2-Methylacetoacetate Reductase and Possible Propionyl Coenzyme A Condensing Enzyme Activity in Branched Chain Volatile Fatty Acid Synthesis by Ascaris lumbricoides*

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The intestinal worm, Ascaris lumbricoides, ferments carbohydrate to a mixture of end products, of which the two major ones are 2-methylbutyrate and 2-methylvalerate. The mode of formation of branched chain acids with a methyl group at C-2 or other even numbered carbon atoms is still not completely understood. It has been proposed that these compounds could be formed either from the fatty acid synthetase reaction involving methylmalonyl-CoA or from propionyl-CoA via a reversal of the β oxidation pathway.

Results of the present study indicate that, in Ascaris, propionyl-CoA may be the direct precursor of the branched chain volatile acids by a pathway similar to, but remarkably different from, a reverse of the β oxidation pathway. Neither fatty acid synthetase nor enoyl-CoA reductase activities were demonstrable in Ascaris muscle preparations by assays that successfully demonstrate these systems in other tissues. Two new enzymes have been partially purified and characterized from Ascaris mitochondria. These are the NADH-linked 2-methylacetoacetate reductase and the NADH-linked propionyl-CoA reductase (propionyl-CoA "condensing enzyme"). The condensing enzyme was assayed spectrophotometrically by coupling the product of the condensation with the 2-methylacetoacetate reductase activity.

The Ascaris 2-methylacetoacetate reductase is unique in that the apparent coenzyme A ester requirement can be substituted for by the ethyl ester. Specificity studies indicated that several substrates are active as the ethyl esters, but only if they also contain a 2-methyl group. Equal reductase activities were obtained when the ethyl esters of either 2-methylacetoacetate or 2-methylpropionate were employed as substrates. These would be possible precursors for 2-methylbutyrate and 2-methylvalerate, respectively. Neither the lithium salts of the branched chain keto acids nor the ethyl ester of acetoacetate were acted upon. However, acetoacetyl-CoA was reduced at approximately one-third the rate of ethyl 2-methylacetoacetate. NADH is the specific reductant. The product of the enzymatic reduction of ethyl methylacetoacetate was isolated and shown by means of NMR spectroscopy to be an erythro isomer of ethyl 3-hydroxybutyrate.

Propionyl-CoA condensing enzyme activity was more than 10 times more active with propionyl-CoA than with acetyl-CoA as substrate. Acetyl-CoA, malonyl-CoA, and methylmalonyl-CoA all inhibited the propionyl-CoA reaction, although the inhibitory effect of methylmalonyl-CoA appeared to be primarily on the reductase rather than on the condensing enzyme per se. The product of the coupled propionyl-CoA condensation and reductase reactions has been tentatively identified as 3-hydroxy-2-methylvaleryl-CoA, as determined by both thin layer chromatography of the hydroxamates and enzymatically by oxidation catalyzed by the Ascaris reductase reverse reaction.

Recently, Buckner and Kolattukudy (1) have demonstrated the synthesis of multibranched long chain fatty acids from methylmalonyl-CoA as catalyzed by purified fatty acid synthetase from the uropygial gland of the goose. Whether this constitutes a major physiological pathway for the synthesis of branched chain fatty acids that contain methyl groups at C-2 or other even numbered carbon atoms in other tissues remains to be determined. The adult parasitic intestinal nematode, Ascaris lumbricoides var. suum, exhibits an essentially anaerobic metabolism and ferments carbohydrate to a mixture of succinate and a variety of volatile fatty acids (2). Two of the predominant fermentation products formed by the parasite are the branched chain acids, 2-methylbutyrate and 2-methylvalerate (3, 4). Evidence based on isotope studies with Ascaris indicated that these two acids, 2-methylbutyrate and 2-methylvalerate, may arise via a condensation of either an acetoacetate unit with the 2-carbon atom of a propionate unit or two propionate units, respectively (5, 6). However, whether propionate units per se or a derivative of this acid, methylmalonate, was the reactant for the condensation reaction in Ascaris could not be distinguished by the 14C labeling patterns, since according to either mechanism, the distribution of isotope would be identical, assuming a subsequent decarboxylation of the methylmalonate-CoA condensation product.
It has been proposed also that the synthesis of branched chain fatty acids in Ascaris may occur via a reversal of the well established $\beta$ oxidation pathway (5, 7). The results of the current study indicate that the presence of the $\beta$ oxidation enzymes cannot, in itself, explain these syntheses and that propionyl-CoA rather than methylmalonyl-CoA is the direct precursor of the branched chain fatty acids in this parasite. In addition, two new enzymatic activities, which appear to participate in the synthesis of Ascaris branched chain acids in preference to straight chain acids, are described, localized, and partially purified. These are the 3-methylalocetate reductase and the presumed propionyl-CoA condensing enzyme activity, both of which were previously described in a preliminary report.1

MATERIALS AND METHODS

Acetyl-CoA and propionyl-CoA were either prepared from the corresponding anhydrides (8) or purchased as the chromatographically pure compounds (P-L Biochemicals, Milwaukee, Wis.). Similar results were obtained with either preparation. Concentrations of acetyl-CoA compounds were determined by the procedure of Lipmann and Tuttle (9). Ethyl 3-keto-2-methylpentanoate (ethyl 3-keto-2methylvalerate) was prepared from the condensation of ethyl propionate in the presence of sodium hydroxide (10).

