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Title: 3-hydroxyacyl-CoA dehydrogenase activity in fasted crayfish (*Orconectes virilis*) hepatopancreas and flexor muscle tissues.

Abstract Approved: Gaylen Uglow

Fatty acids are a major energy source in animal cells. Before fatty acids can be used as an energy source, they must be acylated in the cytosol and oxidized in the mitochondria. This oxidation pathway in the mitochondria is β-oxidation. The enzyme 3-hydroxyacyl-CoA dehydrogenase (HADH) catalyzes the third step of β-oxidation. HADH activity was measured in crayfish, *Orconectes virilis*, to determine the effect of starvation. The samples were taken from the hepatopancreas and muscle of fed, three day fasted, five day fasted, and seven day fasted crayfish. The hepatopancreas and muscle tissue from each animal were homogenized and centrifuged to separate cytosolic and mitochondrial fractions. The HADH activity in the cytosol

and mitochondria was measured. The results showed that no detectable activity in the cytosolic fraction. This suggested that HADH was not a cytosolic enzyme. The HADH activity in the hepatopancreas mitochondrial fractions showed inconclusive differences in the activity level among fasting periods. The mitochondrial fraction in muscle tissue, however, showed a noticeable increase in HADH activity with the length of the fasting period.

Key words: 3-hydroxyacyl-CoA dehydrogenase; β-oxidation; hepatopancreas; *Orconectes virilis*.

3-HYDROXYACYL-COA DEHYDROGENASE ACTIVITY IN FASTED CRAYFISH (*ORCONECTES VIRILIS*) HEPATOPANCREAS AND FLEXOR MUSCLE TISSUES.

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Preface

This thesis was prepared following the publication style of *Comparative Biochemistry and Physiology*.

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INTRODUCTION

Fatty acids are a major energy source in animal cells. The major pathway for fatty acid catabolism and β-oxidation occurs in mitochondria. To yield energy from fatty acids, saturated fatty acids have to be transferred into the mitochondria by carnitine palmitoyl transferase (CPT) before they are broken down and converted into acetyl-CoA units. In mitochondria, the fatty acyl-CoA is cleaved at the β carbon atom and yields a two-carbon fragment in each β-oxidation cycle. This is repeated until the entire fatty acyl-CoA is broken down into acetyl-CoA.

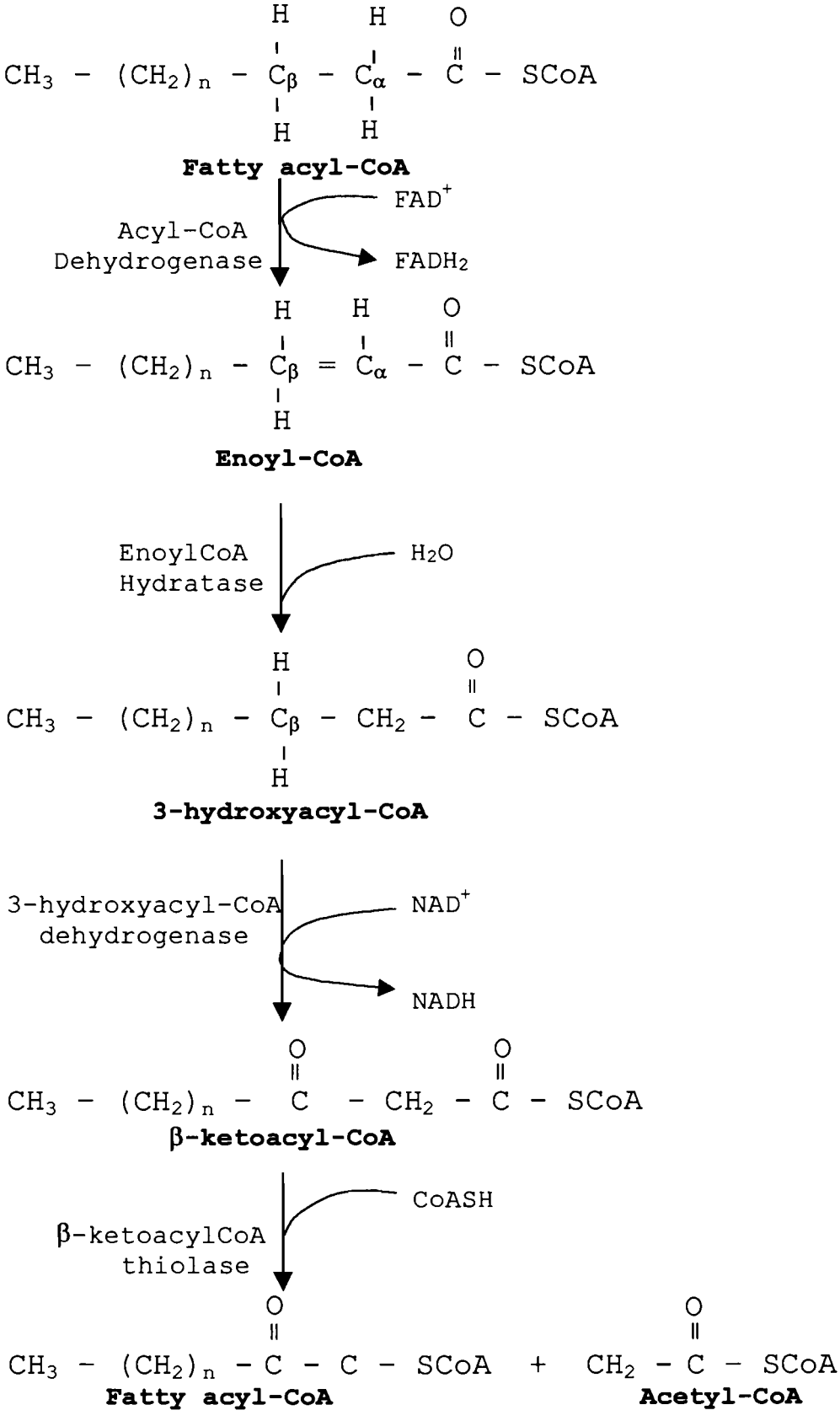
The concept of β-oxidation of fatty acids was first proposed by Franz Knoop in 1904. A benzene ring was attached as a label to the ω (last) carbon of the fatty acid hydrocarbon chain which allowed the metabolic pathway to be traced. In the 1950s, the enzymes involved in fatty acid oxidation were isolated and the enzymatic reactions were verified, confirming Knoop's hypothesis (Voet and Voet, 1990).

There are four enzymes involved in β-oxidation; acyl-CoA dehydrogenase, enoyl-CoA hydratase,

3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (Fig. 1). The β-oxidation enzymes found on the inner mitochondrial membrane are the same as the tricarboxylic acid (TCA) cycle enzymes (Sumegi and Srere, 1984). The first three reactions are very similar to the conversion of succinate to oxaloacetate in the TCA cycle. Both succinate dehydrogenase in the TCA cycle and acyl-CoA dehydrogenase in β-oxidation reduce FAD^+ to $FADH_2$. Malate dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase reduce NAD^+ to NADH. $FADH_2$ and NADH can enter the electron transport chain reaction (Osumi and Hashimoto, 1980; Krahenbuhl et al., 1994). The last reaction of β-oxidation cleaves an acetyl-CoA that can enter the TCA cycle, and newly formed acyl-CoA repeats the cycle. Thus, fats are very efficient in the storage of chemical energy (Karp, 1996).

