Function of Carnitine in the Fatty Acid Oxidase-deficient Insect Flight Muscle

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SUMMARY

The metabolic function of carnitine in the fatty acid oxidase-deficient flight muscle of the blowfly, Phormia regina, was investigated. Mitochondria from flies did not oxidize palmitate or hexanoate in the presence of adenosine triphosphate, coenzyme A, carnitine, and serum albumin. Palmitoyl carnitine was oxidized at an extremely slow rate, QO₂ of 20. The rate of synthesis of the carnitine ester was much slower and is rate limiting in fatty acid oxidation. Hexanoyl carnitine was not metabolized. Flight muscle of flies, however, has a concentration of carnitine of over 4 μmoles per g, wet weight. These findings suggest that the primary function of carnitine in blowfly flight muscle is not related to utilization of fats.

Carnitine increased the rate of oxidative decarboxylation of pyruvate-1-¹⁴C to acetyl-CoA. The malonate inhibition of the rate of production of ¹⁴CO₂ from pyruvate-1-¹⁴C, but not from pyruvate-3-¹⁴C, was relieved by carnitine. The rate of oxidation of pyruvate-3-¹⁴C to ¹⁴CO₂ was inhibited by carnitine. These effects of carnitine may be explained by the formation of acetyl carnitine from carnitine and acetyl-CoA, derived from pyruvate, catalyzed by an active carnitine acetyltransferase. Approximately 90% of the enzyme was found in the mitochondria of the muscle. Acetyl-CoA, in the presence of carnitine, was not oxidized by mitochondria, although acetyl carnitine was oxidized with a QO₂ of over 300. This indicates that blowfly mitochondria are not permeable to acetyl-CoA but are permeable to acetyl carnitine. When both pyruvate and acetyl carnitine were available, pyruvate was used preferentially.

The concentration of acetyl carnitine in flight muscle of blowflies increased 4-fold on initiation of flight, paralleling the increase in pyruvate. After about 1 min of flight, the level of acetyl carnitine decreased and attained a steady state at a concentration twice that in the muscle at rest. Functions for carnitine in carbohydrate utilization, resulting from the direct action of carnitine on pyruvate metabolism, are proposed.

A comparative study of the metabolism of carnitine in blowflies and bees is described.

In pioneering experiments, Friedman and Fraenkel (1) showed that liver extracts mediate the readily reversible acyl transfer between coenzyme A and carnitine (3-hydroxy-γ-trimethylammonium butyrate). The physiological significance of this reaction became apparent with the finding of Fritz (2) that carnitine stimulates the rate of oxidation of fatty acids by liver homogenates. This effect was subsequently extended for other tissues and isolated mitochondria (3-7). These and other studies led to the hypothesis (8) that fatty acyl-CoA thioesters do not readily penetrate mitochondrial membranes, whereas fatty acyl carnitine esters do, and that the formation of carnitine esters by acyl transferases effects the translocation of fatty acyl groups to the site of fatty acid oxidation.

Additional support for the importance of carnitine in fatty acid metabolism came from findings that the oxidation of long chain fatty acids by mitochondria from various skeletal muscles is completely dependent on carnitine (6, 8), and that high activities of carnitine transacetylase in mitochondria from various tissues are correlated with the requirement of carnitine esters for fatty acid oxidation (9). Particularly striking are the differences in transacetylase activity between the flight muscle of two insects, those of the locust, which oxidize fatty acids, and those of the bee, which utilize only carbohydrates in flight. The enzyme is absent from flight muscle of the bee, whereas it is very active in locust flight muscle. This would seemingly suggest that carnitine is of little significance to the metabolism of flight muscle of insects, such as bees and flies, which utilize carbohydrates and not fatty acids during flight (10, 11). On the other hand, it is known that flight muscle of flies is one of the richest sources of carnitine (12). Thus the question to the metabolic function of carnitine in this fatty acid oxidase-deficient tissue prompted the present study. It has been found that carnitine affects carbohydrate utilization, via a role in pyruvate metabolism, in both isolated mitochondria from flight muscle of the blowfly, Phormia regina, and in the flying insect, especially during the transition from rest to rapid contraction.