Ethyl 3-hydroxy-2-methylbutyrate was synthesized by the reduction of ethyl methylacetoacetate with borohydride. The ester was extracted from the reaction mixture with ethyl ether. Water was removed from the ether extract by the addition of anhydrous sodium sulfate, and the ether was subsequently removed by evaporation. The ether remaining was purified by distillation under vacuum. Analyses of the product by gas-liquid chromatography indicated the presence of two peaks, which were present in a 60:40 ratio and which presumably comprised the two expected pairs of diastereoisomers. A pure fraction of the quantitatively minor component was trapped in liquid nitrogen upon preparative gas-liquid chromatography at 150°C. Spectra of each diastereoisomer were as follows. 

**Erythro isomers:** NMR (CDCl$_3$)$\delta$ 1.19 (d, $J = 6.8$ Hz, 3H), 1.20 (d, $J = 7.1$ Hz, 3H), 1.27 (d, $J = 7.3$ Hz, 3H), 2.48 (overlapping q's, $J = 7.3$, 7.2 Hz, 1H), 3.37 (bs, 1H), 3.49 (overlapping q's, $J = 7.2$, 7.3 Hz, 1H), 4.18 (q, $J = 7.3$ Hz, 2F). 

**Three isomers:** NMR (CDCl$_3$)$\delta$ 1.19 (d, $J = 6.8$ Hz, 3H), 1.20 (d, $J = 7.1$ Hz, 3H), 1.27 (d, $J = 7.3$ Hz, 3H), 2.48 (overlapping q's, $J = 4.8$, 7.2 Hz, 1H), 3.05 (bs, 1H), 4.02 (dq, $J = 4.8$, 6.8 Hz, 1H), 4.16 (q, $J = 7.3$ Hz, 2F) 

Muscle strips from adult female Ascaris lumbricoides var. suum were obtained by dissection, and mitochondria were prepared as described previously, employing a medium composed of 0.24 M sucrose, 5 mM EDTA, and 0.15% crystalline bovine serum albumin, pH 7.4 (11). Washed mitochondria obtained from 20 g (wet weight) of muscle were made to 10 ml with 0.01 M potassium phosphate, pH 6.0 (75 mM), and osmotic shock was induced spectrophoto metrically at 340 nm by adding 0.26 mM NADH (0.035 mg), ethyl 3-hydroxy-2-methylbutyrate (2.6 mM), and NAD$^+$ (0.05 mM).

Propionyl-CoA condensing enzyme activity was determined spectrophotometrically by coupling with the 3-methylalocetate reductase reaction. Except where indicated otherwise, preparations of this enzyme contained excess reductase activity as well. Therefore, in addition to enzymes, the condensing enzyme assay system contained the following, in a final volume of 1.0 ml: propionyl-CoA (2 mM), NADH (0.28 mM), and potassium phosphate, pH 6.0 (75 mM).

Enoyl-CoA hydratase was assayed spectrophotometrically by following the appearance of ethyl methylacetoacetate in the presence of acetyl-CoA plus malonyl-CoA (151). Protein was determined according to Lowry et al. (16).

To isolate and identify the product of the enzymatic reduction of ethyl methylacetoacetate, large scale incubations were conducted. One millimole of the chromatographically purified ethyl ester was incubated with propionyl-CoA. The mixture was extracted with ethyl ether. The acyl-CoA compounds were determined by the procedure of Lipmann et al. (16). Activity was determined by following the disappearance of NADPH in the presence of acetyl-CoA plus malonyl-CoA (151). Protein was determined according to Lowry et al. (16).

Isolation of the product of the propionyl-CoA reaction was attempted by incubating 20 μmol of propionyl-CoA with 11.5 μmol of NADH and 8.8 mg of Ascaris mitochondrial-soluble proteins. For these experiments, the mitochondria were disrupted in 20 mM potassium phosphate, pH 6.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. A final exchange was made with the reaction mixture and the protein was removed by centrifugation at 30°C for 2 h. The reaction mixture was extracted with ethyl ether and the product was trapped in liquid nitrogen and identified by means of its NMR spectrum.
unable to demonstrate the oxidation of [14C]palmitate. Unfortunately, the β oxidation enzyme activities were determined in the degradative direction and with only the straight chain substrates.

Re-evaluation of some of these activities by assaying in the direction of synthesis or employing other substrates indicated that the synthesis of the branched chain acids may be more complex than a direct reversal of β-oxidation (Table I). Tiglyl-CoA (2-methyl-3-butenic acid) also is a fermentation product of Ascaris metabolism (23) and appears to be the likely precursor of the corresponding saturated 2-methylbutyrate. With tiglyl-CoA and acetooacetyl-CoA as substrates, appreciable enoyl-CoA hydratase and acetooacetyl-CoA reductase activities were demonstrable. In contrast, all attempts to demonstrate the last step in the synthesis, enoyl-CoA reduction to the saturated acid, were negative. Attempts were made employing both tiglyl-CoA and crotonyl-CoA as substrates with cytosol, sonicated mitochondria, and mitochondrial-soluble or mitochondrial membrane fractions. In addition, four independent methods of assay were utilized. These included coupling with NADH or NADPH in the presence or absence of ATP; coupling with the low potential reduced dye, leukoasarafinine T according to Seubert and Lynen (24); coupling with reduced benzyl viologen (25); and coupling with spinach ferredoxin, which was reduced as described by Mayhew et al. (26). Therefore, the last step in the synthetic chain remains obscure.