During starvation, animals need to use energy from catabolized fatty acids after exhausting primary fuels. High levels of glucagon together with low levels of blood glucose trigger the release of fatty acids from adipose tissue (Voet and Voet, 1990). The free fatty acids immediately bind to albumin in the plasma, resulting in

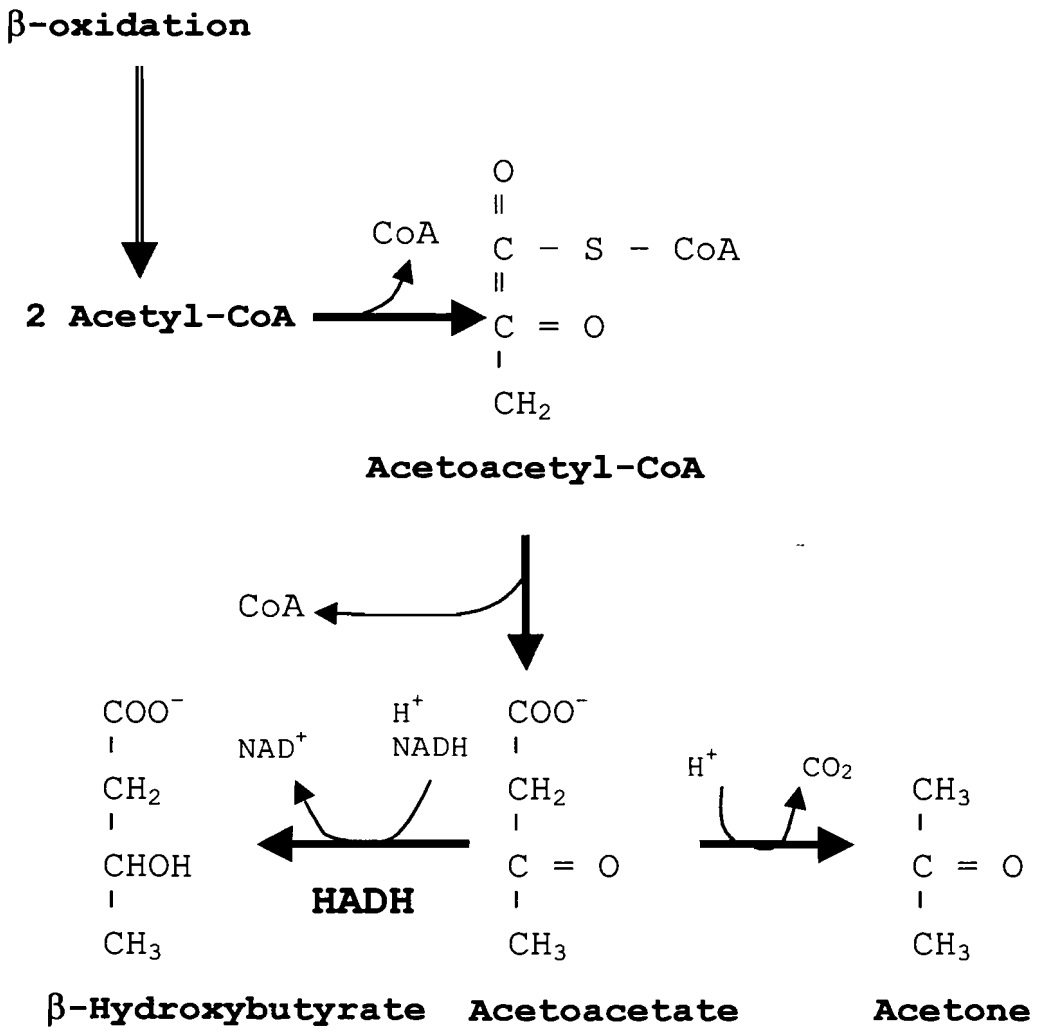
Fig. 1 The β-oxidation pathway of fatty acyl-CoA.


Fatty acyl-CoA
FAD+
Acyl-CoA Dehydrogenase
FADH2
Enoyl-CoA
EnoylCoA Hydratase
H2O
3-hydroxyacyl-CoA
3-hydroxyacyl-CoA dehydrogenase
NAD+
NADH
β-ketoacyl-CoA
CoASH
β-ketoacylCoA thiolase
Fatty acyl-CoA
Acetyl-CoA

their transfer to tissues where fatty acids are used as energy (Champe and Harvey, 1994). Fasting studies have been performed in many systems including rats (Drynan et al., 1996), dogfish (Conlon et al., 1994), carp (Segner et al., 1997), snails (Stuart and Ballantyne, 1996), and plants (Dieuaide et al., 1993). Since most of the β-oxidation product, acetyl-CoA, is converted to ketone bodies in fasted animals (Bremer and Wojtczak, 1972), detecting the activity of the ketone body catalyzing enzyme, β-hydroxybutyrate dehydrogenase (HBDH), is a common way to measure the β-oxidation rate. Ketone body formation is shown in Fig. 2.

On the other hand, McGarry and Foster (1981) suggested that the regulation of CPT by malonyl-CoA controls the rate of fatty acid oxidation in mitochondria. When the malonyl-CoA concentration is high, the cell will go through fatty acid synthesis to store energy in fat. However, a low concentration of malonyl-CoA triggers the increase of CPT so that the fatty acid can enter the mitochondria and start the β-oxidation process. The concept that CPT is a major control of fatty acid oxidation is accepted widely and many scientists use CPT to measure the β-oxidation rate. Thus,

Fig. 2 The formation of ketone bodies.


β-oxidation
2 Acetyl-CoA
CoA
O
C – S – CoA
C = O
CH2
Acetoacetyl-CoA
CoA
COO⁻
CH2
CHOH
CH3
NAD+
H+
NADH
HADH
COO⁻
CH2
C = O
CH3
H+
CO2
CH3
C = O
CH3
β-Hydroxybutyrate
Acetoacetate
Acetone

malonyl-CoA is considered a "rate- limiting" enzyme. However, it is also well established that the steps of the β-oxidation pathway can be rate-limiting for hepatic mitochondrial fatty acid metabolism (Krahenbuhl et al., 1994). Eaton et al. (1994) suggested that inhibition of respiratory-chain activity at complex I, where HADH is linked to the respiratory chain via NAD^+/NADH, may also cause further inhibition of β-oxidation. Stewart et al. (1992) reported that neither CPT nor HBDH were detected in scallops or lobsters, but HADH was detected. These findings suggest HADH can be used for analysis of β-oxidation in cases when CPT or HBDH are not detectable.

The third enzyme of β-oxidation, 3-hydroxyacyl-CoA dehydrogenase (HADH) has been studied in many animals. Hepatic tissues from rat, pig, and cow are often used for studies of the disease caused by a deficiency of this enzyme. There are three kinds of HADH: short-, medium-, and long-chain. Each type of HADH can cleave a different length of fatty acyl-CoA, and long-chain HADH has been associated with fatty acid oxidation deficiency (Pollitt, 1995). Short-chain HADH has been well studied in pig heart muscle (Noyes and Bradshaw, 1973b) and in rat liver (Osumi

and Hashimoto, 1980) for structure and function work. However, HADH studies in non-mammals have been rather rare, especially under fasted conditions. Since fatty acids are a secondary fuel in well-fed animals (Champe and Harvey, 1994), any depletion of the primary energy source, carbohydrate, should increase fatty acid catabolism.