A preliminary account of portions of this investigation has been reported in abstract form (13).

EXPERIMENTAL PROCEDURE

Sodium pyruvate and L-carnitine HCl were obtained from Calbiochem. Uniformly labeled palmitic acid-¹⁴C was purchased from Applied Science Laboratories. Carnitine-methyl-¹⁴C was supplied by TraceLab. Sodium pyruvate-1-¹⁴C, sodium pyruvate-2-¹⁴C, and sodium pyruvate-3-¹⁴C were obtained from New England Nuclear, were checked for purity on Dowex 1-Cl⁻, and
were immediately stabilized by addition of an equivalent amount of HCl (14).

Acetyl, hexanoyl, and palmitoyl carnitines were synthesized as described by Bremer (15, 5), except that, following neutralization of the reaction mixture, the palmitoyl carnitine was extracted with butanol. The butanol extract was evaporated to dryness under reduced pressure. Palmitoyl carnitine was then redissolved in a small volume of warm butanol and precipitated by addition of several volumes of ether. The product was subsequently recrystallized from butanol-ether and several times from water. Acetyl carnitine, m.p. 186°, hexanoyl carnitine, m.p. 145°, and palmitoyl carnitine, m.p. 141°, all reacted positively in the Hestrin test (1). The Hestrin reaction for palmitoyl carnitine was carried out in ethanol because of the insolubility of palmitoyl hydroxamate. Infrared absorption spectra of the acyl carnitines showed strong absorption bands at 5.76 μ, characteristic of an ester linkage, and little or no absorption between 2.8 and 2.9 μ, indicating the absence of a free hydroxyl group. Palmitoyl carnitine showed additional peaks at 3.42 and 3.54 μ, indicative of the presence of a long chain alkane group.

Acetyl-14C carnitine was synthesized enzymatically from pyruvate-2-14C and carnitine by flight muscle mitochondria. The ester was isolated by HCl elution from Dowex 50-H+ and further purified by paper chromatography and recrystallization from ethanol-acetone-ether, following addition of carrier acetyl carnitine to the desired specific activity.

Uniformly labeled palmitoyl-14C carnitine was synthesized enzymatically according to Bremer (16), except that rat liver mitochondria were used. The reaction was stopped with HCl and the reaction mixture was extracted several times with ether. The product was then extracted with butanol and taken to dryness. Carrier palmitoyl carnitine was added and the acyl carnitine was purified on a silicic acid column, followed by paper chromatography and recrystallization from ethanol-acetone-ether, following addition of carrier acetyl carnitine to the desired specific activity. Incorporation of radioactivity from pyruvate into acetyl carnitine by flight muscle mitochondria was measured by passing the neutralized perehloric acid extract of the reaction mixture through a column of Dowex 50-H+ X4, 0.8 x 5 cm, 200 to 400 mesh, followed by 30 ml of H2O. Acetyl carnitine was eluted enzymatically according to Bremer (16), except that rat liver mitochondria with 20 ml of 0.3 M succrose, 1.0 μM EDTA, 10 mM Tris-HCl (pH 7.4), and the reaction mixture was extracted several times with ether. Acetyl carnitine was purified on a silicic acid column, followed by paper chromatography and recrystallization from ethanol-acetone-ether, following addition of carrier acetyl carnitine to the desired specific activity.

Incorporation of radioactivity from pyruvate into acetyl carnitine by flight muscle mitochondria was measured by passing the neutralized perchloric acid extract of the reaction mixture through a column of Dowex 50-H+ X4, 0.8 x 5 cm, 200 to 400 mesh, followed by 30 ml of H2O. Acetyl carnitine was eluted with 20 ml of 0.3 N HCl and lyophilized. The residue was dissolved in 1.0 ml of H2O and chromatographed on Whatman No. 3 paper, 18 hours descending, in methylethylketone-isopropanol-0.1 N HCl (90:30:20) (18). The paper was cut into 1-cm strips and placed in counting vials. Radioactivity was measured in a Tri-Carb liquid scintillation counter following the addition of 0.5 ml of hyamine hydroxide and 10 ml of toluene containing 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene and 0.4% 2,5-di-phenyloxazole. Essentially all the radioactivity on the paper chromatogram was present in acetyl carnitine which cochromatographed with standards. The identity of the product was confirmed by recrystallization to constant specific activity following addition of carrier acetyl carnitine.