Most interesting was the demonstration of the reductive synthesis of the branched chain acids (Table I). One enzyme, which was previously noted (27) but not characterized, catalyzed the reduction of the ethyl ester of methyleacetocetate with NADH. Interestingly, the rate of reduction of ethyl 2-methylacetocetate was approximately 3 times faster than that of the straight chain acetooacetyl-CoA. The second activity that was noted, a propionyl-CoA-dependent oxidation of NADH, might tentatively be assumed to be due to the condensation of two propionyl-CoA moieties, followed by reduction of the condensation product. This activity will be referred to as propionyl-CoA "condensing system."

Substrate Specificity of Ascaris Ethyl 2-Methylacetocetate Reductase and Propionyl-CoA Condensing Enzyme — The ethyl esters of both 2-methylacetocetate and the corresponding C₆ presumed precursor of 2-methylvalerate, 3-keto-2-methylpentanoate, were reduced at essentially identical rates (Table II). This relationship was true even in 120-fold purified preparations. On the other hand, neither the ethyl ester of acetocetate nor the lithium salt of methylacetocetate was reduced by these preparations, indicating a requirement for both the branched methyl and ester groups. Acetooacetyl-CoA was reduced by these preparations, but only at approximately one-third the rate, suggesting the possibility that physiologically the CoA function might substitute for the ethyl ester group.

The ethyl ester of 2-acetolactate also contains a 2-methyl function and is obtained by the limited alkaline hydrolysis of the acetoxy ethyl ester of acetolactate (28). As might be predicted, the former compound is reduced by the system at a slower rate but the acetoxy ester is not acted upon. It was also of interest that all of the reductions catalyzed by the reductase system were specific for NADH; NADPH was inactive.

The reductase reaction was reversible, but the ethyl esters of both 3-hydroxy-2-methylbutyrate and 3-hydroxy-2-methylvalerate were oxidized only at approximately one-tenth the rate of the reverse reduction (Table II). These findings suggest that the major physiological function of this system would be in the reductive or synthetic direction. However, the K₅ value for the reduction of ethyl methylacetocetate at 25° and pH 6.0 was 0.20. The effect of substituting CoA for the ethyl ester group remains to be determined.

Although the activity on acetooacetyl-CoA was approximately 3 times lower than that on ethyl methylacetocetate,

### Table I

<table>
<thead>
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<th>Activity</th>
<th>µmol/min/mg protein</th>
</tr>
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<tr>
<td>Fatty acid synthetase</td>
<td>0</td>
</tr>
<tr>
<td>Enoyl-CoA hydratase (tiglyl-CoA)</td>
<td>3.4</td>
</tr>
<tr>
<td>Acetooacetyl-CoA reductase</td>
<td>1.3</td>
</tr>
<tr>
<td>Enoyl-CoA reductase (tiglyl-CoA)</td>
<td>0</td>
</tr>
<tr>
<td>Enoyl-CoA reductase (crotonyl-CoA)</td>
<td>0</td>
</tr>
<tr>
<td>Ethyl 2-methylacetocetate reductase</td>
<td>3.6</td>
</tr>
<tr>
<td>Condensing enzyme (propionyl-CoA)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

* Activities present in an Ascaris muscle mitochondrial soluble fraction. Details of assay procedures are described in the text.
the $K_m$ for acetoacetyl-CoA (1.4 x $10^{-4}$ M) was approximately 35 times lower than that for ethyl methylacetoacetate (5 x $10^{-3}$ M). Moreover, the $K_m$ for the ethyl ester of 3-hydroxy-2-methylbutyrate (8 x $10^{-4}$ M) was approximately 10 times lower than that for the corresponding keto ester. The high $K_m$ values obtained with the ethyl ester substrates suggest that the esters are not the physiological substrates, since high concentrations of the esters would be required but are not present physiologically in the tissues. On the other hand, the $K_m$ value of 1.4 x $10^{-4}$ M for acetoacetyl-CoA supports the assumed involvement of the CoA derivatives as the physiological substrates for this reaction and is comparable to the $K_m$ values found for the physiological nucleotides NADH and NAD$^+$ (1.0 x $10^{-4}$ and 0.5 x $10^{-4}$ M, respectively). The optimum pH for the reaction in the reductive direction was found to be 8.0. In the oxidative reaction with ethyl 3-hydroxy-2-methylbutyrate as substrate, the optimum activity was found at pH 8.0.

If, in Ascaris, the reduction of methylacetocetate were indeed coenzyme A linked, then it should be possible to determine acetyl-CoA or propionyl-CoA condensing activities by coupling the expected keto-acyl-CoA product with this reductase and following NADH disappearance spectrophotometrically. When this assay was employed, activity was demonstrable in soluble extracts of mitochondria (Table II). In the absence of substrate, there was no oxidation of NADH. In addition, the highest condensing enzyme activity was obtained with propionyl-CoA alone as substrate. Although some acetyl-CoA "condensing" activity also was present in Ascaris mitochondria, this was considerably lower than that of propionyl-CoA. Moreover, acetyl-CoA inhibited propionyl-CoA condensing activity when both substrates were present in equimolar concentration. Neither stimulation nor inhibition was found when the ethyl esters of acetate or propionate or the corresponding free acids were employed as substrates. These findings, together with the possibility of coupling the propionyl-CoA or acetyl-CoA activities (or both) with the Ascaris methylacetocetate reductase, further suggest the involvement of the CoA derivatives in the Ascaris pathway.

