FATTY ACID SYNTHESIS BY CELL-FREE PREPARATIONS OF *ASCARIS LUMBRICOIDES*

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1962

Submitted to the Faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
Master of Science
May, 1965

FATTY ACID SYNTHESIS BY CELL-FREE PREPARATIONS

OF ASCARIS LUMBRICOIDES

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ACKNOWLEDGEMENTS

Deepest appreciation is expressed to Dr. Calvin G. Beames, Jr., for his help and guidance in the selection and the completion of this study and the preparation of this manuscript. The author also wishes to express his gratitude to the members of the staff of the Department of Physiology and Pharmacology for their advice and encouragement during the course of this study. Special thanks are given to Dr. Neil S. Jacobsen for his patience and advice during the preparation of the manuscript.

Finally, the author wishes to express his sincerest thanks to his wife, Norella, for her untiring help in the completion of this study and manuscript.

This investigation was supported in part by Public Health Service Research Grant AI 06047-01 from the National Institute of Allergy and Infectious Diseases and by the Oklahoma Agricultural Experiment Station. The work was carried out while the author was a National Aeronautics and Space Administration Trainee with the Department of Physiology and Pharmacology, Oklahoma State University, Stillwater, Oklahoma.

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CHAPTER I

INTRODUCTION

During the past few years metabolic pathways have been elucidated to the extent that at least a general route of synthesis and degradation is known for most of the well-known chemicals found in an animal's body. However, most of this work has been done with mammalian and bacterial species with little attention having been given to other animals, especially invertebrates. So great has been the desire to work out "generalized" pathways, that the sometimes subtle, sometimes large differences in species have been overlooked in order to establish a firm, broad basis for understanding metabolic pathways. These differences are now being brought to the fore by studies dealing with helminths and more specifically, the parasitic helminths. Within the last decade a great deal of work has appeared in the literature concerning the carbohydrate metabolism of parasitic worms. For example, the blood fluke, Schistosoma mansoni (for review see Timms (102)), has been found to utilize the Embden-Meyerhof scheme for the anaerobic fermentation of glucose (74, 19, 21). The end-product of this fermentation was shown to be lactic acid. From an outward appearance the enzyme system seems to be the same as that found in vertebrates, but upon closer inspection, differences were found in homologous enzymes, which are enzymes that catalyze the same reaction but are found in different species (102).

The homologous enzymes could be distinguished by: (a) immunological procedures, (b) pH optima, (c) substrate affinity, (d) sensitivity to inhibitors (22, 23). Some of these enzymes even appeared to have different active sites (102).

More profound differences in metabolism are found in the case of Fasciola hepatica, the liver fluke of sheep. The main fermentation product from glucose in this animal is not lactic acid, but the volatile fatty acids propionic and acetic (77). In addition to this diverse pattern, it was found that the production of cyclic 3, 5-adenosine monophosphate (which stimulates the production of phosphorylase enzyme) was stimulated by 5-hydroxytryptamine (5-HT, serotonin) while in the mammalian system the synthesis was increased by the action of epinephrine (78).

Although volatile fatty acids are found as fermentation products of bacteria (97-100), their production as end-products of glycolysis in the animal kingdom wasn't thought to be widespread.

The parasitic nematode, Ascaris lumbricoides, produces, as a result of fermentation, a large number of volatile fatty acids the most important being: acetic, propionic, butyric, valeric, α-methyl butyric, α-methyl valeric, and α-methyl crotonic (tiglic) acids, (17, 18, 29, 94).

Most of the parasitic helminths are destined to live a large part of their lives in an essentially anaerobic environment. Therefore, most, if not all, of their available energy must come from anaerobic fermentations. This is not a very efficient type of metabolism and a great deal of energy is lost by the excretion of fatty acids. However, they seem to be well equipped to carry out this type of metabolism. Mansour (77)

reports that Fasciola hepatica utilizes glucose from the external medium at a rate of 110-180 μmoles/g. wet wt./6 hours. Mansour and Bueding (74) state "this process (glycolysis) is more critical for survival of the parasite than is respiration." Most parasites do, however, utilize oxygen when it is present and none seem to be obligatory anaerobes (16). Each species has different requirements for oxygen and as pointed out by Bueding (16), each species must be studied individually before any general statement on oxygen utilization can be made.

The presence and utility of a tricarboxylic acid cycle in these worms is nebulous. Read (88) reported that some parasitic helminths have enzymes which metabolize certain of the tricarboxylic acid cycle intermediates, but he also points out that just because an enzyme seems to be present "does not indicate the extent to which the enzyme may participate in metabolism." Consequently, it cannot be generally stated at the present time how much, if any, of the tricarboxylic acid cycle is utilized by these parasitic worms under aerobic conditions. It is known, however, that Ascaris does not utilize the TCA cycle because approximately half of the necessary enzymes do not seem to be present (88). In addition to this, Ascaris is not the possessor of a complete cytochrome system, which in higher animals is considered necessary for the coupling of high energy phosphorylations to the transport of electrons derived from various biological oxidations (38). Phosphorylations do occur though via a NADH oxidase whose terminal electron acceptor is some type of flavin (22).

The chief consideration of an animal living in an anaerobic environment is the subsequent oxidation of reduced cofactors formed

during glycolysis. Ascaris accomplishes this feat in part by utilizing a portion of the TCA cycle in reverse. It has been found that Ascaris has a succinoxidase which oxidizes succinate to fumarate but the equilibrium favors succinate formation (62). NADH together with fumarate, which arises from a carboxylation reaction of carbon dioxide and pyruvate forming malate then forming fumarate, results in the formation of succinate, with the regeneration of NAD (91). Indeed, succinate has been found to accumulate in the perienteric fluid of Ascaris (20). However, succinate is not excreted in appreciable quantities for this compound is thought to be decarboxylated to carbon dioxide and propionate (91). Another method found in which Ascaris oxidizes reduced cofactors is the production of branched chain volatile fatty acids. The carboxyl carbon of acetate condenses with the α-carbon of propionate thus forming methylacetoacetic acid. This compound is then reduced to α-methylbutyric acid via NADH. NAD is then regenerated in this reaction.

Little is known about the metabolism of lipids in parasitic helminths. Von Brand (12) found no detectable change in the total body fat of Ascaris after 5 days starvation. Passey and Fairbairn (85), who worked with developing Ascaris eggs which were approaching the infective stage, found an increase in the synthesis of carbohydrate with a corresponding decrease in non-volatile fatty acids indicating a conversion of lipid to carbohydrate with a corresponding decrease in non-volatile fatty acids. This pointed to a conversion of lipid to carbohydrate. Jacobsen (60) noted a slight increase in total body fat of Ascaris starved for 6 days. He was also able to demonstrate an oxidation of fatty acids C_2 through C_6 with a questionable oxidation

of C_{16}.

Saz and Weil (92, 93) have uncovered a mechanism of volatile fatty acid synthesis in Ascaris. The synthesis is brought about by way of a condensation of the carboxyl group of one fatty acid with the α-carbon of a second fatty acid, and the subsequent reduction of this compound via NADH to form a branched chain volatile fatty acid.

Other than the above mentioned papers, few works have appeared in the literature regarding lipid synthesis or degradation in Ascaris. According to Fairbairn (38), a female Ascaris excretes enough eggs in 24 hours to amount to approximately 10% of her body weight. The lipid content of the eggs of the worm is 17.5% of the total fresh weight (38). Therefore, this animal has a tremendous ability either to synthesize or assimilate lipids. This raises the question of how the long chain non-volatile fatty acids are synthesized. Is the system similar to that found in mammalian tissues? Does it need the same cofactors and are the pathways similar or apart from the "generalized" scheme? This study was designed to answer, at least in part, these questions.

CHAPTER II

LITERATURE REVIEW

The study of the synthesis of fatty acids by cell-free preparations was begun in 1949 by Stadtman and Barker (97-100) working with a water soluble extract of the bacterium, Clostridium kluyveri. Their system could convert carbon-14 labeled acetate to butyrate. In 1952 Brady and Gurin (10), working with homogenates of pigeon liver, were able to demonstrate synthesis of long-chain fatty acids from acetate. This synthesis was also made possible by utilization of the particle-free supernatant fraction. Van Baalen and Gurin (104) found that with the use of activated charcoal, they could demonstrate various cofactor requirements for fatty acid synthesis. These were: NAD, ATP, Mg, and CoA. They also noted a marked stimulation of synthesis by the addition of citrate. These authors also postulated that synthesis and degradation were the result of 2 distinct enzyme systems. However, their theory was not accepted at the time for the accepted theory of fatty acid synthesis was a simple reversal of beta-oxidation (105, 69). In the late 1950's an enzyme system was isolated from the soluble fraction of cells which wasn't associated with mitochondria (generally believed the location of beta-oxidation enzymes) and that synthesized long chain fatty acids (47, 48, 106, 107, 108). These workers found that the extract from the soluble fraction of the cell plus ATP, NADPH, CoA, Mn^{++}, $KHCO_3$, biotin, and C^{14}

labeled acetate resulted in the synthesis of labeled palmitate. They postulated the following scheme for fatty acid synthesis:

$$CH_3COOH + CoA \xrightarrow{ATP} CH_3COCoA + AMP + P\text{-}P$$

$$CH_3CO\ CoA + CO_2 \xrightarrow[Mn^{++},\ ATP]{biotin} COOHCH_2COCoA$$

$$COOHCH_2COCoA + CH_3COCoA \longrightarrow CH_3COCH(COOH)COCoA \xrightarrow{NADPH}$$

$$CH_3CHOHCH(COOH)COCoA \xrightarrow{-HOH} CH_3CH{=}C(COOH)COCoA \xrightarrow{NADPH}$$

$$CH_3CH_2CH(COOH)COCoA \longrightarrow CO_2 + CH_3CH_2CH_2COCoA$$

The butyryl CoA could then condense with another molecule of malonyl CoA to form the corresponding beta-keto acid which is subsequently reduced, dehydrated, reduced, and finally decarboxylated to form caproyl CoA. However, Bressler and Wakil (14, 15) found in their preparation that palmitic acid was the reaction product of fatty acid synthesis and not palmityl CoA. They also found that certain intermediate acyl CoA derivatives such as; beta-hydroxy butyryl CoA and acetoacetyl CoA, were less effective precursors in the synthesis of palmitate than was acetyl CoA. They concluded that the reacting molecules were not bound to CoA but instead were bound to the enzyme by a thio ester. Lynen and Tada (70) also postulated this type of binding, but they said that one of the reactants was bound to the enzyme and the other was attached to CoA. They were able to isolate an acetoacetyl-S-enzyme complex after they incubated acetyl CoA and malonyl CoA without NADPH.