Several crustaceans including crabs (Schatzlein et al., 1973) and lobsters (Stewart et al. 1992) have been examined for energy production profiles in well-fed states. Stuart and Ballantyne (1996) reported that 16.6% of HADH activity was detected from the cytosolic fraction of the hepatopancreas of terrestrial snails. The cytosolic fraction of mammalian tissues does not contain HADH unless there is peroxisomal or mitochondrial contamination. However, there are several reports that point out the major difference between mammals and non-mammals in their metabolic response (Segner et al., 1997; Boulton and Huggins, 1970).

In this study, effects of carbohydrate deprivation on short-chain 3-hydroxyacyl-CoA dehydrogenase activity were determined in hepatopancreas mitochondria and cytosol from fasted crayfish, *Orconectes virilis*. Measuring HADH is fairly easy and inexpensive. Also, HADH is present in high

concentrations as compared to other β-oxidation enzymes in most animals.

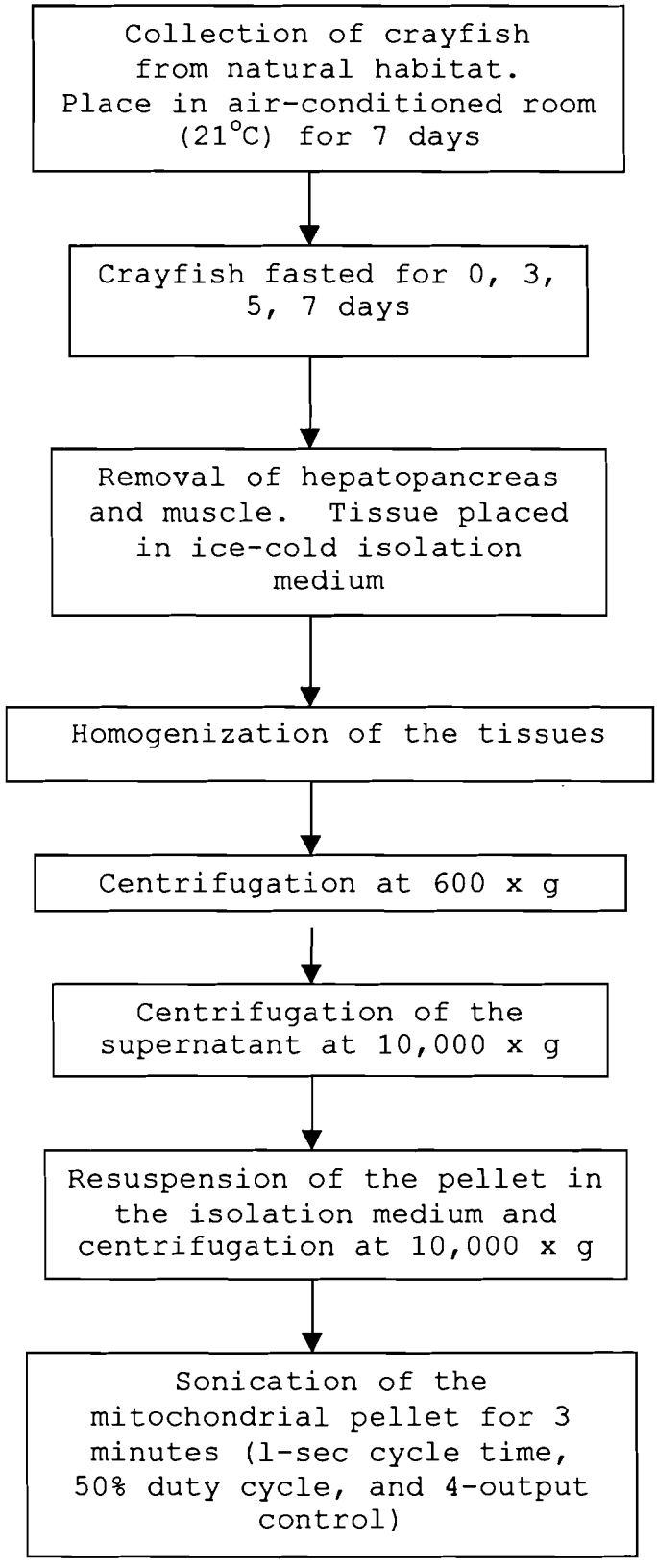
Hepatopancreas was chosen for this study because it is the largest organ in the crayfish body (Hickman and Hickman, 1988), and its role in energy production is still uncertain (Stewart et al., 1992). It appears to be the site of synthesis, detoxification and triglyceride storage (Swift et al., 1980). The flexor muscle was chosen for comparison to HADH activity in hepatopancreas. This muscle is the largest in the crayfish body and is used in the quick backward motion (Storer and Usinger, 1957) which suggests the muscle may have greater energy consumption than does the hepatopancreas.

MATERIALS AND METHODS

Tissue preparation

Males and non-pregnant female crayfish were collected from Wooster Lake at Emporia State University, Emporia, Kansas. The standard tissue preparation procedures are summarized in Fig. 3. The crayfish were kept in a controlled environment (21 ± 1 °C) and were fed with rat-chows (Harlan Teklad) to achieve a standardized physiological condition. The room was maintained on a LD 12:12 photoperiod. The crayfish were divided into four groups: after a 10 day acclimation period, one group was the control (fed) and the other three groups were fasted for 3, 5, and 7 days. The hepatopancreas and muscle tissue were removed and weighed. Both tissues were separately placed in isolation medium which contained 100 mM sucrose and 20 mM N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid] (HEPES) at pH 7.5 (Stuart and Ballantyne, 1996; Weinbach, 1961). The hepatopancreas tissue was placed into isolation medium (1/10 ratio by volume of wet tissue to the isolation medium). The ratio for the muscle tissue was 1/2 since it had much less protein. All procedures were

Fig. 3 Schematic outline of the tissue preparation.


Collection of crayfish from natural habitat. Place in air-conditioned room (21°C) for 7 days
Crayfish fasted for 0, 3, 5, 7 days
Removal of hepatopancreas and muscle. Tissue placed in ice-cold isolation medium
Homogenization of the tissues
Centrifugation at 600 x g
Centrifugation of the supernatant at 10,000 x g
Resuspension of the pellet in the isolation medium and centrifugation at 10,000 x g
Sonication of the mitochondrial pellet for 3 minutes (1-sec cycle time, 50% duty cycle, and 4-output control)

conducted on ice to prevent protein degradation. The tissues were homogenized (Potter-Elvehjem homogenizer) for at least 30 seconds to make sure the tissue was finely dispersed. The muscle tissue was first finely minced with a scissors before the homogenization. The homogenized tissue was centrifuged at 600 x g for 10 minutes at 4°C to remove the tissue fragments and cellular debris. Pellets were discarded and the supernatants centrifuged at 10,000 x g for 20 minutes at 4°C to isolate the mitochondrial fraction. The samples were defatted using cotton swabs (Weinbach, 1961). The mitochondrial pellets were washed by resuspension in isolation medium and centrifuging as above. Mitochondrial fractions were sonicated for three minutes using a W-385 Sonicator with #418 microtip (Heat Systems Ultrasonic Inc.). The cycle time was set for 1-second cycle time at 50% duty cycle and 4-output control (Li, 1996). The separated and sonicated fractions from hepatopancreas and muscle tissue were stored at -70°C until further use.