The rates of palmitoyl carnitine synthesis and carnitine-palmitoyl carnitine exchange were estimated by measuring the incorporation of radioactivity from 14C-carnitine into palmitoyl carnitine (17). The latter compound was extracted from the reaction mixture with butanol and counted.

Fatty acid-activating enzyme (19) and carnitine acetyl transferase (20) were prepared as described. Carnitine was assayed by the method of Pearson and Tubbs (21) and acetyl carnitine by the procedure of Marquis and Fritz (22). It was found to be advantageous to include 5 mM EDTA in the assay reactions to overcome inhibition of the transferase by the perchloric acid extracts.

Mitochondrial protein was estimated by the biuret method (23) on the trichloroacetic acid-precipitated material from an aliquot of the mitochondrial suspension. Oxygen uptake was measured polarographically with the Clark oxygen electrode.

Blowflies, Phormia regina, were reared and maintained in laboratory culture as reported (24). Bees, Apis mellifera, were generously provided by George Abrams, Entomology Department, University of Maryland, and Charles Fries, Magnolia, Maryland.

Flight muscle mitochondria from blowflies were isolated as described previously (25), except that two different isolation media were used. Mitochondria isolated by Procedure A, in 0.25 M sucrose, 1.0 mM EDTA, and 50 mM Tris-HCl (pH 7.4), do not oxidize pyruvate maximally in the absence of added proline, a precursor of oxaloacetate (26). Isolation by Procedure B, in 0.25 M sucrose, 1.0 mM EDTA, 10 mM Tris-HCl (pH 7.4), 0.5% bovine serum albumin, yields mitochondria which are not dependent upon added sparkers for maximal pyruvate oxidation. Bee flight muscle mitochondria were isolated by Procedure B.

The method for measuring concentrations of metabolites in flight muscle during flight was as described (27).

**RESULTS**

The concentration of carnitine in flight muscle of the blowfly is approximately 4.2 μmoles per g, wet weight (Table I), a value in agreement with that in the housefly (12). Essentially all of the carnitine was found in the 10,000 x g supernatant fraction, in accord with the observations of Frenkel (12) and Marquis and Fritz (22) for housefly flight muscle and rat heart, respectively. In view of the permeability of blowfly mitochondria to carnitine, however, the possibility that intramitochondrial carnitine leaked into the washing during isolation of the mitochondria has not been precluded. Carnitine was not detected in the thorax of the bee, indicating a level less than 0.02 μmoles per g, wet weight.

The inability of blowfly mitochondria to oxidize fatty acids at appreciable rates in vitro is shown in Table II. No oxygen uptake was found with free palmitate or hexanoate, in agreement

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**TABLE I**

Concentration and subcellular distribution of carnitine in flight muscle

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Carnitine concentration μmoles/g, wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blowfly</td>
<td></td>
</tr>
<tr>
<td>Whole thorax</td>
<td>4.2</td>
</tr>
<tr>
<td>10,000 x g residue</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>10,000 x g supernatant</td>
<td>4.3</td>
</tr>
<tr>
<td>Bee</td>
<td></td>
</tr>
<tr>
<td>Whole thorax</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

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with the failure of fly muscle homogenates to metabolize free fatty acids (11). Addition of ATP, CoA, and carnitine, singly or in combination, or bovine serum albumin to the mitochondrial system did not increase the respiratory rate in these short term polarographic measurements. Palmitoyl carnitine is oxidized at an extremely low rate, a specific activity of 0.040 pmole per mg of protein per hour, corresponding to a QO, of 20. For comparison, the QO, value for pyruvate is about 650 (26), while that for endogenous substrate is from 5 to 10. In contrast to the oxidation of palmitoyl carnitine, however slow, no evidence was found for the oxidation of hexanoyl carnitine, suggesting acyl specificity by the fatty acyl carnitine transferase. Bee flight muscle mitochondria, in contradistinction to those of the blowfly, did not oxidize any of the fatty acyl carnitine esters or the free fatty acids.