**Cellular Localization of Ascaris 2-Methylacetoacetate Reductase and Propionyl-CoA Condensing Enzyme** — In order to determine the location of both enzymes, cellular and subcellular fractions were obtained from Ascaris muscle, and enzymatic activities were determined (Table III). Both cytosol and mitochondrial fractions showed 2-methylacetoacetate reductase activity. Consistently, however, 60 to 70% of the total activity assayed in the Ascaris crude homogenate was of mitochondrial origin, whereas the remaining 30 to 40% was associated with the cytosol fraction. Within the mitochondrion, activity appeared to be equally distributed between the matrix and the intermembrane space compartments. Almost no activity was associated with the membrane components. In these studies, the markers for the intermembrane space, matrix, inner membrane, and outer membrane fractions were adenylate kinase, citrate synthase, succinate dehydrogenase, and rotenone-insensitive cytochrome c reductase, respectively (13).

On the other hand, a relatively low level of propionyl-CoA condensing enzyme activity was localized in the cytosol fraction (Table III). Almost all of this activity was recovered from the mitochondria as a soluble protein upon centrifugation at 269,245 x $g$ for 1 h. The activity found in both the crude homogenate and the cytosol compartment may be derived from damaged mitochondria, since intact mitochondria would not be expected to be permeable to the CoA derivatives.

**Partial Purification of Ascaris 2-Methylacetoacetate Reductase and Propionyl-CoA Condensing Enzyme** — Isolation of the mitochondrial-soluble fraction from the crude homogenate resulted in a 19-fold purification in terms of 2-methylacetoacetate reductase activity (Table IV). When this fraction was passed through a DEAE-cellulose column equilibrated with 10 mM potassium phosphate buffer (pH 7.5) prior to assay. The mitochondrial soluble fraction was obtained from sonicated mitochondria after centrifugation at 269,000 x $g$ for 1 h. Aliquots of each fraction were assayed as described in the text.

### Table III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Experiment</th>
<th>Cellular fraction</th>
<th>Total activity</th>
<th>$\mu$mol/min x 10 g (wet weight) of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Methylacetoacetate reductase*</td>
<td>1</td>
<td>Crude homogenate</td>
<td>31.25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cytosol</td>
<td>10.0</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intact mitochondria</td>
<td>20.6</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermembrane space</td>
<td>11.95</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Matrix</td>
<td>5.46</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inner membrane</td>
<td>0.080</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Outer membrane</td>
<td>0.020</td>
<td>0.16</td>
</tr>
<tr>
<td>Propionyl-CoA condensing enzyme</td>
<td>1</td>
<td>Cytosol</td>
<td>0.70</td>
<td>4.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonicated mitochondria</td>
<td>4.75</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mitochondrial soluble fraction</td>
<td>3.75</td>
<td>79</td>
</tr>
</tbody>
</table>

* Figures represent $\mu$mol/min x 10 g (wet weight) of muscle tissue.

Ethyl 2-methylacetocetate was employed as substrate in all assays.

**Intact mitochondria** were prepared as described in the text and suspended in mitochondrial media. Sonicated mitochondria were prepared from such suspensions, which were disrupted by sonication. Shocked mitochondria were suspended in hypertonic Triasphate buffer (10 mM pH 7.5) prior to assay. This mitochondrial soluble fraction was obtained from sonicated mitochondria after centrifugation at 269,000 x $g$ for 1 h. Aliquots of each fraction were assayed as described in the text.

**Fatty Acid Synthesis in Ascaris**

Although the reductase and condensing enzyme could not be separated by DEAE-cellulose chromatography, it was possible to separate these activities by means of affinity chromatography (Table V). When a mitochondrial-soluble fraction containing both activities was passed through an NAD-agarose column equilibrated with 20 mM potassium phosphate buffer (pH 6.0), the 2-methylacetocetate reductase activity was bound to the NAD-agarose, whereas the condensing enzyme activity was not. Fractons containing propionyl-CoA condensing activity (Fraction A) were eluted from the column with the equilibrium buffer. Such fractions were devoid of 2-methylacetocetate reductase activity and were inactive in the assay unless reconstituted with propionyl-CoA and Fraction B, which contained the reductase activity. Fraction B, in turn, was obtained after elution from the column with 1.3 mM NADH solution in 20 mM potassium phosphate buffer, pH 8.0,
Enzyme Activities—An indirect indication that the acetyl- substrates for this Ascaris system. No significant activity was
observed. Neither acetyl-CoA nor propionyl-CoA had any observable action. However, the effects of malonyl-CoA and methyl-
ester were employed as substrates. In addition, none of these com-
ponents had any significant effect upon the acetyl-CoA reac-
tion, suggests that the inhibition of the Ascaris re-
condensation activity could only be assayed by the addition of the reductase fraction, the possibil-
ity remains that a necessary nonprotein factor may have been separated rather than the protein itself.