Protein Assay

Protein content was measured by the BioRad assay (Bradford, 1976). The absorbancy was measured with a Spectronic 401 spectrophotometer (Milton Roy, Rochester, NY) at 595 nm using bovine serum albumin as a standard.

Enzyme Assay

The HADH assay solution contained 50 mM imidazole, 0.2 mM NADH, and 0.1 mM acetoacetyl-CoA, adjusted to pH 8.0. To reduce the degradation of acetoacetyl CoA solution in storage, it was prepared separately in 0.1 mM HCl to maintain the acidic condition. The imidazole/NADH solution, pre-adjusted to pH 8.0, and the acetoacetyl CoA solution were mixed just before the enzyme assay. All assay solutions were stored at -70°C and thawed immediately before use. To detect the NADH oxidation by non-HADH, acetoacetyl-CoA was omitted for the negative control which was also adjusted to pH 8.0.

Serial dilutions were conducted with both cytosolic and mitochondrial fractions to determine the adequate concentration for a linear response in the assay to apply the Beer-Lambert law (Boyer, 1993). The diluted cytosolic

or mitochondrial fractions were mixed with HADH assay solution in a cuvette. The ratio of the sample to the assay solution was 1/10 by volume. The HADH activity was measured by observing the absorbancy change at 340 nm to detect the oxidation of NADH to NAD^+. Readings were taken every 2 minutes for 30 minutes.

Analysis

The enzyme assay data from cytosolic and mitochondrial fractions were plotted on individual graphs to determine the slopes which were necessary to calculate the enzyme activity in each sample. The linear part of the curve was used for calculations. The following equation was used (Boyer, 1993):

NADH oxidation rate = slope/6.22 $cm^{-1}mM^{-1}$cm/mg protein.

The slope was determined by subtracting non-NADH oxidation from total NADH oxidation divided by time in minutes. Means and standard deviations were calculated from the data. The NADH oxidation rate was expressed in μmoles NAD^+/min/mg protein. The extinction coefficient of NAD^+ is 6.22/cm/mM.

The data were analyzed by one way Analysis of Variance (ANOVA). The data were organized by the type of tissue and plotted graphically by linear regression to show the correlation between the HADH activity rate and fasting periods.

RESULTS

HADH activity in cytosolic fractions

HADH activity was calculated by taking total NAD^+ production and subtracting non-HADH activity (Fig. 4). HADH activity of cytosolic fractions is shown in Fig. 5. The cytosolic samples were taken from the fed and 3 days fasted groups. Although, NADH oxidation was detected in both groups, the activity was not due to HADH since NADH oxidation by non-HADH (negative control) showed similar rates. The figure shows negligible activity of the enzyme, which suggests there is little enzyme present in the cytosolic fraction.

Concentration of mitochondrial fractions

Fig. 6 shows the mitochondrial HADH activity rates for three concentrations. The samples diluted to 1/5 and 1/10 showed very rapid responses (within 10 minutes) and then a reverse reaction, which indicated that NAD^+ reduction took place immediately. On the other hand, the 1/20 showed relatively slow NADH oxidation for 15 minutes and entered into a stationary phase. This suggested that the 1/20

Fig. 4 NAD^+ production in the hepatopancreas cytosolic fraction.

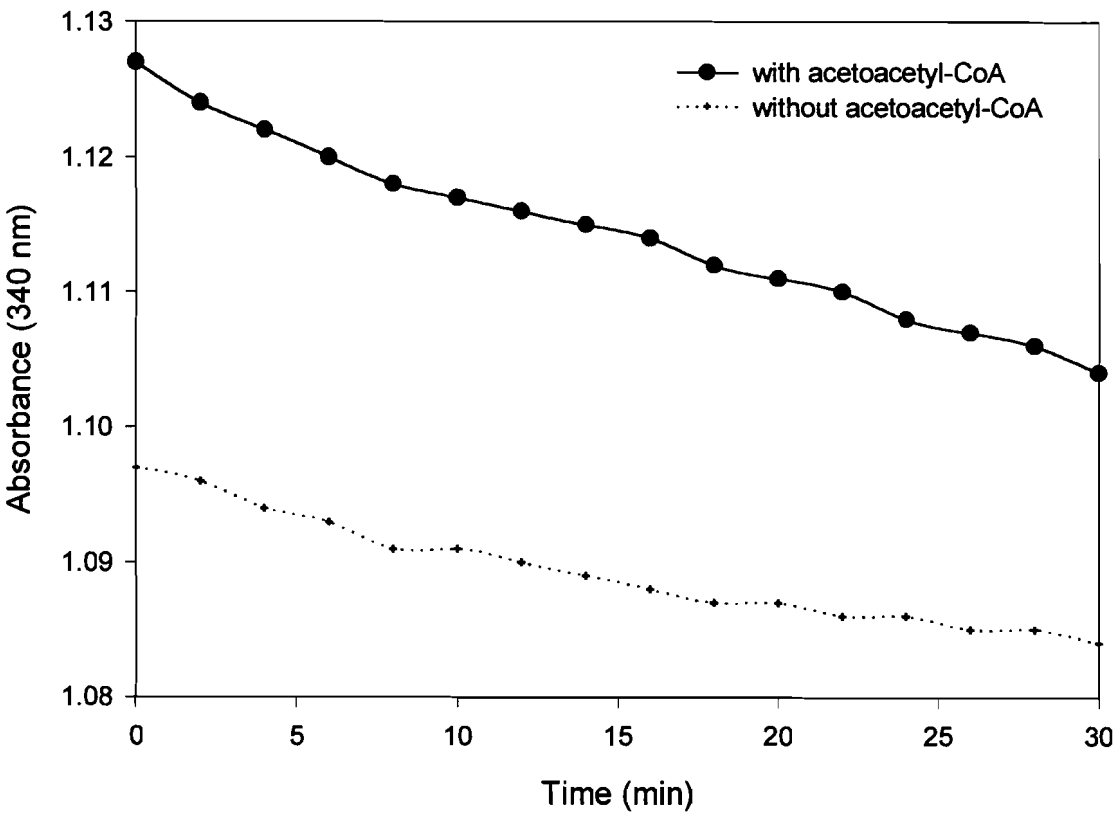

1.13
1.12
1.11
1.10
1.09
1.08
Absorbance (340 nm)
0
5
10
15
20
25
30
Time (min)
with acetoacetyl-CoA
without acetoacetyl-CoA

Fig.5 NAD^+ production of cytosolic fractions from fed and 3-day fasted crayfish. The line was calculated by linear regression analysis.