The activity of the mitochondrial CoA-dependent carnitine-palmitoyl carnitine exchange reactions

\[
Palmoyl carnitine + CoA \rightleftharpoons \text{palmitoyl-CoA} + \text{carnitine}
\]

\[
Palmoyl-CoA + carnitine-^{14}C \rightleftharpoons \text{palmitoyl carnitine-^{14}C} + \text{CoA}
\]

is adequate to account for the rate of palmitoyl carnitine oxidation (Table II). The rate of formation of palmitoyl carnitine by the reaction sequence

\[
\text{Palmitate + CoA + ATP} \rightarrow \text{palmitoyl-CoA} + \text{AMP} + PP_i
\]

\[
Palmoyl-CoA + carnitine-^{14}C \rightleftharpoons \text{palmitoyl carnitine-^{14}C} + \text{CoA}
\]

is only 4% of the rate of palmitoyl carnitine oxidation, indicating that the rate-limiting step in fatty acid oxidation is prior to the mitochondrial oxidation of carnitine ester, in agreement with recent findings with rat liver mitochondria (28). Preliminary efforts to differentiate the rate of palmitoyl-CoA synthesis from that of the extramitochondrial palmitoyl transfer from palmitoyl-CoA to carnitine have been hampered by the finding that the enzymatic activities in flight muscle are less than those required by the sensitivities of the current methods of assay.

Since the data in Tables I and II strongly indicate that the primary function of carnitine in blowfly flight muscle is not related to fatty acid oxidation, a role for carnitine was sought in the metabolism of pyruvate, the end product of aerobic glycolysis in this tissue (27). Carnitine was found to increase the rate of pyruvate decarboxylation of pyruvate-1-14C to acetyl-CoA (Table III). Malonate, which inhibits the utilization of acetyl-CoA via the Krebs cycle, decreased the rate of formation of \(^{14}CO_2\) from pyruvate-1-14C (Table III), presumably by affecting pyruvate decarboxylase by increasing the concentration of acetyl-

### Table II

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation of palmitate</td>
<td>0.000</td>
</tr>
<tr>
<td>Oxidation of palmitate + ATP + CoA + carnitine</td>
<td>0.000</td>
</tr>
<tr>
<td>Oxidation of palmitoyl carnitine</td>
<td>0.040</td>
</tr>
<tr>
<td>Formation of palmitoyl carnitine</td>
<td>0.002</td>
</tr>
<tr>
<td>Carnitine palmitoyl carnitine exchange</td>
<td>0.050</td>
</tr>
<tr>
<td>Oxidation of hexanoyl carnitine</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Values represent the average of at least three determinations.