Lennarz *et al.* (65) and Goldman *et al.* (49) isolated an enzyme system from *Escherichia coli* which contained a heat-labile and a

heat-stable fraction. Both fractions were needed for synthesis of fatty acids. Lennarz and co-workers (65) hypothesized that the heat-stable fraction acted as a cofactor in the synthesis of fatty acids from acetyl CoA and malonyl CoA. Wakil *et al.* (112) have now extended the previous authors works on *E. coli*, and have found that the heat-stable fraction was a protein which acted as a coenzyme in that the acyl reactants were bound to an SH group of the protein and underwent all subsequent reactions while they were still attached to this moiety. Wakil *et al.* (112) have given this heat-stable fraction the name "acyl carrier protein" (ACP) to designate its purpose. Wakil and his colleagues also isolated the heat-labile fraction of the fatty acid synthetase and have further separated it into 3 components, different mixtures of which will synthesize different fatty acids (112). During the reaction they isolated various polar compounds which turned out to be beta-hydroxy acids which were previously bound to the ACP and were intermediates in fatty acid synthesis. From these data Wakil postulated the following theory of soluble fatty acid synthesis.

(1) $CH_3COSCoA + HSACP \longrightarrow CH_3COSACP + CoASH$

(2) $HOOCCH_2COSCoA + HSACP \longrightarrow HOOCCH_2COSACP + CoASH$

(3) $CH_3COSACP + HOOCCH_2COSACP \longrightarrow CH_3COCH_2COSACP + CO_2 + HSACP$

(4) $CH_3COCH_2COSACP + NADPH + H^+ \longrightarrow CH_3CHOHCH_2COSACP + NADP$

(5) $CH_3CHOHCH_2COSACP \longrightarrow CH_3CH{=}CHCOSACP + H_2O$

(6) $CH_3CH{=}CHCOSACP + NADPH + H^+ \longrightarrow CH_3CH_2CH_2COSACP + NADP$

This synthesis, which is carried out within the soluble fraction of the cell, has been designated the *de novo* synthetic route. However, this

system primarily synthesizes saturated C_{16} and C_{18} acids, and leaves the synthesis of unsaturated and branched-chain fatty acids unaccounted for.

In 1960, Wakil and his colleagues (109) were able to demonstrate synthesis of long chain fatty acids from acetyl CoA in a preparation of mitochondria. Their system did not require bicarbonate which is an absolute requirement for synthesis in the non-mitochondrial or de novo system. ATP was needed for synthesis but could be replaced by the addition of acyl CoA derivatives. This led Wakil to conclude that synthesis of fatty acids in the mitochondria occurs via an elongation of various acyl CoA derivatives by the addition of C_2 units as acetyl CoA. Then in 1962 and 1963, Harlan and Wakil (51, 52) found that mitochondria could synthesize fatty acids by three distinct pathways:

(1) a pathway similar to the de novo synthesis in the non-mitochondrial preparation. This system incorporated acetyl CoA primarily into stearic acid.

(2) a pathway for elongation of acyl CoA's by the addition of acetyl CoA.

(3) a pathway by which oleic acid (18:1) is produced by a mitochondrial system which is unlike the de novo system or elongation pathway. This oleic acid thus formed could be elongated and desaturated into C_{20} and C_{22} polyunsaturated acids. The mechanism of this synthesis is unknown.

These studies indicate that the system of desaturation and the synthesis of unsaturated fatty acids reside primarily in the mitochondria and the production of saturated fatty acids is carried out primarily in the soluble fraction of the cell (111).

Wakil (111) also found that most of the unsaturated fatty acids synthesized by the mitochondria were incorporated into the phophoslipid fraction of the mitochondria. This is in agreement with the work of McFarlane et al. (73) and Getz et al. (46) who found that mitochondrial phospholipids of the rat liver contained large quantities (25%-40%) of C_{20} and C_{22} polyunsaturated acids. Wakil (111) postulated that this mitochondrial system was used primarily for the altering of dietary fatty acids or other unsaturated fatty acids manufactured within the cell. He also stated that "the majority of these fatty acids serve structural or transport functions and may have important roles in the function of the active membrane of mitochondria."

The metabolism of branched-chain fatty acids have been studied almost exclusively in bacteria. Polgar and Robinson (86) hypothesized that mycolipenic acid (2,4,6-trimethyl tetracos-2-enoic acid) was synthesized by a reaction of 1 mole of stearic and 3 moles of propionic acid thusly:

$$CH_3(CH_2)_{16}COOH + \underset{CH_3}{CH_3}COOH + \underset{CH_3}{CH_3}COOH + \underset{CH_3}{CH_3}COOH \longrightarrow$$

$$CH_3(CH_2)_{17}\underset{CH_3}{CH}CH_2\underset{CH_3}{CH}CH=\underset{CH_3}{C}COOH$$

Karlsson (61) stated that the synthesis of tuberculostearic acid (10-methyloctadecanoic acid), which is found as one of the lipid components of the tubercle bacillus, "may be derived from oleic acid by methylation at the double bond." In 1952, Hofmann et al. (56) found that the lipids of Lactobacillus arabinosus contained a fatty acid bearing a cyclopropane ring. This compound was named lactobacillic acid. Hofmann et al. (57) elucidated the structure of this acid and found it

to be cis-11,12-methyleneoctadecanoic acid. In 1962, Liu and Hofmann (66) postulated that the biosynthesis of this acid occurred by the addition of a methylene group across the double bond of cis-octaded-11-enoic acid (cis-vaccenic acid). Later O'Leary (84), working with Aerobacter aerogenes and Zalkin and Law (115, 116) working with Serratia marcescens and Clostridium butyricum, found that the methylene group which was added to the carbon involved in the double bond was contributed by S-adenosylmethionine, the activated form of the methyl group carrier, methionine. Recently, Chung and Law (26) have partially purified this enzyme system and have found that the methylene group is added to the monounsaturated fatty acid while the fatty acid forms a component part of phosphatidylethanolamine. Gastambide-Odier and Lederer (45) in 1959 worked out the synthesis of corynomycolic acid (C_{32} H_{64} O_3) found in Corynebacterium diphtheriae. They found that the carboxyl carbon of 1 molecule of palmitic acid ("probably as palmityl CoA") condensed with the α-carbon of another molecule of palmitic acid to form a keto-acid which was then reduced to corynomycolic acid.

From these works one can see that the possibilities of synthesis of branched-chain fatty acids are very numerous. They can be formed from various condensation reactions (45, 86, 92, 93), and by methylating reactions (61, 84). O'Leary states that methylation of double bonds by S-adenosylmethionine may be a mechanism of branched-chain fatty acid synthesis.

Theories of the synthesis of branched chain fatty acids in animals are lacking (except of course, the branched volatile fatty acids, 92, 93). Perhaps the reason for this is that they have been previously

found in only minute quantities. However, since their emergence as significant components of the fatty acids of Ascaris lumbricoides, and with the appearance of the gas-liquid chromatograph, which makes their detection relatively simple, one can only hope that more impetus will be placed on these branched acids.

LIPIDS OF ASCARIS

The study of the lipids of the swine and human parasitic nematode, Ascaris lumbricoides, was begun in earnest in 1912 with the work of Flury (42). In Ascaris he found volatile and non-volatile fatty acids, glycerol, phospholipids, and a compound in the unsaponifiable fraction which he designated as ascaryl alcohol. Working independently, Faure-Fremiet (39) also discovered ascaryl alcohol in the ovaries of the female equine nematode, Parascaris equorum. Faure-Fremiet (39) suggested that ascaryl alcohol existed in vivo as an ester, and also hypothesized that it was present only in the female reproductive system. Schultz and Becker in 1933 (95) worked out the empirical formula for ascaryl alcohol and found it to be $C_{33}H_{68}O_4$. Since that time, Fouquey et al. (43) have elucidated the structure of ascaryl alcohol from Parascaris, and have found it to be not a true lipid, but a group of glycosides having lipid solubility characteristics. They found ascaryl alcohol consisted of 3 ascarosides which they named ascarosides A, B, and C. Ascaroside A was said to consist of a straight-chain aliphatic molecule in glycosidic linkage with a hexose which Fouquey called ascarylose. Ascarosides B and C were somewhat similar in structure to A. Fouquey and workers also found that in the unfertilized eggs the ascarosides were esterified with acetic and propionic

acids therefore confirming Faure-Fremiet's suggestion. Von Brand and Winkeljohn (13) found ascaryl alcohol in male Ascaris muscle tissue, thus refuting the theory of Faure-Fremiet that ascaryl alcohol accurred only in the female reproductive tract and eggs. The function of ascaryl alcohols in somatic tissues isn't clear, however, ascaryl alcohols play an important role in the reproductive tissues. The vitelline membrane of the Ascaris egg has been found to be semipermeable, in that it allows gases to pass into and out of the eggs, but retards the flow of water. Since the metabolism of the developing egg has been shown to be aerobic, this passage of gas and retention of water is very important (34, 38). Fairbairn and Passey (34) also found the vitelline membrane to be lipid in nature and ascaryl alcohol accounted for approximately 70% of the total lipid. Therefore, it seems that the function of ascaryl alcohol is to provide a lipoid membrane which lends semipermeable properties to the egg.

Flury (42) was the first to find phopholipids in Ascaris. Rogers and Lazarus (89) found choline, serine, ethanolamine, and sphingomyelin in the perienteric fluid of Ascaris. These components of phospholipids were also found in the intestine and ovaries but very little sphingomyelin appeared in the ovaries. Fairbairn (32, 35) found that phospholipids comprised about 8.3%, 49% and 41% of the total lipid in the reproductive system, muscle, and integument (cuticle) respectively. Fairbairn (35) also found that phosphoacetals (plasmalogens) comprised about 30% of the total phospholipids in the muscle, but only trace amounts could be found in the intestine.

Beames (4) has studied the phospholipids and plasmalogens in the muscle, reproductive system, and integument extensively. He found

choline in the highest concentration in all tissues except cuticle where ethanolamine was present in higher quantities. Serine was least abundant in all tissues. Beames (4) found 21 acetals of plasmalogens in Ascaris tissues. In cuticle, $C_{18:1}$ comprised 63% of the total lecithin plasmalogen fatty aldehydes while $C_{18:0}$ was the primary aldehyde in the cephalin plasmalogen. It was present as 41% of the total acetals. In the reproductive system $C_{18:1}$ fatty aldehyde was predominate in both the lecithin (40%) and cephalin (62%) plasmalogens.

Phospholipids comprise a large percentage of the lipid in muscle and cuticle in Ascaris. This is also true for muscle tissue in higher animals (50). Phospholipids have many functions attributed to them. One of these is that phopholipids act as a static structural component of living cells. They have a slow turnover rate in these muscle and cuticle (50) which would support this concept. Another theory proposed by Hokin and Hokin (58) is that phospholipids, especially phosphatidic acid, play an important role in the active transport of sodium and potassium ions.

The function of phospholipids in Ascaris is unknown although their concentrations in various tissue indicate that they form a part of the structural make-up of the worm. Unfortunately, phospholipid turnover studies in this animal are lacking.