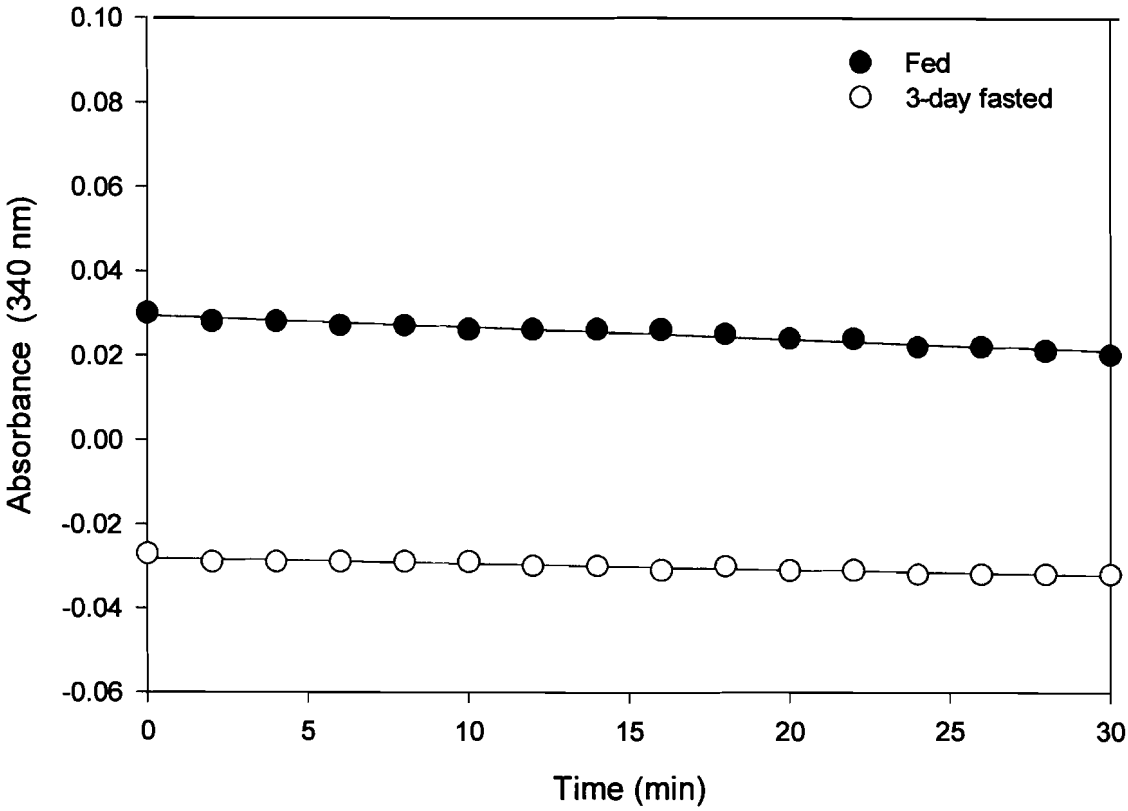
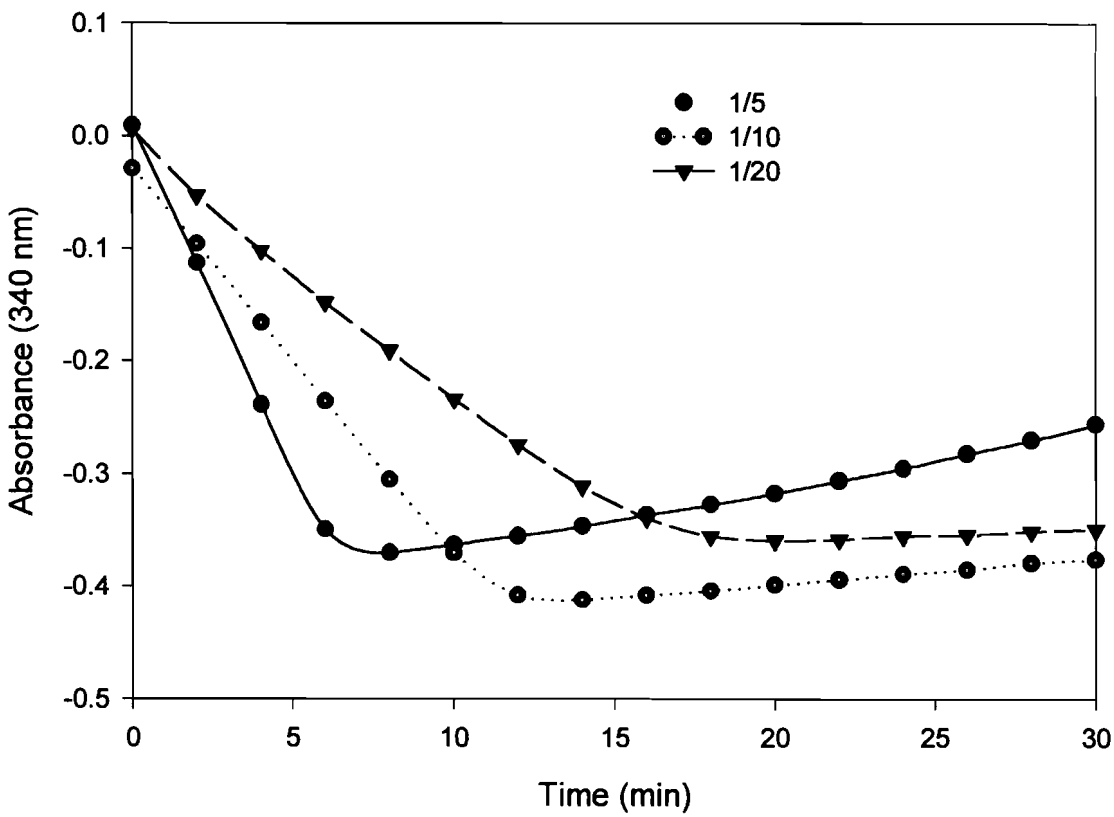

0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
Absorbance (340 nm)
0
5
10
15
20
25
30
Time (min)
Fed
3-day fasted

Fig. 6 NAD^+ production in fed group with three different dilutions of mitochondrial fractions (1/5, 1/10, and 1/20).


0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
Absorbance (340 nm)
0
5
10
15
20
25
30
Time (min)
1/5
1/10
1/20

dilution had an adequate linear response to conduct the enzyme assay. Both overall NAD^+ production rate and NAD^+ production rate by non-HADH were plotted to calculate HADH activity rate (Fig. 7).

HADH activity in hepatopancreas mitochondria

HADH activity in hepatopancreas mitochondria showed no increase with the length of fasting period (Fig. 8). The raw data are shown in Appendix A. The linear regression produced no significant slope which indicated there was not a detectable increase or decrease of the enzyme activity in hepatopancreas. Additionally, the one-way ANOVA showed $P = 0.994$ which confirmed that there were no significant differences among the fasting periods.

HADH activity in flexor muscle mitochondria

The linear regression of HADH activity in flexor muscle mitochondria showed a significant increase in HADH activity with the fasting period (Fig. 9). The equation of the linear plot is:

$$\mu mol\ NAD^+/min/mg\ protein = 5.334 + (0.185 \times days\ fasted)$$

Fig. 7 NAD^+ production in 1/20 diluted hepatopancreas mitochondrial fraction.