### Table III

**Effect of carnitine upon pyruvate oxidation by flight muscle mitochondria**

Reaction media contained 10 mm ADP, 5 mm MgCl₂, 25 mm KPi, 5 mm \(^{14}C\)-pyruvate (150,000 cpm per \(\mu\)mole), 10 mm Tris (pH 7.4), and approximately 2 mg of mitochondrial protein in a total volume of 1 ml. Carnitine and malonate concentrations were 20 mm. Incubations were at 30° for 10 min with shaking. \(^{14}CO_2\) was measured by the method of Cuppy and Crevasse (29). Values represent the mean ± standard deviation for four or more determinations by the use of a different mitochondrial preparation in each replicate. Procedures A and B refer to the method of isolating mitochondria. These are described in the text. Mitochondria isolated by Procedure B oxidize pyruvate at a maximal rate in the absence of added primer. Mitochondria isolated by Procedure A oxidize pyruvate at a slower rate and are deficient in endogenous Krebs cycle intermediates or their precursors (26).
CoA relative to that of CoA, as suggested by Garland and Randle (30). Carnitine completely relieved this inhibition. On the other hand, carnitine did not reverse the malonate inhibition of the rate of $^{14}$CO$_2$ production from acetyl-CoA derived from pyruvate-3-$^{14}$C (Table I). Carnitine also slowed the rate of conversion of pyruvate-3-$^{14}$C, or pyruvate-2-$^{14}$C, to $^{14}$CO$_2$. This inhibition of acetyl-CoA utilization via the Krebs cycle is more evident with mitochondria isolated by Procedure A. These mitochondria are deficient in endogenous Krebs cycle intermediates or their precursors (26). This suggests that carnitine competes more successfully with oxaloacetate for the acetyl-CoA derived from pyruvate under conditions of lower concentrations of intramitochondrial tricarboxylic acid cycle intermediates and, if so, may resolve the discrepancy between the results of Bressler and Katz (31) and Davis (32) on the effect of carnitine on pyruvate-2-$^{14}$C metabolism.

The effects of carnitine, as described in Table III, may be explained by the formation of acetyl carnitine from carnitine and acetyl-CoA, derived from pyruvate, catalyzed by carnitine acetyl transferase (15). Table IV shows the presence of the transferase in blowfly flight muscle. Most of the enzyme is localized in the mitochondria. The specific activity of the transferase in blowfly flight muscle mitochondria is the same as that reported by Marquis and Fritz (22) for rat heart and skeletal muscle mitochondria. Flight muscle from the bee contains no carnitine acetyl transferase activity, confirming the finding of Beenaakkers and Klingenberg (9). The absence of the enzyme in the bee muscle is consistent with the failure of carnitine to affect pyruvate metabolism in this tissue (Table III).

Table V shows, for a representative experiment, the stoichiometric relationship between utilization of pyruvate and formation of acetyl carnitine. Carnitine increased the decarboxylation of pyruvate-1-$^{14}$C. Presumably, the 0.88 mmole of additional pyruvate converted to acetyl-CoA in the presence of carnitine is indicative of the synthesis of acetyl carnitine in an equivalent amount. At the saturating concentration of carnitine used in the experiment, carnitine also inhibited the production of $^{14}$CO$_2$ from pyruvate-2-$^{14}$C by 0.60 mmole. Assuming that this difference is also accounted for by formation of acetyl carnitine, the sum of the values for the increased decarboxylation of pyruvate and the decreased utilization of acetyl-CoA via the Krebs cycle results in a theoretical yield of acetyl carnitine of 1.48 mmoles. In reasonably good agreement with this value, 1.06 mmoles of acetyl carnitine were found. The value of 1.06 mmoles was not corrected for an approximate 30% loss in the recovery of an equivalent quantity of authentic acetyl carnitine added as an internal standard to the perchloric acidified reaction medium. Taking this loss into account, the acetyl carnitine formed in the experiment illustrated in Table V would be 1.5 mmoles, in excellent agreement with the theoretical amount. The data also show that, with the conditions used here, 25 to 40% of the pyruvate metabolized by the mitochondria was converted to acetyl carnitine.

The respiratory activity of blowfly flight muscle mitochondria with acetate, acetyl carnitine, and pyruvate as substrates is presented in Table VI. No oxygen uptake was found with free acetate, or with acetate plus CoA and ATP, in these polarographic measurements. In manometric experiments lasting 1 hour, a respiratory rate slightly greater than that with endogenous substrates was seen with acetate (11). Acetyl-CoA by itself or with added carnitine did not show any oxygen uptake.

### Table IV

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Enzyme</th>
<th>Specific activity</th>
<th>% total</th>
<th>$^{14}$C-acetyl carnitine formed from pyruvate-3-$^{14}$C</th>
<th>Theoretical</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blowsfly</td>
<td>10,000 × g residue</td>
<td>..................</td>
<td>89</td>
<td>25.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bee</td>
<td>10,000 × g residue</td>
<td>..................</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Measured in supernatant following extraction of the residue with 0.1 M KPi and 0.1% deoxycholate (pH 8.1). Following fractionation of the tissue by the use of the medium described by Procedure B, enzyme activity was measured by the coupled spectrophotometric assay described by Marquis and Fritz (22). The value shown for specific activity represents the mean of two determinations, and was calculated on the basis of the original protein content of the mitochondria rather than on the protein content of the deoxycholate extract of the mitochondria.