Fairbairn (32) found both saturated and unsaturated sterols in the reproductive system of Ascaris. Collectively they accounted for 1.7% of the total unsaponifiable lipids. The sterols were present in a ratio of 3 unsaturated to 1 saturated. The saturated sterols were believed to consist of 3-beta hydroxysterols. In a later report, Fairbairn (35)

found that sterols comprised 12 and 17% of the total unsaponifiable lipid in the integument and muscle of Ascaris respectively and Fairbairn and Jones (36) isolated and identified cholesterol from Ascaris tissue. Large amounts of other unsaturated sterols were not found and it was concluded that cholesterol was the primary unsaturated sterol in Ascaris. Cavier and Savel (24) have found sterols in the hemolymph (0.015%) but their method was limited to the detection of unsaturated sterols, so the value for total sterols in the perienteric fluid may be somewhat higher. Again, the function of these lipid components is not known. However, the data from Fairbairn (32, 35) show that the body wall (muscle and integument) sterols are present in greater concentration than those in the reproductive system. These data could lead one to postulate that the sterols are not unlike the phospholipids in that they presumably are stuctural components of the living cell. More work needs to be done on this aspect of Ascaris lipids.

Epps et al. (31) worked out a technique to free Ascaris from most of the bacteria associated with it, and from these animals identified C_5 volatile fatty acids. Bueding and Yale (17) and Bueding (18) identified the following volatile fatty acids from bacteria-free (axemic) Ascaris: acetic, propionic, butyric, α-methylbutyric, α-methyl crotonic (tiglic), and n-valeric acids. Ellison et al. (29) utilized gas chromatography to identify nine volatile fatty acids from bacteria-free culture media in which Ascaris were incubated. They found the following mole percentages: C_2, 0.27%; C_3, 0.77%; C_4, 1.14%; C_5, 38.0%; C_6, 56.2%; C_{7-8}, 3.5%. Ellison and co-workers (29) identified the unknown 6-carbon acid as iso-valeric acid. However, Saz and Gerzon (94) identified this

acid as α-methyl valeric acid. Ellison's paper was corrected in 1963 by Whitlock and Strong (114). The volatile fatty acids in the perienteric fluid were studied by Moyle and Baldwin (83). They identified most of the above volatile fatty acids from this body fluid. However, α-methyl-crotonic acid was not detected in the hemolymph. All of these authors concluded that these volatile fatty acids were excretion products of the worm, but the mechanism of their synthesis was unknown. In an series of recent papers, Saz et al. (91, 92, 93) described possible mechanism for the synthesis of many of these volatile acids. These mechanisms have been discussed in detail in Chapter I, pp. 4 and 5.

The fact that Ascaris produces large quantities of volatile fatty acids is unequivocal. Whether they can be used as an energy source in the presence or absence of oxygen is another matter. Jacobsen (60) has reported dye reduction by C_2 through C_6 volatile fatty acids in a mitochondrial preparation from Ascaris muscle. The chief products of the reaction were acetate and propionate with trace amounts of formate. Passey and Fairbairn (85) demonstrated a net conversion of triglyceride acids (½ volatile, ½ non-volatile acids, (35)) to carbohydrate in Ascaris eggs undergoing embryonation. Since that time, it has been learned that the esterified volatile fatty acids are attached to the ascaryl alcohol moiety and not to glycerol (Fairbairn, personal communication). Therefore, the glucogenic carbon must have arisen from the non-volatile fatty acids. It should be noted that this experiment was carried out on developing eggs which possess an aerobic type of metabolism (37). Whether or not the adult parasite can accomplish this feat is unknown.

Flury (42) reported the presence in Ascaris of palmitic, stearic, and oleic acids. Beames (5) has studied the non-volatile fatty acids in the neutral lipid fractions from Ascaris muscle, cuticle, and reproductive tissue. He found 19 fatty acids with twelve or more carbons. Five of these amounted to 90% of the total non-volatile fatty acids. These were found in the muscle tissue in the following mole percentages: $C_{18:2\&3}$, 33.7%; $C_{18:1}$, 27.3%; $C_{16:0}$, 12.8%; $C_{18:0}$, 10.8%; and an unknown acid, 6.4%. Jacobsen (60) has tentatively identified this unknown acid as tricosanoic acid ($C_{23:0}$). Beames (5) found very little variation in the percentage of each acid in the three tissues. Although the reproductive system accounts for two-thirds of the lipid present in Ascaris, the relative amounts of individual fatty acids in all tissues remained at rather constant values. Jacobsen (60) tentatively identified 24 non-volatile acids representing essentially every carbon number from C_{10} to C_{21} and also C_{23}. Of these 24 acids, 13 were branched isomers, and 14 were acids with odd-numbered carbons. Although the most predominant fatty acids in Ascaris are those which are common to most animals (55), the branched and odd-numbered acids exist in quantities which are too large to overlook (4%, 60). Such acids have been identified in living systems such as: bacteria, wool fat, butter fat, mutton fat, and ox fat; however, these were present only in trace amounts (80).

What the animal gains, in terms of energy and utility, from the synthesis or degradation of fatty acids is unknown. The work of Passey and Fairbairn (85) has already been mentioned in which they demonstrated a net conversion of long-chain fatty acids to carbohydrates. They hypothesized that this conversion occurred after the fatty acids were

degraded to acetyl CoA. They did not, however, demonstrate oxidation of the fatty acids to acetyl CoA. Jacobsen (60) could not show a significant amount of oxidation of palmitate with his mitochondrial preparation of Ascaris muscle. More work needs to be done in this field before any definite statements can be made on the utilization of non-volatile fatty acids by Ascaris.

No work has appeared on a possible mechanism of synthesis of the non-volatile fatty acids in Ascaris. As was mentioned previously, the worm has a tremendous ability either to assimilate or synthesize lipids. Without this ability the parasite cannot possibly survive. One practical application of this phase of Ascaris' metabolism is readily seen. If the mechanism of fatty acid synthesis differs somewhat from the accepted pathway, then the possibility of an anthelminthic drug, based on blocking the fat metabolism of the parasite can be seen.

CHAPTER III

MATERIALS AND METHODS*

Tissue Preparation: Adult female Ascaris lumbricoides var. suum (wt. 3-7g.) were obtained from Wilson and Company, Oklahoma City, Oklahoma and were transported to the laboratory in salt solution (60) maintained at 37°C. The worms were weighed and the muscle strips were dissected out by the method of Laser (64). In order to prepare minced muscle, the tissue was chilled in cold potassium phosphate buffer (0.05 M, pH 7.4), chopped with a Mickle Tissue Chopper, weighed, and incubated at 37°C for 2 hours with sodium-1-C^{14}-acetate (40 umoles 1.5 x 10^6 cpm). Cell-free fractions were prepared as follows: the muscle tissue was weighed, washed in cold 0.05 M potassium phosphate buffer, pH 7.4, minced with scissors, and homogenized in potassium phosphate buffer (0.05 M, pH 7.4, 1:2 w/v) using a Potter-Elvejhem homogenizer with a motor-driven teflon pestle. Care was taken at all times to maintain the temperature of the homogenate below 5°C. The resulting homogenate was centrifuged (International Centrifuge Model HR-1) for 30 minutes at 2°C at 12,000 x g to sediment cell debris, nuclei, and mitochondria. In most of the experiments this

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*ABBREVIATIONS: The following abbreviations are used: NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide; NADP and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate; CoA, coenzyme A; ATP, adenosine triphosphate; G-6-P, glucose-6-phosphate.

fraction (S_{12}) was used for incubation, but the supernatant (S_{100}) remaining from centrifugation at 100,000 x g (Spinco Model L) for 40 minutes was also used for several experiments. The S_{12} supernatant was used either unchanged for incubation, dialyzed in cellophane tubing against 30 volumes of 0.05 M potassium phosphate buffer, pH 7.3, for 6-8 hours at 4°C to remove cofactors, or treated with Dowex I-x8 (200-400 mesh) to remove cofactors (101). When mitochondria were isolated this was done by the method of Kmetec et al. (63). Total protein in all fractions was determined by the method of Lowry et al. (68).

Incubation: The cofactors and buffer (0.05 M phosphate pH 7.3) were added to the screw cap culture tubes in such a manner that the addition of the enzymes as a mince, mitochondria, or supernatant would result in a total volume of 2.5 or 3.0 ml. The reaction was started by the addition of the enzyme and the tubes were flushed with nitrogen for 15-20 seconds before being capped. They were then incubated at 37°C for 1.5 to 2.0 hours in a water bath and shaken frequently to insure proper mixing.

Extraction of Fatty Acids: At the end of the incubation period, the reaction was stopped by the addition of 2 ml 10% KOH in methanol. The mixture was saponified for 1 hour at 90°C. After saponification, 1 ml of 5 N HCl was added to each tube to bring the pH to 1. Five ml of petroleum ether containing 0.75 mg of carrier fatty acid (usually palmitate) was added to each tube and the tubes were then shaken on a Vortex Jr. Mixer for 15-20 seconds. The organic and aqueous phases were separated by centrifugation and the organic phase was transferred to a 125 ml separatory funnel with a Pasteur pipette. This procedure was

repeated twice in exactly the same manner. The organic solution containing the fatty acids was washed 1 time with 2 volumes of water and decanted into a 15 ml graduated centrifuge tube. The petroleum ether was evaporated from each of the tubes in a water bath maintained at 45°C under a stream of nitrogen. The fatty acids were dissolved in 1 ml of chloroform and aliquots were taken for plating on aluminum planchets and counting at infinite thinness in a Baird-Atomic Multiscaler II, Model 132, equipped with a thin end-window G. M. tube. Experiments utilizing palmitate-1-C^{14} as the labeled fatty acid indicated that the use of this extraction procedure resulted in the recovery of 95% of the fatty acids.

Reagents: The sodium-1-C^{14}-acetate was obtained from Nuclear-Chicago, Des Plaines, Illinois; CoA, ATP, NAD, NADH, NADPH, and L-cysteine hydrochloride from Sigma Chemical Co., St. Louis; Biotin and avidin from Nutritional Biochemicals, Cleveland, Ohio; the G-6-P was purchased in the form of the barium salt from Swartz Laboratories, Mt. Vernon, New York, and was converted to the potassium salt by the exchange reaction with potassium sulfate.

CHAPTER IV

RESULTS

Studies on the incorporation of labeled acetate into fatty acids were begun by using chopped strips of Ascaris muscle. The results of these studies are presented in Table I. Acetate incorporation into

TABLE I

INCORPORATION OF ACETATE INTO FATTY ACIDS BY CHOPPED ASCARIS MUSCLE

The chopped muscle was incubated in 100 μmoles of potassium phosphate buffer (pH 7.4) together with 40 μmoles of sodium-1-C^{14}-acetate (1.5×10^6 cpm) for 2 hours at 37°C. Total volume was 2.5 ml.