Relation between Ethyl 2-Methylacetoacetate and Acetoacetyl-CoA Reductions—The finding that, through 130-fold puri-
fication, the ratio of reductase activities on ethyl methylaceto-
acetate and acetoacetyl-CoA remained essentially constant indicated the possibility that both reactions were catalyzed by the same enzyme (Table VI). This was further suggested by results of partial heat denaturation studies, wherein a constant activity ratio was maintained after partial inactivation (Table VI). In agreement with this supposition was the fact that both activities were inhibited by free coenzyme A, which is a noncompetitive inhibitor of the ethyl methylacetoacetate reductase reaction (Fig. 1). This finding, together with the expected formation of free coenzyme A during the condensa-
tion reaction, suggests that the inhibition of the Ascaris re-
ductase system by free coenzyme A may represent a mode for controlling the syntheses of branched chain fatty acids in this muscle.

Effects of Coenzyme A Derivatives on Ascaris Condensing Enzyme Activities—An indirect indication that the acetyl-
CoA- and propionyl-CoA-dependent oxidations of NADH were catalyzed by the same enzyme was the finding that acetyl-CoA inhibited the propionyl-CoA-dependent oxidation of NADH. Neither acetyl-CoA nor propionyl-CoA had any observable effect upon 2-methylacetoacetate reductase. Acetyl-CoA at a concentration of $2 \times 10^{-4} \text{M}$ inhibited the propionyl-CoA (1 x $10^{-4} \text{M}$)-dependent reaction by 40%. A 75% inhibition was observed when both substrates were present in equimolar concentrations (1 x $10^{-4} \text{M}$). The $K_i$ for acetyl-CoA inhibition of the propionyl-CoA-dependent NADH oxidation was $4 \times 10^{-4} \text{M}$.

Other coenzyme A derivatives were examined as possible substrates for this Ascaris system. No significant activity was detected when n-butyryl-, crotonyl-, or valeryl-CoA esters were employed as substrates. In addition, none of these com-
plexes had any significant effect upon the acetyl-CoA reaction. However, the effects of malonyl-CoA and methyl-

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### Table IV

**Partial purification of Ascaris 2-methylacetoacetate reductase and propionyl-CoA condensing enzyme**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme</th>
<th>Fraction</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
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<tr>
<td>1</td>
<td>2-Methylacetoacetate reductase</td>
<td>Crude homogenate</td>
<td>66.3</td>
<td>0.17</td>
<td>0</td>
<td>100</td>
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<tr>
<td></td>
<td>Mitochondrial-soluble</td>
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<td>29.3</td>
<td>3.2</td>
<td>18.8</td>
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<tr>
<td></td>
<td>DEAE-cellulose</td>
<td></td>
<td>17.2</td>
<td>23.0</td>
<td>135</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>Propionyl-CoA condensing enzyme</td>
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<td>9.5</td>
<td>0.13</td>
<td>0</td>
<td>100</td>
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<tr>
<td></td>
<td>Mitochondrial-soluble</td>
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<td>7.5</td>
<td>0.54</td>
<td>4.2</td>
<td>79</td>
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<tr>
<td></td>
<td>DEAE-cellulose</td>
<td></td>
<td>4.8</td>
<td>1.08</td>
<td>8.3</td>
<td>51</td>
</tr>
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</table>

### Table V

**Affinity chromatographic separation of 2-methylacetoacetate reductase and propionyl-CoA condensing enzyme activity**

A mitochondrial-soluble fraction (0.7 ml, 1.19 mg of protein) was applied to a DEAE-cellulose column (1.4 x 13 cm) equilibrated with 20 mM potassium phosphate buffer, pH 6.0. Fraction A was obtained by eluting the column with 10 ml of the equilibration buffer. Fraction B was obtained by eluting the column with 5 ml of 1.3 mM NADH solution in 20 mM potassium phosphate buffer, pH 8.0. 2-

<table>
<thead>
<tr>
<th>Fraction</th>
<th>2-Methylacetoacetate reductase</th>
<th>Propionyl-CoA condensing enzyme</th>
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<tr>
<td></td>
<td>Total activity</td>
<td>Specific activity</td>
</tr>
<tr>
<td></td>
<td>µmol/min</td>
<td>%</td>
</tr>
<tr>
<td>Mitochondrial-soluble</td>
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<td>100</td>
</tr>
<tr>
<td>NAD-agarose Fraction A</td>
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<td>0</td>
</tr>
<tr>
<td>NAD-agarose Fraction B</td>
<td>2.6</td>
<td>92.8</td>
</tr>
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</table>

### Table VI

**Comparison of ethyl methylacetoacetate and acetooctyl-CoA reductase activities on purification and after heating**

In Experiment 2, 1 ml of a mitochondrial-soluble preparation in a test tube fitted with a marble condenser was heated in a water bath at 50°C for 20 min. Immediately thereafter, the preparation was cooled in an ice bath and then centrifuged at 39,100 x g for 10 min. The supernatant obtained was assayed for enzyme activity.
The goose.