### Table V

<table>
<thead>
<tr>
<th>Additions</th>
<th>$^{14}$CO$_2$ formed</th>
<th>Increase in pyruvate decarboxylation</th>
<th>Decrease in acetyl-CoA utilization via tricarboxylic acid cycle</th>
<th>$^{14}$C-Acetyl carnitine formed from pyruvate-3-$^{14}$C</th>
<th>Theoretical</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate-1-$^{14}$C</td>
<td>2.52</td>
<td>0.00</td>
<td>-1.52</td>
<td>0.88</td>
<td>1.52</td>
<td>1.06</td>
</tr>
<tr>
<td>Pyruvate-1-$^{14}$C + carnitine</td>
<td>3.40</td>
<td>0.88</td>
<td>-2.52</td>
<td>1.89</td>
<td>2.52</td>
<td>1.89</td>
</tr>
<tr>
<td>Pyruvate-3-$^{14}$C</td>
<td>1.89</td>
<td>0.60</td>
<td>-1.29</td>
<td>0.88</td>
<td>1.29</td>
<td>1.48</td>
</tr>
<tr>
<td>Pyruvate-3-$^{14}$C + carnitine</td>
<td>1.29</td>
<td>0.60</td>
<td>-1.69</td>
<td>1.06</td>
<td>1.29</td>
<td>1.06</td>
</tr>
</tbody>
</table>

### Table VI

<table>
<thead>
<tr>
<th>Substrate</th>
<th>O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>0</td>
</tr>
<tr>
<td>Acetate + CoA + ATP</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA + carnitine</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl carnitine</td>
<td>330</td>
</tr>
<tr>
<td>Acetyl carnitine + carnitine</td>
<td>220</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>655</td>
</tr>
<tr>
<td>Pyruvate + carnitine</td>
<td>655</td>
</tr>
<tr>
<td>Pyruvate + acetyl carnitine</td>
<td>655</td>
</tr>
</tbody>
</table>
Simultaneous utilization of pyruvate and of acetyl carnitine by blowfly flight muscle mitochondria

Reaction conditions were as described in Table III. Concentrations of pyruvate and of acetyl carnitine were 5 mm. The values shown are data from one representative experiment.

<table>
<thead>
<tr>
<th>Additions</th>
<th>$^{14}$CO$_2$</th>
<th>$^{14}$CO$_2$ obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles/mg protein/20 min</td>
<td>% total</td>
</tr>
<tr>
<td>Pyruvate-$^{14}$C</td>
<td>0.98</td>
<td>100</td>
</tr>
<tr>
<td>Pyruvate-$^{14}$C + unlabeled acetyl carnitine</td>
<td>0.59</td>
<td>60 40</td>
</tr>
<tr>
<td>Acetyl-$^{14}$C carnitine</td>
<td>1.32</td>
<td>100</td>
</tr>
<tr>
<td>Acetyl $^{14}$C carnitine + unlabeled pyruvate</td>
<td>0.28</td>
<td>79 21</td>
</tr>
<tr>
<td>Pyruvate-$^{14}$C + acetyl-$^{14}$C carnitine</td>
<td>1.17</td>
<td></td>
</tr>
</tbody>
</table>

In contrast, acetyl carnitine is one of the best respiratory substrates for blowfly flight muscle mitochondria (Table VI); the carnitine ester is inactive with bee mitochondria, however.