Weight of Muscle (mg)	CPM
Blank	600
115	1638
118	1908
126	2224
152	3200

fatty acids was slow; however, incorporation was found to increase when the weight of chopped muscle increased. These results indicated that Ascaris muscle can incorporate acetate into long chain fatty acids.

Subcellular Site of Fatty Acid Synthesis: To determine the site(s) of fatty acid synthesis from acetate, centrifugal fractionation of muscle

homogenates was carried out. Fractions resulting from centrigufation at 12,000 x g (S_{12}) and 100,000 x g (S_{100}) and the sediment from 12,000 x g (mitochondria) were utilized as the protein source for determinations of fatty acid synthesis. The results of these determinations are seen in Table II.

TABLE II

FATTY ACIDS SYNTHESIS IN CELLULAR FRACTIONS OF ASCARIS MUSCLE

Each tube contained the following additions: Sodium-1-C^{14}-acetate (40 µmoles, 1.5 x 10^6 cpm); ATP (10 µmoles); CoA (0.14 µmoles); NADPH (0.33 µmoles); NADH (0.33 µmoles); $MgCl_2$ (3.3 µmoles); $MnCl_2$ (3.3 µmoles); malonate (10 µmoles); $KHCO_3$ (20 µmoles); biotin (24 µg); potassium phosphate buffer (pH 7.4, 50 µmoles); mitochondria, S_{12}, or S_{100} containing 8-10 mg protein. Total volume 2.5 ml. The tubes were incubated for 2 hours at 37°C.

System	Acetate converted to fatty acids mµmoles/mg protein	
	Experiment 1	Experiment 2
Mitochondria	0.06	——
	0.15	——
S_{12} Supernatant + Microsomes	7.35	3.44
	7.89	3.21
S_{100} Supernatant	6.71	1.95
	7.49	1.67

In both experiments the S_{12} fraction catalyzed a greater synthesis of fatty acids than did the S_{100} fraction. These data are in contrast with

the data of Dils and Popjak (27) who worked with lactating rat mammary gland and found that the presence of microsomes depressed fatty acid synthesis. It is in agreement with the work of Lorch et al. (67) who found that low concentrations of microsomes stimulated fatty acids synthesis in rat liver. The S_{12} fraction was used in all subsequent experiments. The variance between experiments 1 and 2 can be explained only by the fact that the experiments were run on different days and represented worms collected on different days. Considerable variation (up to 100%) was experienced from one particular group of worms to the next.

Cofactor Requirements: Cofactor requirements for the incorporation of acetate into fatty acids by the S_{12} fraction are presented in Table III. There is an absolute requirement for NADH and NADPH or a NADPH-generating system, (i.e., NADP plus G-6-P). Neither NADH nor NADPH could completely replace the other. These results agree with the work of Dils and Popjak (27) with lactating rat mammary glands. They disagree with Abraham et al. (2) who also worked with rat mammary glands but found that the addition of NADH did not increase fatty acid synthesis appreciably. Dils and Popjak (27) concluded that the need for NADH in their system was not clear. NADP plus G-6-P did not completely replace NADPH in the tissue of Ascaris. Entner (30) has studied the pentose phosphate pathway of Ascaris and has found that the activity of the enzymes are very low. It is quite possible that the NADPH generating system (glucose-6-phosphate dehydrogenase) in Ascaris does not function as well as it odes in higher forms. When manganous ions were omitted from the medium there was a significant decrease in the synthesis of fatty acids. However, the absence of magnesium ions did not affect the

uptake of acetate to any great extent. Abraham et al. (2) reported that they achieved maximum synthesis with a magnesium ion concentration of 70 μmoles. No such stimulation was noted with Ascaris tissue in the current experiment. CoA was required by the S_{12} fraction to complete the synthesis of fatty acids.

TABLE III

COFACTOR REQUIREMENTS

Control tubes contained the following additions: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); cysteine (10 μmoles); NADH (0.33 μmoles); NADP (0.33 μmoles); G-6-P (3.3 μmoles); $MnCl_2$ (3.3 μmoles); $MgCl_2$ (3.3 μmoles); malonate (10 μmoles); $KHCO_3$ (20 μmoles); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); S_{12} protein (10 mg) treated with Dowex I (200-400 mesh). Total volume 2.5 ml. Tubes were incubated at 37°C for 2 hours.

Omissions	Additions	Percent of Control Synthesis
NADH	-	73.4 ± 0.5* (3)
NADP, G-6-P	-	70.4 ± 1.8 (2)
NADP, G-6-P	NADPH	110.0 - (1)
$MnCl_2$	-	64.2 ± 15.2 (2)
$MgCl_2$	-	100.1 ± 1.6 (2)
CoA	-	31.0 ± 10.2 (2)
Malonate	-	61.9 ± 4.7 (3)
$KHCO_3$	-	50.9 ± 4.44 (3)
Biotin	-	100.1 - (1)
Cysteine	-	103.1 - (1)
ATP	-	5.1 ± 0.6 (2)
None	Succinate	120.0 - (1)

* Indicates standard deviation; ( ) indicates number of experiments.

A loss of activity of 60% to 70% was experienced when CoA was omitted from the medium. Malonate and bicarbonate were required for incorporation of

acetate. In one experiment in which the supernatant (S_{12}) was dialyzed for 6 hours, the omission of bicarbonate resulted in a 95% decrease in synthesis. The role of malonate is less clear than the role of bicarbonate; however, the rate of incorporation of acetate into fatty acids was decreased when S_{12} was treated with Dowex I ion exchange resin and malonate was omitted from the medium. A requirement for biotin could not be demonstrated by simply leaving it out of the system, but when avidin was used to precipitate endogenous biotin (Table IV), a requirement for it was demonstrated. ATP is an essential cofactor in the system reported here. Without it, fatty acids synthesis proceeds very slowly or not at all. The cofactor requirements reported above are very similar to those for pigeon liver (105, 107), for rat liver (1), and for lactating rat mammary gland (2, 27).

The use of cysteine or glutathione as reducing groups to maintain CoA in a reduced form has been reported by many authors (1, 27, 41, 59). In Ascaris tissue, the presence of cysteine did not affect the rate of fatty acid synthesis. In fact, it caused a slight inhibition when it was present in the medium.

Effect of Malonate on Fatty Acid Synthesis: Figures 1, 2, 3, and 4 illustrate 4 experiments that were designed to determine the effect of malonate on the fatty acid synthesizing system in Ascaris muscle. In the experiments represented by Figures 1 and 2, the supernatant was dialyzed to remove cofactors. In Figures 3 and 4, the supernatant was treated with Dowex I to remove cofactors. In Figure 1, a slight stimulation was noted in the rate of incorporation of acetate into fatty acids when 10 μmoles of malonate were added to the medium. However, there was

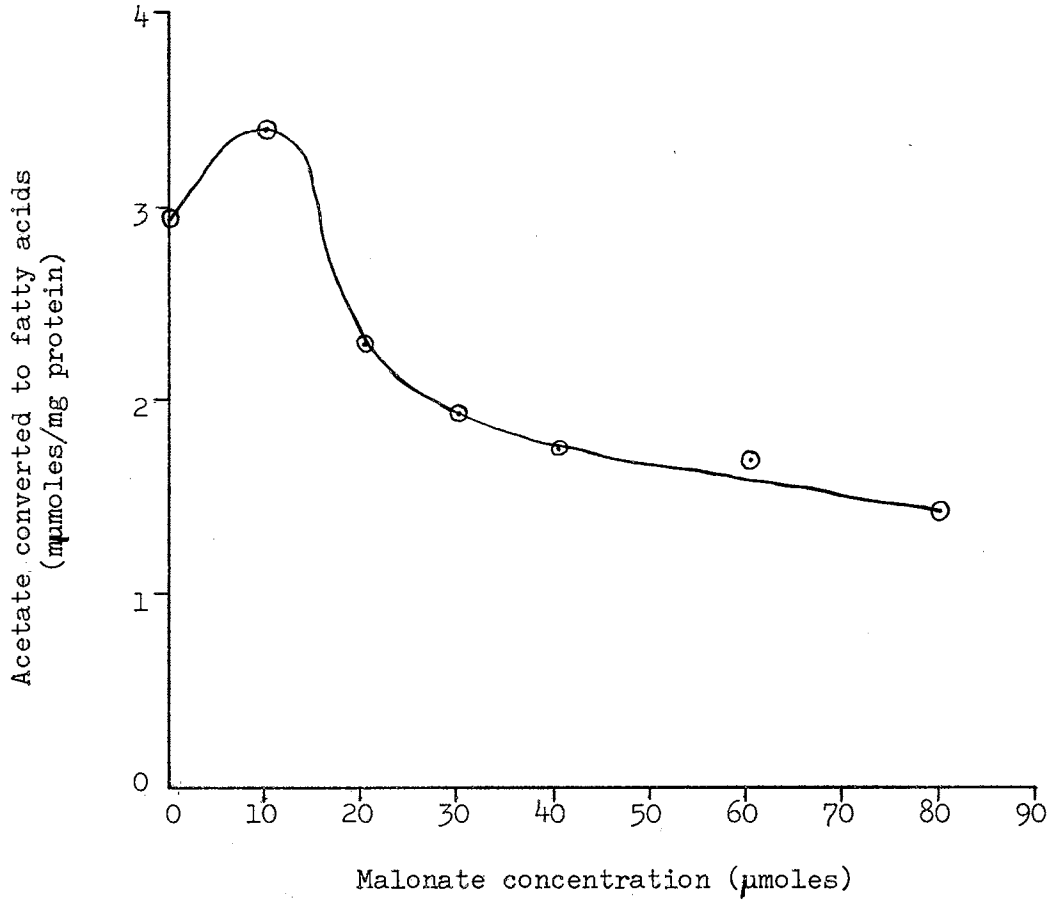

Figure 1. The Effect of Malonate on the Synthesis of Fatty Acids. Sample tubes contained the following additions: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); $MgCl_2$ (3.3 μmoles); $KHCO_3$ (20 μmoles); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); S_{12} protein (10 mg) treated with dialysis for 6 hours. Malonate added as indicated. Total volume was 2.5 ml. Tubes were incubated at 37°C for 1.5 hours.