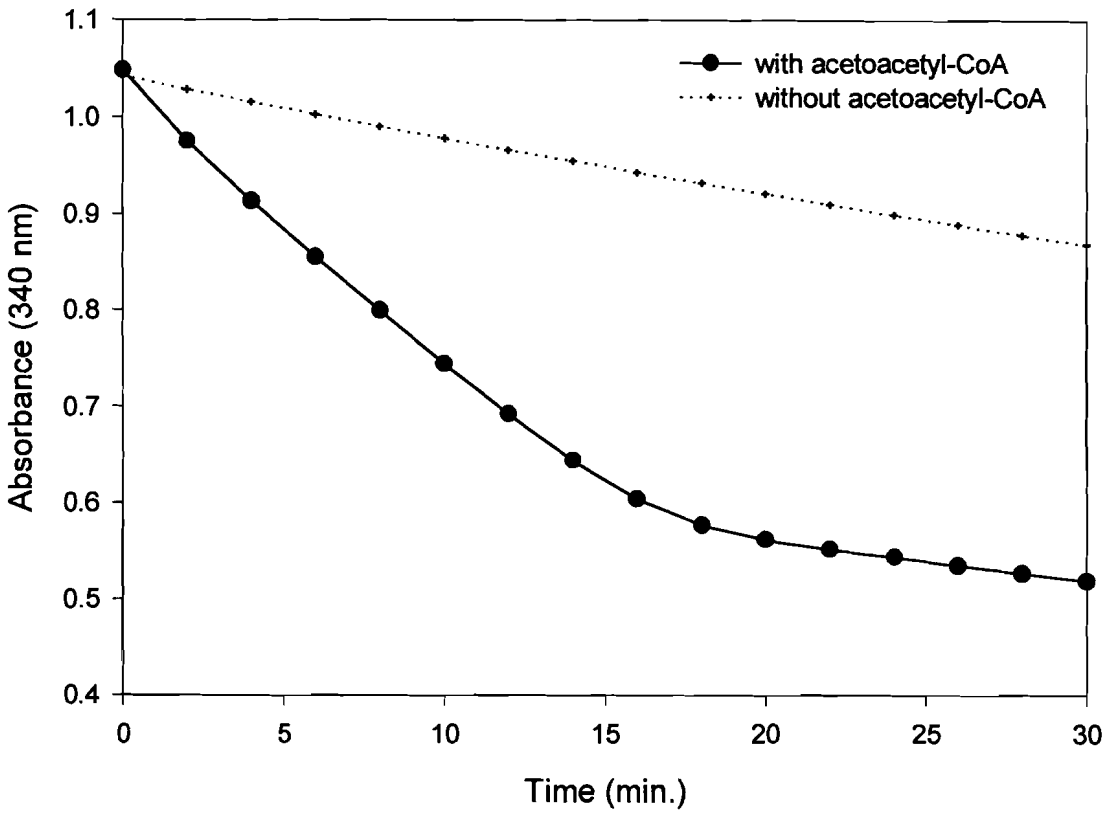

with acetoacetyl-CoA
without acetoacetyl-CoA
Absorbance (340 nm)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0
5
10
15
20
25
30
Time (min.)

Fig. 8 HADH activity in hepatopancreas expressed in μmol NAD^+/min/mg protein. Each group(0,3,5,7 days fasted) had a sample size of at least 11.

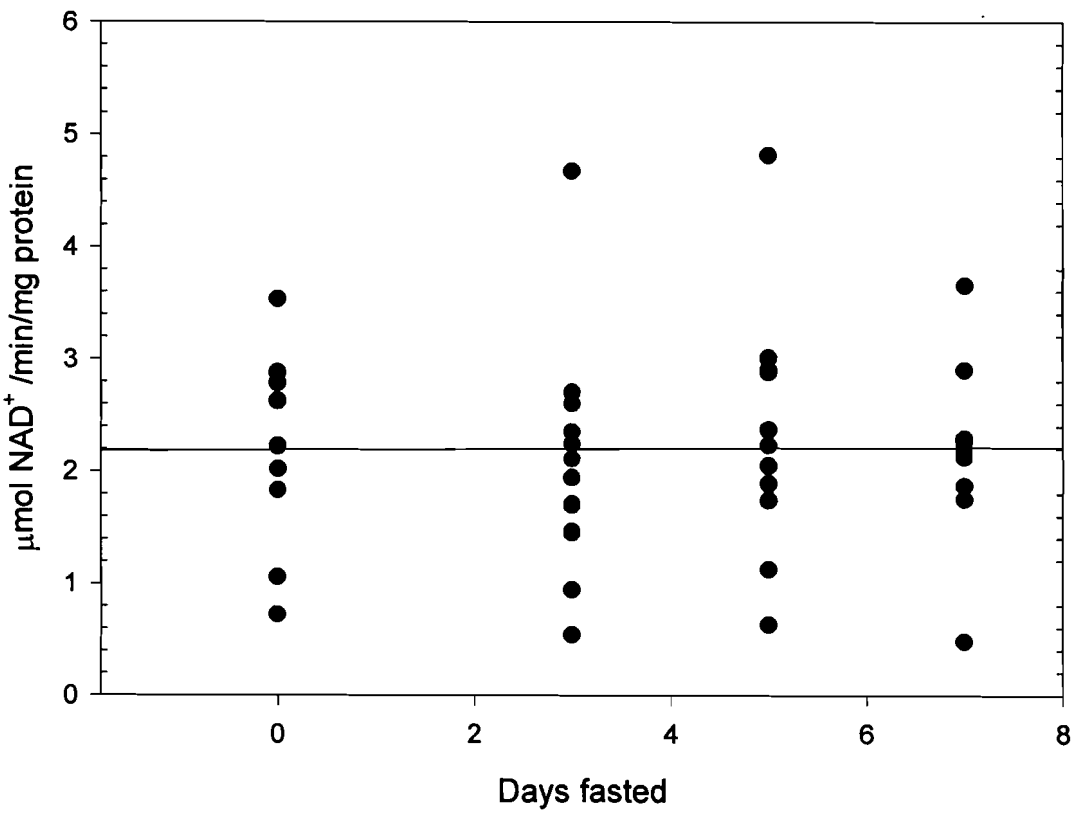
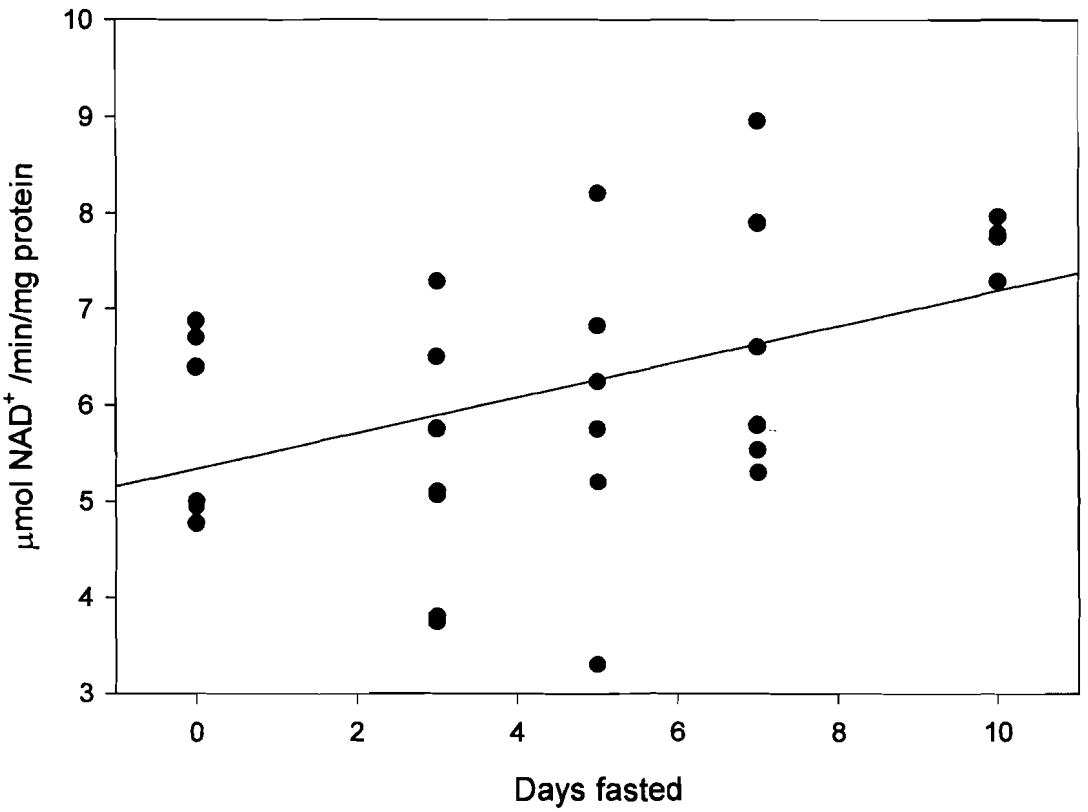

µmol NAD+ /min/mg protein
0
1
2
3
4
5
6
0
2
4
6
8
Days fasted

Fig. 9 HADH activity in flexor muscle tissue expressed in μmol NAD^+/min/mg protein. Each group (0, 3, 5, 7 days fasted) had a sample size of 6. The 10 day fasted group had a sample size of 4. The line was calculated by linear regression analysis.