Table VI also shows that the respiratory rate with the combination of pyruvate and acetyl carnitine is not greater than with pyruvate by itself. In isotopic experiments (Table VII), the presence of both substrates gave about the same rate of $^{14}$CO$_2$ production as either substrate when present alone, indicating additionally that the utilization of acetyl-CoA via the Krebs cycle is rate limiting. When both substrates are available however, pyruvate is utilized preferentially. Approximately 70 and 30% of the total CO$_2$ were derived from pyruvate and acetyl carnitine, respectively (Table VII), perhaps suggesting that acetyl-CoA originating from pyruvate inhibits the conversion of acetyl carnitine to acetyl-CoA or that the acetyl-CoA derived from pyruvate is more available to citrate synthetase. An “acetyl carnitine-sparing” effect of pyruvate was proposed by Bremer (15) for mammalian mitochondria. Table VI shows that in short term (1 to 2 min) measurements of respiratory rates carnitine did not inhibit the rate of pyruvate oxidation. When one considers that the total oxygen uptake before the reaction medium became anaerobic is only sufficient to oxidize 5% of the added pyruvate to acetyl-CoA, then the lack of an effect of carnitine in this kind of experiment is understandable. Carnitine did inhibit the rate of oxidation of acetyl carnitine, however, which is in accord with the readily reversible nature of the carnitine acetyl transferase.

Changes in the concentrations of metabolites in flight muscle in situ during the stationary flight of the blowfly were reported (27, 33). On initiation of flight, the concentration of pyruvate in the muscle increases. Fig. 1 shows that the concentration of acetyl carnitine in flight muscle also increased in flight, with the transition from a resting to a rapidly contracting muscle. The 4-fold increase in acetyl carnitine parallels the 4-fold increase in pyruvate. After about 1 min of flight, the levels of both pyruvate and acetyl carnitine decreased and then attained a steady state at concentrations about twice that in the muscle at rest.

**DISCUSSION**

The importance of carnitine in fatty acid oxidation has been recognized since the report of Frits (2). The observations of Beenakkers and Klingenberg (9) that flight muscle of locusts, which uses fat for flight, has an active carnitine transacetylase, whereas the enzyme is absent from flight muscle of bees, which metabolizes only carbohydrates during flight, reinforced this view. The lack of carnitine-transferring enzymes in the bee has now been confirmed (Table III), and this void is consonant with the absence of carnitine in the thorax of this species (Table I). The suggested correspondence between carnitine and carnitine-transferring enzymes, on the one hand, and fatty acid metabolism, on the other (9), fails, however, in light of the findings presented here with the blowfly. The blowfly, which like the bee does not use fats in flight (10, 11) and has a paucity of enzymes concerned with fatty acid oxidation (Table II), has an abundance of carnitine (Table I) and an active acetyl carnitine transferase (Tables III and IV). Conversely, the evidence developed in this paper indicates that carnitine has a significant function in carbohydrate utilization by affecting pyruvate metabolism, at least in the blowfly.

The data in Tables III and V as well as in Fig. 1 show that acetyl carnitine is formed from pyruvate by mitochondria in vitro as well as by working muscle in vivo. Thus the question arises as to the physiological significance of acetyl carnitine formation in the fly. It has been shown (27) that on initiation of flight glycolytic flux increases many times and 2 eq of pyruvate are formed for each equivalent of hexose metabolized, as indicated by the fact that neither $\alpha$-glycerophosphate nor lactate accumulates. During this initial phase of flight, pyruvate is generated at a rate faster than it is utilized via the Krebs cycle, apparently because of a deficiency in oxaloacetate or its immediate precursors (27, 26). A large share of the pyruvate is transaminated to alanine. Coincidently, proline is converted to $\alpha$-ketoglutarate, via glutamate, generating oxaloacetate via the tricarboxylic acid cycle and relieving the deficiency (27). The formation of acetyl carnitine during this critical period, in which
pyruvate is not metabolized by the Krebs cycle at a maximal rate, can be of advantage to the blowfly in the following possible ways.