Precursor for the synthesis of branched chain acids in Ascaris mitochondrial-soluble fractions can catalyze the formation of propionyl-CoA from methylmalonyl-CoA. The low activity observed with propionyl-CoA alone and one-half of that with methylmalonyl-CoA alone was about one-fourteenth of that late either the propionyl-CoA or the acetyl-CoA condensation reactions. These data are in accord with the supposition that the Ascaris fatty acid-synthesizing system does not involve the fatty acid synthetase enzyme A. The reaction mixtures contained in a total volume of 1.0 ml: 0.13 μmol of NADH, 75 μmol of potassium phosphate buffer (pH 6.0), and 3.3 μg of mitochondrial-soluble proteins. The reaction was started by adding the substrate A, in the absence of coenzyme A; B, in the presence of 2.2 x 10^-4 M coenzyme A.

Methylmalonyl-CoA on the acetyl-CoA- and propionyl-CoA-dependent oxidations of NADH were of interest (Table VII). The addition of malonyl-CoA to either the acetyl-CoA or the propionyl-CoA system resulted in a dramatic inhibition of both and at approximately the same percentage. The additional finding that malonyl-CoA had no effect on the ethyl methylacetoacetate reductase activity (experiments done with ethyl methylacetoacetate as substrate) places the inhibition directly on the presumed condensation reaction. These data support the previous findings, indicating that the Ascaris fatty acid-synthesizing system does not involve the fatty acid synthetase and therefore is different from those systems operating in mammals, yeast, Escherichia coli, and the uropygial glands of the goose.

The possible participation of methylmalonyl-CoA in the syntheses of branched chain volatile acids by Ascaris was examined. Methylmalonyl-CoA did produce a slow oxidation of NADH under the enzyme assay conditions employed (Table VII, Experiment 2). However, the activity obtained with methylmalonyl-CoA alone was about one-fourteenth of that observed with propionyl-CoA alone and one-half of that with acetyl-CoA. These findings would indicate that propionyl-CoA is the preferred substrate for this reaction. The low activity obtained with methylmalonyl-CoA as substrate may be due to the fact that Ascaris mitochondrial-soluble fractions can catalyze the formation of propionyl-CoA from methylmalonyl-CoA (29).

As in the case of malonyl-CoA, it was found that methylmalonyl-CoA inhibited the acetyl-CoA and propionyl-CoA reactions to the same extent. However, in contrast with malonyl-CoA, methylmalonyl-CoA inhibited ethyl methylacetoacetate reductase activity to a considerably greater extent. The inhibitory effect on the presumed condensation, therefore, may be partially or completely due to inhibition of the reductase reaction. In no instance did methylmalonyl-CoA stimulate either the propionyl-CoA or the acetyl-CoA condensation reactions. These data are in accord with the supposition that propionyl-CoA, rather than methylmalonyl-CoA, is the direct precursor for the synthesis of branched chain acids in Ascaris muscle.

**Table VII**

<table>
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<th>Experiment</th>
<th>Substrate</th>
<th>NADH oxidation</th>
<th>% inhibition</th>
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Identification of Product of Enzymatic Reduction of Ethyl Methylacetoacetate—Reduction of ethyl methylacetoacetate with potassium borohydride produced a 60:40 mixture of diastereoisomers of the corresponding hydroxy compounds. The diastereoisomers were separated by preparative gas-liquid chromatography. When each peak of the mixture of diastereoisomers was examined by means of NMR spectroscopy, it was found that, in the NMR spectrum of the chemically obtained major isomer, the methine hydrogens were coupled with a coupling constant of 7.2 Hz, whereas in the minor isomer this coupling had a value of 4.8 Hz. Analysis of the rotational conformations of the erythro and threo isomers indicated that the major isomer, with a coupling constant of 7.2 Hz, is the erythro isomer. Conformation III (Fig. 2) is by far the dominant contributor to the time average structure of the erythro isomer. Conformations I and II are poor competitors because both contain three gauche interactions, including severe gauche methyl-methyl interactions. The 180° dihedral angle between the methine hydrogens of Conformation III results in a large coupling constant between these hydrogens (30). Although Conformation IV, which also contains a 180° dihedral angle between the methine hydrogens, is the most highly populated conformation of the threo isomer, Conformation V, which lacks a severe gauche methyl-methyl interaction, must also be significantly populated. However, a dihedral angle of 60° between the methine hydrogens present in Conformation V results in a much smaller coupling constant between these hydrogens. Therefore, on a time average basis the coupling constant between the methine hydrogens in the threo isomer...
must be smaller than that of the erythro. Support for the above assignment of the stereoisomers is derived by application of Cram's rule to the borohydride reduction of ethyl methylacetoacetate, which predicts that the erythro isomer should be the dominant isomer formed (31).

When ethyl methylacetoacetate was reduced enzymatically with NADH, as catalyzed by the Ascaris reductase, only one of the diastereoisomers was recovered (Fig. 3). The isomer recovered as the product of the enzymatic reaction was analyzed by NMR, and the value obtained for the coupling constant of the methine hydrogen peaks indicated that it corresponded to an erythro isomer of ethyl 3-hydroxy-2-methylbutyrate. In addition, no enzyme activity was observed when a mixture of the two possible three isomers was employed as substrate for the reductase reaction in the reverse (oxidative) direction. However, in the presence of a mixture of the four possible isomers, two three and two erythro, activity was observed. Therefore, Ascaris 2-methylacetoacetate reductase appears to be specific for an erythro isomer (2R, 3R or 2S, 3S) when assayed in either direction.