µmol NAD+ /min/mg protein
10
9
8
7
6
5
4
3
0
2
4
6
8
10
Days fasted

The P value from the one-way ANOVA also indicated significant differences among the groups ($P = 0.015$). The raw data are shown in Appendix B.

DISCUSSION

HADH activity in cytosol

Although there has been a report of HADH activity in the terrestrial snail, *Cepaea nemoralis* (Stuart and Ballantyne, 1996), HADH activity in the hepatopancreas cytosol was not detected in my study. This result was expected because HADH is considered to be a membrane-bound enzyme (Sumegi and Srere, 1984; Kispal et al., 1986). The enzyme binds to a 3-hydroxyacyl-CoA dehydrogenase-binding protein, which is present on the inner mitochondrial membrane, under low ionic strength and in a pH-dependent manner. This binding increases HADH activity, and promotes strong association with other β-oxidation enzymes which eliminates the possibility of intermediate metabolite accumulation (Allmann et al., 1966). Several mitochondrial enzymes also bind to certain proteins of the inner mitochondrial membrane, such as other β-oxidation enzymes (Sumegi and Srere, 1984; Furuta and Hashimoto, 1995).

HADH activity in hepatopancreas

Hepatopancreas is an organ that stores triglycerides in crayfish (Swift et al., 1980). It is often compared to the liver in other animals because the roles are similar as a site of metabolism. However, the hepatopancreas also serves as a center of fat storage (Hickman and Hickman, 1988). When crayfish are fasted, triglycerides are used as fuel. In mammals, fatty acid oxidation is the major source of energy in hepatocytes in the fasted state (Champe and Harvey, 1994). In this study, fat in the hepatopancreas appeared to decrease through the course of fasting (personal observation), suggesting they are utilized as energy.

Wang et al. (1991) stated that the rate of β-oxidation changes in response to changes in the energy demand. However, HADH activity did not increase in the hepatopancreas with the fasting period, which suggests no increase in β-oxidation and no energy demand. To confirm these results, enzyme assays for acyl-CoA dehydrogenase and carnitine palmitoyltransferase (CPT) were necessary since they are also rate-limiting enzymes (Krahenbuhl, et al, 1994; Halestrap, 1989). If β-oxidation rates in

hepatopancreas are not increased by the fasting period, this may suggest that crayfish use fatty acids in the hepatopancreas as a primary energy source even in a fed state.

In dogfish, *Scyliorhinus canicula*, triacylglycerol metabolites circulate as ketone bodies, not as free fatty acids (Conlon et al., 1994). Zammit and Newsholme (1979) reported that CPT was absent from the muscle tissue of four species of elasmobranch fish suggesting that they are unable to use free fatty acids as an energy source in muscle. Meyer et al. (1986) supported a role for ketone bodies as a routine energy source in the freshwater gastropod, *Biomphalaria glabrata*. These reports suggest that mammals and non-mammals have different systems of utilizing fatty acids. If *O. virilis* produces ketone bodies in the well-fed state, this may explain why there was no increase of β-oxidation in fasting periods. To produce ketone bodies, the hepatopancreas has to operate β-oxidation. However, *O. virilis* did not produce any detectable amount of a ketone body catalyzing enzyme, 3-hydroxybutylate dehydrogenase (HBDH), in the well-fed state in this study (data not shown). This suggested

either *O. virilis* did not utilize ketone bodies as lipid-derived energy, or ketone bodies are exclusively produced in the state of starvation. To confirm the ketone body utilization in crayfish, detecting HBDH activity will be necessary since the HBDH activity in the starved state was not measured in the study.

Measuring carbohydrate metabolism rates in the hepatopancreas may reveal some of the answers. In a study of the striped shore crab, *Pachygrapsus crassipes*, Schatzlein et al. (1973) reported that the majority of the glycolytic enzymes of the hepatopancreas were not detectable in the fed state. However, they later concluded the result was due to a technical error. Additionally, Stewart et al. (1992) reported that CPT and 3-hydroxybutyrate dehydrogenase (HBDH), which catalyzes ketone body production, were not detectable in the heart, hepatopancreas, or white muscle in giant scallops or lobster. They also concluded that not being able to detect CPT was due to detergent effects. If their results were in error, then it parallels and could be the explanation for my results in hepatopancreas since CPT was detected in *O. virilis* hepatopancreas (data not shown).

HADH activity in muscle tissue

In mammalian muscle tissue, both free fatty acids and ketone bodies from liver are used as fuels in fasted states (Champe and Harvey, 1994). In my study, *O. virilis* showed an increase in HADH activity in flexor muscle tissue after depletion of the carbohydrate energy source. This suggests that muscle tissue of *O. virilis* can utilize free fatty acids and it metabolizes fatty acids similar to mammals rather than to elasmobranch fish. Also, the increase of HADH activity indicates that depletion of carbohydrate energy affects the metabolism in muscle tissue which means that the primary energy source in muscle tissue is carbohydrate. Boulton and Huggins (1970) concluded that glycolysis alone could supply enough acetyl-CoA to the TCA cycle to account for the respiratory rates observed in well-fed decapod species.

The results from flexor muscle in *O. virilis* did not reveal any ketone body usage (no HBDH activity) as an energy source. However, it did indicate that the free fatty acids were utilized for energy through β-oxidation to compensate for the carbohydrate energy shortage.

HADH and electron transport chain

Mitochondrial β-oxidation is linked to the respiratory chain at two stages; HADH to complex I via NAD^+/NADH and the acyl-CoA dehydrogenase to CoQ via electron-transfer flavoprotein (ETF) and its oxidoreductase (Eaton et al. 1994). β-oxidation and the TCA cycle compete in supplying electrons for the electron transport chain (Bremer and Wojtczak, 1972) which suggests control of β-oxidation by the electron transport chain. If NADH from the TCA cycle were decreased, the β-oxidation rate should increase to supply NADH to the electron transport chain. Since HADH reduces NAD^+ to NADH, this enzyme would be an important factor in the electron transport chain in fasted conditions. Wang et al. (1991) stated that the ratio of NADH/NAD^+ may be important in determining the flux through the β-oxidation cycle in extrahepatic tissues.

