA (Beames, unpublished observations). This implies that something might be happening to the propionate before it is incorporated. The implication was further substantiated by the finding of radioactivity in palmitic acid, because it is difficult to describe a pathway by which a 3-carbon acid could be incorporated into a straight-chain 16-carbon acid. It may also indicate that perhaps the malonyl CoA pathway of fatty acid synthesis is not operative in the ovary-oviduct tissue of Ascaris. The addition of one methyl group to acetate should not change the compound to such an extent that it would not fit the specificity of the enzyme. However, if there is a series of transacylases which transfer the acyl moiety from the CoA to the enzyme (3), the specificity may be imparted at this stage. Wakil et al. (91) have found that intermediates of fatty acid synthesis (acetoacetyl CoA, butyryl CoA) do not function as well as acetyl CoA in promoting incorporation of malonyl-2-C^{14}CoA into fatty acids. This meant that the intermediates did not fit the specificity of the acyl CoA transacylases (91, 3). If one assumes that the malonyl CoA pathway is functional in Ascaris and indication are that it is (6, 7; unpublished observations), and if the assumption is made that fatty acid synthesis is mediated by sulfhydryl enzymes and coenzymes, then the uptake of propionate-C^{14} into odd-numbered fatty acids would be dependent upon a specific propionyl CoA transacylase which would transfer the propionate moiety from CoA to the coenzyme or acyl carrier protein. If this enzyme was not present, then propionate could not be incorporated into odd-numbered fatty acids to any great extent, and the only label from propionate that would appear in the fatty acids would be a small amount which in some manner was converted into acetate. The present data indicates that this might be

the case. In Table VIII, it can be seen that the amount of radioactivity in the odd-numbered fatty acids (3-4% of the total radioactivity) is about the same as the ratio of odd-numbered fatty acids which are found in the tissues of the worm (4-5%, 5). Small amounts of isotope were also found in the 16-carbon acid, which might indicate that a portion of the propionate was being converted into acetate. When the propionate was added to the cell-free system as the sodium salt, greater amounts of radioactivity were recovered in palmitic acid which implies that the CoA moiety may have protected the propionate from degradation. All of these ideas depend on the assumption that there is an acyl carrier protein implicated in the synthesis of fatty acids by Ascaris ovary-oviduct tissue, and that acyl moieties are transferred to this carrier protein by transacylase enzymes. None of these assumptions have been proven by the present study, but they are a possible method for explaining the lack of odd-numbered fatty acids present in the ovary-oviduct tissue of the worm. This tissue is continously bathed in the perienteric fluid which contains large amounts of volatile fatty acids. The lack of acylases either to form the CoA derivative or the acyl carrier protein derivative would shuttle volatile acids (with the exception of acetate, which is readily incorporated into fatty acids) into other pathways. An example of this is propionate which is esterified into tissue lipids and is then available as an energy source for the developing embryo (78). The incorporation of acetate into fatty acids would fulfill yet another function of storage of lipid for the embryo. The primary acid that acetate is incorporated into is palmitate (6, 7), which in turn is readily incorporated into ascaryl alcohols and ascarosides. These compounds form an important portion of the vitelline membrane, which may lend semipermeable properties to the egg (21).

The unknown high molecular weight compounds which contain most of the radioactivity are still unidentified. They do not appear to be fatty acids because they are unsaponifiable and methylation with

diazomethane does not effect their migration on TLC or GLC. Migration on TLC indicated that the compound might be a hydrocarbon. Jezyk (unpublished observations) has tentatively identified hydrocarbons in the lipids of Ascaris ovary-oviduct tissue. However, the identification was only tentative and the function of such compounds in Ascaris is not known. The possibility also arises that they may be sterols of some type, but again the synthesis of sterols in this tissue has not been demonstrated. The compounds may be condensation products of fatty acids. If two molecules of palmitate condensed in the method described by Jezyk and Fairbairn (43), this would form a 31-carbon alcohol. The retention time of this compound on an SE-30 column would be much longer than that experienced with the unknowns. Beames and workers (6) reported these unknowns in their system which used acetat-1-C^{14} as the initial substrate, so their presence was not a manifestation of propionate only. The identity of these compounds are presently being studied.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The present study was designed to ascertain the pathway of synthesis of the odd-numbered fatty acids which are found in the tissues of Ascaris lumbricoides suum. From the data obtained, the following summary and conclusions can be made.

1. Sodium propionate-1-C^{14} was incorporated into fatty acids by the intact ovary-oviduct tissue of Ascaris. Palmitic acid (16-carbons) contained the majority of the activity, while the odd-numbered acids contained very little, if any, activity.
2. Sodium propionate-1-C^{14} was incorporated into the fatty acids by an ammonium sulfate-protein precipitate (25-40% saturation of ammonium sulfate) of the high speed supernatant of the ovary-oviduct tissue. This incorporation was dependent on several cofactors. These were as follows: ATP, NADPH, CoA, manganous ion, malonate, biotin, and $KHCO_3$. The major portion of the radioactivity was again found in the 16-carbon fatty acid, palmitate, with much lesser amounts in 18-, 20-, 22-, and 24-carbon acids. Little or no activity appeared in the odd-numbered acids. An unknown peak of radioactivity was found which contained 27% of the total isotope recovered.
3. Malonate-1-C^{14} and malonate-2-C^{14} were incorporated into the fatty acids 5 to 6 times faster than propionate. Avidin inhibited their uptake and exogenous biotin reversed the inhibition. Carbon dioxide stimulated the incorporation of malonate-1-C^{14} while it had no effect on the uptake of malonate-2-C^{14}.
4. Malonyl CoA and NADPH were definite requirements for the synthesis of fatty acids from propionyl-1-C^{14}CoA. Mercaptoethanol did not

stimulate synthesis. However, the uptake of propionyl CoA was 5 times slower than the uptake of acetyl CoA.

5. Propionyl-1-C^{14}CoA, propionyl-2-C^{14}CoA, and propionyl-3-C^{14}CoA were each incorporated into fatty acids. The labeling pattern indicated that all three were incorporated in much the same manner. Radioactivity was present in palmitate with all three labeled species. Three to four percent of the total radioactivity was present in the odd-numbered acids. This indicates that perhaps the odd-numbered tissue fatty acids are synthesized from odd-numbered precursors such as propionate. The bulk of the radioactivity was found in two unknown compounds which had retention times relative to branched-chain 25- and 27-carbon fatty acids. However, these compounds were unsaponifiable, they were not effected by methylation nor did they migrate with fatty acids on thin-layer chromatograms. Their retention times on gas chromatography did not match that of cholesterol.

Although propionate was incorporated into fatty acids by the ovary-oviduct tissue and by extract from this tissue, it was taken up very slowly. The level of odd-numbered acids in the tissue lipids of the worm can be explained by the rate of uptake. The radioactivity that always appeared in the 16-carbon fatty acid must undoubtedly come from propionate or a metabolite of propionate. The mechanism of this incorporation is not known. The reason that propionate is not easily incorporated into fatty aicds is not known, but several possibilities exist. The absence of an acylating protein derivative would be one possibility. Perhaps the propionate is rapidly esterified into the lipids and is unavailable to the fatty acid synthesizing system. A third pathway imaginable is the metabolism of the molecule to a form such as acetate which could be incorporated into even-numbered acids and thus, into ascaryl alcohols. Another method would be incorporation into the unknown compounds which were found in the present study.

Which, if any, of the aforementioned mechanism are operative in the ovary-oviduct tissue of Ascaris is still a matter of speculation.

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