Identification of Product of Propionyl-CoA Condensation Reaction—Incubation of Ascaris mitochondriol-soluble fractions with propionyl-CoA and NADH allowed a tentative identification of the product as 3-hydroxy-2-methylvaleryl-CoA. The product was isolated from the enzyme reaction mixture as postulated.

The incubation was terminated and deproteinized by the addition of perchloric acid. The neutralized supernatant was obtained after centrifugation, and aliquots were removed and assayed for the presumed product, 3-hydroxy-2-methylvaleryl-CoA, by the addition of NAD\(^+\) and the Ascaris reductase system (acting in the oxidative direction). Alkaline hydrolysis of the product of the propionyl-CoA condensation system recovered in the experimental supernatant was accompanied by the loss of the ability of that product to be oxidized by the Ascaris enzyme, presumably as a consequence of the loss of the coenzyme A function. It was determined also that the amount of NAD\(^+\) disappearing during the reduction of the propionyl-CoA condensation product was approximately equal to the amount of NAD\(^+\) required for the oxidation of the product recovered from the experimental supernatant upon the addition of the Ascaris enzyme (Table VIII).

Additional circumstantial evidence that the product of the propionyl-CoA-dependent oxidation of NADH was a hydroxyacyl-CoA compound was obtained by oxidation of the product back to the keto compound upon the addition of NAD\(^+\) and the Ascaris 2-methylacetoacetate reductase (Table VIII). In these experiments, Ascaris mitochondriol-soluble proteins were allowed to react with propionyl-CoA in the presence of NADH. The incubation was terminated and deproteinized by the addition of perchloric acid. The neutralized supernatant was obtained after centrifugation, and aliquots were removed and assayed for the presumed product, 3-hydroxy-2-methylvaleryl-CoA, by the addition of NAD\(^+\) and the Ascaris reductase system (acting in the oxidative direction). Alkaline hydrolysis of the product of the propionyl-CoA condensation system recovered in the experimental supernatant was accompanied by the loss of the ability of that product to be oxidized by the Ascaris enzyme, presumably as a consequence of the loss of the coenzyme A function. It was determined also that the amount of NAD\(^+\) disappearing during the reduction of the propionyl-CoA condensation product was approximately equal to the amount of NAD\(^+\) required for the oxidation of the product recovered from the experimental supernatant upon the addition of the Ascaris enzyme (Table VIII).

The additional finding that the product of the propionyl-CoA reaction, present in the experimental supernatant, could not be coupled with pig heart L-3-hydroxyacyl-CoA dehydrogenase indicated the absence of a straight chain CoA ester, since the pig heart enzyme rapidly catalyzed the oxidation of hydroxybutyrate (Table VIII). There are, therefore, differences between this enzyme and the Ascaris 2-methylacetoacetate reductase. It would appear that the pig heart enzyme, at least, is specific for the straight chain compound, whereas the Ascaris enzyme reacts with both the normal and branched compounds.

In an effort to compare the rates of formation of straight and branched chain intermediates, a variation of the above experiment was performed. In contrast with the above experiment, both acetyl CoA and propionyl CoA were incubated together as substrates for the Ascaris condensing enzyme. As done previously, after deproteinization, the experimental supernatant was isolated from this incubation mixture, which should contain the reduced condensation products. Incubation of an aliquot of this experimental supernatant with the pig heart re-
with these findings, small amounts of n-butyrate and n-valerate. It was observed that the amount of NAD+ reduced was 7 times higher when the control supernatant was incubated with the Ascaris enzyme preparation in place of the pig heart enzyme suggested that the branched chain hydroxyacyl-CoA derivatives constitute the major end products of the reaction. The nature of the branched chain CoA esters formed cannot be determined from the experiment, since products were not isolated. From data presented above, it appears likely that one of the products would be the C4 branched chain intermediate arising from two propionate units. Whether the corresponding C4 branched chain intermediate, which would arise from a C3 + C3 condensation, was present remains to be determined, although 2-methylbutyrate is one of the major Ascaris fermentation products. Also, when propionyl-CoA alone is employed as substrate (Table VIII), the reaction product is not oxidized by NAD+ in the presence of the pig heart L-3-hydroxyacyl-CoA dehydrogenase, indicating the absence of straight chain products unless acetyl-CoA is present.

### Discussion

In addition to Ascaris, whose major end products include 2-methylbutyrate and 2-methylvalerate, branched chain fatty acids with one or more methyl groups at C-2 or other even numbered carbon atoms occur in a wide variety of biological systems. These include humans (32); the lung fluke, Paragonimus westermani, which infects humans (33); dogs (34); water fowl (1, 33); and Streptomyces erythreus (36). In spite of their wide distribution, the mechanism of synthesis of these branched chain acids is not well understood. Recently, Buckner and Kolattukudy (1) reported that a purified fatty acid synthetase from the urypogial glands of the goose synthesized multibranchned fatty acids from methylmalonyl-CoA and normal straight chain acids from malonyl-CoA. All attempts to demonstrate fatty acid synthetase activity in Ascaris have failed, indicating a different synthetic pathway.

The pathway for the synthesis of the volatile branched chain acids in Ascaris also differs from the previously suggested (5, 7) simple reversal of the β oxidation sequence, since at least three of the enzymes that appear to be involved in these.