There are several β-oxidation enzymes that have been tested for the site of β-oxidation control. 3-ketoacyl-CoA thiolase was suggested as the site where β-oxidation might be controlled by the acetyl-CoA/CoASH ratio (Wang et al., 1991). Acyl-CoA dehydrogenase was described as the rate-

limiting enzyme of β-oxidation under normal conditions (Krahenbuhl et al., 1994). The regulatory mechanism of the β-oxidation pathway has yet to be established.

Biochemistry and molecular structure of 3-hydroxyacyl-CoA dehydrogenase

Understanding molecular structures of the β-oxidation enzymes may reveal their roles in the oxidation regulatory mechanism. Molecular studies of HADH have been done with short-chain HADH for pig heart muscle, rat liver, and cow liver mitochondria (Noyes and Bradshaw, 1973b; Osumi and Hashimoto, 1980; Kobayashi et al., 1996). This enzyme was first isolated from sheep liver, then from cow liver and pig heart (Noyes and Bradshaw, 1973a). Noyes and Bradshaw (1973b) reported that the molecular weight of HADH was approximately 65,000 with two polypeptides of equal molecular weight of approximately 31,000. The sequence of amino acid residues surrounding the single cysteinyl residue, which is the thiol peptide, is His-Pro-Val-Ser-Cys-Lys. Similar molecular structures were reported for the enzyme from cow liver (Osumi and Hashimoto, 1980) and rat liver mitochondria (Kobayashi et al., 1996). Both cow

and rat HADH consist of two identical subunits of 32 kDa (rat), and 35 kDa (cow). Both are similar in their molecular structure, immunochemical reactivity, and carbon chain length specificity (Kobayashi et al., 1996). Kobayashi et al. (1996) also found a new type of HADH that was a homotetramer of 28 kDa polypeptides. The new type of HADH was a monofunctional protein like the classic HADH, and the enzyme activity was comparable to that of classic HADH in cow liver. This type of HADH is also rich in cow, sheep, and horse livers, but it was scarcely detected in other animals (Kobayashi et al., 1996).

Mitochondria also contain a trifunctional protein (also known as long-chain HADH), in addition to a monofunctional protein. This protein contains the enzymatic function of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase (Uchida et al., 1992). This trifunctional enzyme consists as a tetramer or octomer with equal amounts of two subunits: an α-subunit with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities and a smaller β-subunit with thiolase activity. This enzyme has been clinically studied since the first report of the deficiency

in 1989 (Pollitt, 1995). This long-chain HADH deficiency causes hydroxydicarboxylic aciduria (Wanders et al., 1991) and is fatal if the patient is not treated with a diet rich in carbohydrate and low in fat (Wanders et al., 1991).

Peroxisomal β-oxidation

Another β-oxidation system has been found in peroxisomes. Although CPT does not exist in peroxisomes, peroxisomal β-oxidation can catabolize a wide range of substrates (Novikov et al., 1994). Osumi and Hashimoto (1980) reported that peroxisomal HADH is monomeric with a molecular weight of 70 kDa to 77 kDa in rat liver. Dieuaide-Noubhani et al. (1996) characterized five types of peroxisomal HADH based on molecular weight, substrate (L- or D-hydroxyacyl-CoA) stereoisomer specificity, and fatty acid chain length specificity. The peroxisomal β-oxidation was thought to be a small contributor in fatty acid metabolism. However, several studies examined the role of peroxisomal β-oxidation and questioned the distribution of β-oxidation fluxes between mitochondria and

peroxisomes (Rognstad, 1991). The importance of peroxisomal β-oxidation needs to be further investigated.

The peroxisomal HADH can catalyze catabolism of various chain lengths of substrate. Thus, peroxisomal HADH contamination in mitochondrial samples might play a role in my results in hepatopancreas and flexor muscle in *O. virilis*.

Physical conditions of Orconectes virilis

This research was conducted in the summer of 1998. The hepatopancreas in *O. virilis* is typically rich in fat, and *O. virilis* uses fat from this organ in the fasted condition (Personal observation). However, the hepatopancreas stored little fat in the animals captured in winter (Personal observation). This type of seasonal triglyceride content fluctuation was also reported in oysters, *Crassostrea virginica* (Swift et al., 1979). There is little food available for crayfish in winter (Huxley, 1974) forcing a decrease in the metabolic rate to adjust to the condition (Schmidt-Nielsen, 1993). In goldfish, a 10°C drop in temperature causes approximately a two- to three-fold decrease in metabolic rate and energy intake (Rozin

and Mayer, 1961). The fat content from fasted *O. virilis* in winter was measured and found to be slightly more in 5-day fasted *O. virilis* than in fed ones (fed for a week after capture) (data not shown). In pacific oyster, the prolonged storage at 2°C to 4°C caused an anaerobic condition and an inability to catabolize lipids (Swift et al., 1979). The temperature and food availability before capture should influence this result. Interestingly, fasting in house musk shrew, *Suncus murinus*, deposits fat in the liver after 24 hours of fasting and the effect is reversed simply by refeeding (Yasuhara et al., 1991). After *O. virilis* were captured, they were placed in a temperature-controlled room (21°C) and fed for a week. The animals were divided into two groups; fed group and 5-day fasted group. Since the natural habitat was cold, and little food available, the sudden change to the controlled habitat may play a role in the fat content in the hepatopancreas in *O. virilis*. During the week of the control period, copulations were frequently observed. In oyster, fatty acid metabolism change was reported during breeding (Swift et al., 1979). The breeding season may also affect the fatty acid content in hepatopancreas of crayfish.

CONCLUSION

Explaining the results of HADH activity in hepatopancreas and flexor muscle requires a complete study of fatty acid metabolism. Obviously, more than one mechanism for regulation of β-oxidation is involved. β-oxidation is controlled by both intracellular and extracellular (mostly hormonal regulations) mechanisms. My results indicated that fatty acid metabolism in hepatopancreas and flexor muscle operate differently. Hepatopancreas β-oxidation did not increase during fasting, whereas flexor muscle clearly showed an increase of the β-oxidation activity. These results also suggest that crustacean metabolism is different from mammalian fatty acid metabolism.

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APPENDIX A

HADH activity in μmol NAD^+/min/mg protein in hepatopancreas. Each group had a sample size of at least 11.

Sample number	Control	3-day	5-day	7-day
1	2.28	1.46	1.89	3.01
2	2.87	2.70	0.63	0.48
3	1.06	0.54	2.23	1.87
4	0.72	0.94	2.23	2.90
5	2.78	2.24	4.82	2.20
6	3.53	1.45	1.74	1.75
7	1.83	4.68	1.12	2.13
8	2.78	2.11	2.88	2.29
9	2.02	2.60	2.37	2.27
10	2.22	2.35	2.05	2.28
11	2.62	1.70	2.23	3.65
12	--	1.94	2.90	--
Mean	2.25	2.06	2.20	2.26
S.D.	0.82	1.10	1.06	0.81

APPENDIX B

HADH activity in μmole NAD^+/min/mg protein of crayfish muscle tissue.

Sample number	Control	3-day	5-day	7-day	10-day
1	6.40	3.80	8.20	8.95	7.96
2	4.95	7.28	6.24	7.89	7.78
3	5.00	5.07	6.82	5.53	7.28
4	6.87	3.75	5.75	5.30	7.75
5	6.70	6.50	3.30	6.60	--
6	6.38	5.10	5.20	5.79	--
7	4.77	5.75	--	--	--
Mean	5.86	5.32	5.91	6.67	7.69
S.D.	0.91	1.31	1.64	1.45	0.29

Misa Inomata

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