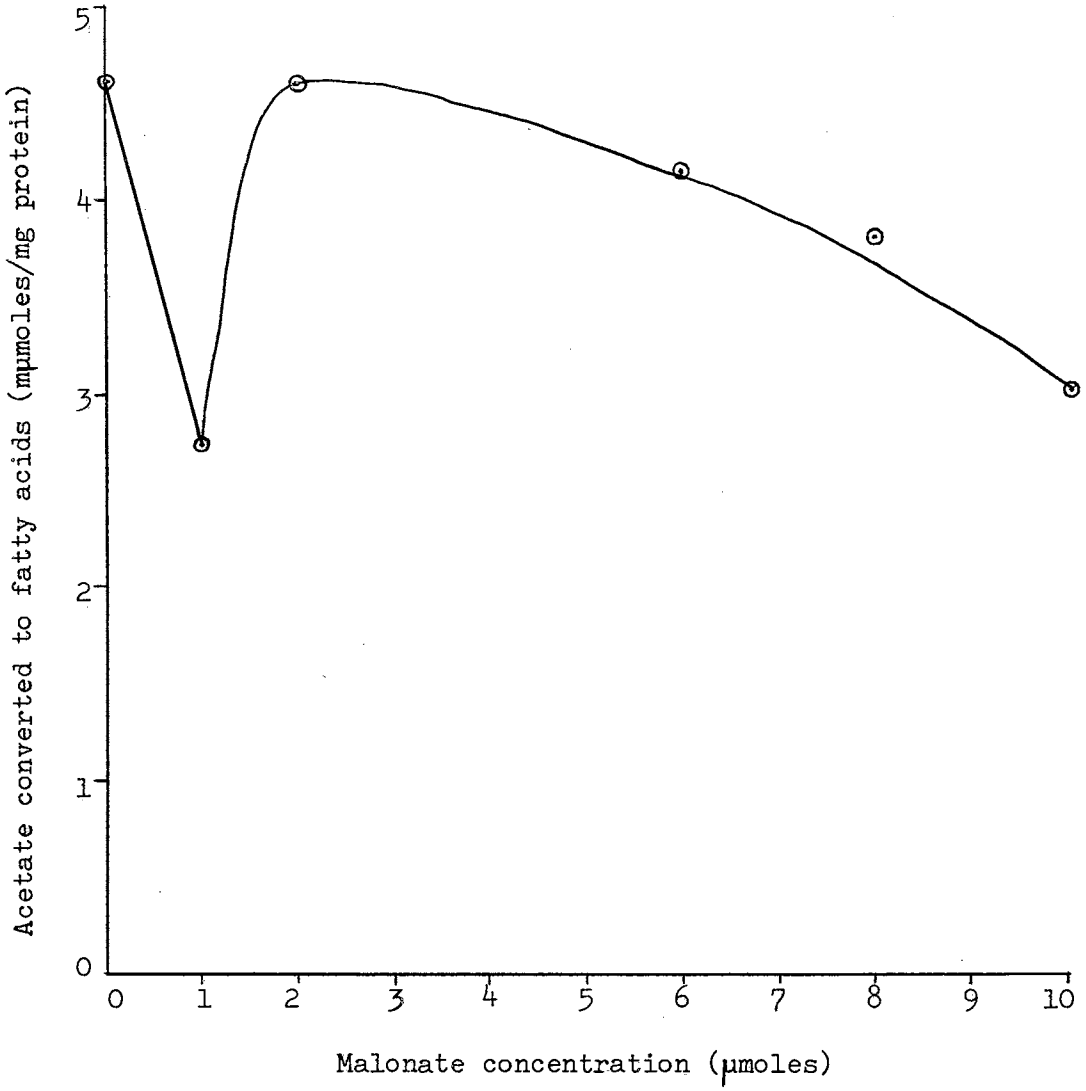

Figure 2. The Effect of Malonate on Fatty Acid Synthesis. Samples contained the following additions: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); $MgCl_2$ (3.3 μmoles); $KHCO_3$ (20 μmoles); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); S_{12} protein (10 mg) treated with dialysis for 6 hours. Malonate added as indicated. Total volume was 2.5 ml. Tubes were incubated at 37°C for 1.5 hours.

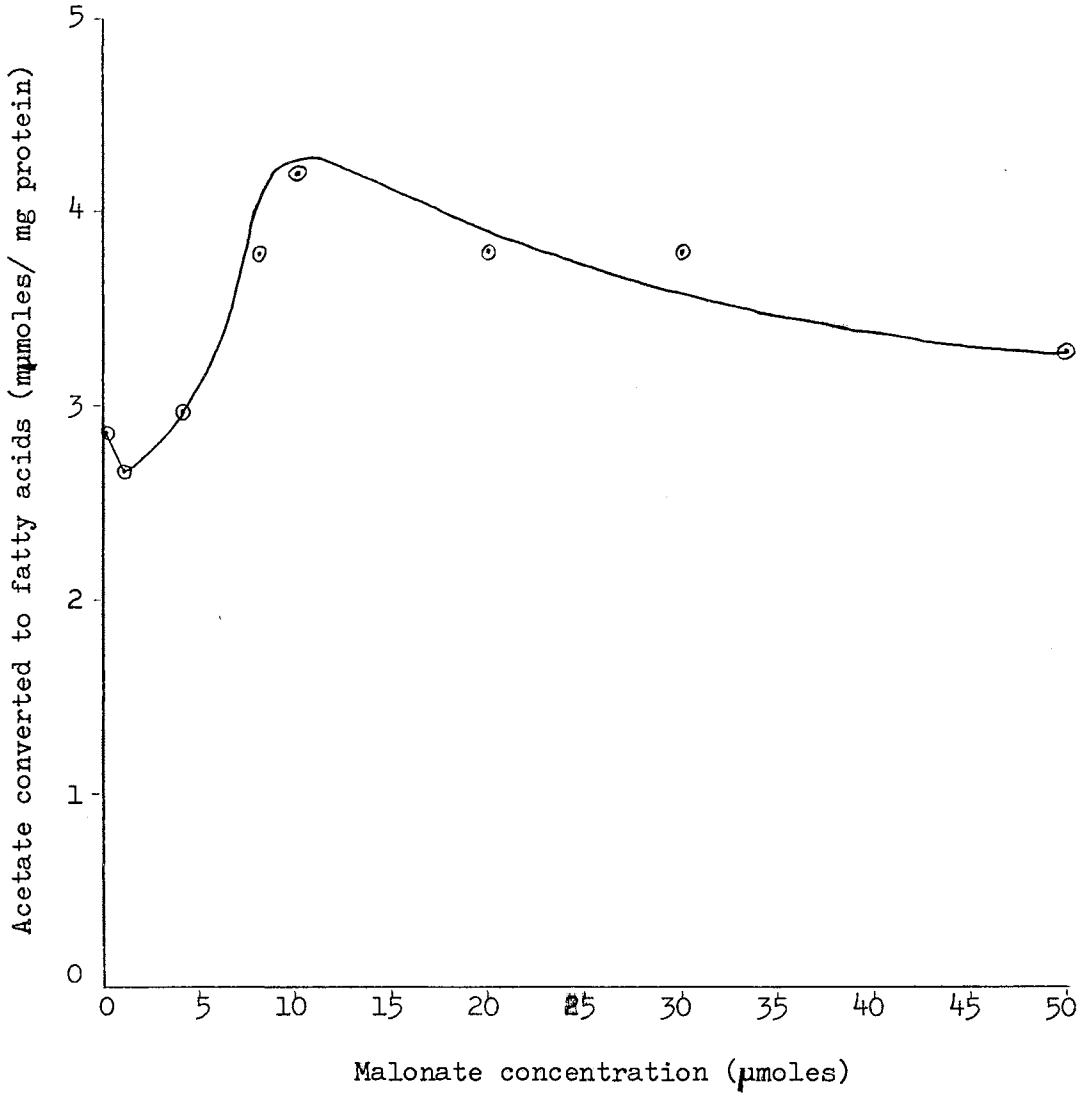

Figure 3. The Effect of Malonate on Fatty Acid Synthesis. Sample tubes contained the following additions: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); $MgCl_2$ (3.3 μmoles); $KHCO_3$ (20 μmoles); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); S_{12} protein (8-10 mg) treated with Dowex I (200-400 mesh). Malonate added as indicated. Total volume was 2.5 ml. Tubes were incubated at 37°C for 1.5 hours.

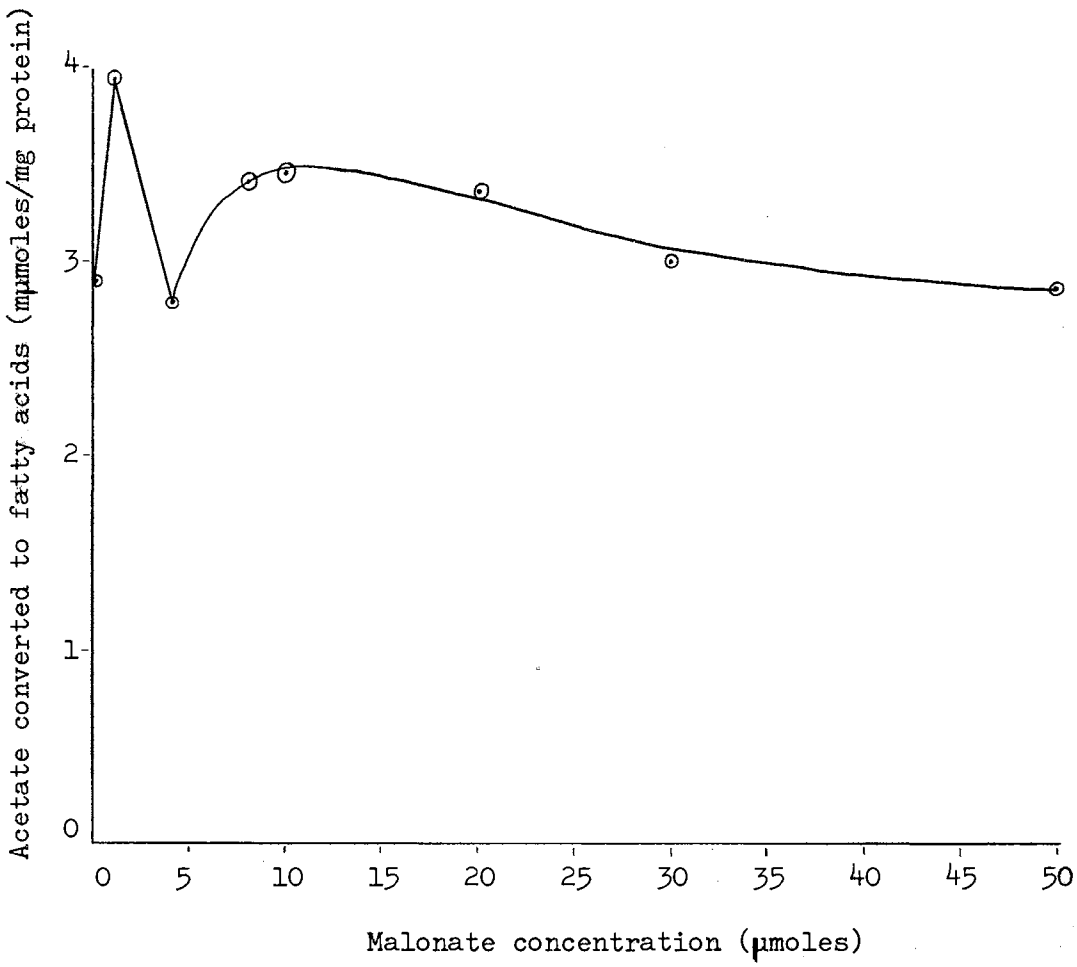

Figure 4. The Effect of Malonate on Fatty Acid Synthesis. Sample tubes contained the following additions: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); $MgCl_2$ (3.3 μmoles); $KHCO_3$ (20 μmoles); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); S_{12} protein (8-10 mg) treated with Dowex I (200-400 mesh). Malonate added as indicated. Total volume was 2.5 ml. Tubes were incubated at 37°C for 1.5 hours.