1. Garland and Randle (30) have suggested that pyruvate decarboxylation is inhibited by an increase in concentration of acetyl-CoA relative to that of free CoA. In the absence of adequate concentrations of oxaloacetate necessary for maximal rates of citrate synthesis, the acetyl-CoA to CoA ratio should increase and inhibit the oxidation of pyruvate. By serving as an acceptor for acetyl groups from acetyl-CoA, carnitine would lower the acetyl-CoA to CoA ratio. This would permit the continuous formation of acetyl-CoA from pyruvate, part of which can then condense with oxaloacetate as the dicarboxylic acid becomes available. Results in vitro (Table III), on the action of the malonate in decreasing the concentration of oxaloacetate and in inhibiting the deacetylation of pyruvate-1 C, and the reversal of this effect by carnitine are analogous to and in accord with this proposed function in vivo of carnitine.

2. Hülsmann et al. (34) have shown that pyruvate inhibits the oxidation of α-ketoglutarate to succinate. This inhibition has also been attributed to a high acetyl-CoA to CoA ratio, resulting in a shortage of free CoA which is necessary for the oxidation of α-ketoglutarate. The inhibition of α-ketoglutarate oxidation by pyruvate is relieved by carnitine (34). This action of carnitine in regenerating CoA and maintaining a low acetyl-CoA to CoA ratio may be of special significance in flight muscle because inhibition of α-ketoglutarate oxidation would prevent the conversion of proline to oxaloacetate. Oxaloacetate formed in this manner is necessary to prime the metabolism of pyruvate via the citric acid cycle at a maximal rate.

3. On initiation of flight, ATP for the contractile process may be in short supply because of low levels of arginine phosphate (33) and because only part of the pyruvate generated by glycolysis can be in short supply because of low levels of arginine phosphate (33).

4. Transamination of pyruvate to alanine on induction of flight is wasteful with respect to carbohydrate-derived carbon for the immediate metabolic needs of the flight muscle because alanine is used by flight muscle during flight only at a slow rate, if at all (27). In contrast, conversion of pyruvate to α-ketoglutarate provides an auxiliary store of “active acetate,” which can be readily transacetylated back to acetyl-CoA for subsequent oxidation (Table VI and Fig. 1). The amount of acetyl carnitine “stored” on initiation of flight, however, is small relative to the amount of alanine accumulated (27).

The inability of blowfly mitochondria to oxidize acetyl-CoA in the presence of carnitine (Table VI) indicates that the mitochondrial membrane is not permeable to the thioester and that the mitochondrial carnitine acetyl transferase does not transfer acetyl groups from extramitochondrial acetyl-CoA to carnitine and, thus, into the mitochondria. Instead, the data (Table V) suggest that the mitochondrial enzyme mediates the transfer of acetyl groups out of the mitochondria although the exit of acetyl carnitine is not essential to the proposed functions of carnitine. On the other hand, the apparent presence of about 10% of the carnitine acetyl transferase activity in the extramitochondrial fraction of the muscle (Table IV) may permit the extramitochondrial acetylation of carnitine, with subsequent transport of the acetyl carnitine into the mitochondria. Also, the experiments reported here in no way rule out the possibility that carnitine functions in intracellular transacetylation reactions associated with cellular processes other than those suggested above on the energetics of flight muscle metabolism.

The striking difference in carnitine metabolism between flies and bees, both of which use carbohydrates and neither of which metabolize fat during flight, serves as an interesting comparative study. An explanation of the difference is not evident at this time. A possible clue in resolving this apparent enigma may be seen in the different effects of malonate on pyruvate metabolism in the two species (Table III). In flies, malonate (20 mM) nearly completely inhibited 14CO2 production from pyruvate-3-14C, indicating a relatively low concentration of endogenous succinate or its precursors in fly mitochondria. In contrast, in bees, the same concentration of malonate inhibited pyruvate-3-14C metabolism in the citric acid cycle by less than 50%. Assuming no difference between the mitochondria of the two species with respect to permeability to malonate, the reduced effectiveness of malonate in bees suggests a larger endogenous content of Krebs cycle intermediates in bee mitochondria. If this is the case, then carnitine would not be required in bees to assure that pyruvate is oxidized at a maximal rate via the Krebs cycle on initiation of flight.

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