#### Table VIII

<table>
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<tr>
<th>Enzyme source</th>
<th>Substrate</th>
<th>Presumed conversion assayed</th>
<th>Total nucleotide transformed</th>
<th>NAD+</th>
<th>NADH</th>
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<tr>
<td>Ascaris muscle</td>
<td>Propionyl-CoA in mixture</td>
<td>9-Propionyl-CoA</td>
<td>NADH 3-Hydrox-2-methylvaleryl-CoA</td>
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<td>Pig heart L-3-hydroxyacyl-CoA dehydrogenase</td>
<td>Experimental supernatant</td>
<td>3-Hydroxy straight chain</td>
<td>NAD+ 3-Keto straight chain</td>
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#### Fig. 4

Comparison of the activities of Ascaris 2-methylacetocetate reductase and pig heart L-3-hydroxyacyl-CoA dehydrogenase on the reduced product(s) of the propionyl CoA plus acetyl CoA condensation reaction. Experimental supernatant + enzyme + NAD+; O, experimental supernatant + pig heart L-3-hydroxyacyl-CoA dehydrogenase + NAD+; 0, experimental supernatant + Ascaris mitochondrial-soluble fraction + NAD+. At the arrow the respective enzyme preparations were added. The experimental supernatant was obtained after removal of the precipitated proteins from incubation mixtures that contained propionyl-CoA (10 μmol) plus acetyl-CoA (10 μmol); NADH (12 μmol); potassium phosphate, pH 6.0 (248 μmol); and 4.3 mg of mitochondrial-soluble proteins in a final volume of 3.3 ml. The control supernatant was obtained from incubation mixtures that lacked the acyl-CoA ester substrates. Other experimental conditions, as well as enzyme assays, were as described in Table VIII.

3-hydroxyacyl-CoA dehydrogenase yielded some reduction of NAD+ (Fig. 4). This indicated the presence of one or more straight chain β-hydroxyacyl CoA compounds, since the pig enzyme is specific for the straight chain derivatives. In accord with these findings, small amounts of n-butyrate and n-valerate, but no detectable n-hexanoate, are formed in the whole worm Ascaris fermentation (5, 6). The additional observation that the amount of NAD+ reduced was 7 times higher when the experimental supernatant was incubated with the Ascaris enzyme preparation in place of the pig heart enzyme suggested that the branched chain hydroxyacyl-CoA derivatives constitute the major end products of the reaction. The nature of the branched chain CoA esters formed cannot be determined from the experiment, since products were not isolated. From data presented above, it appears likely that one of the products would be the C4 branched chain intermediate arising from two propionate units. Whether the corresponding C4 branched chain intermediate, which would arise from a C3 + C3 condensation, was present remains to be determined, although 2-methylbutyrate is one of the major Ascaris fermentation products. Also, when propionyl-CoA alone is employed as substrate (Table VIII), the reaction product is not oxidized by NAD+ in the presence of the pig heart L-3-hydroxyacyl-CoA dehydrogenase, indicating the absence of straight chain products unless acetyl-CoA is present.

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The pathway for the synthesis of the volatile branched chain acids in Ascaris also differs from the previously suggested (5, 7) simple reversal of the β oxidation sequence, since at least three of the enzymes that appear to be involved in these...
The propionyl-CoA condensing enzyme appears to exhibit a marked preference for catalyzing the condensation of the carboxyl carbon of one propionyl-CoA moiety with the 2-carbon atom of another propionyl-CoA moiety, giving rise to the C1 branched chain skeleton. The possibility exists that the same enzyme also may catalyze the condensation of acetyl-CoA in a similar manner with the 2-carbon atom of propionyl-CoA to form the corresponding C3 branched chain skeleton, at a slower rate. The condensation of two acetyl-CoA molecules to form the straight chain C3 acid may also be catalyzed by the same enzyme, but only at a much slower rate. These results would agree well with the physiological finding of only very small quantities of n-butyrate in the Ascaris fermentation and would explain why the fermentation products are primarily the branched chain acids. The inhibition of propionyl-CoA condensing enzyme activity by acetyl-CoA indicates that propionyl-CoA and propionyl-CoA condensations may be catalyzed by the same enzyme.

The findings that methylmalonyl-CoA is not only a poor substrate for condensation but also an inhibitor of the Ascaris propionyl-CoA condensing system indicate that propionyl-CoA and not methylmalonyl-CoA is the direct precursor for the synthesis of the branched chain acids in Ascaris. In agreement with this, the product of the propionyl-CoA reaction appears to be the branched chain acyl-CoA, 3-keto-2-methylvaleryl-CoA. This apparent direct involvement of propionyl-CoA, together with the failure to demonstrate fatty acid synthetase activity in Ascaris muscle, indicates that the Ascaris pathway for the synthesis of branched chain acids is different from that proposed for the synthesis of multibranched chain acids by the uropygal glands of the goose (1). Whether this system is unique to Ascaris remains to be determined.

REFERENCES

4224 Fatty Acid Synthesis in Ascaris

2-methylacetooacetate reductase and possible propionyl coenzyme A condensing enzyme activity in branched chain volatile fatty acid synthesis by Ascaris lumbricoides.

Z S de Mata, H J Saz and D J Pasto


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