as inhibition of synthesis above this level. This led to the experiment on the affect of low concentrations of malonate on fatty acid synthesis (cf. Figure 2). A sharp inhibition of synthesis was observed with the addition of 1 μmole of malonate. The addition of 2 μmoles of malonate brought the level of incorporation back to the same value as when the system contained no exogenous malonate. There was a gradual decrease in synthesis with malonate concentrations greater than 2 μmoles. With such inconsistent results it was deemed necessary to treat the supernatant with Dowex in order to try to effect a removal of malonate and any cofactors associated with it. In Figures 3 and 4, the malonate level again stimulated synthesis at a concentration of 10 μmoles. However, at 1 μmoles malonate concentration these two experiments varied in opposite direction. In Figure 3, 1 μmole of malonate inhibited synthesis while in Figure 4, 1 μmole stimulated synthesis. It is possible that the results shown in Figure 4 represent a technical error because stimulation of synthesis at the 1 μmole level could not be demonstrated again. As in Figure 1 and 2, malonate concentration above 10 μmoles caused a gradual decrease in synthesis. These data are in disagreement with that of Abraham and coworkers (1) who found, while working with rat liver supernatant, that malonate in concentrations of 40-80 μmoles stimulated the incorporation of acetate into fatty acids. Dils and Popjak (27) also reported as much as a 50-fold increase in fatty acids synthesis with the addition of malonate to mammary gland preparation.

Effect of Bicarbonate on Fatty Acid Synthesis: Figure 5 shows the requirement for bicarbonate by the fatty acid synthesizing system isolated

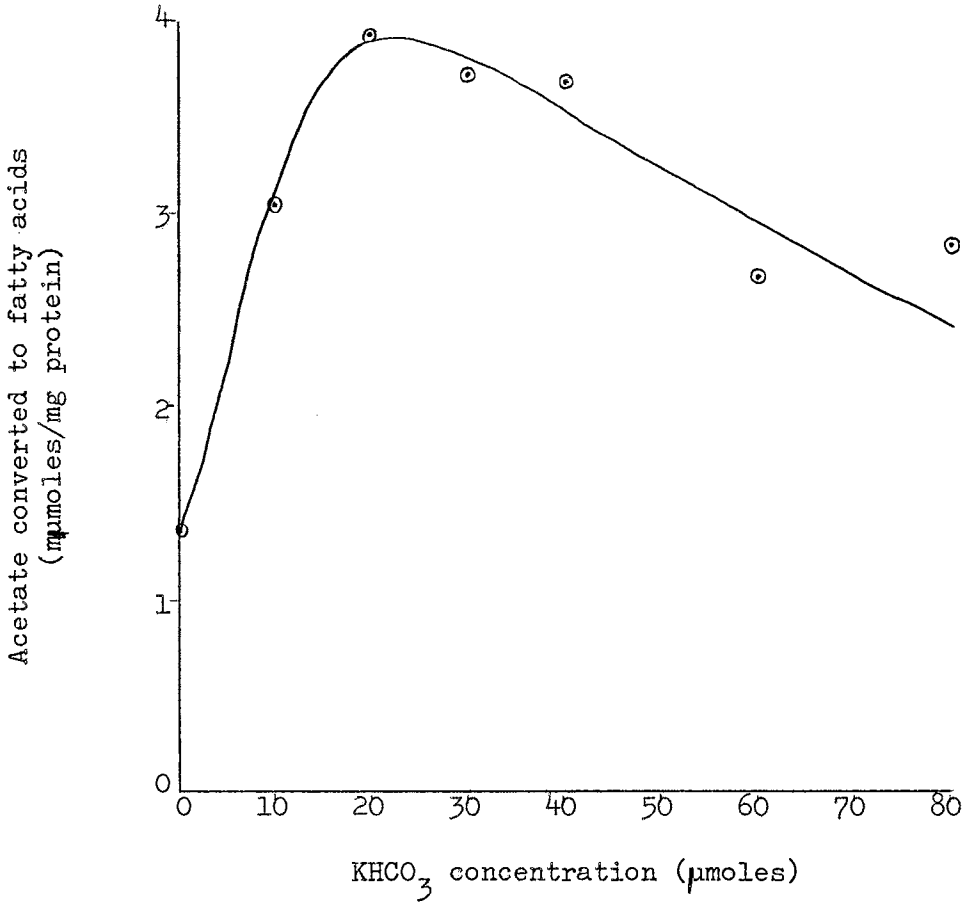

Figure 5. The Effect of Bicarbonate on Fatty Acid Synthesis. Sample tubes contained the following additions: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); malonate (1 μmole); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); S_{12} fraction contained 8-10 mg protein (untreated). $KHCO_3$ added as indicated. Total volume was 2.5 ml. Tubes were incubated for 1.5 hours at 37°C.

from Ascaris. The supernatant fraction (S_{12}) was used without dialysis or Dowex treatment. Optimum incorporation was acheived at a bicarbonate concentration of 20 μmoles. Concentrations above or below this level resulted in a decrease in synthesis. Dils and Popjak (27) reported a stimulation of fatty acid synthesis by the addition of bicarbonate, but they did not observe a decrease at higher concentrations.

Effect of Biotin and Avidin on Fatty Acid Synthesis: Table IV denotes the effect of biotin and avidin on incorporation of acetate into fatty acids. When biotin was omitted from the medium and the concentration of avidin was increased, the rate of incorporation decreased. With the addition of increasing quantities of biotin, fatty acid synthesis increased. Wakil et al. (107) first reported a biotin requirement for fatty acid synthesis in a rat liver preparation. Evidently, biotin is needed by Ascaris tissue to aid in fatty acid synthesis.

TABLE IV

EFFECT OF BIOTIN AND AVIDIN ON FATTY ACID SYNTHESIS

The complete system contained the same additions as in Table II.

System	Acetate Converted to Fatty Acids (mμmole/ mg Protein
Complete	2.53
+Avidin (.2 mg) - biotin	2.48
" (.4 mg) - "	1.36
" (.6 mg) - "	0.72
" (.4 mg) + " (24 μg)	1.26
" " + " (48 μg)	1.51
" " + " (72 μg)	1.70

Effect of Incubation Time: The data presented in Figure 6 correspond very closely to those reported by Wakil et al. (105). There is an initial lag period for the first 30 minutes and then a sharp increase in the rate up to 120 minutes. An incubation time of 90 to 120 minutes was utilized in all experiments.

Effect of Protein Concentration: In the experiment illustrated by Figure 7, it was found that the synthesis of fatty acids increased with increasing concentrations of protein up to a value of 8 mg protein. Dils and Popjak (27) observed lower value (5-6 mg) in mammary gland preparations. Above the 8 mg level, fatty acid synthesis in Ascaris declined and at a level of 22 mg of protein, the synthesis was approximately 50% of the maximum observed rate. Factors such as lack of substrate and end-product inhibition could have brought about the depressed rate of synthesis.

ATP Requirement: Addition of 1 to 20 μmoles of ATP to the medium resulted in a linear increase of fatty acids elaborated by the supernatant fraction which had been treated with Dowex (cf. Figure 8). Concentrations above 20 μmoles caused a rather sharp decrease in synthesis. Abraham et al. (1) reported similar results with a mammary gland S_{100} fraction with the exception that their peak activity was at 10 μmoles of ATP. They felt that the increase in ionic strength of the medium brought about by the addition of large quantities of ATP caused the inhibition of synthesis.

pH Effects: Optimal fatty acid synthesis was achieved at a pH of 7.5 (Figure 9). Fatty acid yields decreased at higher or lower hydrogen ion concentrations. A pH of 7.4-7.5 was used in all subsequent experiments.

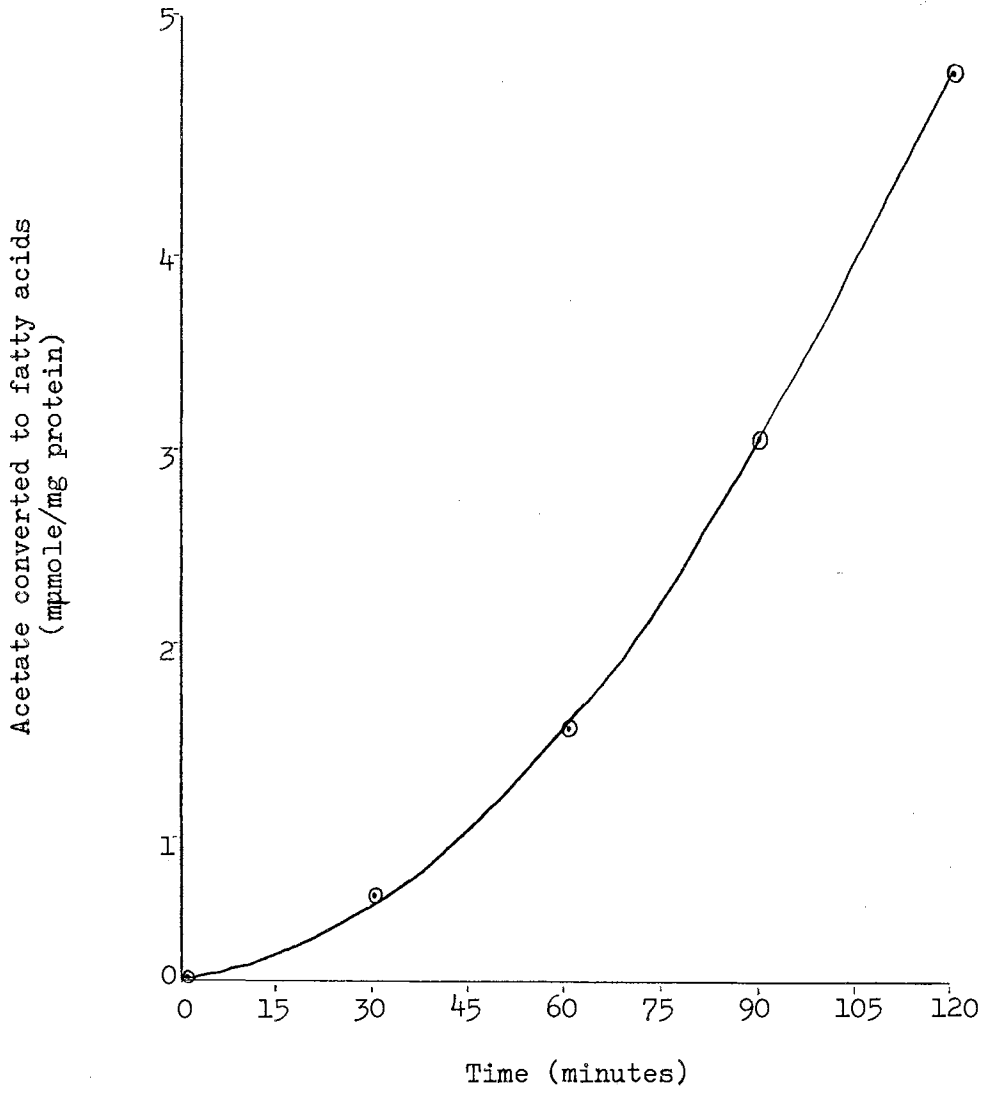

Figure 6. The Effect of Time on the Synthesis of Fatty Acids. Each tube contained the following cofactors: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); malonate 1 μmole); $KHCO_3$ (20 μmoles); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); S_{12} fraction contained 12 mg untreated protein. Total volume was 3.0 ml. Incubated the indicated time at 37°C.

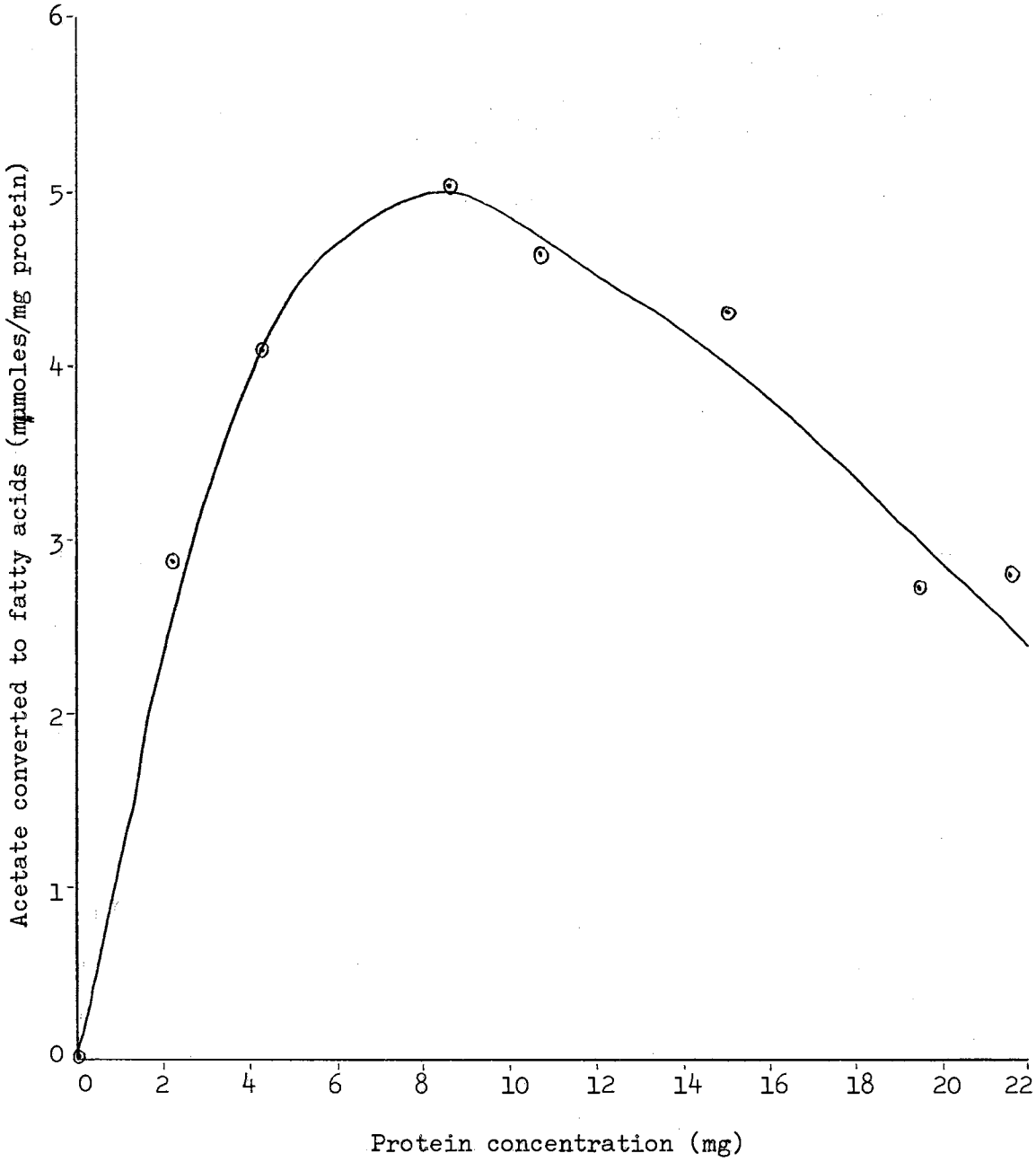

Figure 7. The Influence of Protein Concentration on the Uptake of Acetate into Fatty Acids. Each tube contained the following cofactors: Sodium-1-C^{14}-acetate (40 μmoles, 1.5×10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); malonate (1 μmole); $KHCO_3$ (20 μmoles); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); untreated S_{12} fraction added as indicated. Total volume was 3.0 ml. Tubes were incubated for 2 hours at 37°C.

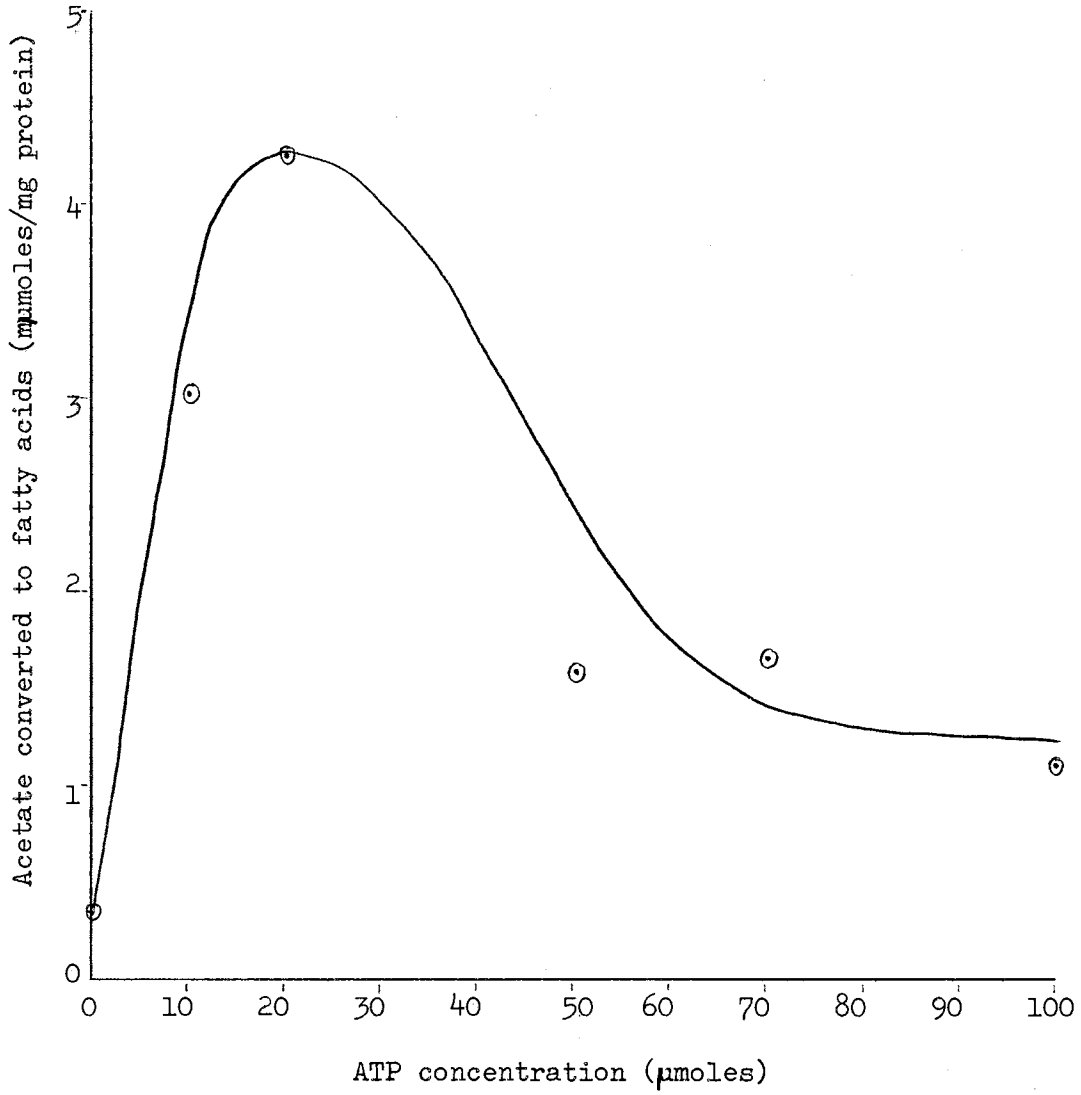

Figure 8. The Effect of ATP Concentration on the Synthesis of Fatty Acids. Sample tubes contained the following cofactors: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP was varied as indicated; CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); malonate (1 μmole); $KHCO_3$ (20 μmoles); biotin (24 μg); potassium phosphate buffer (50 μmoles, pH 7.4); S_{12} fraction was treated with Dowex I and contained 9.5 mg protein. Total volume was 2.5 ml. Samples were incubated at 37°C for 1.5 hours.

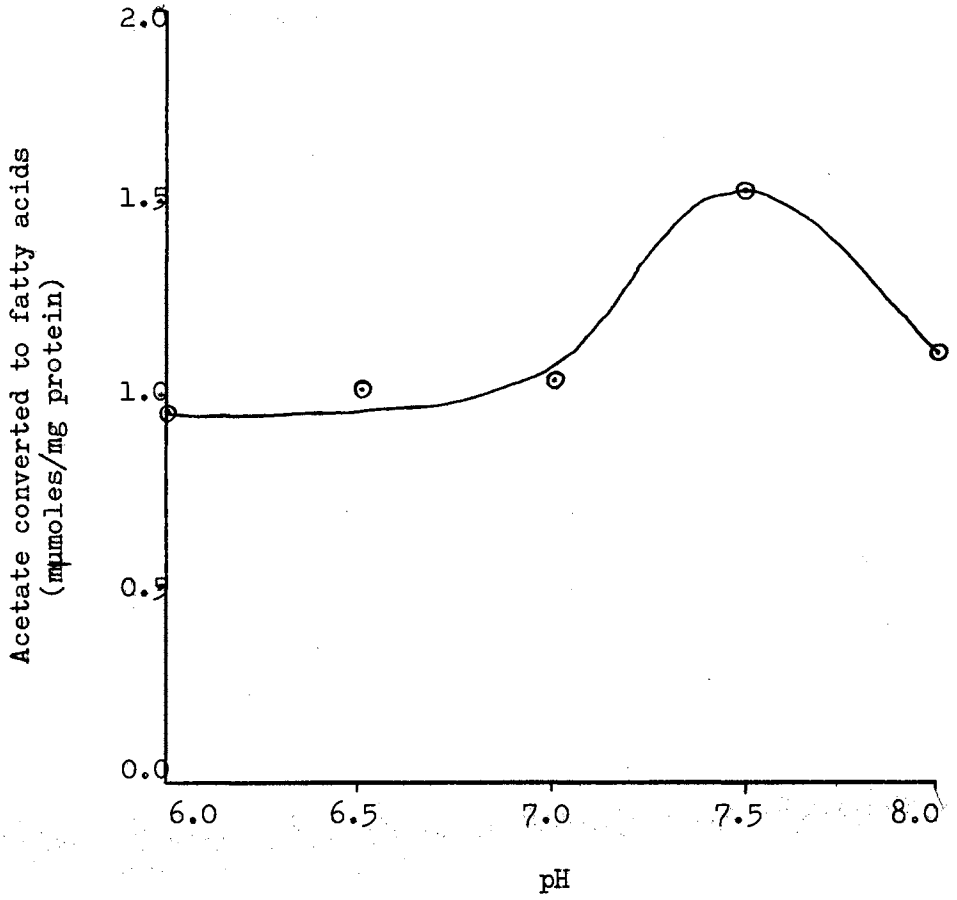

Figure 9. The Influence of pH on the Synthesis of Fatty Acids. The muscle tissue was homogenized and centrifuged in 0.25 M sucrose and 50 μmoles of potassium phosphate buffer at the appropriate pH was added. All tubes contained: Sodium-1-C^{14}-acetate (40 μmoles, 1.5 x 10^6 cpm); ATP (10 μmoles); CoA (0.14 μmoles); NADH (0.33 μmoles); NADPH (0.33 μmoles); $MnCl_2$ (3.3 μmoles); malonate (1 μmole); $KHCO_3$ (10 μmoles); biotin (24 μg); untreated S_{12} fraction contained 10 mg protein. Total volume was 3.0 ml. Samples were incubated at 37°C for 1.5 hours.

CHAPTER V

DISCUSSION

The cell-free fraction of the muscle tissue of Ascaris lumbricoides var. suum has the ability to incorporate acetate into long chain non-volatile fatty acids. This incorporation was found to be dependent on several factors. The fraction of the cell chiefly responsible for this incorporation was found to be what is commonly referred to as "supernatant + microsomes". Microsomes as such, have not been described in the muscle tissue of Ascaris, therefore, an affect of microsomes on acetate incorporation into fatty acids cannot be definitely stated. When the fraction, which is usually designated as containing the highest concentration of microsomes (S_{100}), was removed by centrifugation, acetate incorporation by the system was inhibited. This effect isn't clear and more work needs to be done in this area. Cofactors required for the system indicated that the possible route of incorporation was by way of the malonyl CoA pathway. ATP, Mn^{++}, and CoA were needed for the activation of acetate to acetyl CoA; ATP, $KHCO_3$, biotin, and Mn^{++} for carboxylation of acetyl CoA to malonyl CoA, and NADH and NADPH for the subsequent reduction of the condensation products. This pathway is commonly found in some bacteria (65), pigeon liver (105), heart sarcosomes (59), lactating rat mammary gland (2, 27), and rat liver (1). However, the nucleotide used in these systems was NADPH, and NADH contributed very little to the

systhesis. NADH was required by the system isolated from Ascaris muscle. A NADH dehydrogenase system has been described in Ascaris muscle tissue (62). This system primarily converts fumarate into succinate which can be decarboxylated to form propionate (91). Saz and Weil (92, 93) have described a mechanism by which propionyl CoA can condense with acetyl CoA or another molecule of propionyl CoA to form an α-methyl β-keto acid which is reduced via NADH to form a branched volatile fatty acyl CoA. Perhaps long chain fatty acid synthesis can occur by elongation of this fatty acyl CoA into long branched chain fatty acids. NADH would then be required: (1) to furnish hydrogen for the reduction of fumarate and (2) for the reduction of the branched moiety. Thus the animal could synthesize long straight-chain fatty acids via the malonyl CoA pathway which requires NADPH and synthesize branched-chain fatty acids by way of elongation of volatile fatty acids. The latter system would require NADH. The postulate concerning the latter pathway is highly speculative and no data have been collected to support it, other than the need for NADH. In one experiment, succinate stimulated the incorporation of acetate into fatty acids. In Ascaris, where succinate is a precursor of propionate, an increase in the succinate concentration could conceivably stimulate fatty acid synthesis. Dils and Popjak (27) found that various members of the tricarboxylic acid cycle lost their stimulatory effect on fatty acid synthesis with the addition of pure reduced pyridine nucleotide. This was not found in the afore mentioned experiment. Experiments in which labeled succinate is used would possibly answer these questions.

The stimulatory effect of malonate on the synthesis of fatty acids from acetate was first observed by Popjak and Tietz (87) and later

confirmed by Fletcher and Myant (40), Abraham *et al.* (2) and Dils and Popjak (27). A strong stimulation of acetate incorporation into fatty acids by malonate was not noted in the fatty acid synthesizing system isolated from *Ascaris* muscle tissue. With varying concentrations of malonate, inconsistent results were obtained. Malonate is generally known to inhibit the TCA cycle in higher animals by competing with succinate for the active site on the enzyme, succinic dehydrogenase. Realizing that the volatile fatty acids can be derived from succinate via propionate (91, 92, 93), it is conceiveable that malonate could compete with succinate on the succinate decarboxylase enzyme. This inhibition of the branched chain fatty acid synthetase would override the stimulatory effect of malonate on the malonyl CoA pathway with the result that only slight stimulation of acetate incorporation would appear. These statements are also highly speculative and more work needs to be done to complete this picture by using labeled malonyl CoA and labeled volatile fatty acids.

The function carried out by bicarbonate is that of furnishing CO_2 which is reacted with acetyl CoA via biotin to form malonyl CoA (106). Therefore the presence of bicarbonate in a fatty acid synthesizing system utilizing the malonyl CoA pathway is obligatory. All soluble systems studied thus far in higher animals and bacteria require bicarbonate. This need can be alleviated by beginning the synthesis with malonyl CoA instead of acetate or acetyl CoA (112). The fatty acid synthesizing system in *Ascaris* also requires bicarbonate or CO_2 for the incorporation of acetate into fatty acids. The mechanism may or may not be the same as that found in higher systems. The worm has the ability to affix CO_2

into succinate (91) and this might be an alternative pathway for CO_2 utilization. CO_2 is readily available to the animal *in vivo* for it lives in an environment rich in CO_2 (88), and it would not be surprising to learn that the animal could utilize large quantities of CO_2.

The presence of large quantities of unsaturated fatty acids (5) in the lipids of *Ascaris*, Acanthocephala (6) and in tapeworms (54) poses a question of synthesis. A major purpose for the desaturation of a saturated fatty acid is the production of energy by the transfer of 2 hydrogens and 2 electrons along a respiratory chain to the ultimate hydrogen acceptor, oxygen, thus forming water (8). A source of oxygen is needed for the terminal acceptor. The intestinal parasites abide in an environment where the oxygen tension is very low (88). Therefore, the concentration of unsaturated fatty acids would be expected to remain at a rather low value if they were synthesized at all. Beames (5), Beames and Fisher (6), and Harrington (54) found large quantities of unsaturated acids in the intestinal parasites mentioned previously. In most cases these unsaturated fatty acids comprised the greatest percentage of the fatty acids present. One theory is of course, that the parasite derives all of these unsaturated acids from the food supply of the host. But why should the parasite assimilate more unsaturated acids than saturated? Does the parasite synthesize these unsaturated acids, and if it does, is this synthesis accomplished by the known pathways? If this is true what happens to the hydrogen ions and the electrons that are released during the desaturation reaction? Do the animals utilize the small amount of oxygen present in the intestine? All these questions remain unanswered at the present time.

CHAPTER VI

SUMMARY AND CONCLUSIONS

This study was designed to determine the extent of acetate incorporation into fatty acids by the muscle tissue of Ascaris lumbricoides var. suum, and to elucidate some possible mechanisms by which this incorporation was accomplished. The following results were found.

1. The fraction of the muscle cell responsible for acetate incorporation into fatty acids was found to be the soluble supernatant + microsomes. Synthesis by the mitochondrial fraction was negligible. Removal of the microsonal fraction inhibited incorporation in all experiments.

2. The following cofactors were required for acetate incorporation: NADH, NADPH, Mn^{++}, CoA, ATP, malonate, HCO_3^-, biotin. These results indicated that the muscle tissue of Ascaris utilized the malonyl CoA pathway for incorporation of acetate into fatty acids. NADP plus G-6-P was not an effective substitute for NADPH in this preparation. It is concluded that the activity of the pentose phosphate pathway was too low to generate NADPH effectively. The possibility of a new pathway based on data obtained with NADH is discussed.

3. A stimulatory effect of high concentration of malonate on incorporation of acetate was not noted. In four experiments in which both Dowex I and dialysis were used to remove cofactors, malonate stimulation was never greater than 15-20% above control values, and in higher con-

centration it inhibited incorporation. The results of these experiments were inconclusive and no definite statements can be made at the present time on the role of malonate in fatty acid synthesis in Ascaris.

4. A stimulation of synthesis by bicarbonate was noted. This stimulation was effective up to a bicarbonate concentration of 20 μmoles. Levels greater than this elicited a slight inhibition of synthesis.

5. A requirement for biotin was demonstrated with the use of avidin. This indicates that a carboxylation reaction involving biotin occurs and acetyl CoA is converted to malonyl CoA via this reaction.

6. ATP stimulated acetate incorporation up to a concentration of 20 μmoles. Concentrations above this level caused an inhibition of synthesis.

The muscle tissue of Ascaris has the ability to incorporate acetate into fatty acids. This incorporation can be demonstrated in a mince or in a centrifuged homogenate fraction. However, the rate of incorporation is very low. This would be expected because muscle tissue is usually thought of as a rather static tissue which is composed mainly of proteins. The concentration of lipids in Ascaris muscle is much lower than that found in other tissues of the animal (37). Whether fatty acids synthesis is important to the animal in vivo cannot be stated at this time. The next step would be to investigate the ability of the reproductive tract to incorporate acetate into fatty acids. This tissue is primarily responsible for the excretion of lipids as components of the egg. Thus, it must have a tremendous ability to synthesize lipids.

The problems confronting an anaerobic organism have been known for a long time. The lack of oxygen in their environment has brought about drastic changes in their metabolism which no doubt deviate from the bio-

chemical unity concept. This study has answered some questions regarding lipid biosynthesis in Ascaris. It has also created many new ones on this aspect of the parasites metabolism.

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